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The Impact of Primary Renal Epithelial Senescence Induction in the Murine Kidney

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Declaration

I declare that this thesis has been composed by myself and that the work has not been submitted for any other degree of qualification. I confirm that the work presented in this thesis is my own, except where stated/acknowledged in the manuscript.

Marie-Helena Docherty

Abstract

Senescence is a cellular process resulting in irreversible growth arrest, an altered transcriptome and a pro-inflammatory, pro-fibrotic secretome.

Senescent cells are a highly heterogeneous cell group with distinct context dependent roles. The induction of cellular senescence is a physiological response to acute stimuli in several contexts *in vivo* including cancer defence, wound healing, and organogenesis during embryonic development. However, the persistence and accumulation of senescent cells in response to cell stress, ageing and tissue injury associates with tissue dysfunction and impaired regeneration. In experimental models of ageing and/or injury, depletion of long-lived senescent cells both before and after injury improves organ function and regenerative capacity. Much of this heterogeneity of senescent cell behaviour remains unexplained.

A key reason for this pertains to the fact that senescence itself, by virtue of its induction by genotoxic damage and cell stress, is induced by injury and age. Therefore, senescent cells associate very strongly with both. Without separating senescence from injury and from age, it is not possible to determine the exact contribution of senescent cells themselves to tissue injury or fibrosis. To understand if senescent epithelial cells can directly cause tissue dysfunction vs being bystanders to dysfunction requires a separation of senescence and injury.

This PhD examined the hypothesis that epithelial senescence in the absence of age and injury was sufficient to initiate renal fibrosis. By using *Pax8*, a gene highly expressed in the renal epithelium but very lowly outside of the kidney to drive an inducible cre-recombinase system, it was possible to generate renal cortical epithelial senescence without injuring mice and to show that isolated renal epithelial senescence in young immunocompetent female mice was sufficient to initiate fibrosis in the absence of injury.

This model allowed us to define the onset of senescence, and to study the evolution of senescent cells *in vivo* over time. This has not been possible in other models of induction such as injury or age as it is impossible to understand exactly when a cell became senescent in response to a senescence-inducing stimulus, and it is furthermore not possible to delineate what component of any observed inflammatory cell infiltrate is due to injury vs that due to senescence. We showed that in young female uninjured mice, there was a suggestion that senescent epithelial cells were cleared over time and this is in contrast to the persistence of senescent cells after injury which we have observed in other models of senescence induction.

These results demonstrate that epithelial senescence induction is profibrotic in the absence of injury to other cell lineages. Of importance, these results also suggest mechanisms in the healthy adult kidney which allow detection and physiologic clearance of acutely senescent cells and prevent ongoing fibrosis. Understanding how these pathways are lost with ageing and chronic injury may lead to new routes to promote clearance of profibrotic senescent epithelia.

Lay summary

Chronic Kidney Disease (CKD) affects over 850 million people worldwide. CKD is incurable, with progressive kidney scarring (fibrosis) a feature common to multiple different kidney diseases. There are no available treatment options to directly treat or reverse kidney fibrosis.

People who have experienced an episode of AKI are at significantly higher risk of developing CKD than those who have never suffered any kidney injury. This suggests that even when the original insult which resulted in AKI resolves, the underlying kidney remains vulnerable to future damage, and there are processes which continue to take place in the aftermath of injury which cause on-going tissue dysfunction, and which impair the ability of the kidney to undergo appropriate repair.

We are particularly interested in a group of cells known as senescent cells. These are cells which have entered into a state of permanent growth arrest in response to cell stress or DNA damage. They are metabolically active cells and in the process of becoming senescent, these cells develop the ability to produce signalling molecules which can promote kidney fibrosis and inflammation, and which can prevent kidney tissue from regenerating appropriately following injury.

Senescent cells are strongly associated with injury and with age. They are attractive candidates to explain a potential link between a previous injury and ongoing tissue damage. An overarching goal of scientific research is to understand human disease and to identify novel therapies. In mice, senescent cell depletion has been demonstrated to be safe and extends mouse lifespan. It has been shown across many organ systems, and in many different injury models that removing senescent cells improves the ability of an organ to regenerate after injury and lowers the levels of fibrosis.

Whilst this provides a strong suggestion that senescent cells which persist after injury are harmful and contribute to fibrosis, it has proven difficult to study senescent cells in isolation because they occur so frequently in response to injury and age. Therefore, it has not been possible to define their individual contribution to tissue damage and scarring, separate to the damage caused by the initial injury itself.

By utilising a transgenic mouse line in which we can genetically switch on senescence in a group of kidney cells without physically injuring the kidney, we show that senescent cells themselves are capable of inducing kidney fibrosis and kidney inflammation. Our results also suggest that senescent cells seem to be cleared over time in young healthy uninjured kidneys, and this is different to what we observe in the aftermath of injury, when senescent cells seem to persist.

We are now looking to identify mechanisms in a healthy kidney which allow the detection and clearance of senescent cells and to determine how these mechanisms are lost in the presence of injury and age. Understanding these pathways may uncover new ways to remove senescent cells in a targeted manner and may prove to be of therapeutic benefit.

Publications

The following publications relate to work undertaken just prior to and during this PhD. Where relevant they are referenced. The first three are of particular relevance to the work presented within this thesis and they can be found in Appendix B.

Cellular Senescence in the Kidney. **Marie-Helena Docherty**, Eoin D. O’Sullivan, Joseph V. Bonventre and David A. Ferenbach. *Journal of the American Society of Nephrology* (2019).

Cellular Senescence and Senotherapies in the Kidney: Current Evidence and Future Directions. **Marie-Helena Docherty**, David P. Baird, Jeremy Hughes and David A. Ferenbach. *Frontiers in Pharmacology* (2020).

Cellular senescence inhibits renal regeneration after injury in mice, with senolytic treatment promoting repair. *Sci. Trans Med.* Katie Mylonas, Eoin D. O’Sullivan, Duncan Humphries, David P. Baird, **Marie-Helena Docherty**, Sarah A. Neely, Paul J. Krimpenfort, Annette Melk, Roland Schmitt, Sofia Ferreira-Gonzalez, Stuart Forbes, Jeremy Hughes, David A. Ferenbach (2021).

The Role of Ageing and Parenchymal Senescence on Macrophage Function and Fibrosis. *Front Immunol.* Ross A Campbell, **Marie-Helena Docherty**, David A Ferenbach, Katie J Mylonas.

Single-cell analysis of senescent epithelia reveals targetable mechanisms promoting fibrosis. *JCI Insight.* Eoin D. O’Sullivan, Katie J. Mylonas, Rachel Bell, Cyril Carvalho, David P. Baird, Carolynn Cairns, Kevin M. Gallagher, Ross Campbell, **Marie-Helena Docherty**, Alexander Laird, Neil C. Henderson, Tamir Chandra, Kristina Kirschner, Bryan Conway, Gry H. Dihazi, Michael Zeisberg, Jeremy Hughes, Laura Denby, Hassan Dihazi, and David A. Ferenbach (2022).

Indian Hedgehog release from TNF-activated renal epithelia drives local and remote organ fibrosis. Sci. Trans. Med. Eoin D. O'Sullivan, Katie J. Mylonas, Cuiyan Xin, David P. Baird, Cyril Carvalho, **Marie-Helena Docherty**, Ross Campbell, Kylie P. Matchett, Scott H Waddell, Alexander D Walker, Kevin M Gallagher, Siyang Jia, Steve Leung, Alexander Laird, Julia Wilflingseder, Michaela Willi, Maximillian Reck, Sarah Finnie, Angela Pisco, Sabrina Gordon-Keylock, Alexander Medvinsky, Luke Boulter, Neil C. Henderson, Kristina Kirschner, Tamir Chandra, Bryan Conway, Jeremy Hughes, Laura Denby, Joseph V Bonventre, and David A. Ferenbach

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Abbreviations

ADPKD	Autosomal dominant polycystic kidney disease
AKI	Acute kidney injury
ANOVA	Analysis of variance
CAG	Chicken actin globin
CDK	Cyclin dependent kinase
CKD	Chronic kidney disease
DAPI	4', 6'-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DN	Diabetic nephropathy
DDR	DNA damage response
DTL	Descending thin limb
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FOXO	Forkhead box O
IL	Interleukin
LTL	Lotus tetragonolobus lectin
MOMP	Mitochondrial outer membrane permeabilization
MDM	Mouse double minute
NK	Natural killer
PiSR	Picrosirius red
PTEC	Proximal tubular epithelial cell
PUMA	p53 upregulated modulator of apoptosis
RB	Retinoblastoma
RNA	Ribonucleic acid
RPM	Revolutions per minute

RPMI	Rosewell park memorial institute
RT	Room temperature
SASP	Senescence associated secretory phenotype
scRNA	Single cell ribonucleic acid
TNF	Tumour necrosis factor
TGF	Transforming growth factor
qPCR	Quantitative polymerase chain reaction
UMAP	Uniform manifold approximation and projection
UUO	Unilateral ureteric obstruction

Suppliers

10X Genomics, California, USA

Abcam, Cambridge UK

Bio-Rad Hercules, California, USA

Miltenyi Biotec, Auburn, California, USA

Parse Biosciences, Seattle, USA

Qiagen, Hilden, Germany

Sarstedt, Leicester, UK

Sigma-Aldrich, St Louis, MO, USA

Thermo-Scientific, Massachusetts, USA

Vector Laboratories, San Francisco Bay Area, USA

Zeiss, Oberkochen, Germany

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Chapter 1: General Introduction

Introduction

Senescence is a cellular process which results in irreversible growth arrest coupled with widespread alterations at a transcriptional, metabolic and secretory level, in addition to modified cell morphology and chromatin organisation (Sturmlechner, Durik et al. 2017, Gorgoulis, Adams et al. 2019).

The word itself derives from the Latin “Senex” meaning “old”. The association between ageing and senescence is well established and in the recent past, the terms were used interchangeably. It is now clear that these terms are not synonymous. There are recognised examples where senescence occurs regardless of biological age such as organogenesis during embryonic development (Munoz-Espin, Canamero et al. 2013), wound healing (Demaria, Ohtani et al. 2014) and cancer defence (Martin-Caballero, Flores et al. 2001). There are also very clear examples where the induction of senescence drives an ageing phenotype (Xu, Pirtskhalava et al. 2018) and indeed where the elimination of senescent cells results in increased health span (Baker, Wijshake et al. 2011, Baker, Childs et al. 2016, Xu, Pirtskhalava et al. 2018). I will define the process of cellular senescence in detail in the chapters to follow but in short, it is a cellular mechanism which although associates strongly with age and tissue dysfunction, is very clearly also seen in early life. Why senescence associates so strongly with age is subject to debate. There is a suggestion that it relates the accumulation of cellular stress (a key trigger for senescence) over time, and/or relates to immunosenescence and defective clearance of senescent cells as we age (van Deursen 2014)

A seminal description by Leonard Hayflick and colleagues in 1961 of what is now recognised as a telomere shortening with prolonged cell culture *in vitro* began to shape our modern understanding of the term, as an entity distinct to that of biological ageing (Hayflick and Moorhead 1961).

Whilst age and senescence are distinct, the persistence and accumulation of senescent cells in response to cell stress, ageing and tissue injury associates strongly with tissue dysfunction and impaired regeneration. In murine experimental models of ageing and/or injury, depletion of long-lived senescent cells both before and after injury improves organ function and regenerative capacity and extends healthy lifespan. (Schafer, White et al. 2017, Xu, Pirtskhalava et al. 2018, Mylonas, O'Sullivan et al. 2021).

Thus, senescence is a complex and diverse process. Senescent cells are a highly heterogeneous cell group with distinct context dependent roles. Key factors purported to influence this heterogeneity include the mode of senescence induction and the duration of time a senescent cell remains resident within a tissue. (Hernandez-Segura, de Jong et al. 2017)

Cellular characterisation in the kidney

The kidney is recognised as having a high degree of cellular complexity. It is an organ with multiple functions. These functions range from filtering blood and excretion of waste products, to regulating water homeostasis and blood pressure. The kidney is also responsible for the maintenance of haemoglobin (through renal production of erythropoietin) and plays a central role in healthy bone biochemistry.

The human kidney is made up of an average of one million nephrons (Murawski, Maina et al. 2010). Individual nephrons comprise predominantly epithelial cells and are surrounded by vascular, stromal and immune cells (Wallace 1998). The anatomical structure of a nephron is shown below in Figure 1. (Novella-Rausell, Grudniewska et al. 2023).

The proximal tubule has enormous resorptive capacity. It is approximated that over 150L of filtrate are reabsorbed per day per kidney. For efficient reabsorption to

occur, a sodium gradient is generated by the Na^+/K^+ -ATPase channels located in the proximal tubule. This gradient drives key components such as water, essential amino acids, glucose, and salts back into the circulation. This is an energy demanding process and hence, the proximal tubule is recognised as being metabolically very active. (Curthoys and Moe 2014, Bhargava and Schnellmann 2017)

The filtrate is then further concentrated as it travels through the loop of Henle (Marcoux, Tremblay et al. 2021) before arriving at the distal convoluted tubule and collecting duct where further concentration of the filtrate (urine) occurs. (Kokko 1987)

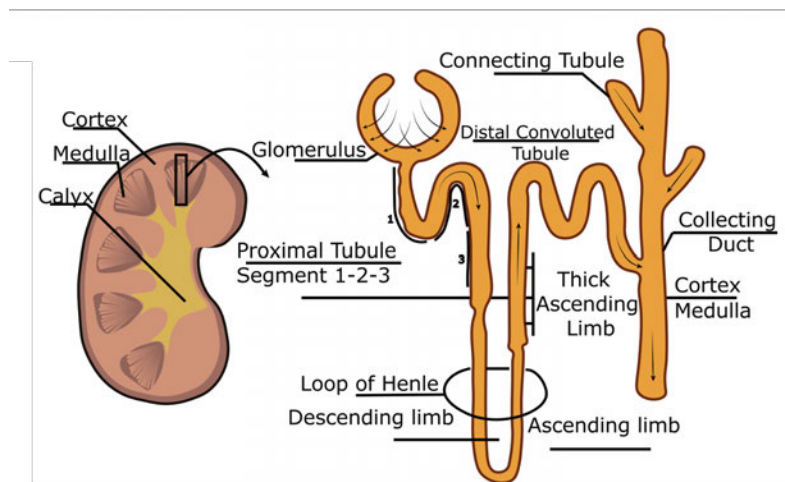


Figure 1 - The Nephron (Novella-Rausell, Grudniewska et al. 2023)

The function and nomenclature of the cells which form the nephron.

The ageing kidney

It is recognised that as kidneys age, the total nephron number declines. (Nyengaard and Bendtsen 1992) Other changes also occur, such as thickening of the glomerular basement membrane and tubulointerstitial fibrosis. This age-related appearance is sometimes termed 'nephrosclerosis'. It is a histological finding which clearly associates with age. In youth (<30 years), it is found in less than 3% of human kidney biopsies, compared to over 70% in those over 70 years of age. (Rule, Amer et al. 2010)

Ageing involves a progressive loss of tubular function. As discussed above, the archetypal role of the proximal tubule is that of reabsorption. As we age, the ability to reabsorb key salts such as sodium decreases, as does our ability to concentrate urine effectively. (Sands 2012)

Beyond the tubule, there are also important changes in blood vessel structure and function in the aged kidney. There is increased intimal cell proliferation in preglomerular arterioles and increased extracellular matrix deposition. (Takazakura, Sawabu et al. 1972) There is increased renal sympathetic tone and hence increased vasoconstriction. (Hajduczuk, Chapleau et al. 1991) and renal vasodilators are less effective. (Mulkerrin, Brain et al. 1993)

Despite there being a strong association between age and senescence, the specific mechanisms through which senescent cells impact an aged kidney are not fully understood. They are likely to be multifactorial, involving the loss of replicative function, disordered repair, and local inflammatory influence of the SASP.

The Identification of Senescent Cells

Whilst physical and functional alterations of senescent cells *in vitro* are well characterised, the identification of senescent cells *in vivo* is difficult because there is no single feature which uniquely identifies a senescent cell. Moreover, some markers that are used to define senescence are specific to the mode of induction, and some markers are also found in non-senescent healthy replicating cells. Defining senescent cells therefore requires a careful multi-faceted approach, usually involving two or more identifying features.

Well recognised markers of senescence include two key cyclin dependent kinase inhibitors which are involved in enforcing cell cycle arrest at the G1/S checkpoint; p16^{INK4A} and p21^{cip1}. An assay looking for senescence-associated- β -galactosidase (SA- β -gal) activity is often used as an additional marker. This reflects increased lysosomal

activity in senescent cells, though SA- β -gal itself is not required for senescence to occur. (Childs, Durik et al. 2015, Gorgoulis, Adams et al. 2019) Lysosomal β -Gal is an enzyme thought to be increased in senescent cells, which cleaves galactose residues. In the assay used to detect SA- β -gal activity, the compound X-gal is hydrolysed in a reaction which leads to the release of a visible deep blue insoluble product (Dimri, Lee et al. 1995).

Various international groups have proposed multi-step workflows for the identification of senescent cells.

The Van Deursen group proposed that quantifying tissue senescence should comprise three elements: the identification at a protein level of markers of cell cycle arrest, an assessment of the effects of senescence-associated protein release and the exclusion of concomitant cellular proliferation (Childs, Durik et al. 2015).

More recently, the International Cell Senescence Association (ICSA) provided an alternative but complementary approach, which acknowledged that the specificity of markers of senescence is context dependent, and is influenced by factors including cell type, and organismal developmental stage. The ICSA pipeline for senescence detection is shown below in Figure 2 A. When defining senescent cells in my own project, I stuck to this definition as closely as possible, and I have outlined this in Figure 2 B. Using fixed tissue precluded the use of SA- β -Gal staining. For reasons which related to the genetic pathways induced in the transgenic mouse which I used, and which I will go on to describe in detail, I focussed on the expression of p21^{cip1} and the gene which encodes p21^{cip1}; *Cdkn1a*. I looked for the absence of Ki67 in cells which expressed p21^{cip1} as a marker that these cells had undergone growth arrest.

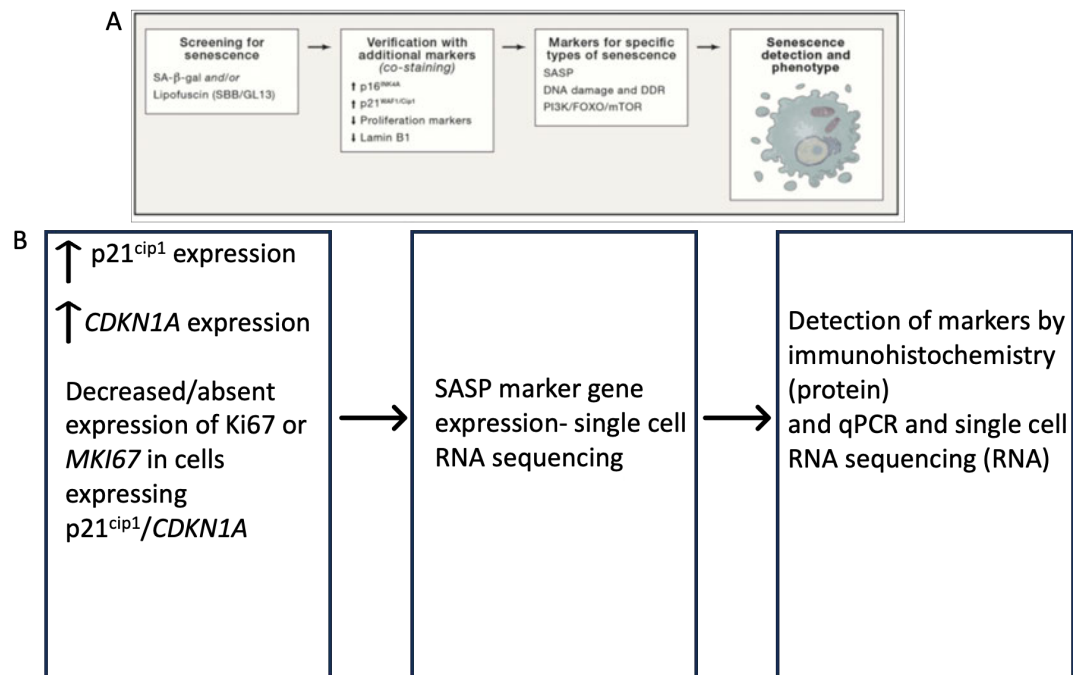


Figure 2 -The Identification of Senescent Cells: adapted from (Gorgoulis, Adams et al. 2019)

Figure 2. A) The identification of senescent cells as per the recommended ICSA pathway (Gorgoulis, Adams et al. 2019). B) The approach I used to define senescent cells in my project; an amended version of the recommended ICSA approach.

The first step in this approach is to screen for SA-β-gal activity. It is worth noting that the use of SA-β-gal is restricted as it cannot be used in live cells, nor in paraffin embedded tissue sections. A proposed alternative is to quantify lipofuscin which can be visualised in lysosomes *via* histochemical staining (Gorgoulis, Adams et al. 2019).

The second step is to identify protein markers of senescence; p16^{INK4A} and p21^{cip1} and to look for the absence of cell proliferation markers, and other intracellular changes such as loss of lamin-B1 (LMNB1), a recognised nuclear envelope protein.

The third step is to identify markers which might reflect the mode of induction of senescence. Genotoxic stress is a well-recognised inducer of senescence and markers of DNA damage such as genes associated with the DNA damage repair pathway (DDR) can be used to identify senescent cells. In addition, FOXO, a transcription factor and phosphoinositide 3-kinase (Pi3K), an intracellular signal transducer, both of which enable senescent cells to resist apoptosis are also recognised as potential senescent

cell identifiers (Liu, Liu et al. 2011, Baar, Brandt et al. 2017). Finally, the secretome of senescent cells; termed the senescence associated secretory phenotype (SASP) can be quantified.

The induction of senescence

Cellular senescence can be triggered by a broad number of different insults, including DNA damage, oncogene activation, metabolic stress, hypoxia, and critical telomere shortening. These insults indicate that the cellular conditions are not favourable for healthy replication and as such, a hallmark of senescent cells is irreversible cell cycle arrest.

Most senescence-inducing stimuli result in increased production of the cyclin dependent kinase inhibitors p16^{INK4A} and p21^{cip1} as demonstrated below in Figure 3.

p21^{cip1} inhibits cyclin dependent kinases (CDK) 1, 4 and 6 (Bertoli, Skotheim et al. 2013). A p21^{cip1} mediated arrest can act as a temporary repair step and if DNA damage is successfully repaired, cells can resume cycling (Rodriguez and Meuth 2006, Hsu, Altschuler et al. 2019).

p16^{INK4A} inhibits CDK 4 and 6 (Hara, Smith et al. 1996). In mammalian cells, the retinoblastoma (RB) family of proteins and p53 protein are important for establishing senescence. In cells undergoing replication, RB is phosphorylated by specific cyclin dependent kinases including CDK4, CDK6 and CDK2. Once phosphorylated, RB is less able to repress E2F family transcription factor activity (Brown, Phillips et al. 1999). The E2F family of transcription factor play an integral role in cell proliferation by activating genes crucial for G1/S progression (Xie, Pei et al. 2021). Because of the accumulation of cyclin dependent kinase inhibitors in senescent cells, this cyclin dependent kinase mediated RB phosphorylation is reduced, resulting in the activation of RB family proteins and the inhibition of E2F activity which promotes cell cycle arrest. This effect is maintained by the heterochromatinisation of E2F target

genes which inhibit E2F responsive genes, even in response to mitogenic signalling. This effect is not observed in temporarily arrested quiescent cells (Narita, Nunez et al. 2003).

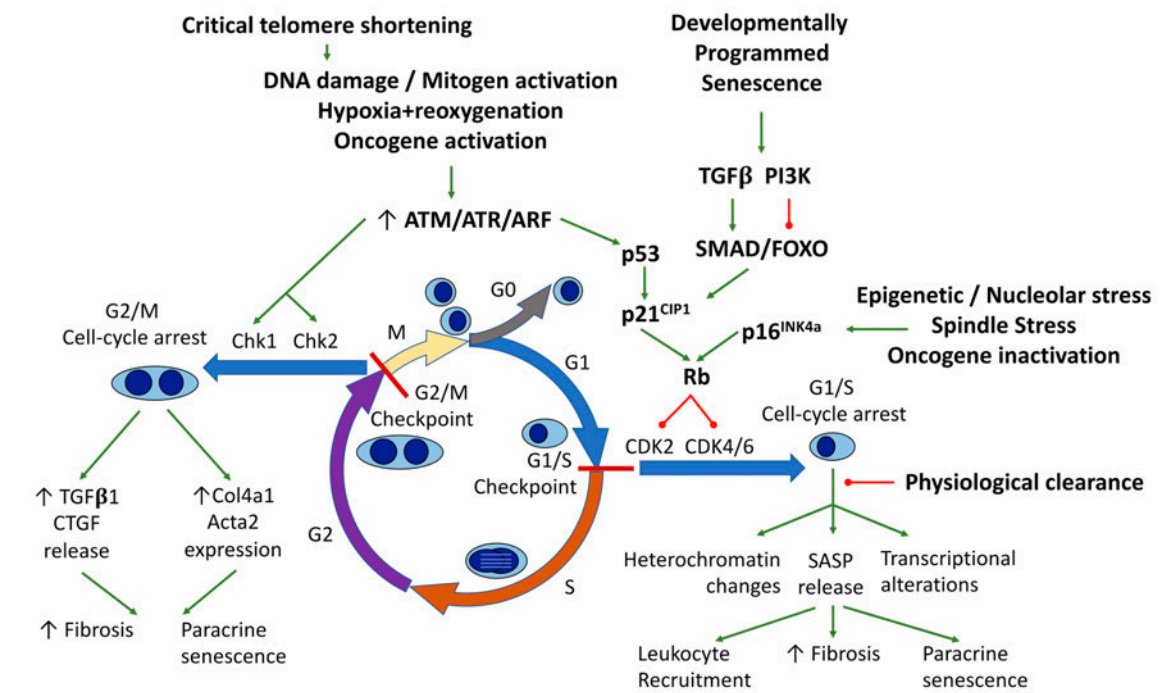


Figure 3 - Pathways involved in the induction and maintenance of senescence (Docherty, O'Sullivan et al. 2019)

Pathways to cellular senescence in eukaryotic cells. Multiple discrete cellular insults act via distinct signalling mechanisms to induce cell-cycle arrest in the kidney at either the G1/S (via inhibition of cdk2 and/or cdk4/6) or G2/M checkpoints (via Chk1/2 activation or cdc2/25c inhibition). Inactivation of oncogenes and spindle/epigenetic/nucleolar stress trigger activation of the cyclin-dependent kinase inhibitor p16^{ink4a}. Telomere shortening, DNA damage, mitogen or oncogene activation, and hypoxia/reoxygenation also result in G1/S cell-cycle arrest, via a pathway dependent on p53 and p21^{cip1} activation. In contrast to this, developmental senescence appears to induce p21^{cip1} by pathways mediated by TGFβ/PI3K and independent of p53. ATM/ATR/ARF, Ataxia–Telangiectasia Mutated/Ataxia Telangiectasia and Rad3-related protein/p14 Alternate Reading Frame (human).

Senescence is distinct from quiescence, in which proliferation can be re-instated by appropriate stimuli. Senescence is also distinct from terminal differentiation; a state of durable cell cycle arrest, in cells that have acquired specific cellular functions. In contrast to becoming terminally differentiated, in the process of becoming

senescent, cells conversely acquire a phenotype distinct to their original function within a tissue (Korotchkina, Leontieva et al. 2010).

It is not fully understood why damaged or stressed cells do not undergo apoptosis instead of senescence. The expression levels, kinetics and transcriptional activity of p53 are all proposed to play a role, with higher levels of p53 observed with apoptosis (Chen, Liu et al. 2000). P53 mediated activation of senescence or apoptosis specific genes can also direct cell fate. For example, transgenic mice with altered p53 acetylation sites who lack the ability to upregulate the pro-apoptotic proteins PUMA and NOXA retain the ability to undergo senescence via p21^{cip1} activation (Li, Kon et al. 2012). It is well recognised that senescent cells up-regulate members of the anti-apoptotic Bcl-2 family of proteins including Bcl-2 and Bcl-XL which prevent mitochondrial outer membrane permeabilization (MOMP) (Zhu, Tchkonja et al. 2015).

p21^{cip1} expression has been proposed to negatively regulate p53-dependent apoptosis (Gartel and Tyner 2002). *In vitro* studies of doxorubicin induced senescence have demonstrated an inverse relationship between p21^{cip1} expression and apoptosis (Zhang, Gao et al. 2011). In p21^{cip1} knockout mice, a lack of ability to senesce is partially compensated by apoptosis.

The Senescence Associated Secretory Phenotype

A key feature of senescent cells is the acquisition of a secretory phenotype termed the senescence associated secretory phenotype (SASP). There is significant heterogeneity in the SASP composition depending on the senescence inducer and the cell type involved and the relationship of timing to the original insult. (Hernandez-Segura, de Jong et al. 2017, Basisty, Kale et al. 2020).

Though the SASP is context dependent, it typically contains pro-inflammatory and pro-fibrotic cytokines, chemokines, growth factors and proteases. Classical SASP

proteins noted across different methods of induction and different biological contexts include IL1- β , IL-6, IL-8 and TGF β 1 (Coppe, Patil et al. 2008, Ito, Hoare et al. 2017).

Through the SASP, senescent cells can exert effects on the surrounding tissue including the induction of paracrine senescence in neighbouring cells (Ferreira-Gonzalez, Lu et al. 2018), the re-enforcement of senescence in an autocrine manner (Acosta, Banito et al. 2013), the recruitment of immune cells (Bird, Muller et al. 2018, Sturmlechner, Zhang et al. 2021), and activation of myofibroblasts (Ferreira-Gonzalez, Lu et al. 2018).

One of the most powerful actions of the SASP is the induction of paracrine senescence. Young healthy mice who received an intraperitoneal implantation of senescent cells developed systemic physical dysfunction such as reduced walking speed and grip strength. Injecting senescent pre-adipocytes, but not healthy adipocyte controls, resulted in a 50-fold increase in total body senescent cell number. Consistent with the distant spread of senescence, increased p16^{INK4A} staining, and SASP proteins such as IL-6 were observed in the quadriceps muscles, distant to the intraperitoneal injection site of transplanted senescent cells. (Xu, Pirtskhalava et al. 2018).

Emerging evidence indicates that in addition to proteins, extracellular vesicles (EVs) and microRNAs are important mediators by which senescent cells impact on their local environment. EVs are membrane bound structures and have a crucial role in maintaining cellular homeostasis by excreting damaged DNA. Inhibition of exosome secretion results in apoptosis. Senescent cells secrete higher numbers of EVs (Lehmann, Paine et al. 2008, Takahashi, Okada et al. 2018). Extracellular vesicles isolated from human fibroblasts induced to undergo senescence *in vitro* have been shown to induce paracrine senescence (Borghesan, Fafian-Labora et al. 2019).

Transfection of MiR-126 into human glomerular mesangial cells (HGMCs) *in vitro* resulted in decreased levels of senescence (Cao, Jiang et al. 2018) whereas miR 1468-3p induced human cardiac fibroblast senescence *in vitro*. (Lin, Rahtu-Korpela et al. 2020).

The Physiological Roles of Senescence

Senescence in embryonic development

Developmental senescence is a tightly regulated and evolutionarily conserved cellular process. Senescent cells have been identified in the developing embryos of multiple species including humans (Munoz-Espin, Canamero et al. 2013), mouse (Munoz-Espin, Canamero et al. 2013, Storer, Mas et al. 2013), birds (Gibaja, Aburto et al. 2019), and amphibians (Davaapil, Brockes et al. 2017).

In mouse embryos, senescent cells have been found in the apical ectodermal ridge of the developing limb, the mesonephros, the neural tube, and the endolymphatic sac of the inner ear (Munoz-Espin, Canamero et al. 2013, Storer, Mas et al. 2013). Senescent cells have also been described in the mesonephros and the endolymphatic sacs of human embryos in early (9 weeks) gestation (Munoz-Espin, Canamero et al. 2013).

In the context of embryonic development, senescence is thought to play a role in organogenesis. The importance of this role depends on the chronological stage of embryogenesis. In p21^{cip1} knockout mice that have a genetically altered capacity to induce senescence, a compensatory increase in apoptosis was observed. Appropriate formation of structures such as the mesonephric tube was delayed in mice who lacked the ability to induce senescence, although mesonephric structures ultimately appeared similar between wild-type and knockout mice at later stages of development. Conversely, despite a compensatory rise in apoptosis, p21^{cip1} knockout female mice demonstrated permanent vaginal septal abnormalities (Munoz-Espin, Canamero et al. 2013)

In the context of development, senescence appears to be dependent upon p21^{cip1} and TGFβ1 signalling. and is independent of p53, and p16^{INK41}. p21^{cip1} knockout mesonephric tubules were found to have significantly less TGFβ1 expression by microarray analysis and RT-PCR. Wild-type mice treated with a TGFβ receptor inhibitor demonstrated lower levels of SA-β-gal in the mesonephros and endolymphatic sac, in addition to lower levels of the p21^{cip1} protein (Munoz-Espin, Canamero et al. 2013).

Of note, the induction of embryonic senescence was followed by an infiltration of macrophages, which subsequently cleared senescent cells (Munoz-Espin, Canamero et al. 2013).

Senescence in tissue repair

Senescence is a vital component of appropriate tissue repair and in this context, cell lineage is an important consideration.

Depletion of senescent cells after skin injury in mice led to delayed wound closure. In wild-type mice with appropriate wound healing, senescent endothelial cells and fibroblasts were noted at the site of the wound. These cells expressed high levels of PDGF-α (platelet derived growth factor alpha) and vascular endothelial growth factor (VEGF). Other 'classical' SASP factors such as IL-6 and TGFβ1 were not observed. (Demaria, Ohtani et al. 2014)

The SASP protein cellular communication network factor 1 (CCN1) has also been shown to be important in wound healing. CCN1 deficient mice demonstrated lower numbers of senescent myofibroblasts at the sites of wounding and demonstrated an enhanced fibrotic response, demonstrating higher levels of collagen deposition which extended past the window of appropriate wound closure and granulation. In this context, senescence in myofibroblasts serves to limit inappropriate collagen deposition and fibrosis (Jun and Lau 2010).

Senescence of hepatic stellate cells has been shown to limit fibrosis in the context of liver injury (Krizhanovsky, Yon et al. 2008), again with CCN1 playing a key role in promoting induction of senescence in hepatic stellate cells (HSCs) (Kim, Chen et al. 2013). Much like developmental senescence, this appears to be a tightly controlled process. Mice lacking the Natural Killer (NK) activating receptor Nkg2d which binds to ligands expressed by senescent cells (MICA, ULBP1 and 2) demonstrated higher numbers of senescent HSCs and twice as much fibrosis as measured by picrosirius red staining compared to wild-type mice (Sagiv, Burton et al. 2016).

Development and tissue repair both demonstrate the context-dependent nature of senescence with respect to tissue outcomes, i.e., advantageous vs deleterious. Cell lineage, the timing of onset of senescence, the composition of the SASP and the subsequent immune mediated clearance of senescent cells determine how beneficial the effect of senescence is at tissue level. When one such factor is lost, e.g., NK-mediated cell clearance in the example of liver senescence, senescent cells persisted, and tissue fibrosis was more marked.

Prevention of malignancy

Oncogene activation is a recognised inducer of senescence, with senescence acting as a barrier to prevent inappropriate replication of abnormal cells. Both p16^{INK4A} and p21^{cip1} deficient mice have increased susceptibility to multiple cancers when compared to age matched wild-type mice (Krimpenfort, Quon et al. 2001, Martin-Caballero, Flores et al. 2001). In humans with renal cell carcinoma (RCC), lower expression of senescence markers was associated with advanced tumour stage, higher grade malignancy and higher likelihood of metastatic disease (Macher-Goeppinger, Bermejo et al. 2013).

It has also been demonstrated that senescent cells can promote their own clearance *via* secreted chemokines and cytokines present in the SASP; this is also known as ‘senescence surveillance’. Oncogene activation in murine hepatocytes results in CD4+

T cell immune mediated clearance of these premalignant cells whereas impaired removal of senescent hepatocytes results in the development of hepatocellular carcinoma (HCC) (Kang, Yevsa et al. 2011).

Whilst senescence provides a barrier to replication of malignant cells, it is worth noting that the SASP can also act to drive tumour growth. Senescent human fibroblasts stimulate pre-malignant and malignant epithelial cells to form tumours in mice (Krtolica, Parrinello et al. 2001). It has been demonstrated that IL-6 and IL-8 isolated from human senescent cell SASP can induce epithelial to mesenchymal transition which is strongly associated with tumour progression and tissue invasion (Ortiz-Montero, Londono-Vallejo et al. 2017). Mice with induced osteoblast senescence demonstrate a higher metastatic bone tumour burden in the context of breast cancer compared to mice with no osteoblast senescence, suggesting that senescent cells create a favourable microenvironment for tumour growth within the bone. Treatment with an anti-IL-6 antibody led to a 30-fold reduction in tumour growth (Luo, Fu et al. 2016).

Finally, whilst senescence has historically been considered as irreversible growth arrest, there is some evidence to suggest that this is not always the case. Senescent murine embryonic fibroblasts with lentiviral induced p53 suppression are capable of reverting to a replicative phenotype. (Dirac and Bernards 2003). *In vitro* studies in malignant or pre-malignant cells have demonstrated that in the absence of p53, p21^{cip1} expressing cells can escape growth arrest and resume replication (Galanos, Vougas et al. 2016). Senescent non-small cell lung cancer cells were demonstrated to re-enter a replicative cycle and the level of p21^{cip1} was important in determining a senescent vs a proliferative cell fate (Hsu, Altschuler et al. 2019). In the context of Hodgkin lymphoma, a higher percentage of senescent Reed-Sternberg cancer cells has been shown to be associated with treatment failure and higher mortality. Whilst further work is required to explain this, one suggested reason is that these cells have escaped from senescence and have re-entered the cell cycle, having already acquired chemotherapy resistance (Myrianthopoulos, Evangelou et al. 2019). In senescence

induced by injury or irradiation, we observed the persistence of senescent, growth arrested cells at a late time point post-injury, thus fitting the classical description of senescent cells being irreversibly growth arrested and pro-survival (Mylonas, O'Sullivan et al. 2021). Given that the expression level of p21^{cip1} has been reported to be a key determinant in the potential reversibility of senescence (Hsu, Altschuler et al. 2019) and that an MDM2 targeted senescence induction focusses on this particular cell cycle arrest protein, I was open to the possibility we might observe reversal of senescence. For reasons that I will describe in the following results chapters (particularly chapter 5), my own view from the data I have available to me at the time of writing this thesis is that this was not the case in my model, and a reversion to a proliferative phenotype did not occur.

Senescence in kidney disease

Senescent cells are found in numerous kidney diseases in humans as shown in Figure 4.

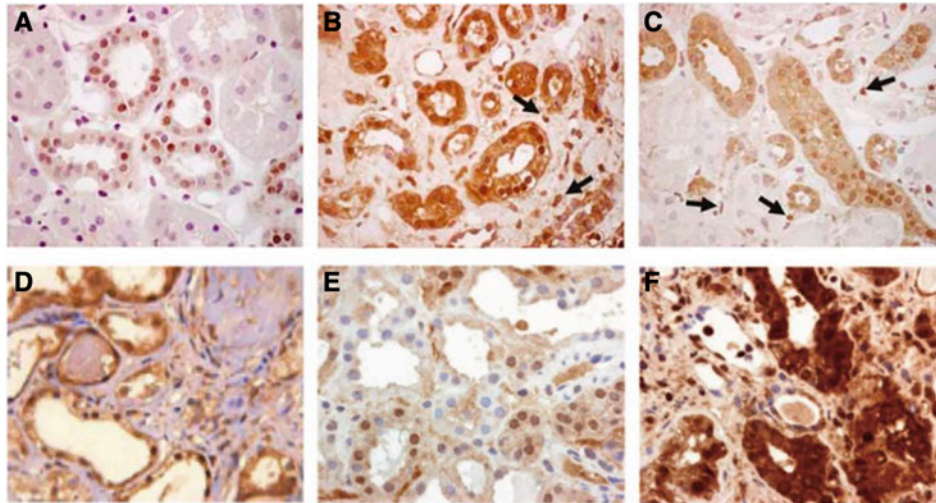


Figure 4-Senescent cells in human kidney biopsies (Docherty, O'Sullivan et al. 2019)

A) Normal kidney biopsy specimen from a 42-year-old living related donor with p16^{INK4A} staining in some tubular epithelial cell nuclei. B) Membranous nephropathy from a 64-year-old man demonstrating cytoplasmic and nuclear p16^{INK4A} in tubular epithelium and interstitial nuclei. C) FSGS biopsy specimen from a 48-year-old man, showing increased p16^{INK4A} staining in some tubules and interstitial cell nuclei (arrows). D) Grade 5 IgA nephropathy, with p16^{INK4A} staining demonstrating increased cytoplasmic and nuclear staining E) Implantation biopsy specimen from a 45-year-old donor with expected low levels of p16^{INK4A}-positive staining (F) Repeat biopsy specimen of the same kidney 7 years post-transplantation demonstrating intense nuclear and cytoplasmic staining. A-D adapted from (Sis, Tasanarong et al. 2007), E, F adapted from (Melk, Schmidt et al. 2005).

Senescent cells are found at higher number in patients with glomerular disease compared to healthy aged-matched controls (Sis, Tasanarong et al. 2007). In patients with IgA nephropathy, a common cause of kidney failure, shorter telomere lengths were found in mononuclear cells extracted from the urinary sediment of patients with the disease vs healthy aged match controls. Furthermore, shorter telomere length predicted worse kidney function and higher serum creatinine values (Szeto, Poon et al. 2005).

Increased numbers of senescent cells were seen in renal epithelial cells in biopsy samples from patients with diabetic nephropathy (DN) (Verzola, Gandolfo et al. 2008). This is supported by murine data which showed that p21^{cip1}/p27^{kip1} knockout mice that are also diabetic had reduced proteinuria, less glomerular hypertrophy, and less tubulo-interstitial damage, suggesting that senescence induction contributes to disease progression (Wolf, Schanze et al. 2005).

In the context of renal transplantation, the presence of pre-implantation senescent cells correlated with poorer graft outcomes in humans. (Ferlicot, Durrbach et al. 2003, Melk, Schmidt et al. 2005, Sis, Tasanarong et al. 2007). Post-transplant, senescent cell number in human transplant biopsies correlated significantly with worsening interstitial fibrosis and tubular atrophy (IFTA), so called 'chronic allograft nephropathy'. (Halloran, Melk et al. 1999, Ferlicot, Durrbach et al. 2003). P16^{INK4A} knock-out mice appeared to have significantly less tubular atrophy and interstitial fibrosis in response to warm ischaemia time when compared to age-matched wild-type mice. Mice that received kidney transplants from a p16^{INK4A} knockout donors had significantly better survival and appeared to have healthier, less fibrotic kidneys post transplantation (Braun, Schmidt et al. 2012). This has also been shown in the liver transplantation. p21^{KO} mice had preserved biliary architecture during cold storage (Ferreira-Gonzalez, Man et al. 2022).

One exception to the association of senescent cells and worsening of renal disease is in the setting of autosomal dominant polycystic kidney disease (ADPKD) where levels of p21^{cip1} appeared lower in human patients with ADPKD and in murine models of cystic renal disease. Furthermore, treatment with the cyclin dependent kinase inhibitor roscovitine restored p21^{cip1} levels, increases SA-β-gal expression and slowed disease progression in murine PKD model. This implies that in contrast to the disease examples outlined above, here the induction of senescence appeared beneficial in limiting cyst progression (Park, Schutzer et al. 2007, Park, Park et al. 2009).

Renal epithelial senescence

The tubular epithelium has been implicated as a key site of renal senescence in both human and murine data. In a series of transplant patients who were biopsied in the context of tubular atrophy and interstitial fibrosis, p16^{INK4A} positive cells were observed in distal tubular nuclei and in the collecting ducts of all biopsies, with some staining also seen in parietal epithelial cells, podocytes and vascular smooth muscle cells (Melk, Schmidt et al. 2005).

In humans with glomerular diseases such as focal segmental glomerular sclerosis (FSGS), minimal change disease (MCD) and membranous nephropathy, p16^{INK4A} staining was significantly higher in glomerular, tubular and interstitial cell nuclei. Despite these being diseases which classically affect the glomerulus, the most notable difference between healthy controls and diseased kidneys was observed in tubular epithelial cell as p16^{INK4A} staining which was present in 80% of cases vs 21% of healthy donor controls (Sis, Tasanarong et al. 2007).

Validating these findings in a murine experimental context, mice were subjected to unilateral ureteric obstruction (UUO) and SA- β -gal staining was observed predominantly in cells of the proximal tubule and collecting duct (Wolstein, Lee et al. 2010). In the context of ischaemia reperfusion injury (IRI), p21^{cip1} expression was noted in proximal and distal tubular epithelial cells, with no glomerular staining found (Megyesi, Andrade et al. 2001). In aged kidneys, higher numbers of senescent cells are seen in the proximal tubule (Baker, Childs et al. 2016).

Senescence and fibrosis

Clearance of senescent cells in murine models has now been demonstrated to be of significant benefit, improving organ function and increasing lifespan. This has been consistently demonstrated across organs and across a wide range of injury models. (Baker, Childs et al. 2016, Chang, Wang et al. 2016, Schafer, White et al. 2017,

Ferreira-Gonzalez, Lu et al. 2018, Xu, Pirtskhalava et al. 2018, Mylonas, O'Sullivan et al. 2021, Zhang, Pearsall et al. 2022).

As discussed, multiple pro-fibrotic molecules have been described in the SASP. It seems plausible therefore that senescent cells can exert a pro-fibrotic effect on their surrounding environment. It is worth noting that this relationship between senescence and fibrosis appears to depend at least in part on cell lineage. In the context of wound healing, fibroblast senescence appears beneficial in limiting tissue fibrosis (Jun and Lau 2010, Demaria, Ohtani et al. 2014). p16^{INK4A} knockout mice have higher levels of fibrosis after unilateral ureteric obstruction (UUO). Again, mesenchymal cell senescence seems to be required to limit inappropriate tissue fibrosis.

There are clear examples in the literature of senescent cells being associated with tissue fibrosis. Senescence has been strongly implicated in the pathogenesis of idiopathic pulmonary fibrosis (IPF) in both humans and mice (Schafer, White et al. 2017, Hernandez-Gonzalez, Faner et al. 2021). Gene mutations related to telomere maintenance and telomere attrition are well described in familial cohorts with inherited IPF (Armanios, Chen et al. 2007). p21^{cip1} and p16^{INK4A} have been described in the fibroblasts and epithelial cells in human lung tissue (Kuwano, Kunitake et al. 1996, Lomas, Watts et al. 2012). In bleomycin-induced pulmonary fibrosis in mice, senescent cell depletion, either by pharmacological or transgenic means, resulted in improved lung compliance and exercise capacity (Schafer, White et al. 2017).

Whilst hepatic stellate cell senescence (HSC) has been shown to limit fibrosis (Krizhanovsky, Yon et al. 2008) there is a clear association between hepatocyte and cholangiocyte senescence and fibrosis, reinforcing the importance of cell lineage. Hepatocyte telomere length has been shown to correlate with increasing levels of fibrosis (Wiemann, Satyanarayana et al. 2002). p21^{cip1} knockout mice had less fibrosis after treatment with carbon tetrachloride (CCL₄), a recognised murine model of fibrotic liver injury (Yosef, Pilpel et al. 2017). Mice lacking the DNA repair

endonuclease protein ERCC1-XPF demonstrated significantly higher levels of hepatic steatosis (Gregg, Gutierrez et al. 2012); a recognised precursor to cirrhosis and depletion of senescent cells reversed this (Ogrodnik, Miwa et al. 2017).

In a transgenic murine model of cholangiocyte senescence induced by the loss of *mdm2* (K19-Mdm2^{flox/flox}), senescent cholangiocytes were noted to be spatially adjacent to activated myofibroblasts and collagen deposition progressively increased following senescence induction. In this model, increased levels of fibrosis were also noted in the kidneys and intestines of induced transgenic mice, suggesting that the actions of the SASP are not limited to the organ in which primary senescence is induced. (Ferreira-Gonzalez, Lu et al. 2018)

An association between epithelial senescence and fibrosis has also been demonstrated in the kidney, where depletion of senescent cells prior to injury limited tissue fibrosis post-injury (Mylonas, O'Sullivan et al. 2021). Here, cellular senescence was induced by injury, irradiation and age. Having undergone induction of senescence, mice were treated with ABT-263. Senescent cells are known to up-regulate pro-survival factors such as *Bcl-2* and *Bcl-xL* (Zhu, Tchkonja et al. 2015). ABT-263 targets these up-regulated pathways and effectively drives senescent cells into apoptosis (Chang, Wang et al. 2016).

The findings of this work are highly relevant to our understanding of renal senescence. As shown in Figure 5, treatment with ABT-263 clearly reduced renal epithelial senescence and mice who had undergone such depletion (Figure 3B) had better post-injury outcomes with less tissue fibrosis (Figure 3C) and significantly higher kidney function as measured by cystatin C (Figure 3A). (Mylonas, O'Sullivan et al. 2021)

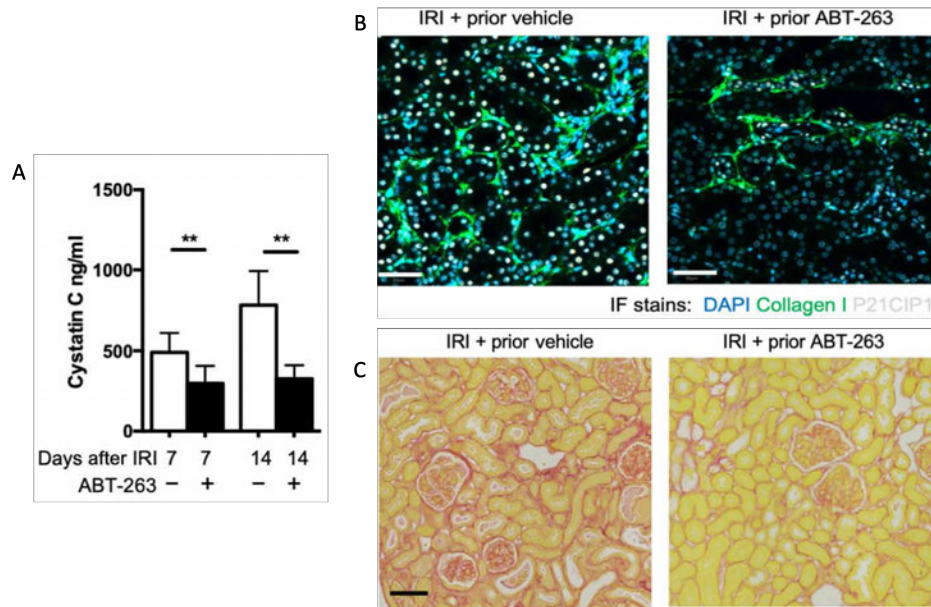


Figure 5 -Senescent cell depletion improves renal outcomes (Mylonas, O'Sullivan et al. 2021)

A) Significant improvement in renal function at day 7 and day 14 in old female mice treated with ABT-263. Mann-Whitney test. $n = 7-8$ per group. $**p < 0.01$. B) Reduction in p21^{cip1} and collagen I staining by immunofluorescence after treatment with ABT-263 prior to IRI, p21^{cip1} nuclei are shown in white and collagen I is shown in green. C) Reduction in all collagen as measured by PiSR after treatment with ABT-263 prior to IRI. Collagen appears red. (Mylonas, O'Sullivan et al. 2021)

There are two caveats to note with respect to these findings, which provide the experimental rationale for the body of work presented within this thesis.

Firstly, senescent cell depletion with ABT-263 is non-specific and will target any cell with high levels of *Bcl-2* and *Bcl-xL* expression and could thus result in the depletion of senescent cells of different cell lineages, i.e., epithelial vs mesenchymal and senescent cell types, i.e., 'acute' vs 'chronic', 'primary' vs 'secondary'. Additionally, there is published literature to suggest that ABT-263 itself is anti-fibrotic and through inhibition of *Bcl-2* can induce fibroblast apoptosis. (Lagares, Santos et al. 2017, Cooley, Javkhlan et al. 2023).

Secondly, the methods of induction of senescence used here; age, irradiation and tissue injury, are themselves injury phenotypes and will have wider tissue effects than that of induction of senescence alone.

Therefore, whilst these findings have significantly advanced our understanding of renal epithelial senescence, it is not possible to determine the exact contribution of senescent cells themselves to ongoing tissue injury, impairment of tissue repair, and generation of fibrosis. To understand if senescent epithelial cells can directly cause tissue dysfunction vs being bystanders to dysfunction requires a separation of senescence and injury.

To investigate this, a system whereby epithelial senescence can be generated in the absence of either injury or age is required and this led to the generation of a novel transgenic mouse; Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL}, discussed in Chapter 3.

Immune cell mediated senescent cell clearance

The SASP is recognised to contain chemokines and cytokines. There are clear examples of senescent cell immune mediated clearance such as those described in the prevention of malignancy where recruited CD4⁺ T cells clear senescent hepatocytes (Kang, Yevsa et al. 2011) and in tissue repair, where NK cells clear senescent fibroblasts (Krizhanovsky, Yon et al. 2008). In embryonic development, the induction of senescence is followed by increased macrophage numbers (Munoz-Espin, Canamero et al. 2013) (Sturmlechner, Zhang et al. 2021)

The mechanisms behind immune cell mediated clearance are not fully understood. However, it has recently been shown that the secretory phenotype produced specifically in response to p21^{cip1} mediated senescence contains factors such as CXCL14 and IL-34, which in turn may promote immune cell recruitment and subsequent clearance of senescent cells. Conditioned media derived from mouse embryonic fibroblasts which express p21^{cip1} was sufficient to induce macrophage migration in cell culture, whereas this effect was lost with conditioned media from irradiated p21^{cip1} depleted fibroblasts. Similarly, *in vivo*, p21^{cip1} positive hepatocytes were more likely than p21^{cip1} negative hepatocytes to attract macrophages at an early timepoint after senescence induction. The number of p21^{cip1} positive hepatocytes fell significantly at day 8 post senescence induction which coincided with high levels of M1-polarised macrophages, and CD4⁺ and CD8⁺ T lymphocytes. This immune cell recruitment effect was found to be specific to p21^{cip1} mediated cell cycle arrest and was not observed in p16^{INK4A} induced cell cycle arrest. (Sturmlechner, Zhang et al. 2021).

To induce senescence, I have used a p21^{cip1} driven approach and these p21^{cip1} immune cell recruitment effects are potentially relevant to my data.

Therapies to deplete senescent cells

I have not used any senescent cell depletion strategies in the experiments presented within this thesis. A complete review of the literature pertaining to senolytic therapies is out with the scope of this introduction, but I have included a published literature review on senotherapies which I co-authored in Appendix B. It is worth noting that numerous pharmacological approaches to deplete or to modulate senescent cell behaviour exist. These strategies have been used successfully in murine experiments (Chang, Wang et al. 2016, Ferreira-Gonzalez, Lu et al. 2018, Xu, Pirtskhalava et al. 2018, Mylonas, O'Sullivan et al. 2021) and are now being translated into human clinical trials (Hickson, Langhi Prata et al. 2019, Justice, Nambiar et al. 2019). A key question of this PhD has been to define the role that senescent cells play in the induction of tissue fibrosis. Senescent cell depletion, and any subsequent effect this has on tissue fibrosis and inflammation, may prove an insightful adjunct in future experiments.

An overview of some of the approaches to deplete or modify the behaviour of senescent cells is shown below in Figure 6.

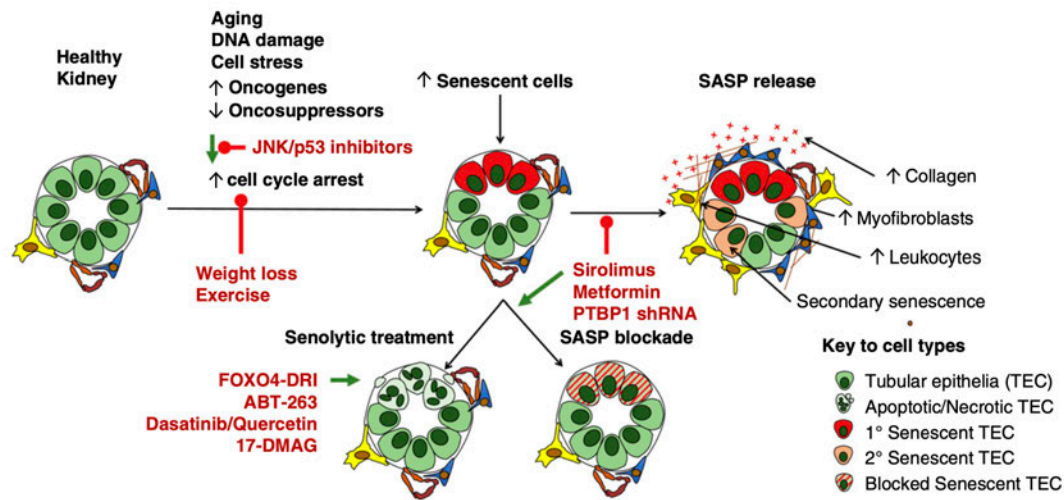


Figure 6 - Interventions to target senescent cells (Docherty, O'Sullivan et al. 2019)

Current and potential future interventions to target growth arrested cells in the kidney *in vivo*. Multiple points in the generation of senescent cells are under investigation as potential windows to alter their accumulation and/or pathogenic effects. Interventions are being trialled to prevent senescent cell formation (e.g., weight loss, exercise), promote senescent cell apoptosis (e.g., FOXO4-DRI, ABT- 263), inhibit SASP release (e.g., sirolimus, metformin), or utilize the metabolic activity of senescent cells to activate compounds (e.g., galacto-oligosaccharide–conjugated drugs). shRNA, short hairpin RNA.

Hypothesis and thesis outline

Senescent cells are a highly heterogeneous cell group with distinct context dependent roles. Their persistence and accumulation in response to cell stress, ageing and tissue injury associates with tissue dysfunction and impaired regeneration. To understand if senescent epithelial cells can directly cause tissue dysfunction vs being bystanders to dysfunction requires a separation of senescence and injury.

My key hypothesis was that senescent renal epithelia were sufficient to induce fibrosis in the absence of any other renal insult. To test this, I used a transgenic mouse which allowed conditional epithelial cell-restricted senescence.

The aims of this thesis are to:

1. Describe a novel transgenic mouse, Pax8CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} which allows the induction of epithelial senescence in absence of injury.
2. Determine if the induction of epithelial senescence alone is sufficient to initiate tissue fibrosis.
3. Characterise the changes observed at an early and late timepoint after senescence induction.

Chapter 2: Materials and methods

Mouse Work

Mouse age and rearing conditions

Mice were bred and maintained in specific pathogen free facilities at the University of Edinburgh, UK. All animal work was in accordance with the UK Government Animals (Scientific Procedures) Act 1986. Mice were housed in conventional barrier unit facilities with conventional bedding, a 12:12h light:dark cycle, ambient temperature control and access to food and water ad libitum. All animal work was approved by the University of Edinburgh Ethical Review. All experimental work was carried out under the project licence PP1773530. Mouse age was defined as being 'young' for mice aged below 6 months and 'old' for mice aged over 18 months. I defined 'age-matched' as mice being within 2 months in age of one another.

The data presented in the following chapters were derived predominantly from young mice. The main reason for this was that when I undertook the nephrectomy study in aged mice, many mice had to be culled early; primarily because of wound breakdown, though several old mice were also lost in the immediate post-operative period. Decisions pertaining to early cull were taken in close discussion with animal facility staff and the overseeing vet. Animals were observed twice daily by me for 72 hours post-operatively and were then weighed weekly for the duration of any study (maximum 6 weeks). A loss of 10% of body weight also resulted in early cull. Again, this was a problem predominantly for older mice. Whilst it would have been powerful and fascinating to repeat this study in aged mice, animal welfare was correctly prioritised. Over time, we hope to complete a meaningful dataset of old mice. For the reasons outlined above, this is likely to take several years.

Power calculations were undertaken at the time of costing for the MRC grant which funded this PhD. These calculations were performed by a university statistician. I have not included them in this thesis, since I did not perform them and I was given

the pre-calculated information which suggested I use a minimum of 6 mice per experimental group. I have used the largest number of mice available to me. Where I used less than 6 replicates per group it was either because of animal death, lack of suitable transgenic mice or lack of appropriate fixed tissue; i.e. misplaced or damaged blocks.

Mouse lines

C57BL/6J mice

C57BL/6J Mice were purchased from the Centre for Regenerative Medicine (CRM) at the University of Edinburgh.

Ai14Ho mice

All mice were bred on a C57BL/6J background. Ai14 reporter (Stock numbers 024327-C57BL/6J) were purchased from the Jackson Laboratory (MI, USA).

Pax8-CreER^{T2} mice

Pax8-cre/ER^{T2} mice (Stock number 10364 – C57BL/6J background) were purchased from the European Mutant Mouse Archive (Munich, Germany).

Mdm2 fl/fl mice

Mdm2 fl/fl mice were purchased from the Jackson Laboratory (MI, USA). Strain number 031614.

Pax8CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice

Pax8-CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice were bred by crossing Pax8-CreER^{T2} mice with Ai14Ho mice. The data presented are from mice homozygous for *mdm2* LoxP sites and either heterozygous or homozygous for cre-recombinase and tdTomato. The breeding strategy and genetic constructs are discussed further in Figure 20 (Chapter 3).

CDKN1AiTomato mice

CDKN1AiTomato mice were bred from Ai14Ho mice and a Cdkn1a-mNeonGreen mouse purchased from Cyagen. The genetic construct is discussed in Figure 28 (Chapter 3).

Tamoxifen administration

Tamoxifen (T5648-1G, Sigma Aldrich) was prepared as a stock 20mg/ml solution, diluted in corn oil. The solution was prepared 24 hours in advance of need and was shaken in the dark at 37°C, 240 revolutions per minute (RPM) for a minimum of 18 hours prior to administration. Mice received tamoxifen by oral gavage only. The dosing regimens are stated in each results chapter.

Experimental mouse models

Unilateral nephrectomy

Unilateral nephrectomy was performed by Dr David Ferenbach. Mice were anaesthetised with 2% isofluorane and underwent fur clipping before being placed under a sterile drape and the exposed skin cleaned with iodine. Body temperature was maintained throughout at 37°C using a heat mat placed under the mouse. The kidney was accessed through a posterior flank incision. The renal artery, renal vein and ureter were all identified, clamped and ligated. Haemostasis of the vessels was achieved through application of a surgical clip and the kidney was removed once haemostasis ensured. The peritoneum was closed with 5.0 sutures and the skin wound subsequently closed with surgical clips. All mice received post operative buprenorphine analgesia and 1mL 0.9% Normal Saline as fluid resuscitation.

Mice underwent unilateral nephrectomy seven days after drug administration (tamoxifen or corn oil) and were culled five weeks post-nephrectomy, six weeks post drug administration. Kidneys were either removed by unilateral nephrectomy or at cull. Mice were culled by cervical dislocation and removal of vital organs. The kidney was removed, decapsulated, and divided into sections. The two timepoints in this experiment (one week and six weeks post induction) were chosen arbitrarily. We wanted to allow sufficient time for senescence induction and potential effect hence the choice of seven days rather than a shorter timepoint of one or two days (though with all the available mice and resources that would also have been interesting).

Likewise, a later timepoint of six weeks was felt to be sufficient time for the longer-term effects of any senescence induction to be observed, whilst still being a sensible timepoint in relation to preservation experimental numbers. We suspected at the time of designing this experiment that a longer timepoint of ten or fourteen weeks would potentially have led to higher numbers of mice being culled; most especially in older mice, where we were concerned that loss of a kidney would lead to

significant longterm effects. As it transpired, we lost older mice at a much earlier timepoint due to poor wound healing; something which we had not anticipated. Younger mice were well with no health concerns in the majority by six weeks. Therefore, it would be possible to run this experiment to a later timepoint in younger mice with no additional welfare concerns.

There are recognised compensatory mechanisms which take place in the remaining (contralateral) kidney post-nephrectomy. (Kikuchi, Chou et al. 2023) I will describe how these mechanisms might have affected my results in the relevant results chapters to follow. I have controlled for these mechanism in a larger dataset of mice (not presented within this thesis due to time constraints) where I have included an experimental group of non-nephrectomised mice.

Ischaemia Reperfusion Injury (IRI)

Ischaemia reperfusion injury was performed by Dr David Ferenbach. Mice were anaesthetised with 2% isoflurane. Body temperature was maintained throughout at 37°C using a homeothermic heat mat placed under the mouse controlled by a rectal probe placed prior to surgery. The kidney was accessed via a posterior flank incision. The renal pedicle was identified and clamped for 15 minutes. After 15 minutes the clamp was removed; the peritoneum closed with a 5-0 suture and the skin closed with surgical clips.

The same compensatory mechanisms described in the contralateral kidney left after nephrectomy are also relevant in the setting of IRI. We chose to perform unilateral IRI because it was considered by our animal facility and overseeing vet to be an acceptable injury from an animal welfare point of view. It would be interesting to repeat this study using bilateral IRI (within the confines of acceptable animal welfare). However, the results generated would need to be interpreted in the context of this being a more severe injury phenotype.

The use of female mice

It is recognised that hormonally intact female mice display greater tolerance of ischaemia reperfusion injury (IRI). (Park, Kim et al. 2004) This is reported to be an oestrogen-related effect with ovariectomised and oestrogen-receptor α knockout (ER α KO) female mice being less protected.

Female mice who received exogenous oestrogen prior to injury demonstrated less severe injury than vehicle-treated female mice. Orchiectomised male mice demonstrated a trend towards reduced injury. In the context of kidney transplantation from male mice to female mice, and *vice versa*, where transplanted kidneys were subjected to ischaemia post-transplantation, recovery was determined by host sex, not donor sex. (Aufhauser, Wang et al. 2016) These results suggest that testosterone and oestrogen contribute differently in injury and that the oestrogen receptor is required for the maintenance of protection in females. (Wyatt, Coates et al. 2016)

Even if male and female mice were subjected to an equivalent level of renal injury secondary to ischaemia reperfusion (by increasing the ischaemia time in females), female mice demonstrated less cardiac dysfunction one year post injury, with significantly less diastolic dysfunction and less hypertension. (Soranno, Baker et al. 2022)

In order not to introduce any confounding gender effect, I used female mice. There were two male mice in the scRNA sequencing dataset and are explicitly referenced where any data from those mice are presented.

Tamoxifen and fibrosis

Tamoxifen is a selective oestrogen receptor modulator which is widely used as a small molecule effector for cre-recombinase inducible mouse models (Feil, Valtcheva et al.

2009). Tamoxifen is both an agonist and an antagonist of oestrogen receptor signalling (Falke, Broekhuizen et al. 2017).

Both male and female mice possess oestrogen receptors α and β (ER α , ER β) in renal tissue (Irsik, Carmines et al. 2013). Murine renal tissue is particularly oestrogen sensitive as possesses a high number of ER α receptors (Jelinsky, Harris et al. 2003).

Tamoxifen has been demonstrated to be anti-fibrotic in murine models, both in terms of renal injury such as UUO and in terms of progressive renal disease such as hypertensive nephrosclerosis (Delle, Rocha et al. 2012, Mao, Xu et al. 2014).

In male mice, high dose tamoxifen around the time of UUO resulted in significantly fewer interstitial macrophages and significantly lower renal expression of ICAM-1 and TNF. The anti-fibrotic effect of tamoxifen was purported to be via TGF β 1 and downstream Smad signalling, with lower levels of TGF β 1, Smad 3 and Smad 7 proteins observed in the kidneys of treated vs vehicle control mice (Kim, Lee et al. 2014).

This apparent anti-fibrotic effect of tamoxifen is affected by the treatment regimen used, specifically, the relationship between discontinuation of the drug and assessment of fibrosis. In the example outlined above in male mice, mice were treated for 14 days post UUO and then culled with no washout period. In a comparison of male and female mice undergoing UUO pre-treated with a lower dose of tamoxifen, male mice showed no difference in levels of fibrosis when a washout period of 14 days between tamoxifen treatment and assessment of tissue was applied. Female mice, on the other hand, still demonstrated significantly less fibrosis even 14 days after discontinuation of tamoxifen. Neither male nor female mice included within this study had any detectable tamoxifen in either blood or tissue, suggesting that the effect of tamoxifen in female mice is potentially an indirect ER α effect rather than one relating directly to tamoxifen *per se* (Falke, Broekhuizen et al. 2017).

The fibrosis data presented within this thesis are derived entirely from young female mice aged less than six months of age. Though the day seven 'early' timepoint is within the recommended washout period, the inclusion of wild-type tamoxifen-treated mice allows for a fair comparison of any directly anti-fibrotic effect attributed to the drug itself.

Tissue processing and staining

Tissue processing

For all experiments, kidneys were processed in 4% paraformaldehyde (4% PFA: paraformaldehyde, PBS, pH 7.4), methacarn (methanol: 60mL, acetic acid, glacial: 10mL, chloroform: 30mL) and 10% formalin and were switched from fixative into 70% ethanol between 18 to 24 hours. Kidneys were embedded into paraffin blocks and sliced by microtome by the SURF core histology facility at the Queen's Medical Research Institute (QMRI), University of Edinburgh.

A section of kidney was also snap frozen on dry ice or liquid nitrogen and stored at -80°C and a further section embedded in OCT (Optimal Cutting Temperature compound) and snap frozen in liquid nitrogen and stored at -80°C.

Picrosirius Red (PiSR) staining

Picrosirius red staining was performed by SURF core histology services at the QMRI. Methacarn fixed paraffin embedded slides were deparaffinised and rehydrated before treatment in haematoxylin for 8 minutes. After washing, these slides were stained in PicroSirius Red (PSR) for 1 hour before acidified water washing, dehydration and mounting.

Immunofluorescence reagents

Table 1 details the primary and secondary antibodies used for immunofluorescence staining. All secondary antibodies and all animal sera were purchased from SURF core histology facility, QMRI, University of Edinburgh.

Primary Antibody	Species	Dilution	Secondary	Block
p21 Abcam (188224)	Rabbit	1:200	Donkey anti-rabbit Alexa Fluor (AF) 555	5% Donkey serum
Collagen I Southern Biotech (1310-01)	Goat	1:100	Donkey anti-goat AF488	5 % Donkey serum
mCherry Sicgen (AB0081-500)	Goat	1:400	Donkey anti-goat AF488	5% Donkey serum
Mki67 Bio-technie (AF7649)	Sheep	1:200	Donkey anti-sheep AF488	5% Donkey serum

Table 1 -Primary and secondary antibodies used for immunofluorescence staining

All immunofluorescence images presented are from tissue fixed in 4% PFA and have undergone pressure-cooker based antigen retrieval in 10mM citrate buffer pH 6.0 (Table 2). All secondary antibodies used were donkey, and thus a species-specific donkey block (200 μ L 5% normal donkey serum + 0.3M glycine in PBS) was used throughout. All staining was performed in a slide staining tray.

Sodium Citrate Buffer	Volume/Weight	Supplier
Tri-sodium citrate (dihydrate)	2.94g	Sigma-Aldrich 6132-04-3
Distilled H ₂ O	1L	Laboratory supply
Tween 20	0.5ml	Sigma-Aldrich P1379
Hydrochloric Acid	To pH 6.0	Laboratory supply

Table 2- Sodium citrate buffer

Reagent	Cat No.	Supplier
TrueView	SP-8400	Vector
DAPI	D1306	ThermoFisher
ProLong Diamond Antifade Mountant	P36961	ThermoFisher

Table 3 - Reagents used for immunofluorescence staining

p21^{cip1} staining

Paraffin-embedded slides were immersed in xylene for two distinct 5-minute periods. Slides were then moved sequentially through ethanol concentrations (100%, 95%, 80% and 70%) for a duration of 1 minute per ethanol concentration. For the subsequent staining protocols listed below, I will refer to this process as 'deparaffinisation and rehydration'.

Antigen retrieval was achieved with 10mM sodium citrate buffer and 30 minutes in a pressure cooker. Slides were rinsed in 200 µL in 0.05% PBST (phosphate buffered saline, 0.5mL Tween 20). Tissue was blocked with donkey block. Slides were blocked for 2 hours at room temperature then washed in PBS before overnight incubation at 4°C with the primary antibody, rabbit anti-mouse p21 antibody. Slides were washed twice with PBS for 2-minute durations and incubated at room temperature with the secondary antibody for 2 hours.

Slides were washed and quenched using Vector Laboratories TrueView Autofluorescence quenching kit and then washed again in PBST for 2 minutes, repeated four times. Slides were stained with DAPI for 20 minutes before a final 2, 2-minute washes in PBST. Slides were mounted with ProLong Diamond Antifade Mountant. (Table 3)

Slides were air dried for 1 hour and stored overnight/indefinitely at 4°C in tinfoil.

Collagen I staining

Paraffin-embedded slides were deparaffinised and rehydrated. Antigen retrieval was achieved with 10mM sodium citrate buffer and 30 minutes pressure-cooker. Slides were rinsed in 200 µL in PBST. Tissue was blocked with donkey block. Slides were blocked for 2 hours at room temperature then washed in PBS before overnight incubation with primary antibody, goat anti-mouse collagen 1 antibody at 4°C. Slides

were washed twice with PBS for 2-minute durations and incubated at room temperature with the secondary antibody for 2 hours.

Slides were washed and quenched, then washed again in PBST for 2 minutes, repeated four times. Slides were then stained with DAPI for 20 minutes before a final 2, 2-minute washes in PBST before being mounted, air dried for 1 hour and stored indefinitely at 4°C.

tdTomato staining

Paraffin-embedded slides were deparaffinised and rehydrated. Antigen retrieval was achieved with 10mM sodium citrate buffer and 30 minutes pressure-cooker. Slides were rinsed in 200 µL in PBST. Tissue was blocked with donkey block. Slides were blocked for 2 hours at room temperature then washed in PBS before overnight incubation with primary antibody, goat anti-mCherry antibody at 4°C. Slides were washed twice with PBS for 2-minute durations and incubated at room temperature with the secondary antibody for 2 hours.

Slides were washed and quenched and then washed again in PBST for 2 minutes, repeated four times. Slides were stained with DAPI. for 20 minutes before a final 2, 2-minute washes in PBST. Slides were mounted, air dried for 1 hour and stored indefinitely at 4°C.

Ki67 staining

Paraffin-embedded slides were deparaffinised and rehydrated. Antigen retrieval was achieved with 10mM sodium citrate buffer and 30 minutes pressure-cooker. Slides were rinsed in 200 µL in PBST. Tissue was blocked with donkey block. Slides were blocked for 2 hours at room temperature then washed in PBS before overnight incubation at 4°C. with the primary antibody, sheep anti-mouse anti-Ki67 antibody.

Slides were washed twice with PBS for 2-minute durations and incubated at room temperature with the secondary antibody for 2 hours.

Slides were then washed and quenched, then washed again in PBST for 2 minutes, repeated four times. Slides were then stained with DAPI for 20 minutes before a final 2, 2-minute washes in PBST. Slides were mounted, air dried and stored indefinitely at 4°C.

OCT-embedding for tdTomato epifluorescence

Sections of kidney were stored in 4% PFA for 2 hours before being switched into 15% sucrose for 1 hour and 30% sucrose overnight. Kidneys were then flash frozen in an ethanol slurry and embedded in OCT (Optimal Cutting Temperature compound) before either being frozen (in tinfoil for darkness and signal preservation) or being sliced by core histology facilities.

Imaging and analysis

Images were taken either on the Axioscan Z1 slide scanner (Zeiss) or the Z1 Axio observer inverted fluorescence microscope. All large group analyses presented including PiSR staining, dual p21^{cip1}/collagen I staining, dual p21^{cip1}/Ki67 staining have been taken on the Axioscan Z1 to permit identical image acquisition settings per-experiment and to allow whole kidney images to be obtained.

Picrosirius Red imaging and analysis

Whole slide images were acquired using the bright-field function on an Axioscan Z1 slide scanner (Zeiss) and .czi images analysed in QuPath and ImageJ. Three random equally sized areas of cortex and medulla were selected and exported to ImageJ as shown in Figure 8. The percentage of picrosirius red staining per area of cortex and per area of medulla was quantified in ImageJ using the macro detailed in Figure 7. Where possible, I measured two areas per anatomical area and averaged them (10X

lens magnification). Some slides had sufficient tissue to take a single reading for the medulla.

```
//first we need to pull the name of the slide, to allow us to select it later on..
slide_name=getTitle();

//then we split it into it's 3 red/green/blue channels from the .czi file
run("Split Channels");

selectWindow(slide_name+" (red)");
close();

selectWindow(slide_name+" (green)");
close();
setThreshold(0, 100);

//these thresholding values will vary depending upon images
//to set them appropriately compare the 'thresholded' appearance with the original image
//once set within an experiment these values cannot be changed

run("Measure");

//the measurement will be a % of picosirius red of the total area contained within the exported snapshot
close();
```

Figure 7 -Picosirius red Image-J macro

Details of macro used for PiSR quantification after images exported to ImageJ

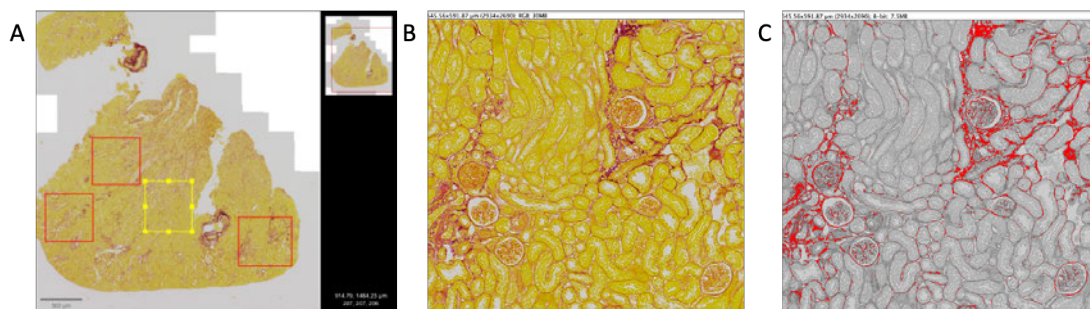


Figure 8 – Brightfield PiSR images exported to ImageJ for quantification of total collagen

A) A representative .czi file in QUPath with two randomly selected areas of cortex and outer medulla. B) A region 'snapshot' of cortex exported to ImageJ. C) Representative example of the thresholding applied to brightfield images to select the picosirius red staining (shown in red).

I excluded any obvious blood vessels or renal capsule as these stain strongly for picosirius red but are not relevant to collagen quantification when quantifying interstitial fibrosis.

Immunofluorescence imaging and analysis

p21^{cip1} analysis

Whole slide images were acquired using the immunofluorescence camera on an Axioscan Z1 slide scanner (Zeiss). The acquisition settings were identical for each experiment. Images were analysed in QUPath.

Three representative areas of cortex and outer medulla (where tissue allowed) were analysed per kidney. p21^{cip1} is predominantly a nuclear stain and I used 'Positive Cell Detection' in QUPath to identify positive nuclei. This process is outlined in Figure 9 and Figure 10.

Owing to the inherent variable nature of fluorescence, a minimum of 6 images were examined by me before the brightness and contrast values per channel and nuclear detection thresholds were set. Once set, these were not amended.

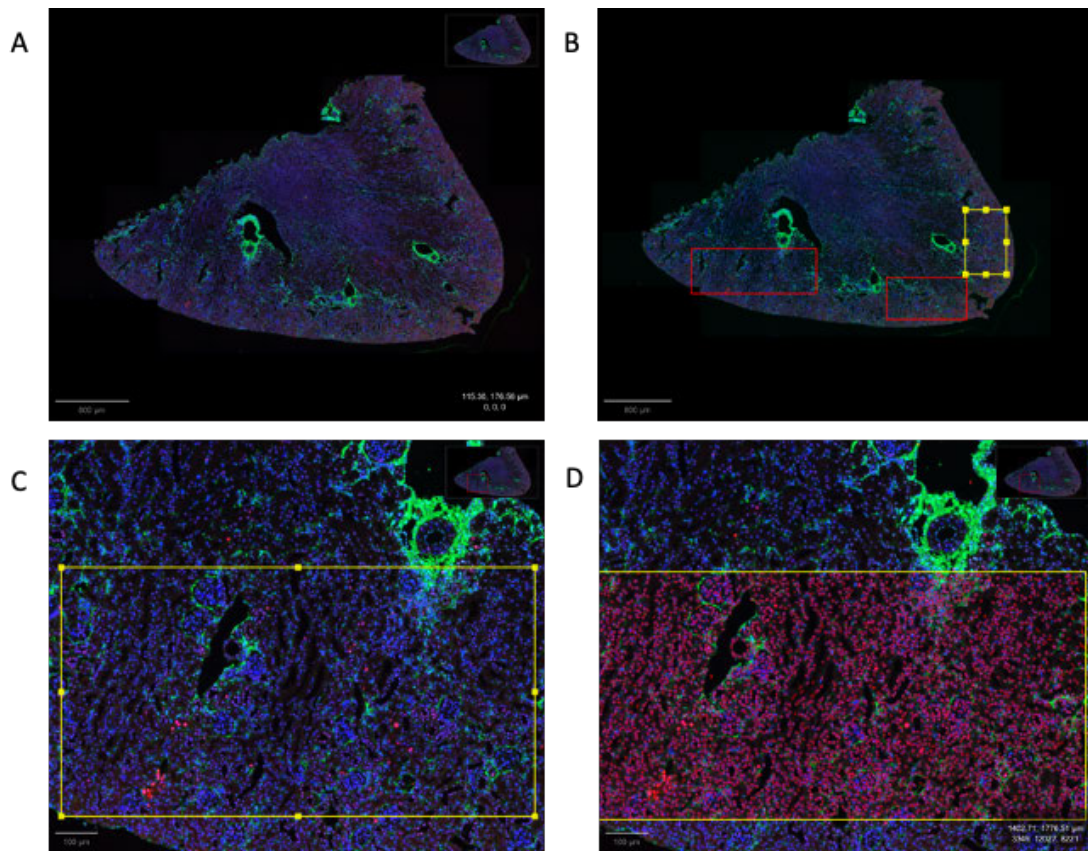


Figure 9 -Immunofluorescence staining for p21^{cip1}

A) Whole kidney section in QUPath. Fluorescence observed in three channels: DAPI, FITC, Cy3. B) Three representative areas of cortex. C) An area of cortex at higher magnification. D. Cell detection function on QUPath. Nuclei are detected in DAPI and all nuclei detected here are outlined in red giving a total nuclear count for this area of cortex.

Using 'Cell Detection', I identified all nuclei within a particular field as shown in Figure 9 and Figure 10. Nuclei were selected in the DAPI channel. The secondary antibody used to detect the p21^{cip1} antigen was conjugated to an AF555 secondary antibody. I used 'Positive Cell Detection' as shown in Figure 10 to identify nuclei which were above a particular level of intensity in Cy3 (Rhodamine) by setting a mean nuclear Rhoda threshold.

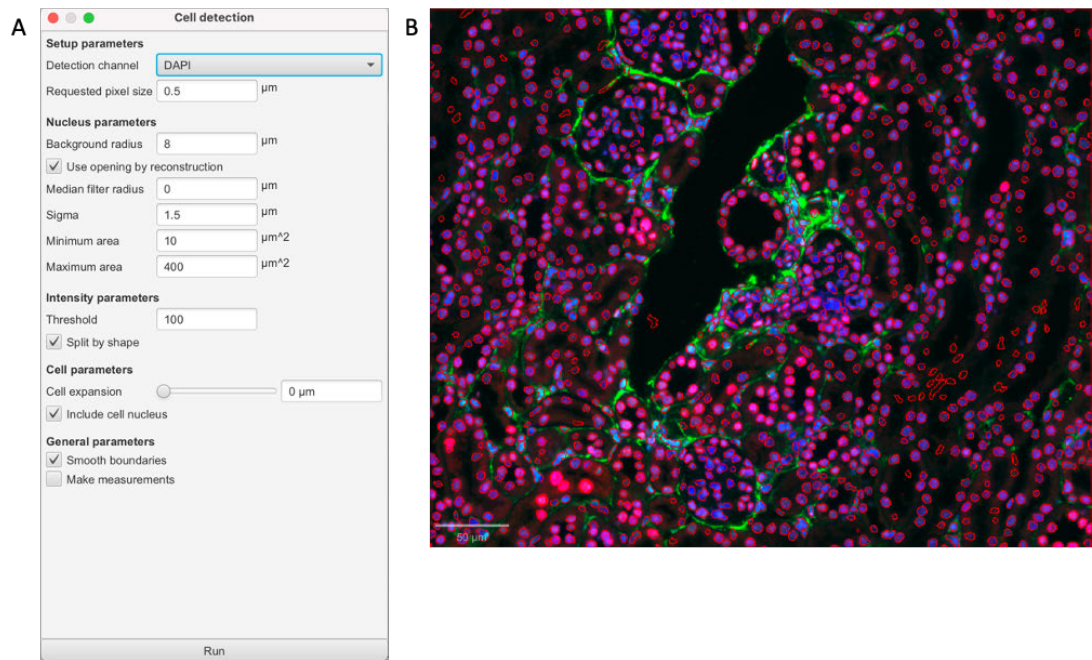


Figure 10 -Identification of nuclei in $p21^{cip1}$ staining

A) Cell detection function. As $p21^{cip1}$ is nuclear the detection channel was DAPI. B) All detected DAPI-positive nuclei are outlined in red in this image.

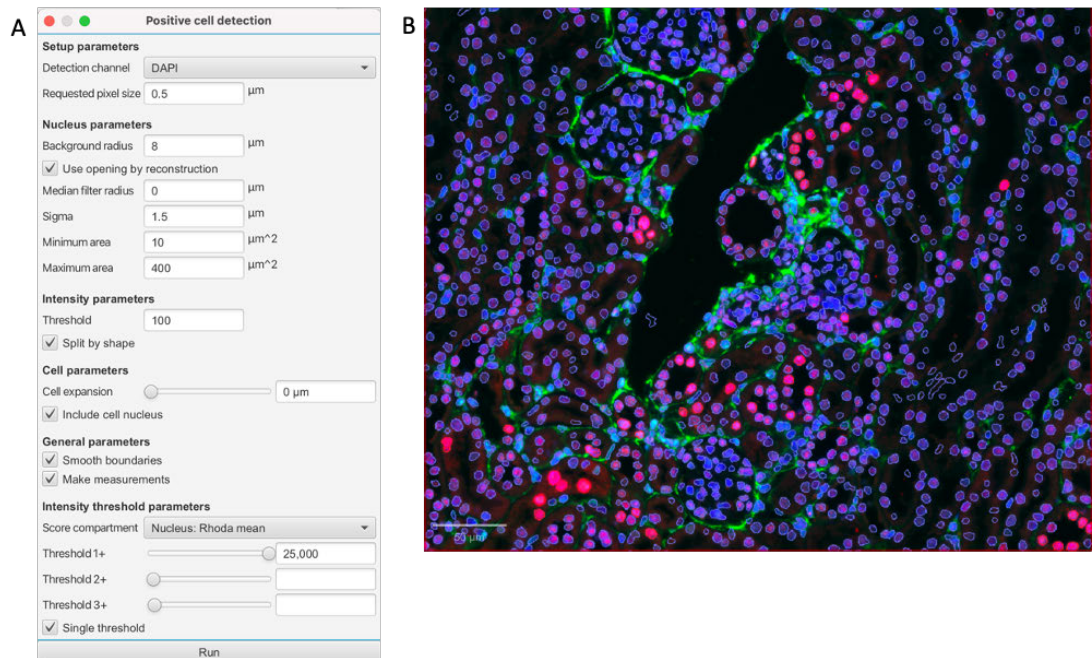


Figure 11 -Identification of positive nuclei in $p21^{cip1}$ staining

A) Positive cell detection function. B) Nuclei with a Rhodamine (Cy3) mean value < 25,000 are outlined in purple. 'Positive' nuclei with a Rhodamine (Cy3) mean value > 25,000 are outlined in red.

The 'Positive Cell Detection' function generated a measurement of positive nuclei expressed as a percentage of the total nuclei measured in the area of interest. Three areas of cortex were averaged to generate the final result.

Collagen I analysis

Whole slide images were acquired using the immunofluorescence function on an Axioscan Z1 slide scanner (Zeiss) and analysed in QUpath and ImageJ. Two areas of cortex and outer medulla were selected at random. These randomly selected snapshots were exported to ImageJ (Figure 12).

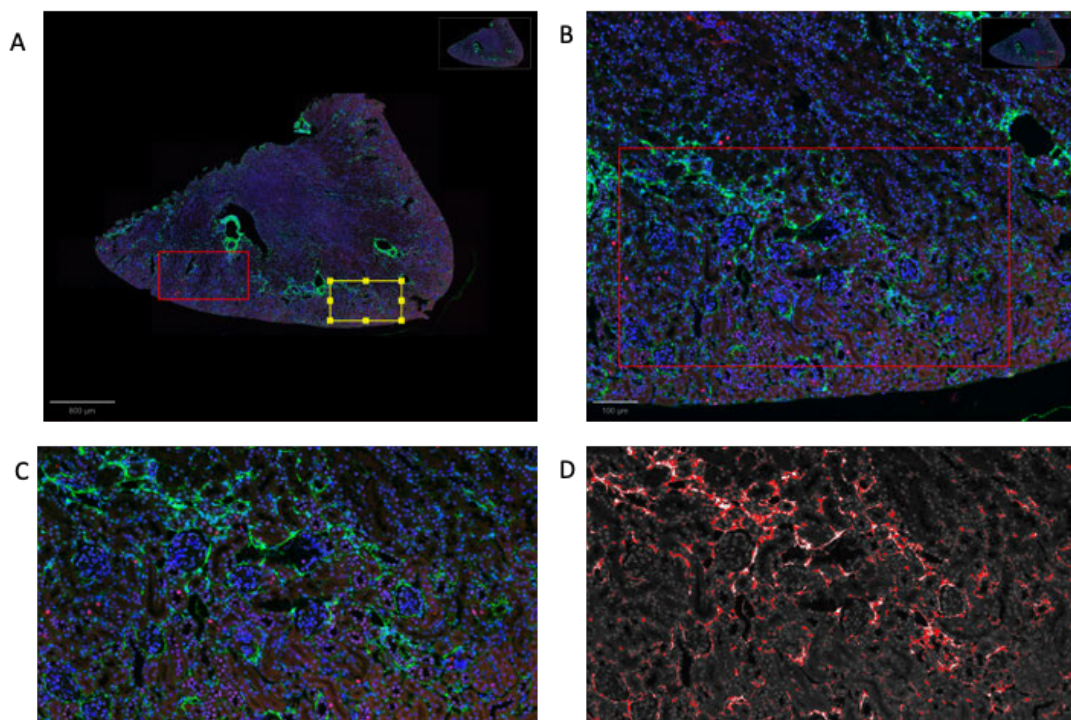


Figure 12 -Immunofluorescence staining for Collagen I

A) Whole kidney .czi in QUPath. B) Randomly selected areas of cortex. C) Region of interest exported to ImageJ. Collagen I is seen in green. D) Identification of Collagen I staining by macro where collagen I is shown in red.

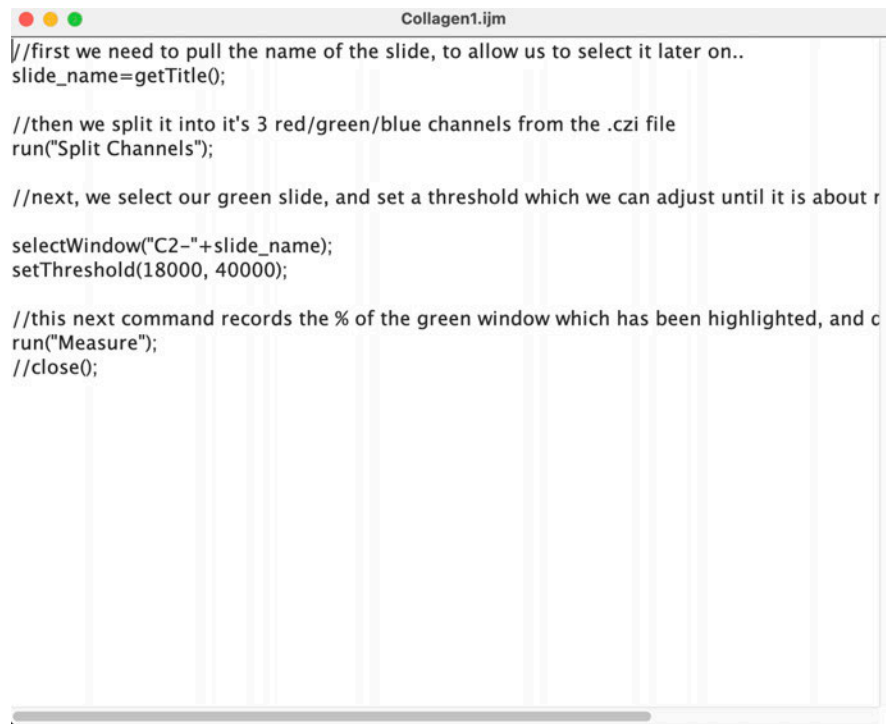


Figure 13 -Collagen I Image J macro

Macro for Collagen 1 quantification where Collagen I is detected in channel 2 and thresholding was set after examining several images. Once set, thresholding was not adjusted, and all images are analysed across identical settings.

The macro outlined in Figure 13 generated a measurement of Collagen I staining expressed as a percentage (%) of the exported area. Three areas of cortex or medulla were averaged where possible.

Ki67 analysis

Whole slide images were acquired using the immunofluorescence function on an Axioscan Z1 slide scanner (Zeiss). Images were analysed in QUPath. Three areas of cortex and medulla were selected. Both p21^{cip1} and Ki67 are nuclear stains. Cell detection function was used initially to count the number of nuclei in the area of interest. (Figure 14).

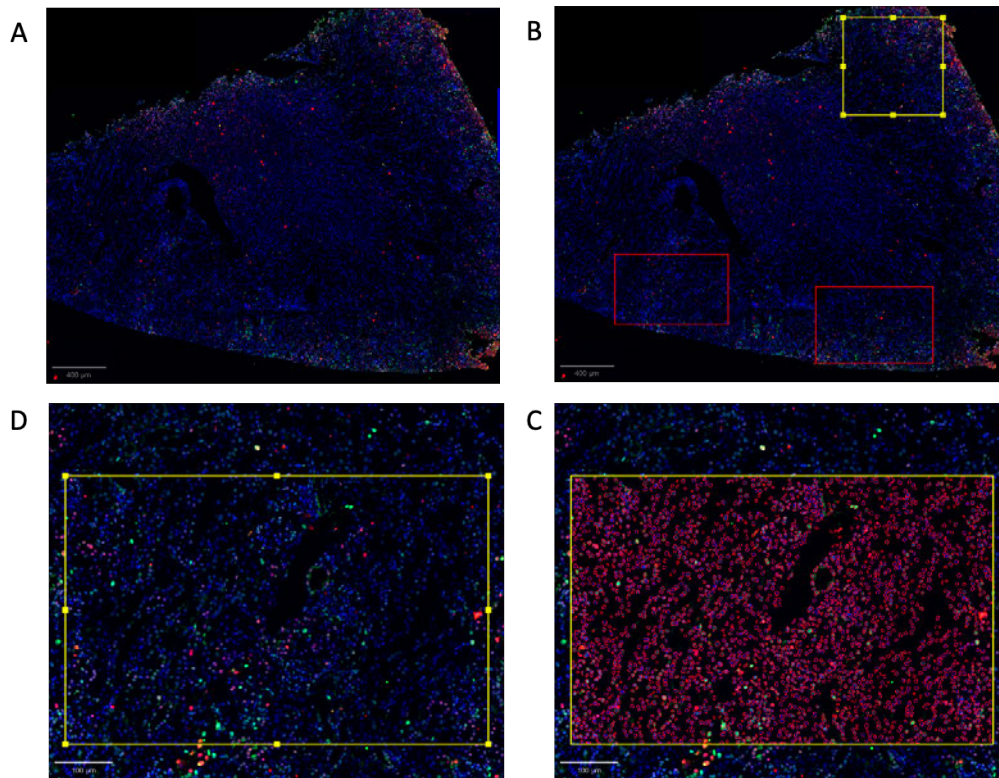


Figure 14 - Immunofluorescence and analysis for $p21^{cip1}$ and mKi67

A) Whole kidney .czi in QUPath. B) Randomly selected area of cortex. C) Randomly selected area of cortex at higher magnification. D) Identification of cell nuclei by cell detection function- nuclei outlined in red.

To analyse these nuclear stains individually, I used two separate single measurement classifiers in QUPath. A single measurement classifier was used to detect nuclei which are positive for a particular threshold intensity in a single channel. I used a single channel classifier in Cy3 for $p21^{cip1}$ as shown in Figure 15, and a separate single classifier in FITC for Ki67 as shown in Figure 16. These reflected the secondary antibodies used: AF555 and AF488 respectively and their channels of detection. In order to set a particular threshold that I felt was reliable and reflective of the image dataset in its entirety, I examined between 4 to 8 images. The images had unique tracking numbers that did not reveal the experimental group and I was blinded until analysis in GraphPad Prism. Once set, the single channel classifier was not amended, nor the pixel value settings in brightness and contrast and all images were analysed across identical settings.

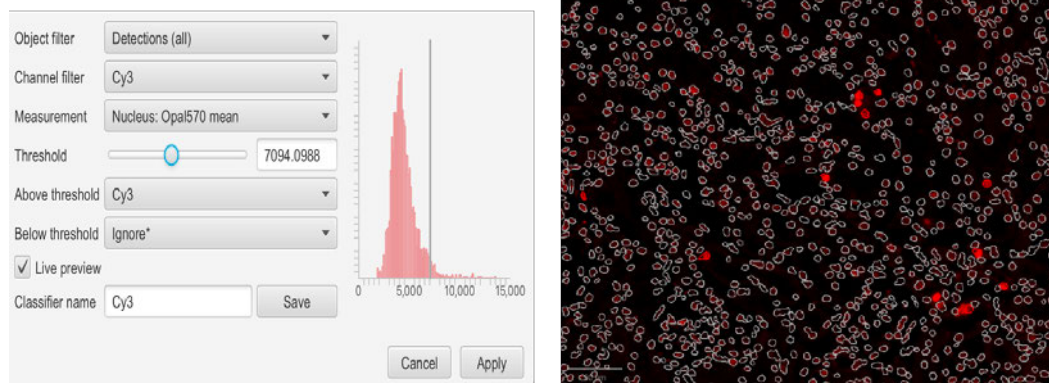


Figure 15 - Single measurement classifier for Cy3

Threshold setting in Cy3 to determine positive nuclei. The image is displayed only in Cy3 for ease of viewing. Positive nuclei appear red and are circled in red. Negative nuclei appear black and are circled in grey. These negative nuclei are termed 'Ignore'. Here, 'Opal570 mean' is used because this was the name of Cy3 on the slidescanner profile used.

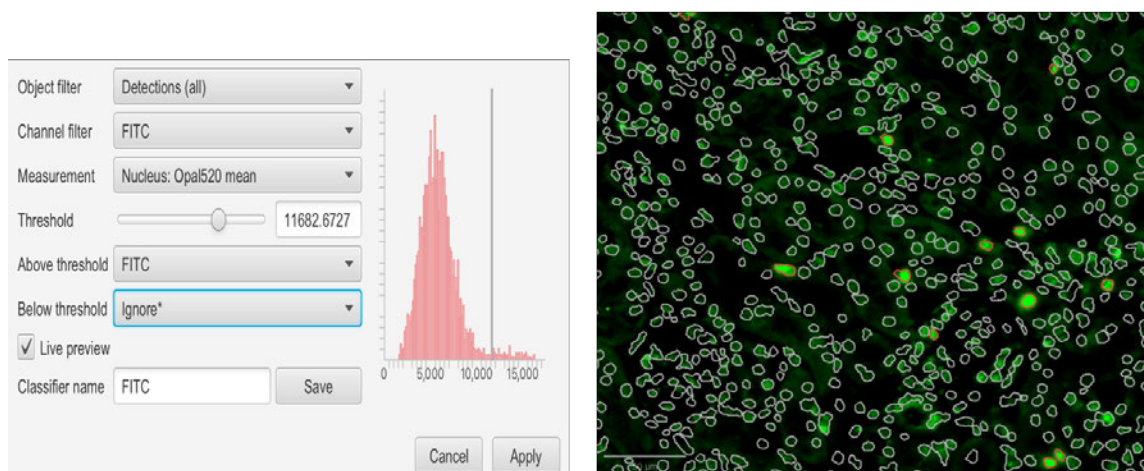


Figure 16 - Single measurement classifier for FITC

Threshold setting in FITC to determine positive nuclei. The image is displayed only in FITC. Positive nuclei appear green and are circled in red. Negative nuclei appear black and are circled in grey. These negative nuclei are termed 'Ignore'. Here, Opal520 mean used because this was the name of FITC on the slidescanner profile used.

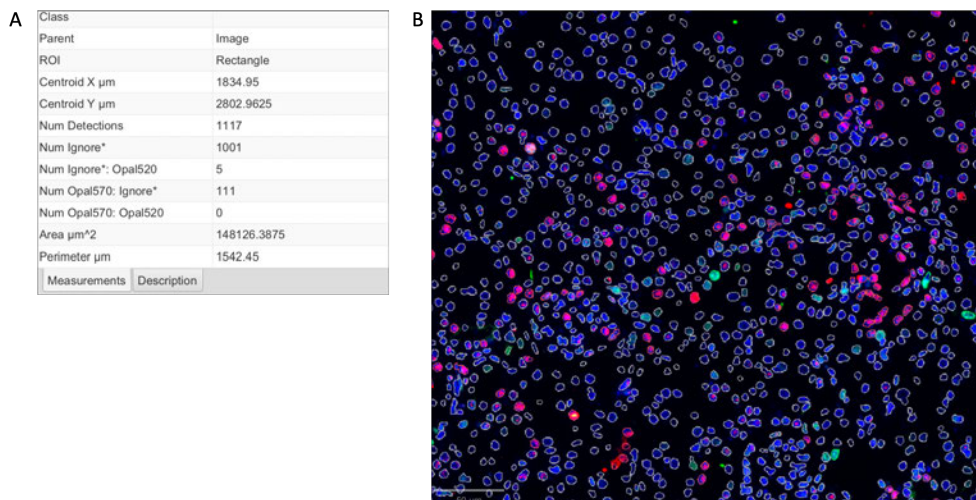


Figure 17 -Example of p21^{cip1} and Ki67 analysis

A) An example of the measurements generated by QUPath. Total number of nuclei in measured area was 1117. Number of nuclei not positive for either p21^{cip1} or Ki67 was 1001. Number positive for Ki67 was 5. Number positive for p21^{cip1} was 111. No dual positive nuclei. Therefore p21^{cip1} positivity here was expressed as $111/1117 \times 100 = 9.93\%$ and Ki67 $5/1117 \times 100 = 0.44\%$. B) The image used in this example.

An example of the measurements generated by the single channel classifier function is shown in Figure 17. These measurements included the total number of nuclei, the number of nuclei positive in both individual respective channels and the number of double positive nuclei. From these measurements, I generated a percentage of nuclei positive in Cy3 (p21^{cip1}), FITC (Ki67) and dual positive.

In-Situ Hybridisation (ISH)

BaseScope Duplex Assay

This assay was performed by following the BaseScope™ Duplex Detection Reagent Kit (ACD bio-technique). Owing to the long nature of this assay, I have outlined an abridged version below and further details can be found in the manufacturer's protocol which is available on the ACD bio-technique website (www.acdbio.com).

Probe	Cat No.
Cdkn1a	717291
tdTomato	824101
Mdm2	1128401-C2

Table 4 -Probes used for in-situ hybridisation

This assay was used to detect three mRNA transcripts: *mdm2*, *CDKN1A* and *tdTomato*. I will refer to *mdm2* in relationship to BaseScope as '*mdm2 full-length*'. Both the *Cdkn1a* and *tdTomato* probes were purchased from ACD bio-technique as a stock supply. The *mdm2* full-length probe was custom designed. These probes are shown in Table 4. In the *mdm2* fl/fl mouse, the LoxP recombination sites flank exons 5 and 6 of the native *Mdm2* gene. Therefore, if recombination occurs, this transgenic strain of mouse is still capable of producing a truncated protein which lacks the key p53 binding domain. Hence, unless specifically targeting a genetic sequence contained within those Lox-P binding sites, it would have been possible to detect a shortened *Mdm2* transcript by ISH which would have confounded any result.

Reagents	Cat No.	Supplier
RNAScope Hydrogen Peroxide	322335	ACD bio-techne
RNAScope Protease III	322337	ACD bio-techne
RNA Scope antigen retrieval buffer	328408	ACD-bio-techne
RNA Scope wash buffer	312402	ACD-bio-techne
AMP 1-12	2017277	ACD bio-techne
Ethanol absolute	Laboratory supply	VWR International
Xylene	Laboratory supply	VWR International
Vectamount	H-5000-60	2B-Scientific

Table 5 - Reagents used for in-situ hybridisation BaseScope Duplex assay

SSC Buffer 20X	Cat No.	Supplier
dH ₂ O	N/A	Laboratory supply
Sodium chloride	S9888	Sigma Aldrich
Sodium citrate	6132-04-3	Sigma Aldrich

Table 6 - Reagents for SSC Buffer

This assay was performed on tissue fixed in 10% formalin for 16-32 hours at RT and slides were sectioned maximum 14 days before staining. Slides were baked at 60°C for one hour and then deparaffinised. Slides were treated with hydrogen peroxide for 10 minutes at RT before undergoing antigen retrieval in a steamer at a minimum temperature of 99°C (Daewoo) (Figure 18). Antigen retrieval with the pre-made buffer from the RNAScope kit was performed over 20 minutes (Table 5).



Figure 18 – BaseScope antigen retrieval process

Antigen retrieval in a Daewoo steamer at 100°C measured throughout by a thermometer

After antigen retrieval slides were rehydrated in 100% alcohol and baked at 60°C for 5 minutes. The tissue was drawn around with a hydrophobic pen and was treated with RNA Scope Protease III for 40 minutes. After treatment with Protease III, the slides were incubated for 2 hours at RT with the probes of interest: *Mdm2*, *Cdkn1a* and *tdTomato* (Table 4). After incubation with probes, the slides were stored in X5 SSC (Table 6) buffer overnight at RT.

Slides then underwent a 12-step amplification process. In the process of optimisation, the following changes were made to the protocol. For amplification steps 7 and 11, the slides were incubated at RT for 60 minutes rather than 30 minutes. Slides were washed in PBS rather than dH₂O for the final wash. Slides were counterstained in Haematoxylin for 10 seconds rather than 1 minute at RT. Slides were taken immediately from Haematoxylin and rinsed in standard tap water. Slides were baked and mounted and dried overnight at RT.

Fluorescence activated cell sorting

Sample collection and processing

Kidneys were removed from mice and placed immediately into Roswell Park Memorial Institute (RPMI) media containing fetal calf serum (FCS) and penicillin/streptomycin (P/S). The reagents used to make the collection RPMI media are shown in Table 7.

RPMI (sterile)	Volume	Cat no.	Company
RPMI 1640 (1X)	500mL (100%)	31870-025	Gibco
FCS	50mL (10%)	FCS-SA	Labtech.com
Pen/Strep (10,000 U pen/10mg/mL strep)	5mL (1%)	P4333-100mL	Merck

Table 7 -RPMI

Ingredients used to make RPMI buffer prepared in sterile tissue culture hood.

FACS Buffer	Volume	Cat no.	Company
Phosphate Buffered Saline(-CaCl ₂ /MgCl ₂)	500mL	14190-094	Gibco
FCS	10mL	FCS-SA	Labtech.com
0.5M EDTA	2mL	AM9260G	Invitrogen

Table 8 -FACS Buffer

Ingredients used to make FACS buffer used in all digests except for cells being sorted for scRNASeq analysis using 10X platform prepared in sterile tissue culture hood.

Depending on the experiment in question and the cell yield required, I typically digested either $\frac{1}{2}$ to $\frac{3}{4}$ of a mouse kidney. Unless otherwise specifically stated, all centrifugation steps were carried out at 300xg at 4°C for 5 minutes and the FACS buffer used is outlined in Table 8.

Once on ice, kidneys were transported from the animal facility to the laboratory and the cell digest commenced. The cell digest mixture was always made fresh on the day of digesting and the reagents are shown in Table 9.

Digestion Solution (mg)	mg/mL RMPI	Cat no.	Company
Collagenase II	0.425	C6885	Sigma Aldrich
Collagenase D	0.625	11088882001	Roche
Dispase	1	17105-041	Gibco
DNase	0.03 (30 μ g)	10104159001	Roche

Table 9 -Reagents used for digest mix for FACS

FACS digestion mix

For digest, kidneys were placed into a weighing boat and finely cut up using a scalpel. Per ½ kidney digested, 4mL of RMPI digest mixture was used. From this point onwards in the digest, care was taken to prevent kidneys being exposed to light to preserve tdTomato fluorescence and so where possible samples were wrapped in tinfoil.

Samples were homogenised using the 'GentleMACS' tissue dissociater and were then shaken and incubated for 30 minutes at 37 °C, 150 RPM. Once shaken, the tissue was homogenised further using a second 'GentleMACS' dissociation step. Samples were then filtered through a 70 μ m mesh filter into 50ml falcon tubes. Samples were pelleted by cold centrifugation and resuspended 1ml of RBC lysis buffer per ½ kidney, incubated for 1 minute at room temperature and washed with 15ml FACS buffer. Samples were re-spun and were processed differently depending on the experiments outlined below.

Fluorescence activated cell sorting (FACS)

After red cell lysis, cells were pelleted and resuspended in FACS buffer (volume dependent on experiment and the number of FMO (fluorescence minus one) and single stain controls required). Samples were incubated in Fc block (CD16/CD32 Mononuclear Antibody (93), eBioscience: (ThermoFisher Cat: 14-0161-86) at 1:50 for 30 minutes at 4°C. Next samples were incubated for 40 minutes at room temperature in combination of antibodies from Table 6. Samples were then washed twice in FACS buffer, re-pelleted and finally resuspended in a final volume of 200µL FACS Buffer. Samples were sorted on the day of staining.

Mouse Flow Antibodies	Conjugated Fluorophore	Supplier/Cat No	Concentration
Lotus Tetragonolobus Lotus (LTL)	FITC	Vector FL-1321	1:50
Lotus Tetragonolobus Lotus (LTL)	Cy5	BioWORLD 21761117	1:50
Artocarpus integrifolia (Jacalin)	FITC	Vector FL-1151	1:50
Artocarpus integrifolia (Jacalin)	Cy5	BioWORLD 21761091	1:50
Rat anti-mouse Anti-CD31	BV421	BD Horizon 1354011	1:100
Mouse anti-mouse Anti-CD45.2	BV605	BioLegend 109841	1:100
Rat anti-mouse Anti-CD90	APC/Cy7	BioLegend 140331	1:50

Table 10 -Flow cytometry antibodies

Samples were processed using the FACS Aria III (BD) through a 100µm nozzle at 4°C. For each experiment requiring antibody staining a series of single stains and FMO controls was always used. The gating strategies are presented within the relevant results chapters. Flow cytometry data were analysed using FlowJo.

Flow cytometry and cell sorting for single cell RNA sequencing analysis

Kidneys were obtained and digested in the same method as that outlined above and digested using a standard collagenase digest mixture. After red cell lysis, cells were pelleted, washed and re-suspended in FACS buffer (Table 8) *minus* EDTA. The purpose of excluding EDTA was that it is non-compatible with the sequencing reagents used in the 10X sequencing workflow.

Cells were either sorted according to cell surface antibodies or whole kidney digest sorting for live cells only. Once resuspended, cells were processed on the FACS Aria III (BD) with the shortest possible intervening time between digest, re-suspension and sorting. Sorted cells were transported on ice to the CRM (Centre for Regenerative Medicine) where they were processed further for either 10X or Parse scRNA sequencing.

RNA Extraction – Qiagen Column method

All RNA extraction was performed using the RNeasy® Mini-Kit in accordance with the manufacturing protocol. The bench was cleaned and prepared with RNase AWAY™ Decontamination spray (ThermoFisher). RNA extraction was performed on sorted cells which had been collected into RLT Buffer/ β -mercaptoethanol and stored at -80°C. Cells were thawed and 350 μ l Buffer RLT Plus added. Cells were vortexed for 15 seconds. The homogenised mixture was transferred to a gDNA Eliminator column and an equal volume of 70% ethanol to mixture was added, followed by pipetting. The cell suspension/ethanol mixture was transferred in volumes of 700 μ L to an RNeasy spin column and centrifuged for 15 seconds at $>8000 \times g$. The flow through was discarded each time. This process was repeated until all homogenised cell suspension had been passed through the RNeasy column. 700 μ L of Buffer RW1 was added to the RNeasy MinElute spin column and centrifuged for 15 seconds at $>8000 \times g$ and the flow through discarded. 500 μ L RPE was added to the column and spun for 15 seconds at $>8000 \times g$ and the flow through discarded. 500 μ L of 80% ethanol was added to the column and centrifuged for 2 minutes at $>8000 \times g$ and the flow through discarded. The column was placed in a fresh 2ml collection tube and spun at full speed of 21,000 g for 5 minutes. The flow through was discarded. The column was placed into a final 1.5ml collection tube and RNA eluted with 14 μ L of RNase free water. RNA concentration was determined on the Nanodrop 1000 Spectrophotometer (ThermoFisher).

cDNA synthesis

Variable amounts of RNase free water were used to achieve the same concentration of RNA in each tube. cDNA synthesis was performed on ice using the (Qiagen: QuantiTect Reverse Transcription Kit). Briefly, 2µL of gDNA Wipeout was added to each PCR tube, along with 12µL of RNA. This was incubated at 42°C for 2 minutes and returned to ice. An RT master mix was made at a ratio of RT: RT Buffer: Primer Mix of 1:4:1 and 6µL of this pre-prepared mixture was added to each sample. This was incubated for 15 minutes at 42°C, followed by 3 minutes at 95°C. Samples were diluted at 1:2 or 1:3 (depending on the number of planned genes for RT-qPCR) by adding either 20µL or 40µL of RNase free water. An RNase free water alone sample was included to ensure no RNA contamination in the RNase free water.

Reverse transcription quantitative polymerase chain reaction (RT q-PCR)

A single well plate on a 384 plate (Sarstedt, Leicester, UK) contained 5µL of probemaster (Quanta Perfecta FastMix II), 2.75µL of RNase free H₂O and 0.25µL of TaqMan® gene expression assay and 2µL cDNA. The plate was sealed with clear sealing tape (Sarstedt 95.1994) and was analysed on the LightCycler ® 480 Instrument II (Roche) under the FAM hydrolysis protocol.

RT-qPCR analysis

A relative quantification approach using the $\Delta\Delta$ CT-Method was used. *Hprt* and *Ppia* were used as housekeeping reference genes. TaqMan® gene expression assays were used throughout (. mRNA expression levels for genes of interest were normalised to the housekeeper genes and presented as a fold increase compared to the control group.

Gene	TaqMan© Assay (ThermoFisher)
Cdkn1a	Mm04205640_g1
Tgfb1	Mm01178820_m1
Hprt1	Mm03024075_m1
Ccl2	Mm00441242_m1
Tnf	Mm00443258_m1
Hprt	Mm03024075_m1
Ppia	Mm02342430_g1

Table 11- TaqMan® gene expression assays

scRNA sequencing

Kidneys were harvested, decapsulated and placed immediately into RPMI on ice. Standard collagenase digest was used for all samples. All FACS was performed by the QMRI FACS facility, and all single cell preparation was performed by Dr Andrea Corsinoti, Centre For Regenerative Medicine (CRM).

Cells were sorted based on being DAPI negative (live cells) or by surface antibody expression if sorting by population of interest, on the BD FACS ARIA II and collected into FACS buffer *minus* EDTA, followed by centrifugation at 500 x *g* for 5 minutes at 4°C. Single-cell suspensions were prepared as outlined by the 10x Genomics Single Cell 3' v2 Reagent kit user guide, or by the Parse Biosciences kit user guide v1.

Single cell RNA Sequencing analysis was performed using either the 10x Genomic platform, or Parse. Sequencing was undertaken by the Edinburgh Clinical Research Facility, Western General Hospital. All single sequencing analysis was undertaken by Maximillian Reck in the Conway/Denby Group, Centre for Cardiovascular Sciences (CVS), University of Edinburgh using the methods outlined below. The decision to use both sequencing technologies was made by Professor David Ferenbach and Max Reck. This was made with the intention to move exclusively to Parse in future experiments. Therefore, part of the aim of this project was to run a pilot experiment comparing both technologies. Hence, we have data derived from both scRNA approaches in the same mouse; allowing a head to head comparison. The power of Parse technology is that it allows fixation of cells prior to library preparation and

sequencing. This allows higher numbers of biological replicates to be sequenced because the experiment can run over a longer period. It also allows reliable timepoint experiments to be run because samples from different time points can be fixed, stored, and prepared and sequenced on the same day; mitigating the batch effect observed with 10X technology where libraries are prepared immediately.

When describing my scRNA sequencing data in the results chapters to follow, I have been explicit about what technology has been used, and if the sequencing data has been combined. For example, I have termed the explanation 'Combined 10X and Parse data' or 'Parse data alone'. The combining and preparation of the sequencing data was performed solely by Maximillian Reck who has an established bioinformatics background.

10x scRNA-seq (scCv3)

10x Genomics scCv3 libraries were sequenced at a mean read depth of 320 million reads per library on an Illumina NextSeq 2000 P3 flowcell. For read alignments a custom reference genome based on GRCm38 (mm10) genome was created by insertion of tdTomato (2100bp) and CreErt2 (2886bp) sequences followed by Cell Ranger mkref. Sample demultiplexing, barcode processing and gene expression quantifications were performed using the 10x Cell Ranger (7.0.1) pipeline. For gene counting intronic reads were considered. All cell barcodes passing 10x Cell Ranger filters were initially forwarded for downstream analysis. Barcodes were further filtered excluding all cells <1000 UMIs or >25000, <500 or >4000 unique genes and cells with mitochondrial transcripts >5%. Particularly, mitochondrial transcripts were enriched in all scCv3 libraries, leading to removal of a significant proportions of cells.

Parse Evercode WT v1

Library DNA from the parse experiment was divided into 8 sub-libraries which were sequenced at a mean depth of 175 millions reads per library on an Illumina NextSeq 2000 P3 flowcell. Similarly to the 10x experiment, reads were aligned to a custom reference genome including tdTomato and CreErt2 sequences which was generated using Parse Biosciences split-pipe mkref. Reads for individual sub-libraries were further processed and quantified using split-pipe mode all and merged using split-pipe mode comb. All cell barcodes passing pipeline filters were considered for further analysis. Initial cell filtering consisted of removal of all cells <500 or >12000 Unique Molecular Identifiers (UMIs), <400 or >4000 unique genes and >5% mitochondrial content. The workflow for this is shown below in Figure 19.

QC

Filter:

```
cells_to_keep_10x <- meta[(meta$nCount_RNA > 1000 & meta$nCount_RNA < 25000 &
  meta$nFeature_RNA > 500 & meta$nFeature_RNA < 4000 &
  meta$percent.mt < 50 & meta$LibraryType=="10x"),]
cells_to_keep_parse <- meta[(meta$nCount_RNA > 500 & meta$nCount_RNA < 12000 &
  meta$nFeature_RNA > 400 & meta$nFeature_RNA < 4000 &
  meta$percent.mt < 5 & meta$LibraryType=="Parse"),]
```

Cell numbers after:

10x	Parse
14406	25393

A1_10x	A1_Parse	A2_10x	A2_Parse	A3_10x	A3_Parse	A4_10x	A4_Parse
2970	3684	2936	4861	2879	3834	2667	3565
A5_10x	A5_Parse	A6_10x	A6_Parse	BL_Parse	IRI_Parse	Pax8_Parse	WT_Parse
1498	1861	1456	2463	823	1489	1198	1615

Figure 19- Workflow for refinement of scRNA sequencing data

The workflow for refinement of the scRNA sequencing data. Performed by Max Reck. Refined for UMIs, genes and mitochondrial content. Total cell number per mouse after refinement.

Combined clustering analysis

Clustering analysis was performed by merging the filtered scCv3 and Evercode datasets using Seurat (v.4.3.0) with default settings unless mentioned. The combined data was normalised and scaled using `NormalizeData()` and `ScaleData()` and principal components were computed using `RunPCA()`. Harmony v.1 was used to integrate datasets grouping cells by dataset with lambda parameter of 0.2. The adjusted principal components were used to compute a Uniform Manifold Approximation and Projection (UMAP) using `RunUMAP()` and cluster cells using `FindNeighbors()` and `FindClusters()` with a resolution of 0.5.

This procedure was repeated for each major cluster (level 1 annotation) identified in order to define finer sub-clusters (level 2 annotation) in the data. Sub-clusters were removed if the number of cells was very low (<10), the cluster was only populated by cells from one library type indicating technical artefacts, or express marker genes of different cell lineages (e.g. *Ptprc* and *Slc34a1*) suggesting doublets.

Cluster identities were annotated based on known markers based on published single-cell datasets (Park, Shrestha et al. 2018, Conway, O'Sullivan et al. 2020, Kirita, Wu et al. 2020, Lu, Liao et al. 2021, O'Sullivan, Mylonas et al. 2022).

Data collection and statistical analysis

Data from individual experiments were recorded in Microsoft Excel for Mac. All data were analysed, and graphs generated in GraphPad Prism. This is with the exception of scRNA sequencing data which were analysed and plotted in R Studio. Details of statistical tests used are found in individual figure legends.

Details of all programs used are found below.

Microsoft® 365 Office

QU Path v0.4.2 (Open source) Bankhead, P et al. QUPath: Open source software for digital pathology image analysis. *Scientific Reports* (2017)

FlowJo v5.0 (©Becton Dickinson & Company 2006-2022)

GraphPad Prism v9.5.1 (© 1994 - 2023 GraphPad Software, LLC)

FIJI v2.9.0 (Open source)

R Studio Version 2022.12.0+353. (© 2022 Posit Software)

Chapter 3: Transgenic induction of epithelial restricted cellular senescence at an early timepoint

Introduction

The Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse is a novel transgenic strain with no published literature in the kidney. The breeding strategy used to generate this mouse is shown below in Figure 20. All strains of mice used to generate the final Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse were bred on a C57/B6 genetic background. As discussed in the previous chapter, I used exclusively female mice (with the exception of two male mice used in the scRNA sequencing dataset, and I have referenced these mice explicitly where relevant).

The choice of female mice related purely to the numbers of mice I had available to me at the time of performing my initial experiments. As discussed in the previous chapter, there are documented sex differences; both with respect to a response to injury and to tamoxifen treatment in mice (Kim, Lee et al. 2014, Falke, Broekhuizen et al. 2017) With the number of mice I had available to me at the time of performing my experiments, I was concerned that working with a mixed sex dataset would introduce potentially confounding results. I started this PhD project with a relative abundance of female mice and hence I stuck to this throughout. I have now repeated this entire experiment in young male mice (results not included due to timing). This serves as a sex control for the results presented in this thesis. Tamoxifen was chosen because I used a Pax8CreER^{T2} mouse. The reasons why I chose this promoter (*Pax8*) and this transgenic model are discussed below.

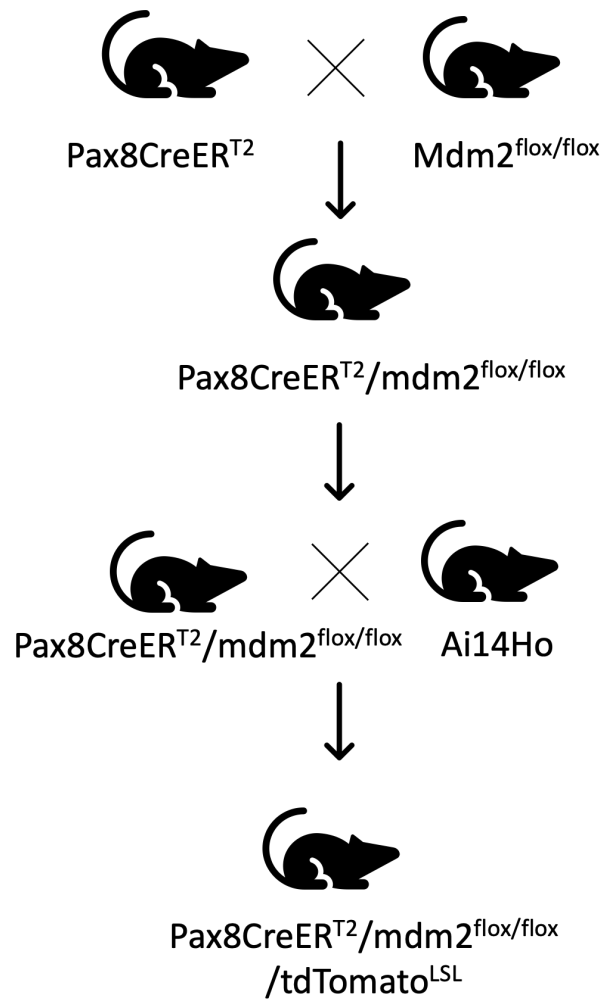


Figure 20 -Breeding strategy to generate the *Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL}* mouse

The generation of a triple transgenic mouse: *Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL}* from three antecedent mouse lines.

In order to selectively target renal epithelial cells, we used the *Pax8-CreER^{T2}* mouse which generates a conditionally activatable cre-recombinase under the control of the *Pax8* promotor. This has been reported to result in widespread cre- expression within *Pax8* expressing renal tubular epithelia (Espana-Agusti, Zou et al. 2016). *Pax8* is expression is restricted to renal epithelia with little expression seen outside of the kidney as demonstrated by Figure 21 (Tabula Muris, Overall et al. 2018).

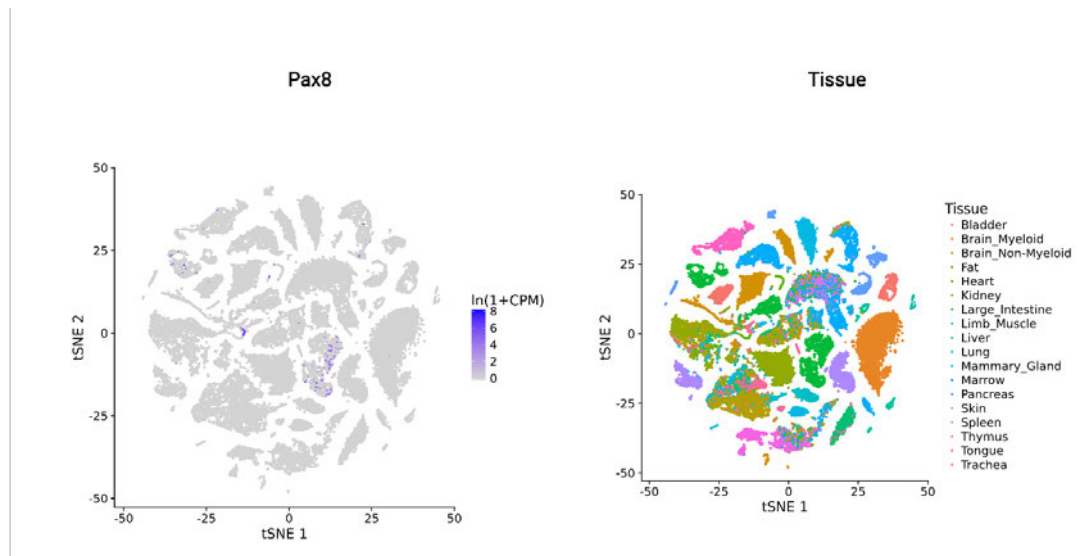


Figure 21 -Tabula muris whole body expression of Pax8 (Tabula Muris, Overall et al. 2018)

Pax8 expression in mouse tissue by scRNA sequencing. *Pax8* expression is low outside of renal tissue, with some expression seen in the gut and the thymus.

Figure 22 shows that within the kidney *Pax8* is expressed most widely in proximal tubular epithelial cells (PTECs) but is also expressed in other epithelial cells such as cells of the collecting duct (Tabula Muris, Overall et al. 2018). Studies which have looked at immunohistochemical renal staining have demonstrated *Pax8* expression in glomerular parietal epithelial cells (Ozcan, Shen et al. 2011). This expression pattern makes *Pax8* an attractive promoter to use in the targeting of a renal epithelial specific effect whilst limiting any off-target non-renal and non-epithelial effects.

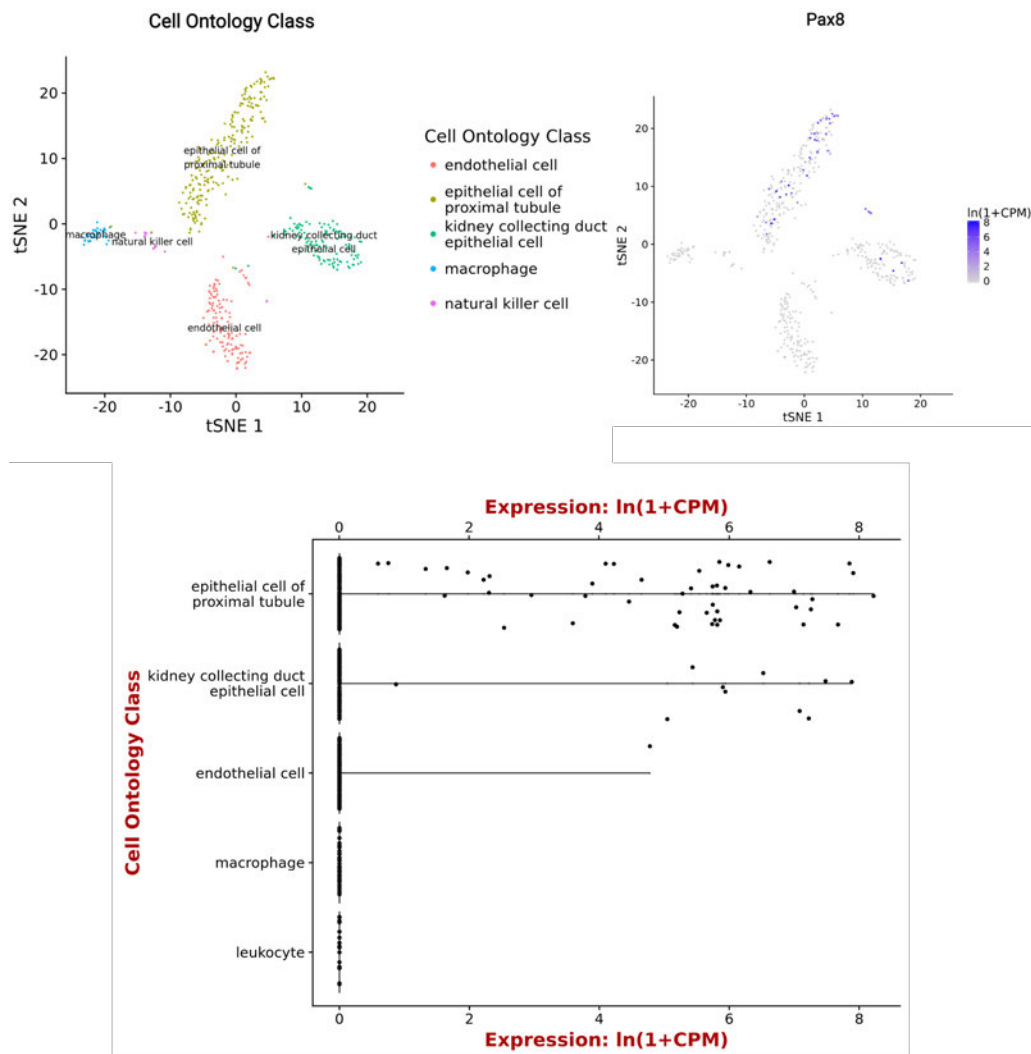


Figure 22 -Tabula Muris Pax8 expression within the kidney (Tabula Muris, Overall et al. 2018)

Pax8 expression in renal tissue by scRNA sequencing. *Pax8* is predominantly expressed in epithelial cells of the proximal renal tubule with some expression in the collecting ducts.

Pax8 is located on chromosome 2 in both humans and mice. With respect its functionality, it is nephric-cell-lineage transcription factor which is recognised as having a key role in renal organogenesis (Tong, Yu et al. 2009). In both human and murine kidneys, *Pax8* is expressed in the primitive structures which ultimately form the developed kidney; the mesonephros, metanephros and Wolffian duct (Narlis, Grote et al. 2007). Mice with deletions in *Pax8* have functionally normal kidneys and it is thought that *Pax2*, an additional transcription factor which is often co-expressed with *Pax8* has a more important role in organogenesis (Eccles, He et al. 2002).

The roles of *Pax8* in the developed/adult kidney are less well understood, though owing to its epithelial restricted expression pattern, it is felt likely to play a role in maintaining the differentiated feature of a renal epithelial cells (Narlis, Grote et al. 2007). It is also expressed widely in a variety of adult human renal epithelial tumours (Tong, Yu et al. 2009). Despite the role of *Pax8* not being fully elucidated in the developed kidney, its epithelial restricted expression pattern made it an ideal choice for our model; in which we wanted to induce senescence, specifically in renal epithelial cells. As discussed above, the use of the *Pax8* promoter to direct expression of CreER^{T2} is recognised as an effective transgenic model in published literature (Espana-Agusti, Zou et al. 2016). Our model neither does not affect the expression pattern of *Pax8* (i.e. it does not cause up or down regulation). It simply uses the *Pax8* promoter to drive the expression of cre-recombinase.

We crossed the Pax8Cre-ER^{T2} mouse with an *mdm2*fl/fl mouse: a transgenic mouse in which LoxP sites flank a portion of the *mdm2* gene as shown below in Figure 23. Figure 23 is taken from the original paper which reported this mouse (Grier, Yan et al. 2002). This shows that the LoxP sites flank exons 5 and 6 of the native *Mdm2* gene. In the presence of Cre, these exons are excised and the result is a truncated mRNA formed from exons 1-4 and 7-12 of the full 12 exon sequence.

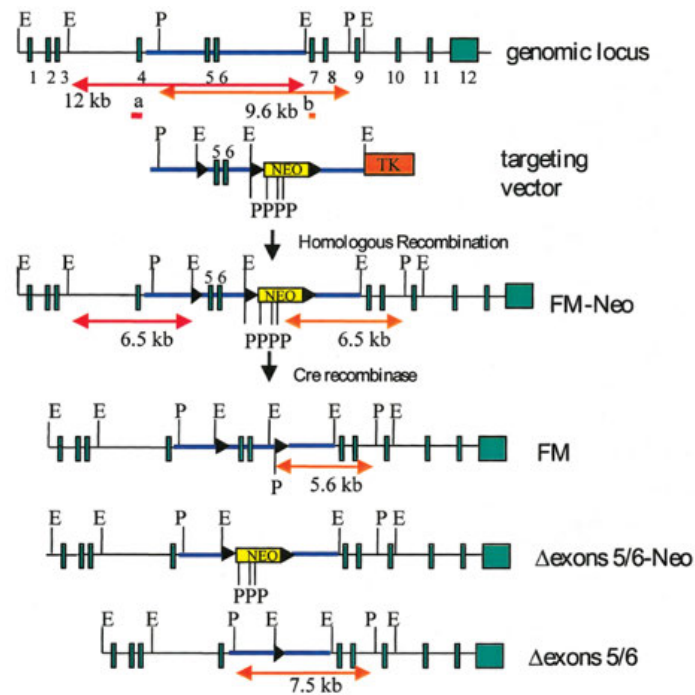


Figure 23 - LoxP binding sites in the *mdm2* fl/fl mouse flanking exons 5 and 6 (Grier, Yan et al. 2002)

LoxP sites flank exons 5 and 6 of the native *Mdm2* gene. In the presence of Cre, these exons are excised and the result is a truncated mRNA formed from exons 1-4 and 7-12 of the full 12 exon sequence.

MDM2 is a ubiquitin ligase which forms a tightly controlled negative feedback loop with p53 whereby p53 induces the expression of MDM2, which in turn targets p53 for proteosomal degradation (Moll and Petrenko 2003, Nag, Qin et al. 2013). p53 strongly positively regulates the cyclin dependent kinase inhibitor p21^{cip1} (Engeland 2022). Therefore, cells with higher levels of p53 also have increased p21^{cip1} expression, which in turn promotes cell cycle arrest (Abbas and Dutta 2009, Jung, Qian et al. 2010, Sturmlechner, Zhang et al. 2021).

Having generated a double transgenic Pax8Cre-ER^{T2}/mdm2^{flox/flox} mouse, this strain was subsequently crossed with an Ai14Ho reporter mouse. The Ai14Ho mouse contains a floxed-Stop cassette controlled tdTomato reporter construct, preceded by a strong 'CAG' (chicken, beta-actin, globin) promoter sequence as shown below in Figure 24.

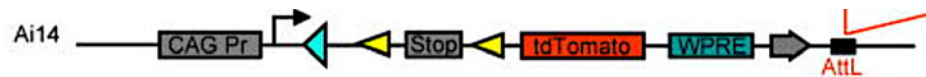


Figure 24 - Ai14 tdTomato construct (Madisen, Zwingman et al. 2010)

The Ai14Ho transgenic construct showing the floxed STOP cassette preceding the tdTomato sequence

This whole construct is situated within the Rosa26 locus. This locus is recognised to be a preferred site for the integration of transgenes and reporter constructs as it can be easily targeted for recombination. This locus is active in most cells and thus supports strong ubiquitous expression of inserted sequences and is not subject to gene silencing effects (Strathdee, Ibbotson et al. 2006, Irion, Luche et al. 2007).

My hypothesis was that in the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} model, cells expressing *Pax8* would express cre-recombinase, which would be bound to a mutant oestrogen receptor -so called 'ERT2'. This mutated oestrogen receptor would only be activated when bound to the tamoxifen metabolite 4-OHT. When active, the cre-recombinase-ERT2 complex would translocate to the nucleus and execute its function by recognising LoxP sites (Feil, Wagner et al. 1997).

As described, these LoxP sites are located in two places within this model: flanking a portion of the *mdm2* gene, and also flanking a STOP codon preceding a tdTomato reporter sequence as shown below in Figure 25.

Therefore, if recombination occurred at both LoxP sites, a portion of the *mdm2* gene would be excised with the resultant rise in p53 and p21^{cip1}, and additionally the STOP codon preceding the tdTomato reporter construct would also be excised, allowing

tdTomato transcription and resulting in cells undergoing recombination being labelled with tdTomato.

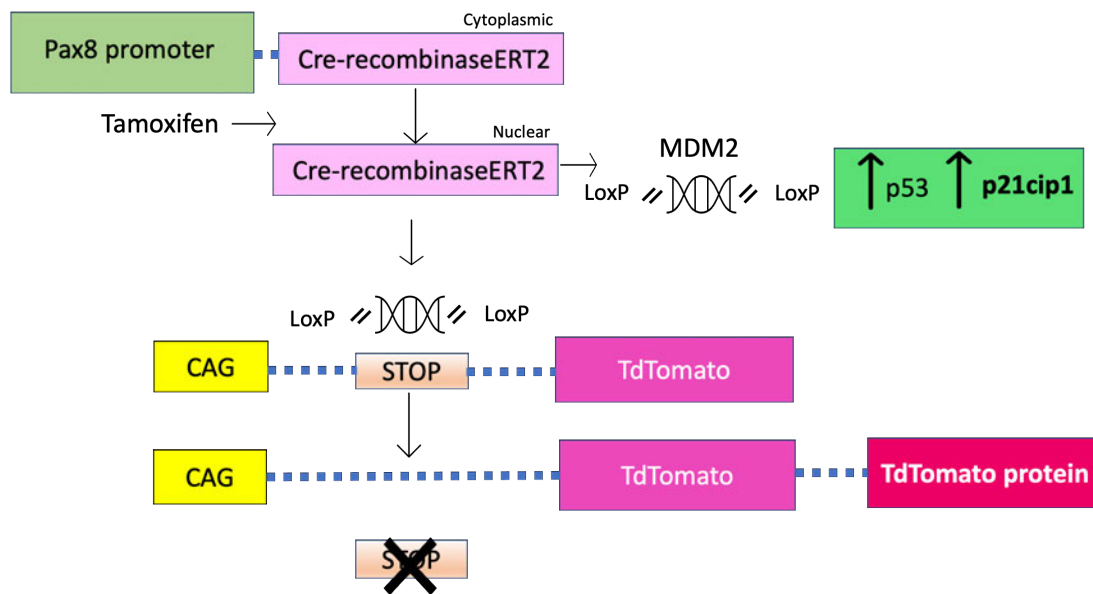


Figure 25 -Schematic of the *Pax8-CreERT^{T2}/mdm2^{flox/flox}/tdTomato^{LSL}* mouse

In cells expressing the *Pax8* promoter, cre-recombinase and a mutant oestrogen receptor complex is transcribed and the cre/oestrogen complex is held within the cell cytoplasm. Tamoxifen is administered and is metabolised to its active metabolite 4OHT by the liver. 4OHT binds to the mutant oestrogen receptor, allowing cre-recombinase/ER to translocate to the cell nucleus. In this *Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL}* mouse there are two key sites of cre-mediated recombination. There are LoxP sites flanking exons 5 and 6 of the native *mdm2* gene and there are LoxP sites flanking a STOP codon which lies between a strong CAG promoter and tdTomato reporter construct. In the presence of cre- this STOP codon is excised and the tdTomato mRNA is transcribed and subsequently translated into tdTomato protein.

This is a novel transgenic mouse. Therefore, it was not clear at the start of this project what the effect of an MDM2-targeted induction of senescence would be in the kidney. The models used in my laboratory group to date have focussed on injury and irradiation. These models described upregulation of SASP genes which are also well described in the literature; *Il6*, *Tnf*, *TGFβ1* (Mylonas, O'Sullivan et al. 2021). The closed transgenic example comes from the liver

where MDM2 targeted senescence resulted in TGF β 1 mediated paracrine senescence and upregulation of a typical SASP gene signature; including *Tgf β 1*, *Tnf*, *Nfkb*, *Il6*, and *Il6*. I did not observe a typical SASP genetic signature in my scRNA sequencing dataset. The potential reasons for this are discussed in the discussion to this chapter

Experimental design

To test if tamoxifen administration resulted in cre-mediated recombination, mice were treated with either tamoxifen or vehicle (corn oil). Mice were categorised into four groups. Wild-type mice who received corn oil or tamoxifen, and transgenic mice who received corn oil or tamoxifen. Unless otherwise stated, mice received a standard dose of tamoxifen 200mg/Kg twice by oral gavage on alternate days. Mice were female and aged less than 6 months. Mice were culled seven days after the first dose of tamoxifen.

Figure 26 shows a schematic of this experimental design.

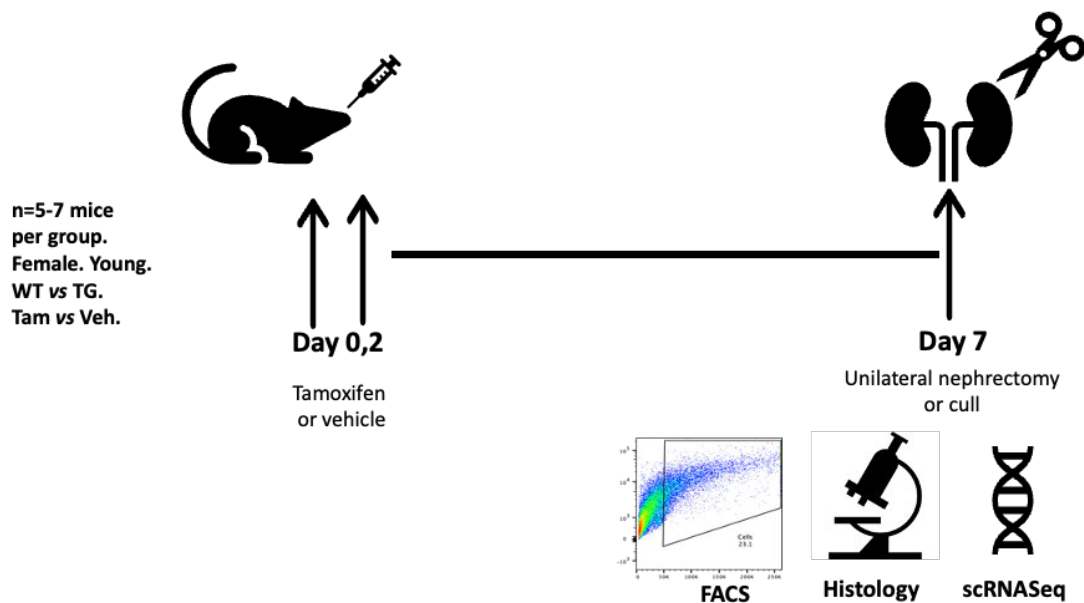


Figure 26 - Experimental design to investigate the effect of tamoxifen induction at an early timepoint

Experimental design used to define the effect of induction of epithelial senescence at early (day 7) timepoint. Young female Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} transgenic mice or control mice (littermates lacking the full genetic construct or C57BL/6J mice) were dosed with either tamoxifen or vehicle. At day 7 mice underwent unilateral nephrectomy or cull. Tissue was taken for histological analysis, for flow cytometry and cell sorting followed by downstream scRNA sequencing analysis.

To test if transgenic induction with tamoxifen resulted in tdTomato expression I used fluorescence assisted cell sorting (FACS). I wanted to test the hypothesis that tdTomato positive cells are senescent, hence the use of cell sorting in addition to flow cytometry as this offered me the possibility of RNA extraction from sorted cells and qPCR for key markers of senescence. To test if tamoxifen mediated recombination resulted in the development of cellular senescence, I used histology to look for expression of p21^{cip1} and the absence of Ki67 and qPCR and single cell RNA sequencing to look for the expression of *Cdkn1a* and other markers of senescence.

From a genetic perspective, 'wild-type' mice were either C57BL/6J mice, littermates of Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice who lacked a key element of the genetic construct or mice from the antecedent groups e.g., Ai14Ho mice.

The use of single cell RNA sequencing to define the evolution of a senescent cell over time

By inducing and marking the onset of senescence, we could investigate the evolution of a senescent cell population at a transcriptomic level over time. The role of senescent cells, and their properties are highly context dependent (Krizhanovsky, Yon et al. 2008, Jun and Lau 2010, Munoz-Espin, Canamero et al. 2013, Storer, Mas et al. 2013, van Deursen 2014, Sturmlechner, Zhang et al. 2021). Understanding the transition from an acute, potentially physiologically cleared senescent cell, to a chronic senescent cell which persists within the tissue is important and is currently unknown. It is not possible to study this trajectory without the ability to mark the onset of senescence.

As this is a novel model of senescence induction, we designed this experiment to compare any transcriptomic changes observed in induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice to that of an established injury model; ischaemia reperfusion injury (IRI). Figure 27 outlines the experimental design used to sort cells for downstream scRNA sequencing.

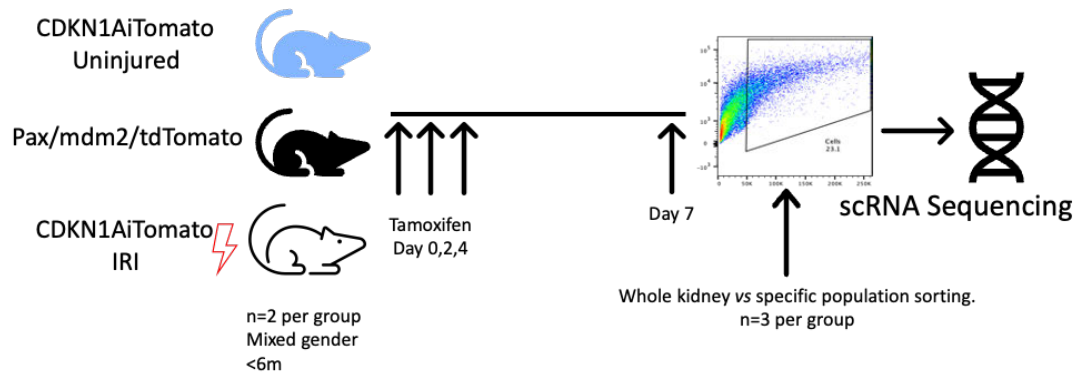


Figure 27 -Experimental design for scRNA Sequencing

The experimental schematic for sorting cells for scRNA sequencing. Tamoxifen dosing used in this experiment was 200mg/Kg x3 for Pax8/mdm2/tdTomato mice and tamoxifen 200mg/Kg x2 for CDKN1AiTomato mice. Both the uninjured and the injured CDKN1AiTomato mouse groups were dosed on day 4 and 5 after injury/sham, equating to 48-72 hours prior to cull and cell sorting. Mice were culled at day 7 post IRI/sham or in the case of the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse day 7 after the first dose of tamoxifen. Cells were sorted and underwent downstream scRNA sequencing analysis.

The CDKN1AiTomato mouse used is described below in Figure 28. The reason for its inclusion in this experiment (vs for example a C57BL6J wild type that could have undergone injury) is that it also permitted the sorting of a tdTomato positive population of cells with the hypothesis that these tdTomato expressing cells would also express *Cdkn1a* as explained below.

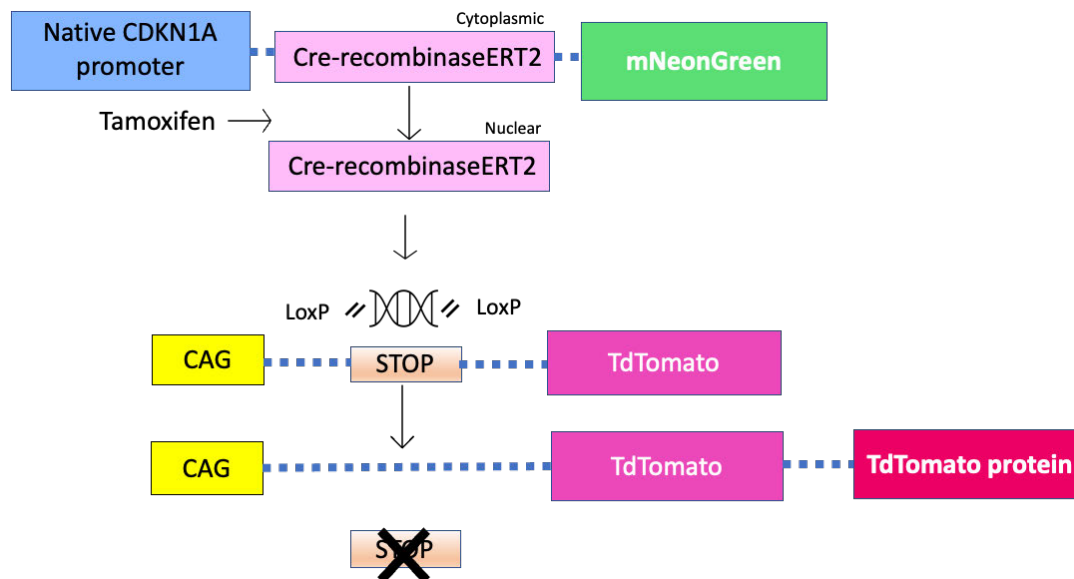


Figure 28 -CDKN1AiTomato mouse

In cells expressing the *Cdkn1a* promoter, cre-recombinase and a mutant oestrogen receptor complex is transcribed in addition to the mNeonGreen protein. Tamoxifen is administered and is metabolised to its active metabolite 4OHT by the liver. 4OHT binds to the mutant oestrogen receptor, allowing cre-recombinase/ER to dissociate and to translocate to the cell nucleus where it is active. When active, it cleaves a stop codon which lies between a strong CAG promoter and tdTomato reporter sequence which then drives the expression of tdTomato.

In this CDKN1AiTomato model, rather than using the renal epithelial promoter *Pax8*, the native promoter of the *Cdkn1a* gene is used to drive the expression of cre-recombinase. As *Cdkn1a* is the gene which encodes for the protein p21^{cip1}, we would expect cells which express sufficient and prolonged *Cdkn1a* to become senescent (Rodriguez and Meuth 2006). An important caveat of this transgenic model is that *Cdkn1a* is known to be expressed in non-epithelial cells e.g., macrophages. In these

non-epithelial contexts, its expression does not necessarily signify cellular senescence. (Muhleder, Fernandez-Chacon et al. 2021, Allouch, Voisin et al. 2022).

Given the transgenic design of this mouse there is a significant chance that these non-senescent *Cdkn1a* expressing cells would concomitantly express tdTomato. Including these cells in any downstream analysis might have confounded any result because these cells are not truly senescent. Moreover, some *Cdkn1a* expressing cells are not epithelial in origin. The primary aim of this PhD has been to define the relationship between senescent epithelial cells and their environment. To overcome this potential confounder, when selecting a population of epithelial cells at FACS for subsequent analysis by scRNA sequencing, I selected CD31 and CD45 negative cells, thus allowing me to exclude endothelial cells and cells of the immune system.

Chapter aims and hypotheses

Aims: To characterise the phenotype seen at an early timepoint after tamoxifen induction in the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse.

Hypotheses:

- 1) Tamoxifen administration would induce a senescent phenotype including expression of p21^{cip1} and *Cdkn1a* expression by day 7 in transgenic, but not control mice.
- 2) Tamoxifen administration would induce tdTomato expression by day 7 in transgenic mice but not control mice.

Results

Epithelial tdTomato expression is increased by day 7 in young female transgenic mice who have undergone tamoxifen mediated recombination

To assess the proportion of renal epithelia expressing tdTomato, I used the epithelial cell marker lotus tetragonolobus lotus (LTL). LTL is a lectin which binds avidly to the surface of proximal tubular epithelial cells (Hennigar, Schulte et al. 1985) and is used routinely across experimental modalities to identify the proximal tubule. (Forbes, Thornhill et al. 2011, Kusaba, Lalli et al. 2014).

Shown below in Figure 29 is the gating strategy for FMO and single stain controls for LTL which demonstrate a clear, easily identifiable LTL+ population; in this example detected in FITC because I have used an LTL-FITC conjugated antibody.

Figure 30 shows the gating strategy for the identification of a dual tdTomato positive and LTL positive population.

Figure 31 shows the gating strategy for the FMO control for tdTomato. Figure 32 shows this same FMO but repeated in different control mice. Figure 32 demonstrates that no inherent tdTomato fluorescence was seen without tamoxifen induction. I felt it particularly important to exclude this both in the Ai14Ho mice and in a full transgenic uninduced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse. These additional FMO controls shown in Figure 32 confirmed that tdTomato was not expressed at a protein level outside of the context of a transgenic mouse treated with tamoxifen which had undergone recombination.

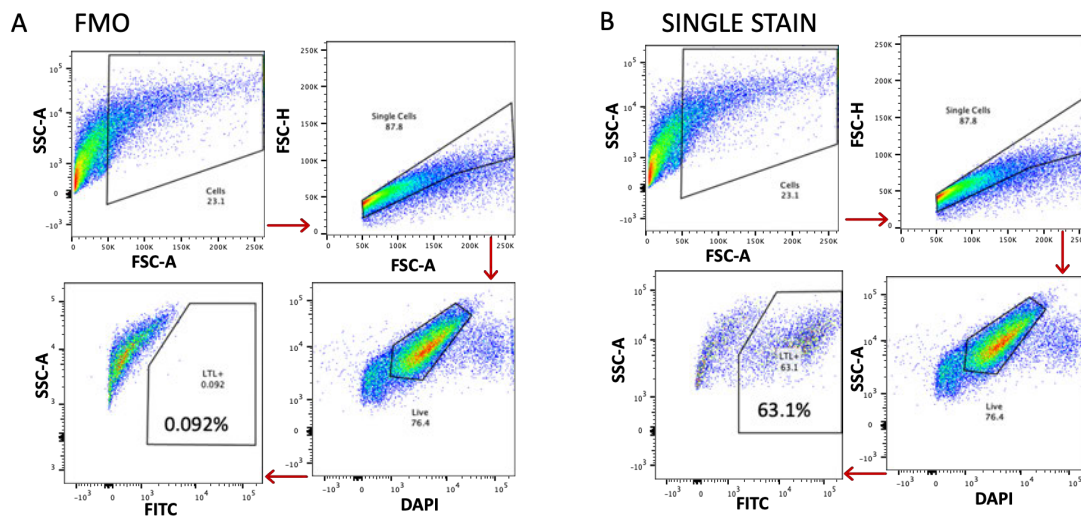


Figure 29 - FMO and single stain controls for LTL flow gating

A) Proximal epithelial cell gating strategy showing the FMO control for LTL B) Proximal epithelial cell gating strategy showing the single stain population for LTL. Young female Pax8Cre-ER^{T2}/mdm2^{fllox/fllox}/tdTomato^{LSL} mouse aged <6months treated with tamoxifen 200mg/Kg x2 7 days prior to cell sorting. **Proximal epithelial cells:** Cells/Single cells/Live cells/LTL+ cells.

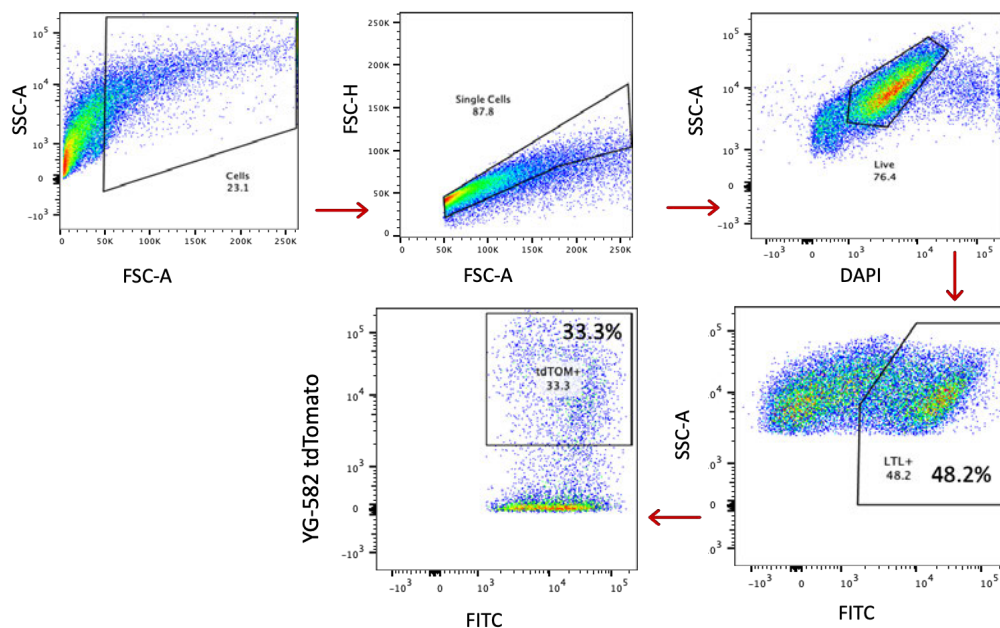


Figure 30 -Flow gating for LTL and tdTomato

Flow gating strategy for identifying tdTomato positive proximal epithelial cells. **tdTomato positive proximal tubular epithelial cells:** Cells/Single Cells/Live cells/LTL+ epithelial cells/tdTomato positive LTL+ epithelial cells. Young Pax8Cre-ER^{T2}/mdm2^{fllox/fllox}/tdTomato^{LSL} female mouse aged <6 months treated with tamoxifen 200mg/Kg x2 7 days prior to cell sorting.

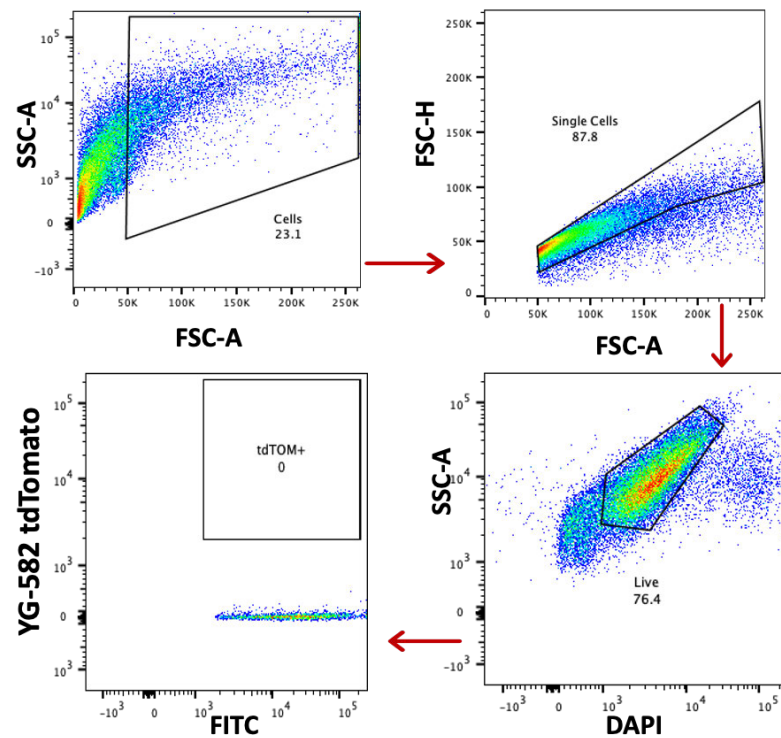


Figure 31 - FMO for tdTomato in a wild-type mouse

The FMO control for the gating strategy for the identification of tdTomato positive proximal epithelial cells. Young female C57BL/6J mouse aged <6 months treated with tamoxifen 200mg/Kg x2 7 days prior to cell sorting.

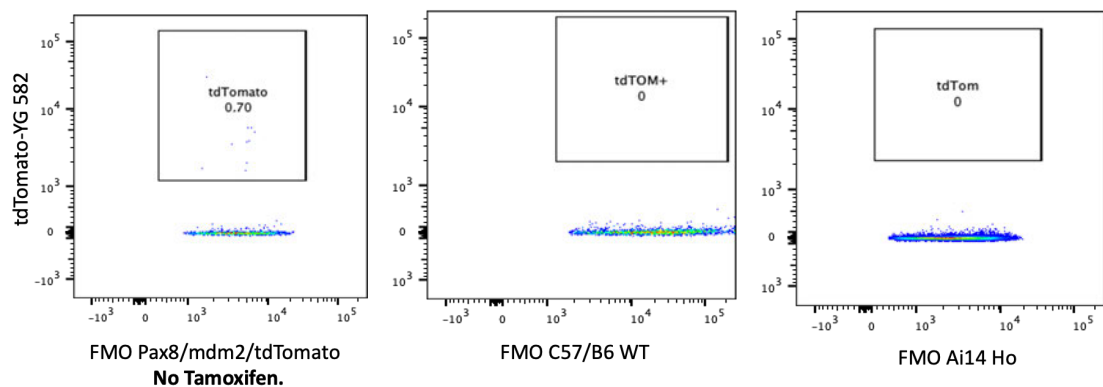
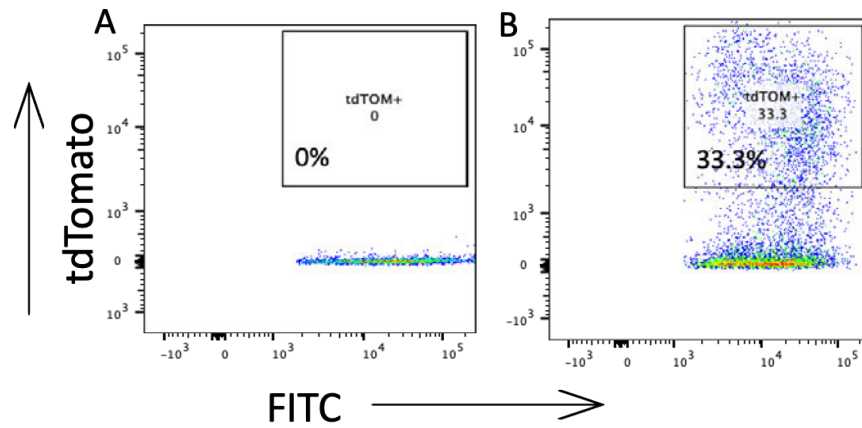


Figure 32 - FMO for tdTomato using additional controls

Gating strategy for identification of tdTomato positive proximal epithelial cells, using different control mice as FMO controls. Mice as labelled; an uninduced full transgenic mouse which had not received tamoxifen prior to cull, a wild-type C57BL/6J mouse and an Ai14Ho mouse, both of which had received tamoxifen at standard dose of 200mg/Kg x2.

Figure 33 shows the levels of tdTomato fluorescence detected at FACS between groups and shows an easily identifiable tdTomato positive epithelial cell group seen

in an induced transgenic mouse and not seen in a wild-type control mouse. As described above, the epithelial marker used to define my population is LTL and as LTL binds with significant predominance to proximal tubular cells, these data show tdTomato positivity specifically in a population of proximal tubular epithelial cells (PTECs).



C Induction of tdTomato in *Proximal* Tubular Epithelial Cells in mice treated with Tamoxifen 200mg/Kg x2

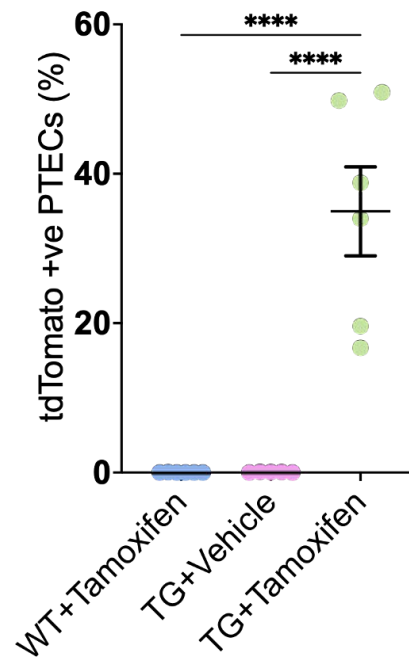


Figure 33 - Induction of tdTomato in PTECs

A) tdTomato fluorescence by FACS analysis in a wild-type mouse treated with tamoxifen 200mg/Kg x2. B) tdTomato fluorescence by FACS analysis in Pax8Cre-ERT²/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with tamoxifen 200mg/Kg x2. C) Quantification reveals a significant difference in tdTomato fluorescence in PTECs in transgenic (TG) mice treated with tamoxifen vs wild-type (WT) mice treated with tamoxifen and TG mice treated with vehicle control. n=5-6 per group, female mice aged <6 months treated with tamoxifen 200mg/Kg x2. One-way ANOVA with Tukey's multiple comparisons test. All error bars are mean $\pm$ SEM, ****P<0.0001.

Pax8, though expressed most highly in the proximal tubule, is also expressed in distal and parietal epithelial cells (Traykova-Brauch, Schonig et al. 2008, Ozcan, Shen et al. 2011). Given that LTL binds with high specificity to proximal tubular cells, it was possible with this early gating strategy which focussed solely on the proximal tubule that I was missing a population of *Pax8* expressing epithelial cells outside of the proximal tubule.

When gating on LTL alone, I noted a small, but consistent population of cells which were negative for LTL but positive for tdTomato ($3.9 \pm 0.9\%$ of all live cells). To investigate this population further, I added in a second epithelial marker; jacalin. Jacalin is also a lectin, known to bind to the luminal border of distal tubular epithelial cells and cells of the collecting duct. (Engel, Breborowicz et al. 1997).

My hypothesis was that if this small, but potentially relevant population of cells was epithelial in origin but not captured by LTL, then the addition of a second epithelial marker with binding capacity outside of the proximal tubule would result in a reduction of this population.

Were this population to have remained at the same level, it would have raised the possibility of a non-epithelial tdTomato positive population. This would have been unusual and unexpected given that *Pax8* expression is confined to the epithelial cell compartment. However, it would have merited further investigation.

With the addition of jacalin, this 'LTL negative tdTomato positive' population significantly and consistently reduced in size (3.9 ± 0.9 vs 0.9 ± 0.15 % of all live cells).

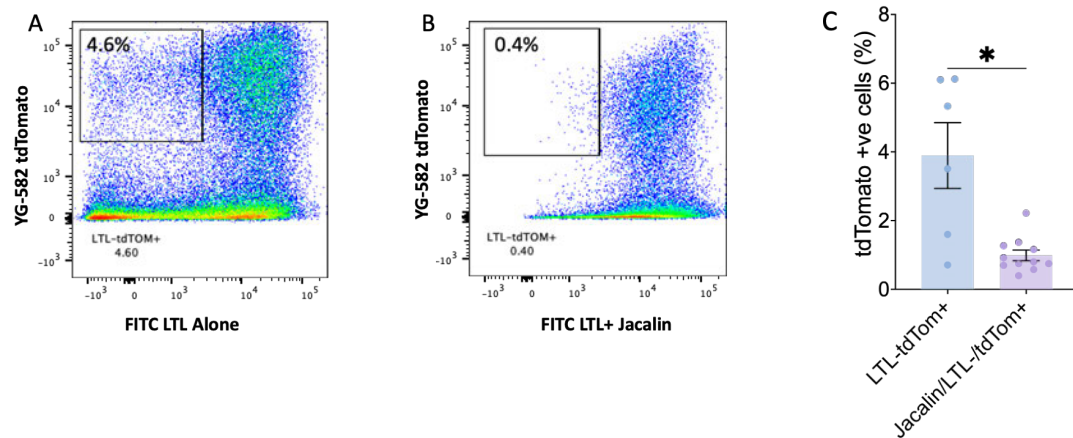


Figure 34 – A second epithelial cell marker: jacalin

A) An induced transgenic mouse with a population of LTL-/tdTomato+ cells (4.6%) when gated on all live cells in an experiment where only LTL was used to select any epithelial population B) Visible reduction in this LTL-/tdTomato+ population (0.4%) with the addition of a second epithelial marker: jacalin, in addition to LTL C) Comparison of tdTomato+ cells when comparing gating with one (LTL) vs two (LTL+jacalin) epithelial markers where tdTomato positivity is expressed as a percentage of all live cells. n=6-11 mice. Female <6 months of age. Mann-Whitney. Error bars are mean $\pm$ SEM *p<0.05.

Having established that this model allowed me to reliably detect tdTomato positive cells after tamoxifen administration as shown in Figure 33, I optimised a protocol for sorting tdTomato positive cells for downstream scRNASeq analysis. This involved refining the gating strategy through the addition of CD31 and CD45 cell surface antibodies, thus allowing me to select CD31 and CD45 negative cells. I defined my epithelial cell population as being live cells which were negative for both CD31 and CD45 cell surface antibodies, and positive for jacalin and/or LTL lectins and the gating strategies are shown in Figure 35 and Figure 36. The definition of the tdTomato population is demonstrated in Figure 37 and Figure 38.

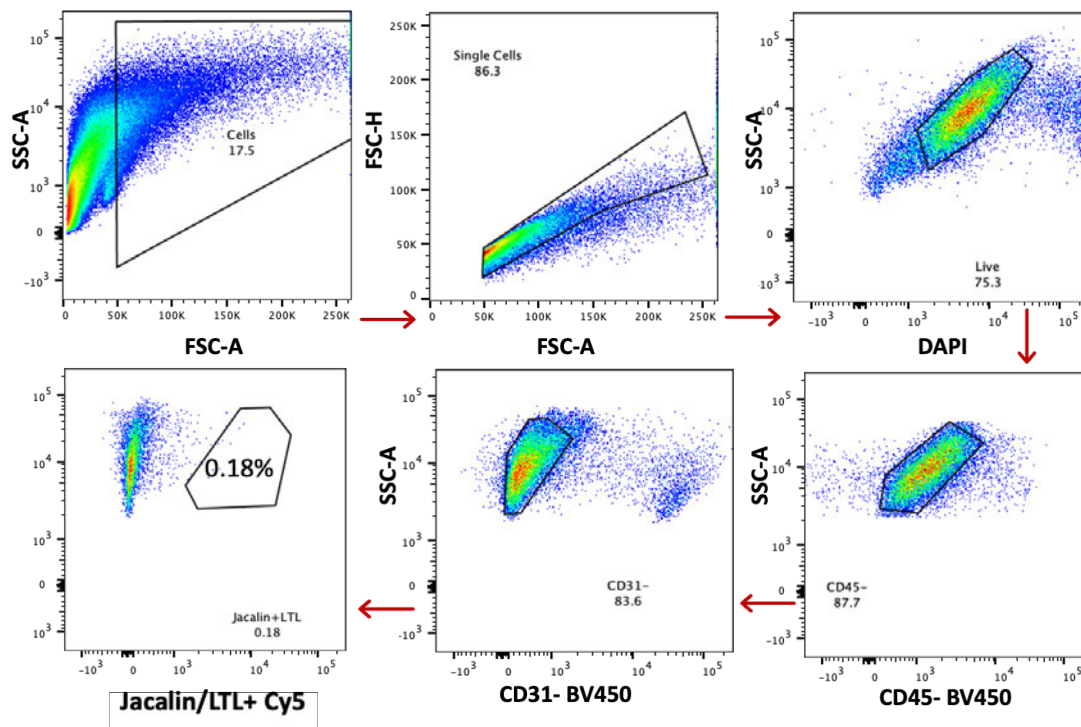


Figure 35 - Jacalin and LTL FMO

Flow gating FMO control for the identification of Jacalin/LTL+ cells. Young Pax8Cre-ER²/mdm2^{flox/flox}/tdTomato^{LSL} female mouse <6 months treated with tamoxifen 200mg/Kg 3 7 days prior to cell sorting.

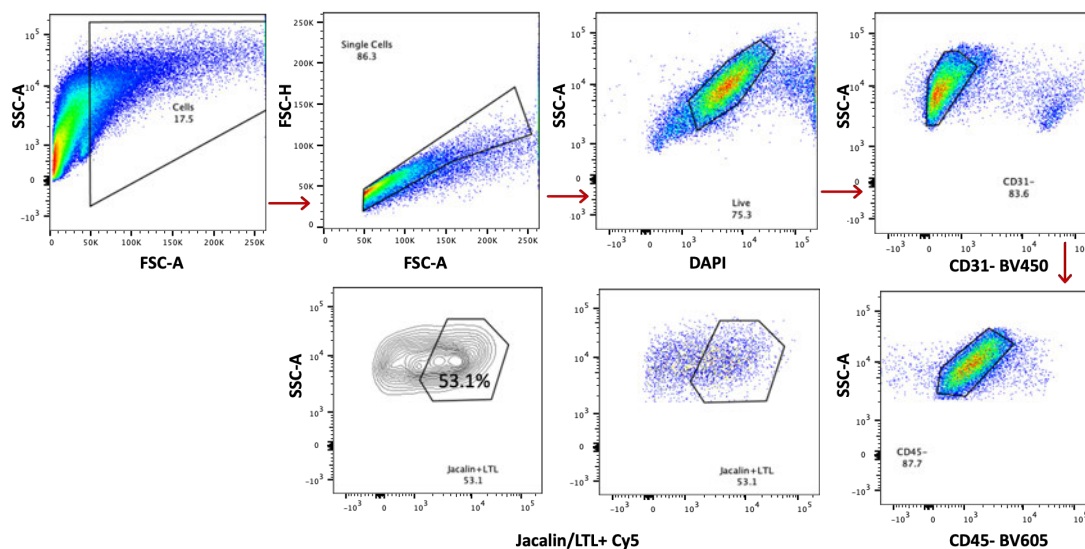


Figure 36 -Single stain Jacalin and LTL

Flow gating strategy for the identification of a Jacalin/LTL+ epithelial cell population. Young female C57/B6 mouse aged <6 months treated with tamoxifen 200mg/Kg x3 7 days prior to cell sorting. **JacalinLTL+ epithelial cells:** Cells/Single cells/Live cells/CD31 negative cells/CD45 negative cells/JacalinLTL+ cells.

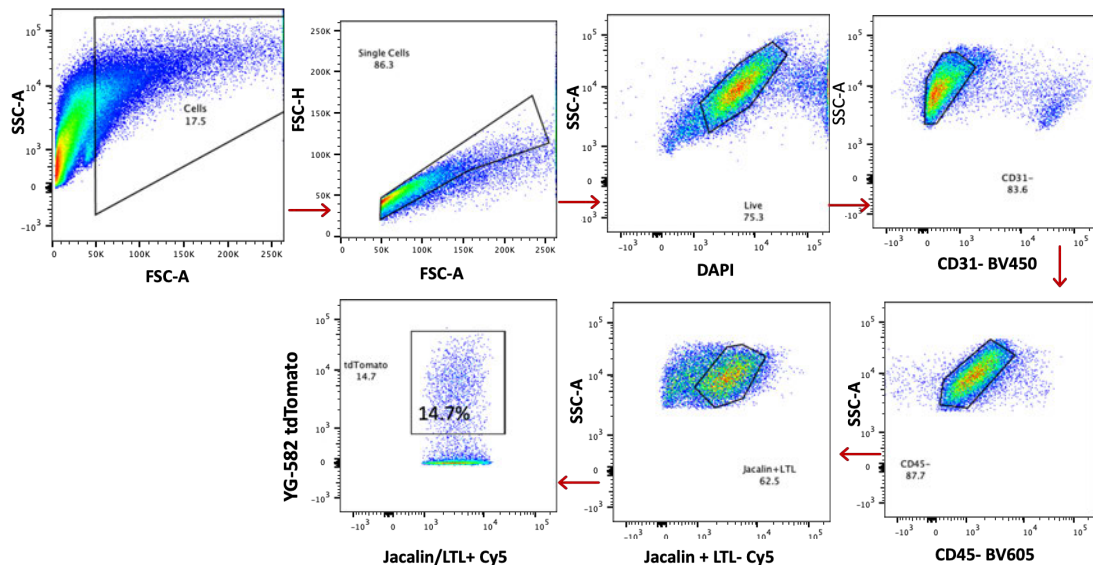


Figure 37 - *tdTomato* fluorescence with LTL and Jacalin

Flow gating strategy for the identification of a *tdTomato*⁺/*Jacalin*LTL⁺ population. ***tdTomato*⁺/*Jacalin*LTL⁺ cells:** Cells/Single cells/live cells/CD31 negative cells/CD45 negative cells/*Jacalin*LTL⁺ cells/*tdTomato*⁺/*Jacalin*LTL⁺ cells. Young Pax8Cre-ER^{T2}/*mdm2*^{flox/flox}/*tdTomato*^{LSL} female mouse, aged <6 months treated with tamoxifen 200mg/Kg x3 7 days prior to cell sorting.

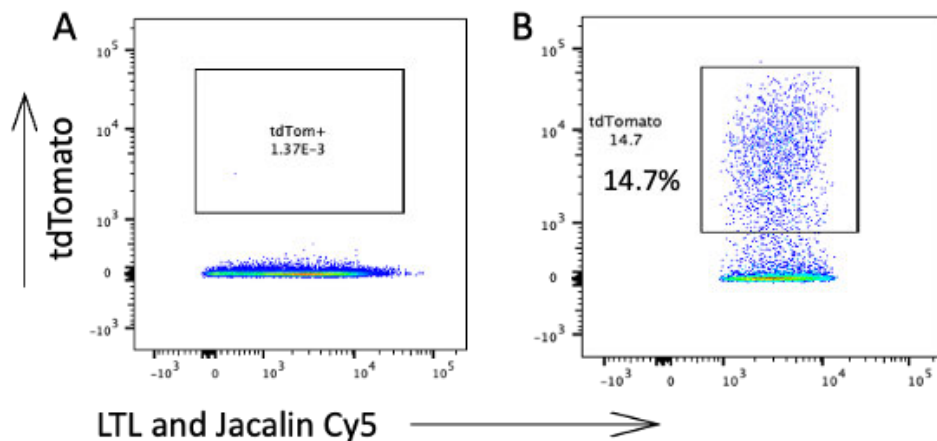


Figure 38 - Wild-type and transgenic expression of *tdTomato*

A) *tdTomato* fluorescence in a C57BL/6J wild-type mouse and (B) Pax8Cre-ER^{T2}/*mdm2*^{flox/flox}/*tdTomato*^{LSL} mouse both treated with tamoxifen 200mg/Kg x3 7 days prior to cell sorting. Young female mice, aged <6 months.

This optimisation process also involved a change to the tamoxifen dosing regime and a subset of mice were dosed with higher amounts of tamoxifen at a dose 200mg/Kg x3. As shown in Figure 39, treatment with a higher dose of tamoxifen also induced

significant levels of tdTomato expression at day 7. Figure 40 shows that an increased dose of tamoxifen did not result in any significant difference to the levels of observed tdTomato expression and as such, I have pooled data derived from both dosing regimens to generate the data shown in Figure 41.

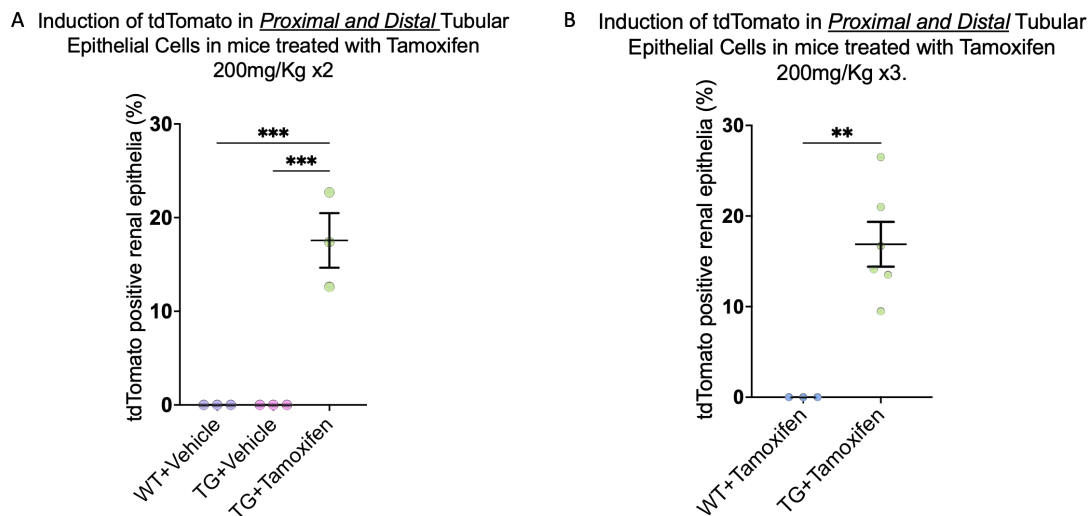


Figure 39 - tdTomato fluorescence with different doses of tamoxifen

A) tdTomato expression in proximal and distal renal epithelia by FACS at day 7 in mice treated with 200mg/Kg x2 of tamoxifen and (B) tamoxifen 200mg/Kg x3. n=3 per group, young female mice, <6 months of age. One-way ANOVA with Tukey's multiple comparisons. Error bars are mean $\pm$ SEM ***p<0.001, **p<0.005.

%tdTomato positivity in Proximal and Distal Tubular
Renal Epithelia

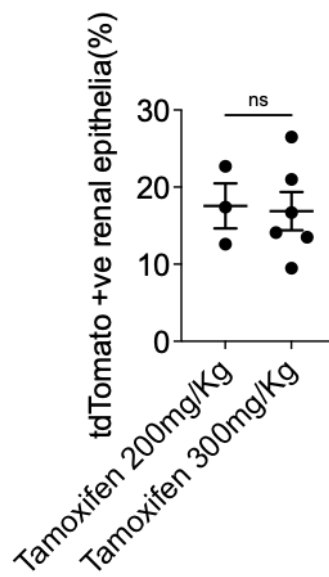


Figure 40 - Tamoxifen dosing comparison

A comparison of tdTomato expression in proximal and distal renal epithelial cells in Pax8/mdm2/tdTomato mice treated with tamoxifen dosing regimens of 200mg/Kg x2 vs x3. n=3-6, young female mice aged <6 months of age. Error bars are mean $\pm$ SEM. Mann-Whitney. p=0.92.

Induction of tdTomato in Proximal and Distal Tubular Epithelial Cells. Tamoxifen doses *combined*.

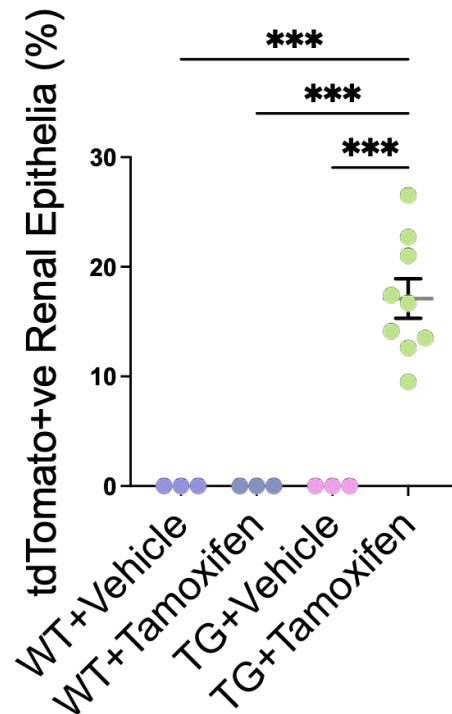


Figure 41 - tdTomato fluorescence across different tamoxifen doses

Pooled analysis of tdTomato expression in proximal and distal renal epithelia as assessed by FACS at day 7 in young female mice who have been dosed with either tamoxifen 200mg/Kg x2 or x3. n=3-9 per group, female, <6 months of age. One way ANOVA with Tukey's multiple comparison tests. All error bars are mean $\pm$ SEM, ***p<0.001.

Transgenic induction leads to significant cortical expression of p21^{cip1} by day 7 in young female mice

As discussed, p21^{cip1} is a protein marker of growth arrest and as shown in Figure 42 it was upregulated by day 7 in young female transgenic mice who had undergone induction with tamoxifen and not in the relevant control groups.

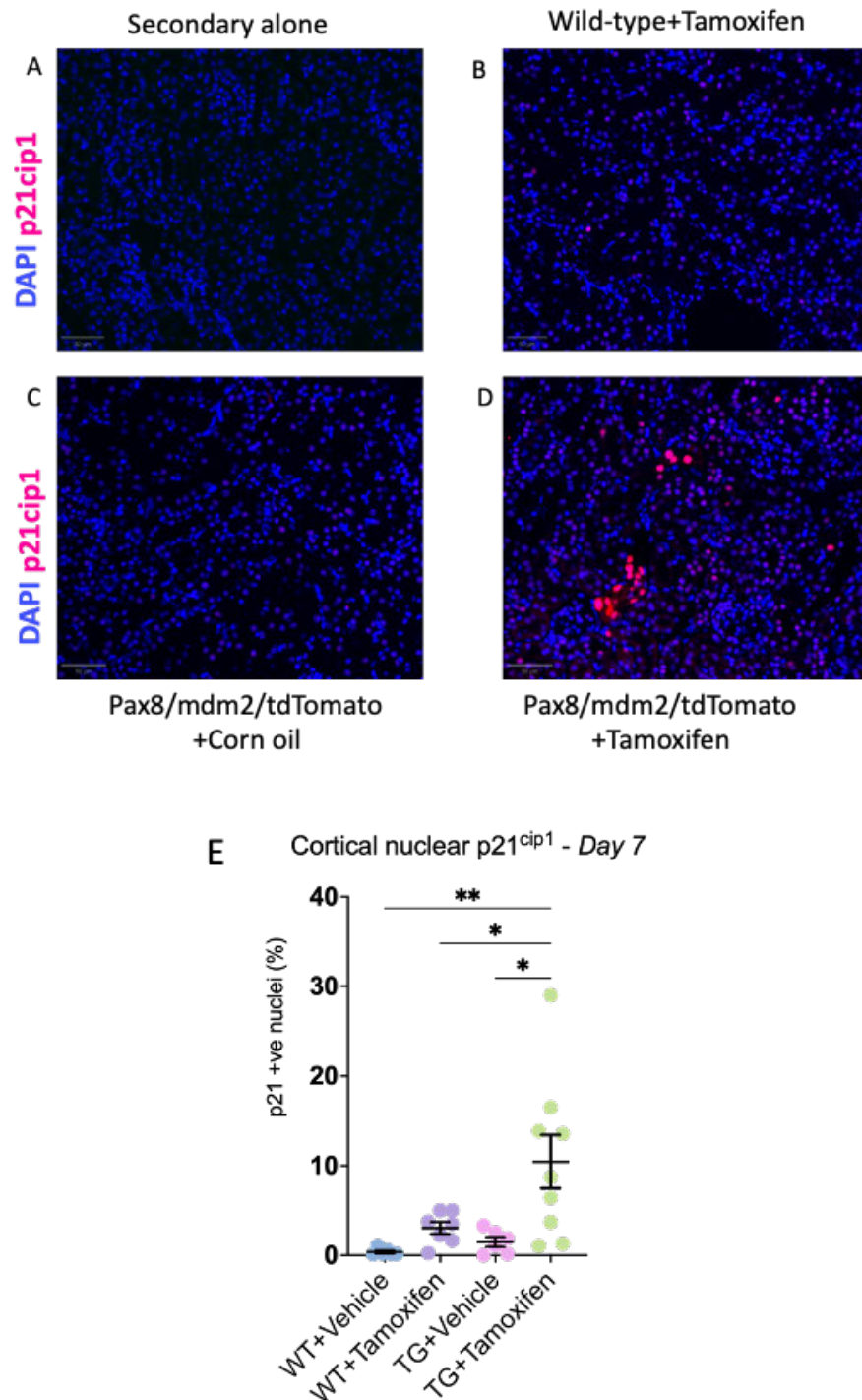


Figure 42 – Cortical nuclear p21^{cip1} expression 7 days after tamoxifen vs vehicle treatment

A) Secondary antibody alone control. B) p21^{cip1} expression 7 days after tamoxifen treatment in a wild-type mouse. C) Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} treated with corn oil. D) Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} treated with tamoxifen. p21^{cip1} positive nuclei appear dark pink. E) Comparison of p21^{cip1} positive nuclei across groups. p21^{cip1} positive nuclei are expressed as a percentage of all nuclei in a representative area of renal cortex. Three cortical readings have been taken and averaged per individual mouse. n=7-9 per group. Female mice,

<6months of age. Tamoxifen 200mg/Kg x2. One way ANOVA with Tukey's multiple comparisons. All error bars are mean $\pm$ SEM *p<0.05, **p<0.01.

Figure 43 demonstrates that there was no significant difference across groups in medullary expression of p21^{cip1} at an early timepoint after tamoxifen administration.

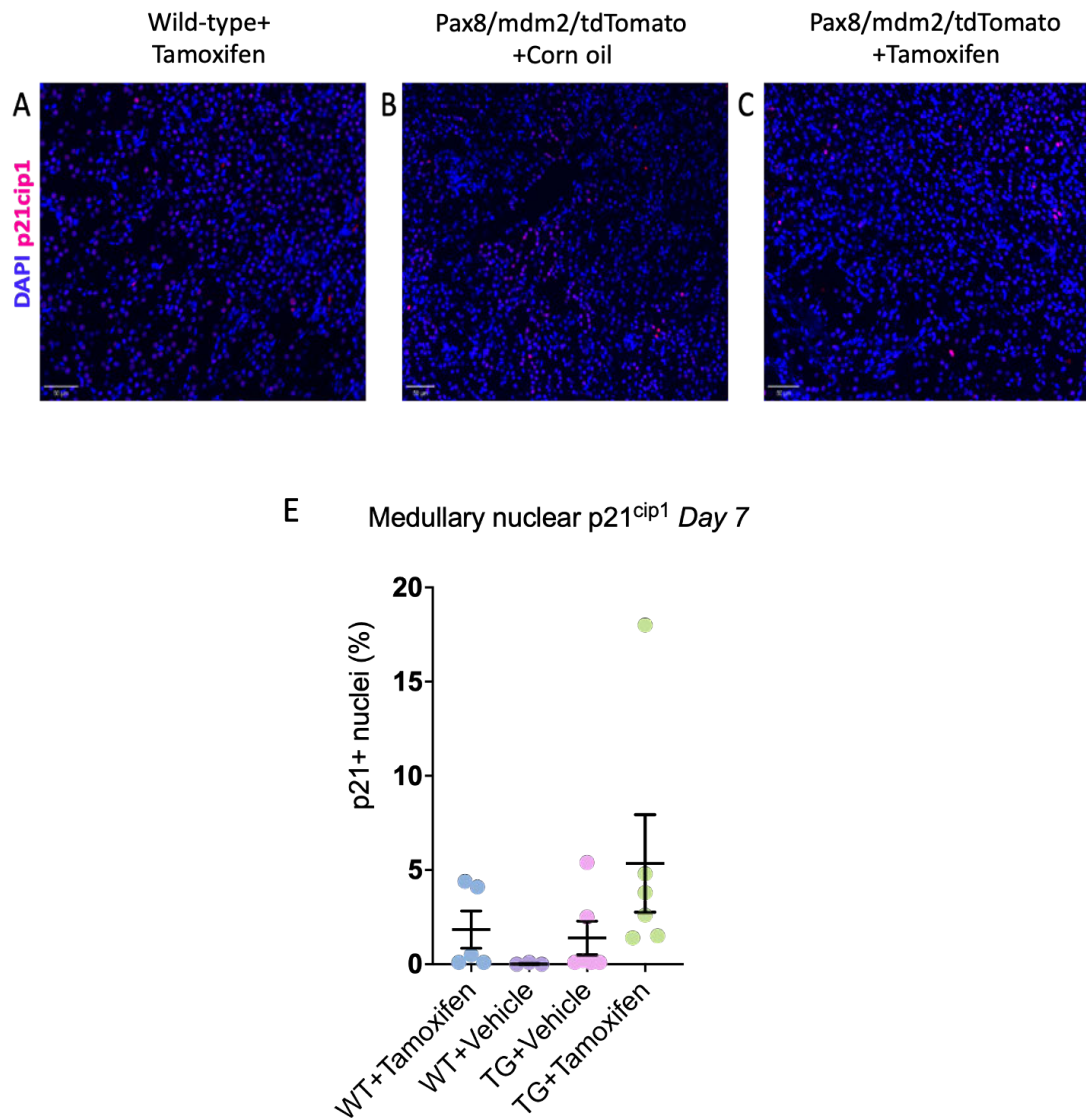


Figure 43-Medullary p21^{cip1} expression early after tamoxifen

Expression of medullary p21^{cip1} in (A) Wild type treated with tamoxifen (B) Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with corn oil and (C) Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with tamoxifen. Young female mice aged <6 months of age treated with tamoxifen 200mg/Kg x2 7 days prior to cull. E) Comparison of p21^{cip1} positive nuclei across all groups 7 days after treatment with tamoxifen 200mg/Kg x2 or vehicle. p21^{cip1} positive nuclei are expressed as a percentage of all nuclei in a representative area of renal medulla. n=3-6 per group. Young female mice. One way ANOVA with Tukey's multiple comparisons.

I looked for the absence of Ki67 in cells expressing p21^{cip1} as a second marker of growth arrest. Figure 44 shows that in induced transgenic mice there is a highly

significant difference in cell populations between p21^{cip1} positive cells and dual positive cells and the two markers; p21^{cip1} and Ki67 do not colocalise. Although induced transgenic mice appear to show trending higher levels of Ki67 in general this is not significant (WT+Tam, WT+Veh, TG+Veh: p=0.9992, 0.9952, 0.9965). Figure 44 is consistent with the p21^{cip1} analysis presented in Figure 42 with p21^{cip1} levels being significantly higher in treated transgenic mice vs all other control groups.

Figure 44 and Figure 45 show low numbers overall of Ki67 positive cells across experimental groups and a high number of p21^{cip1} positive cells in the induced transgenic mouse, with little co-localisation between both markers.

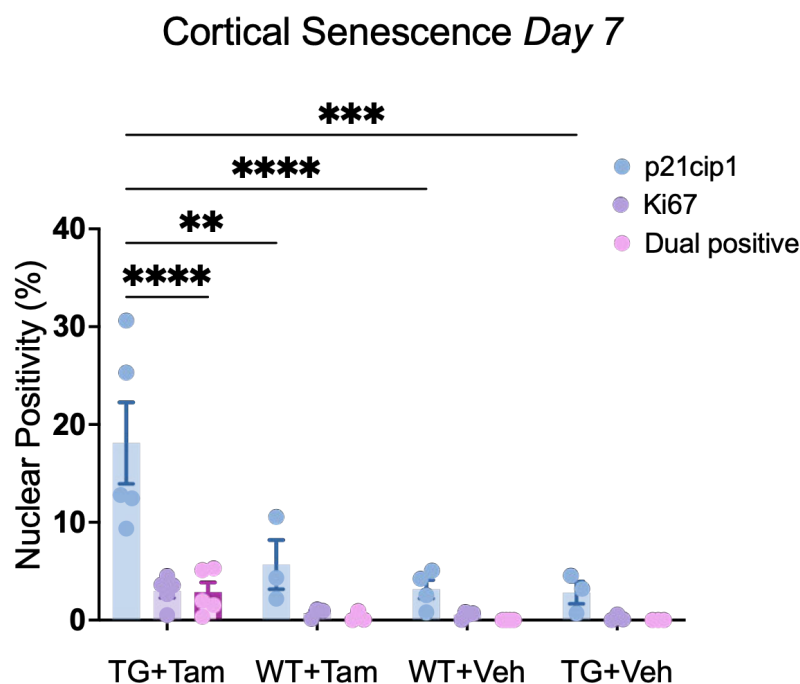


Figure 44 -Expression of p21^{cip1} and Ki67 across groups

A comparison of p21^{cip1} positive nuclei alone, Ki67 positive nuclei alone and dual positive p21^{cip1}/Ki67 nuclei. These percentages are calculated by dividing the number of positive nuclei for each respective class by the number of entire nuclei for a given area of cortex. Example 1000 nuclei per cortical area, 100 nuclei p21^{cip1} positive: 10% p21^{cip1} positivity. n=3-5 per group. Female, <6 months. 2-way ANOVA with Tukey's multiple comparisons. All error bars are mean $\pm$ SEM, ****p<0.0001, **p<0.001.

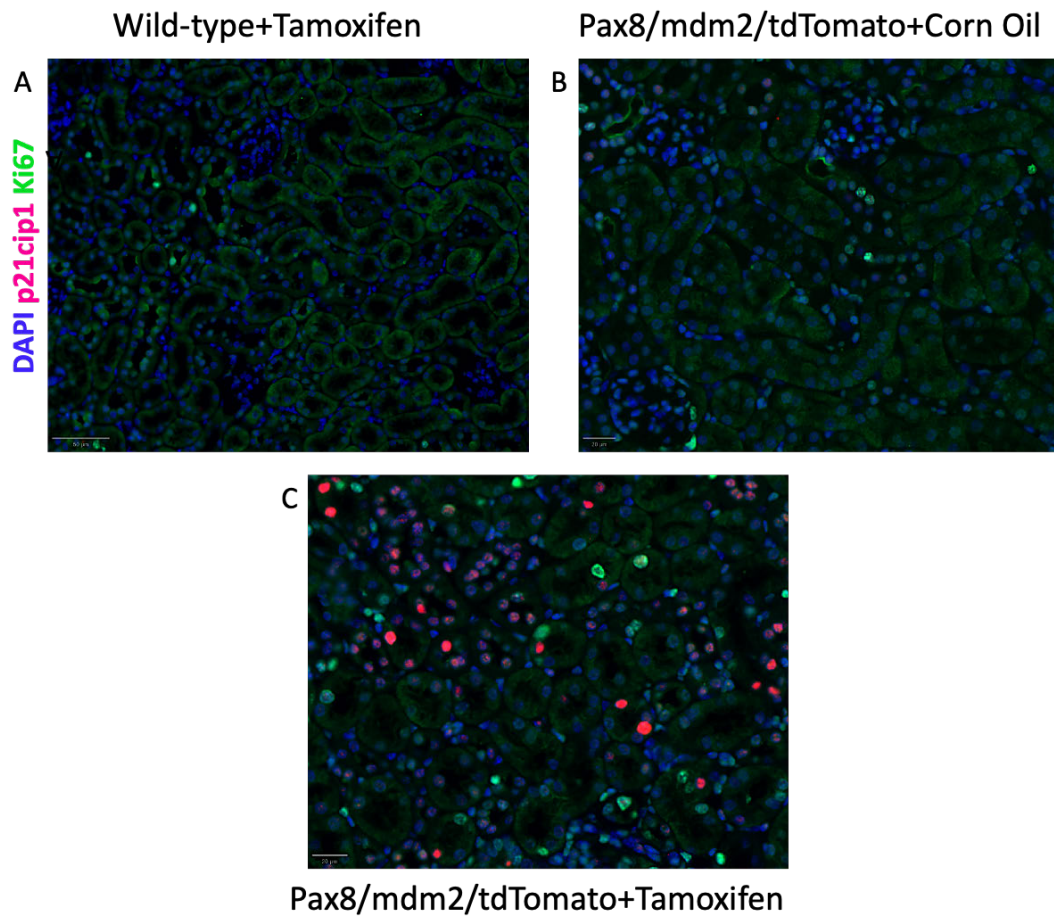


Figure 45 - Cortical expression of p21^{cip1} and Ki67

p21^{cip1} and Ki67 immunofluorescence in the renal cortex in (A) Wild-type mouse treated with tamoxifen 200mg/Kg x2 (B) Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with corn oil and (C) Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with tamoxifen 200mg/Kg x2 7 days prior to cull.

Figure 46 shows tubular nuclei staining positively for either p21^{cip1} or Ki67 and shows a single nucleus demonstrating positive immunofluorescence for both p21^{cip1} and Ki67. As can be seen in Figure 42, Figure 43 and Figure 44, there is some co-expression of p21^{cip1} and Ki67. This was expected as p21^{cip1} is a cell cycle marker and thus the cells expressing both proteins most likely represent cycling cells. The important finding was that the vast majority of p21^{cip1} expressing cells did not express Ki67 as shown in Figure 42. If the contrary had been shown; i.e. most p21^{cip1} expressing cells also expressed Ki67 then I would not have been confident to use p21^{cip1} as my key marker of senescence in this project.

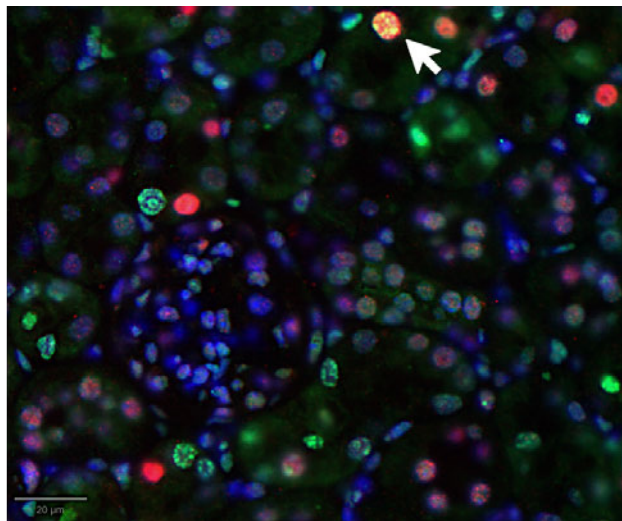


Figure 46 - Nuclear expression of p21^{cip1} and Ki67: an example of co-localisation

p21^{cip1} and Ki67 immunofluorescence in the renal cortex showing easily observed separation of both stains. p21^{cip1} positive nuclei are red and Ki67 positive nuclei are green. At the top of this immunofluorescence image there is an orange nucleus highlighted with a white arrow. This is a nucleus which is positive for both stains: this is an example of co-localisation of both antigens.

Single cell RNA sequencing confirms distinct senescent populations in an induced transgenic mouse vs post-injury senescence

The experimental design is described in Figure 27. This experiment was designed to identify any transcriptomic changes observed at an early timepoint after senescence induction in the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse vs senescence induction by injury. This experiment contained induced transgenic mice, injured mice and baseline uninjured controls. The injury model used was ischaemia reperfusion injury.

I excluded CD31 and CD45 positive cells and the gating strategy for the identification of my final epithelial population is shown in Figure 47. Because I wanted to define the relationship between senescent epithelial cells and the development of fibrosis, I enriched for CD90+activated fibroblasts as shown in Figure 48.

In total, cells were sorted from six individual mice. Three of these mice underwent whole kidney sorting and cells were sorted on live/dead only as shown by Figure 49. Three mice underwent sorting by cell surface antibody approach as shown by Figure 47 and Figure 48.

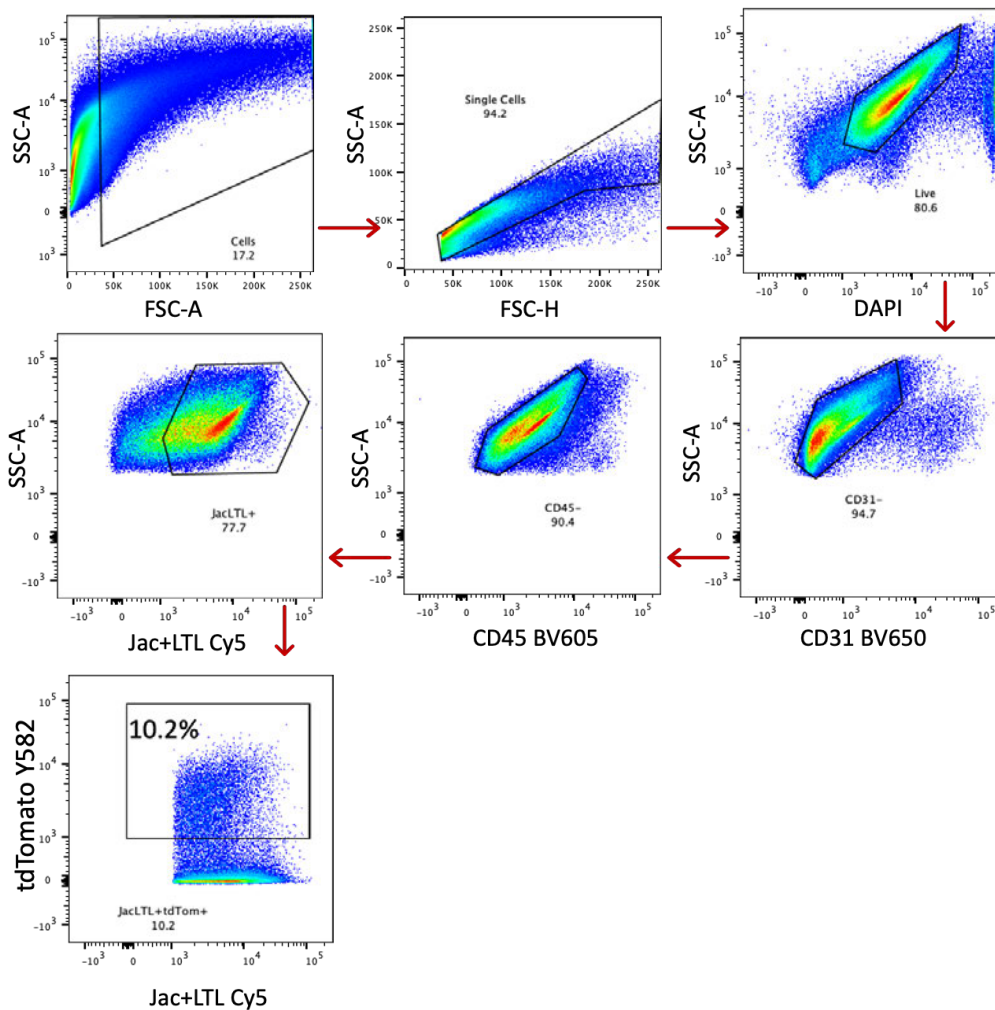


Figure 47 -Flow gating strategy for population-based sorting for scRNA sequencing

Flow gating strategy for the identification of a tdTomato+/JacalinLTL+ population.
tdTomato+/JacalinLTL+ cells: Cells/Single cells/live cells/CD31 negative cells/CD45 negative cells/JacalinLTL+cells/tdTomato+/JacalinLTL+cells.
 Young female Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse aged <6 months treated with tamoxifen 200mg/Kg x3 7 days prior to cell sorting.

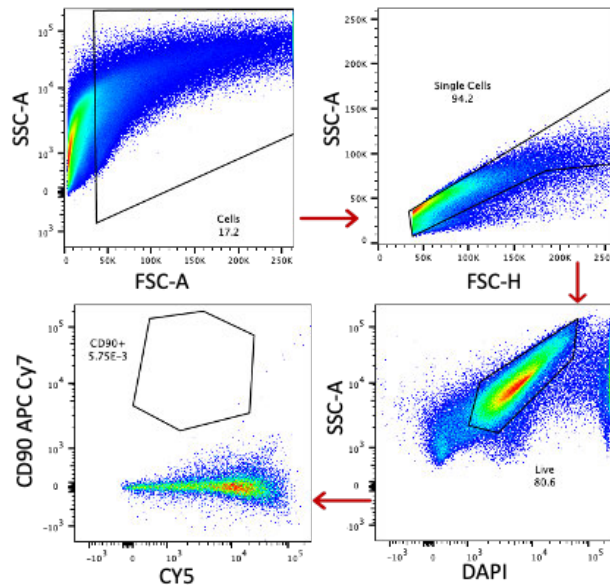


Figure 48 - FMO for CD90

Flow gating strategy for identification of CD90+ cells using a CD90 FMO control. Young female Pax8/mdm2/tdTomato mouse treated with Tamoxifen 200mg/Kg x3 7 days prior to sorting

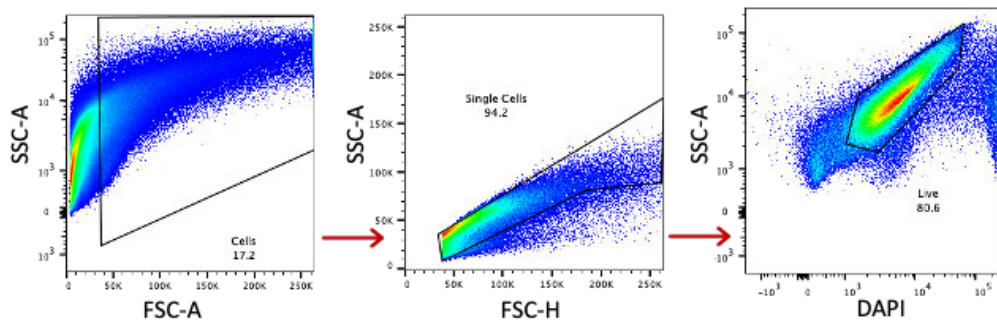


Figure 49 -Flow gating for scRNA sequencing on live/dead only

Flow gating strategy for sorting of live cells in whole kidney sorts. Young female Pax8/mdm2/tdTomato mouse aged <6 months treated with tamoxifen 200mg/Kg x3 7 days prior to cell sorting.

tdTomato expression in epithelial cells across mouse groups is shown below in Figure 50. Whilst the group size does not allow statistical comparison, the population of tdTomato positive proximal and distal tubular epithelial cells in an induced transgenic mouse was similar to that of an injured CDKN1AiTomato mouse (13.8% vs 10.2%). The baseline level of tdTomato positive epithelial cells found in an uninjured CDKN1AiTomato mouse was 1.44%.

In the context of tamoxifen administration in the CDKN1AiTomato mouse, only cells expressing *Cdkn1a* at the time of tamoxifen administration undergo potential recombination. This is why tamoxifen was given on days 4 and 5 post-injury in these mice. Administering tamoxifen simultaneously to injury would not reflect senescence induced by injury because any cell induced to senesce because of injury would not yet have undergone that process at the time of tamoxifen administration.

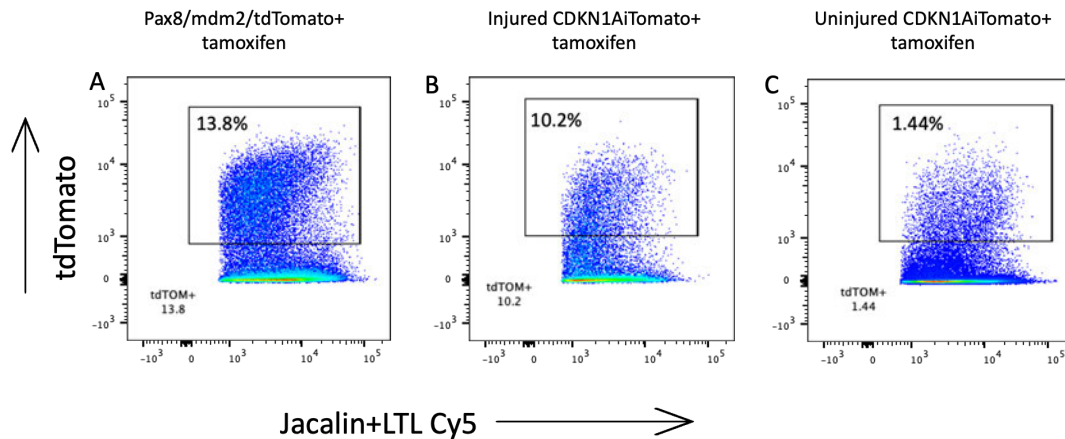


Figure 50 - tdTomato expression at FACS in CDKN1AiTomato and Pax8/mdm2/tdTomato mice

A) tdTomato expression in an induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with tamoxifen 200mg/Kg x3 7 days prior to cell sorting. B) tdTomato expression in an injured CDKN1AiTomato mouse which underwent unilateral IRI 7 days prior to sorting (of the injured kidney) and was treated with tamoxifen 200mg/Kg x2 48 and 72 hours prior to cell sorting. C) tdTomato expression in a non-injured 'baseline' CDKN1AiTomato mouse treated with tamoxifen 200mg/Kg x2 48 and 72 hours prior to cell sorting.

Figure 51 shows the populations of CD90+ cells across mouse groups. The population of CD90+ cells is low overall but higher in both the induced transgenic Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse and in the injured CDKN1AiTomato mouse (0.39% vs 0.31% vs 0.13%).

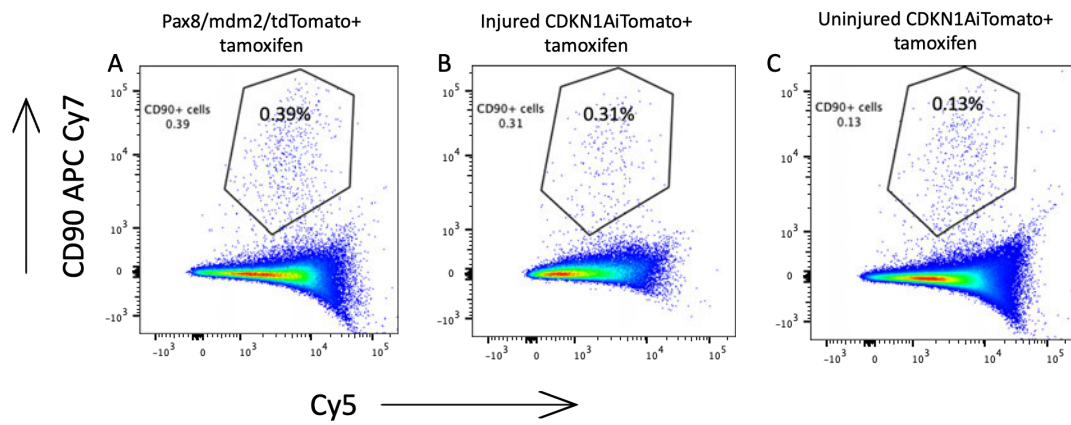


Figure 51 - CD90 expression at FACS in CDKN1AiTomato and Pax8/mdm2/tdTomato mice

A) CD90 expression in an induced Pax8Cre-ER^{T2}/mdm2^{fllox/fllox}/tdTomato^{LSL} mouse treated with tamoxifen 200mg/Kg x3 7 days prior to sorting. Young male mouse, aged <6 months of age B) CD90 expression in an injured CDKN1AiTomato mouse treated with tamoxifen 200mg/Kg x2 48 and 72 hours prior to sorting. Young female mouse, aged < 6 months of age. C) CD90 expression in a non-injured 'baseline' CDKN1AiTomato mouse treated with tamoxifen 200mg/Kg x2 48 and 72 hours prior to sorting. Young male mouse, aged <6 months of age.

As discussed in chapter 2, the scRNA sequencing analysis was performed by Maximillian Reck (Conway/Denby group. Centre for Cardiovascular Sciences. University of Edinburgh). With bioinformatics support throughout, I performed the biological interpretation of this dataset and the generation of all the scRNA sequencing figures presented.

Figure 52 shows a simplified UMAP of cell clustering from ten murine kidneys. In this figure, three populations of senescent cells highlighted by black arrows can be seen but only two are annotated; 'Senescent cell Mdm2' and 'Senescent Mdm2 c'. The unannotated population lies close to 'Senescent Mdm2 c' on the UMAP below and is called 'Senescent Mdm2 b'. Some labels have been omitted for ease of viewing.

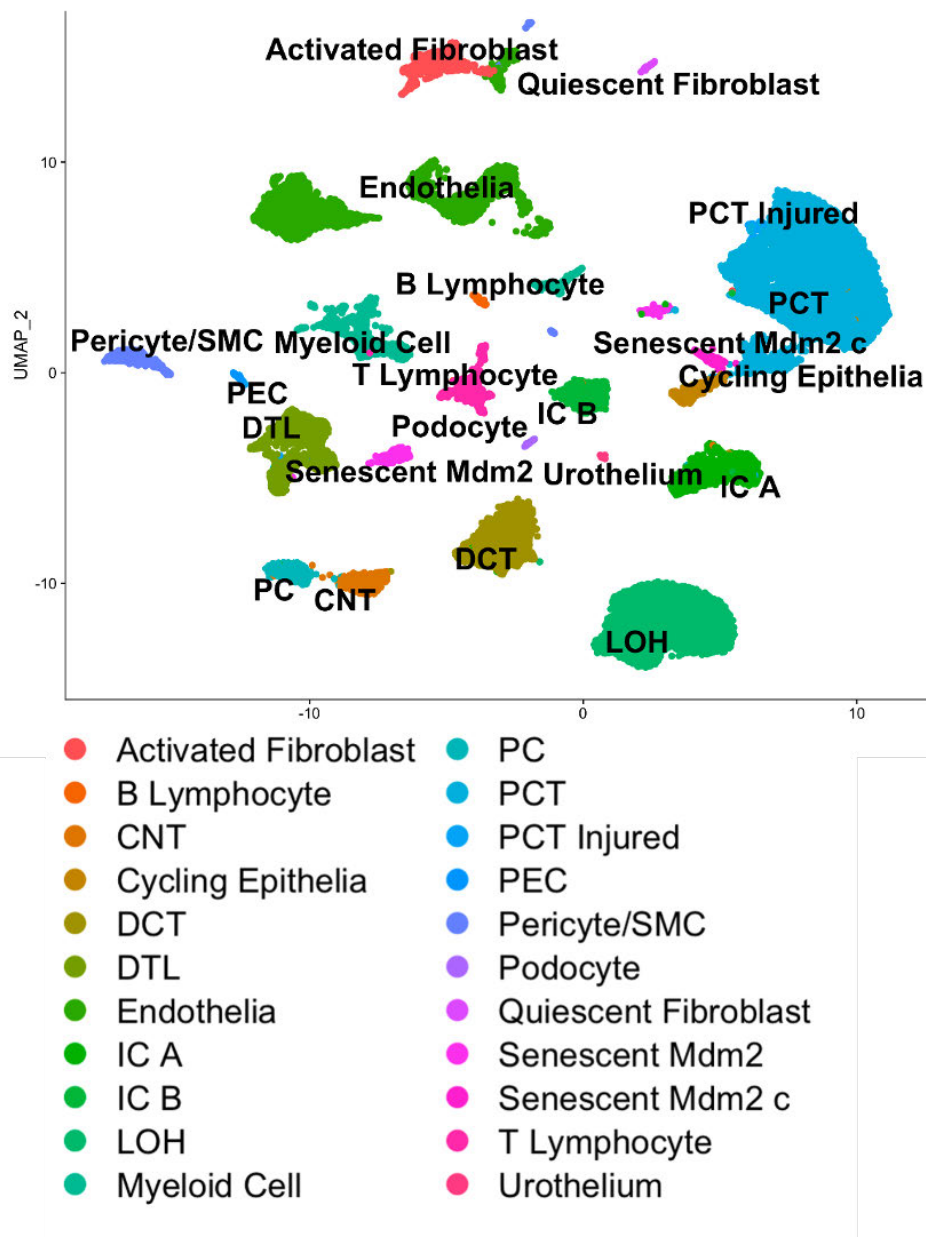


Figure 52 - Clustering and cell type identification

Cell clusters identified in mouse kidney sorts. Sorted by population based or whole kidney sorts. Mixed gender. n=10 total, aged <6 months. Broad mouse groups: CDKN1AiTomato uninjured, CDKN1AiTomato injured, and induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL}. n=2 per group, culled and sorted at day 7 post injury/post induction. Tamoxifen 200mg/Kg x2 given 48 and 72 hours prior to sorting (day 4 and 5 post injury) in the CDKN1AiTomato mice and tamoxifen 200mg/Kg x3 7 days prior to sorting in the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice. Contained within this UMAP are data derived from four additional mice culled at day 42 post injury/induction. n=4. Young mice, aged <6 months, mixed genders.

Figure 52 also includes data derived from four mice which were assessed at later day 42 timepoint after injury or transgenic induction. These mice will be discussed in the

final results chapter. In this early time-point experiment, two sequencing platforms were used; 10X and Parse. The UMAP shown in

Figure 52 is for all cells sequenced and the data for both sequencing platforms has been combined to generate this figure. The decision to use both sequencing technologies was made by Professor David Ferenbach and Maximillian Reck. This decision related primarily to the fact that newer technologies such as Parse allow the fixation of cells prior to library preparation-a feature which is not possible with 10X. Fixation allows sample storage for months before library preparation and sequencing. Therefore, it opens the possibility of significantly larger numbers of experimental replicates than 10X because samples can be obtained over a much longer period and fixed; but crucially prepared and sequenced at the same time; therefore, mitigating any 'batch effect'. This is a relatively new technology. As such, combining sequencing data obtained from two different sequencing platforms is not fully understood. This was directed exclusively by Max Reck and any populations which he felt to be an artefact of the technology were excluded.

As shown below in Figure 53, Senescent Mdm2 a and b were identified with both sequencing technologies. The third population Senescent Mdm2 c was only apparent in the 10X dataset. This was felt to be a potential artefact of the 10X data or an alignment issue between both sequencing datasets. Senescent Mdm2 c highly expressed *Cdkn1a* (avg_log2FC 2.87, $p=6.9 \times 10^{-251}$) and it is likely that this population belongs to one of the other populations. However, as we cannot be sure of this at present and after discussion with Max Reck, I have only considered the two senescent populations which were clearly identified with both sequencing approaches.

In the final results chapter, I will present data from a later time-point post induction (day 42). At that later timepoint we only used Parse sequencing. Therefore, from a scRNA sequencing perspective, for any direct comparisons made between day 42 and

day 7 I have used Parse data only and I have been explicit when discussing results which exclude 10X data.

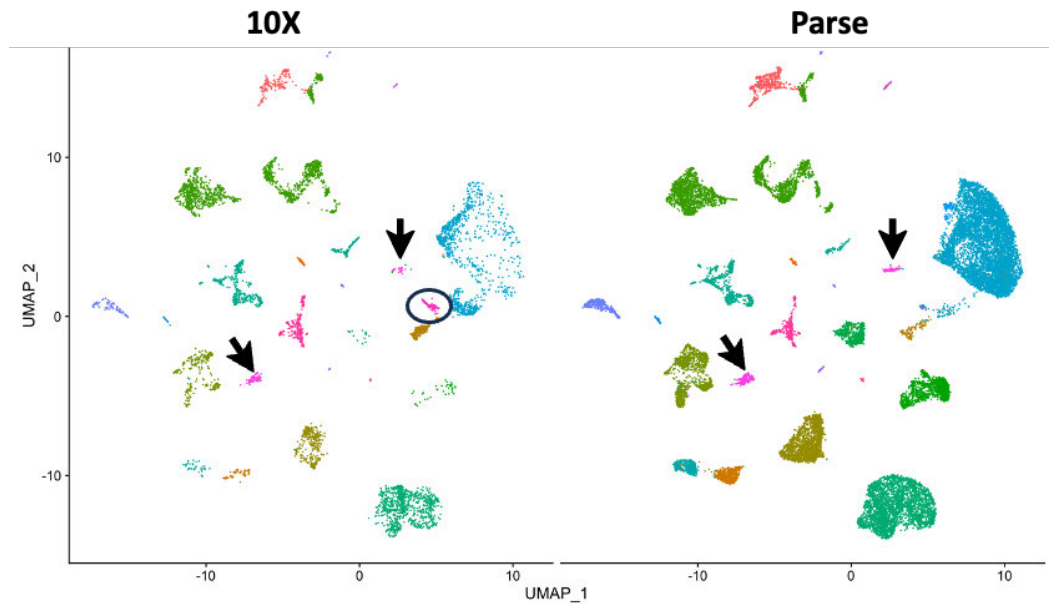


Figure 53 - 10X and Parse sequencing

Cell clusters identified in mouse kidney sorts shown by sequencing platform. Sorted by population based or whole kidney sorts. Mixed gender. n=10 total, aged <6 months. Broad mouse groups: CDKN1AiTomato uninjured, CDKN1AiTomato injured, and induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL}. n=2 per group, culled and sorted at day 7 post injury/post induction. Tamoxifen 200mg/Kg x2 given 48 and 72 hours prior to sorting (day 4 and 5 post injury) in the CDKN1AiTomato mice and tamoxifen 200mg/Kg x3 7 days prior to sorting in the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice. Contained within this UMAP are data derived from four additional mice culled at day 42 post injury/induction. n=4. Young mice, aged <6 months, mixed genders.

Figure 54 shows that *Pax8* expression was limited to the epithelium as was expected.

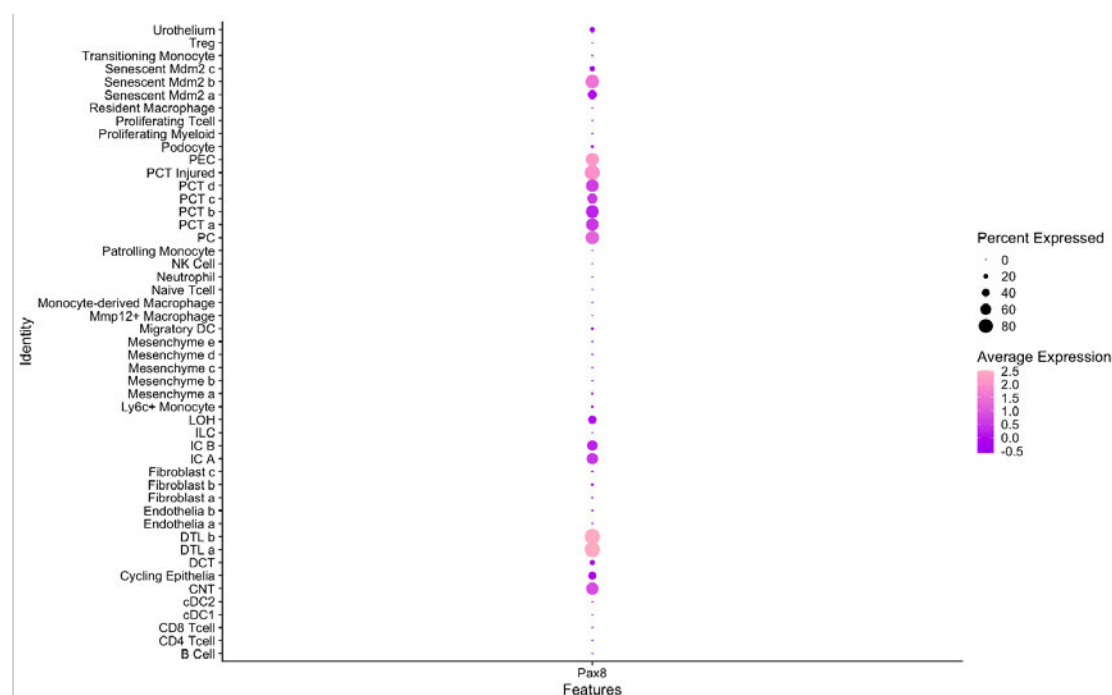


Figure 54 - *Pax8* expression by scRNA sequencing

Pax8 expression across all mice. Sorted by population based sorting or whole kidney sorts. Mixed gender. n=10 total, aged <6 months. Mouse groups: CDKN1AiTomato uninjured, CDKN1AiTomato injured, and induced *Pax8*Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL}. n=2 per group, culled and sorted at day 7 post injury/post induction. Tamoxifen 200mg/Kg x2 given 48 and 72 hours prior to sorting (day 4 and 5 post injury) in the CDKN1AiTomato mice and tamoxifen 200mg/Kg x3 7 days prior to sorting in the *Pax8*Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice. Contained within this UMAP are data derived from four additional mice culled at day 42 post injury/induction. n=4. Young mice, aged <6 months, mixed genders.

Senescent cell populations in the Pax8CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse

Two distinct populations of senescent epithelia were observed in the induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice: one originating from cells of the thin descending limb of Henle (DTL), and one from proximal tubular epithelial cells (PCT) as shown in Figure 55 and Figure 56.

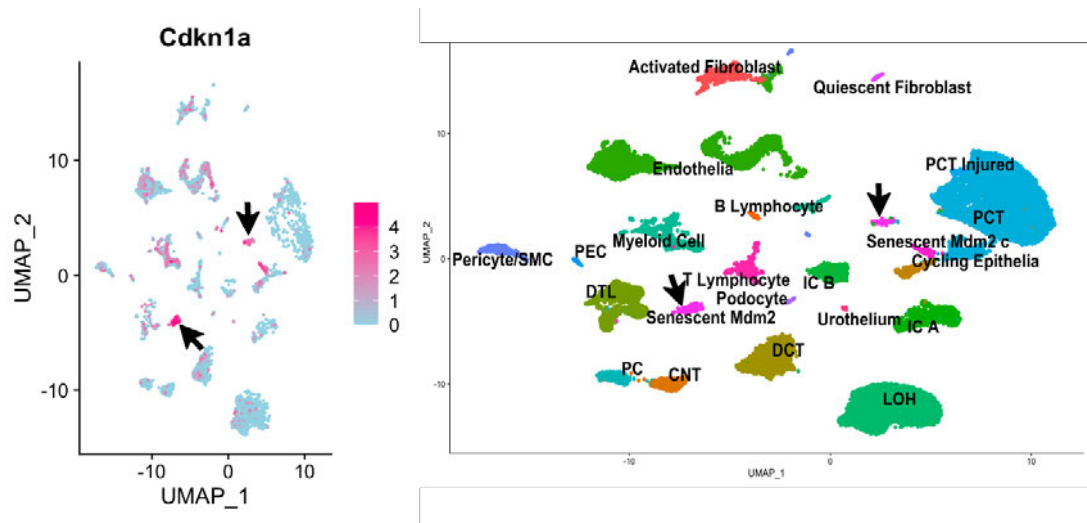


Figure 55 -Senescent cell populations in an induced Pax8/mdm2/tdTomato mouse

Cdkn1a expression in an induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with tamoxifen 200mg/Kg x3 7 days prior to cell sorting. Whole kidney sort. Young female mouse. This is shown beside the UMAP for all cells for reference. **Senescent cell populations are identified by black arrow.** For ease of viewing the labels are truncated. Senescent Mdm2 lies next to the DTL population and Senescent Mdm2 b is adjacent to the PCT population.

As shown below in Figure 56, 'Senescent Mdm2 a' expressed markers associated with the proximal tubule such as *Slc5a2* and *Slc13a3*. 'Senescent Mdm2 b' expressed markers associated with the thin descending limb of Henle (DTL) such as *Bst1* and *Aqp1* (Park, Shrestha et al. 2018, Lu, Liao et al. 2021).

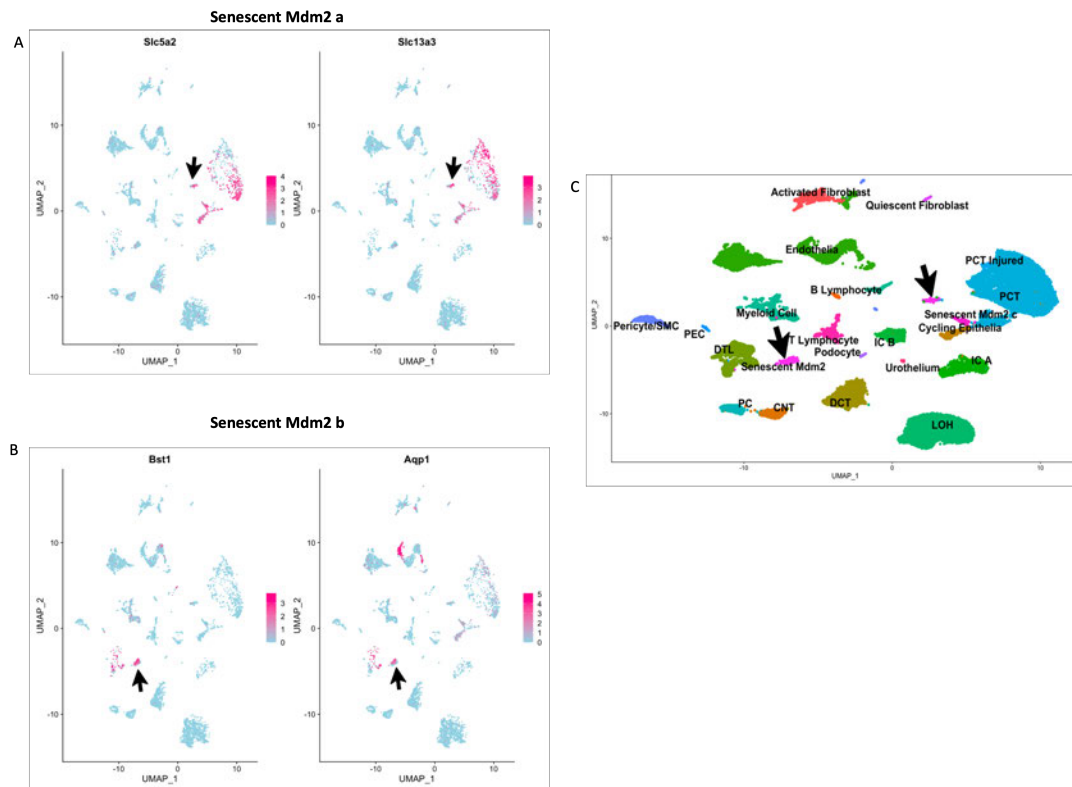


Figure 56 -Tubular markers and senescent epithelia

A) Expression of *Slc5a2* and *Slc13a3* in 'Senescent Mdm2 a'. B) Expression of *Bst1* and *Aqp1* in 'Senescent Mdm2 b'. C) UMAP of all cells for reference. (A) and (B) are taken from a Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with tamoxifen 200mg/Kg x3 7 days prior to cell sorting. Whole kidney sort. Young female mouse.

Figure 57 shows *Cdkn1a* expression in whole kidney sorts from different mice. The black arrows highlight the two populations of senescent epithelia discussed above. These were not seen at baseline nor after injury. In the injured CDKN1AiTomato mouse, there was a population which demonstrated high *Cdkn1a* expression which is highlighted with a black asterix. This population is not seen at baseline and appears less obvious in the induced transgenic mouse. These are neutrophils and cell cycle protein expression is a recognised feature of normal neutrophil biology (Amulic, Knackstedt et al. 2017).

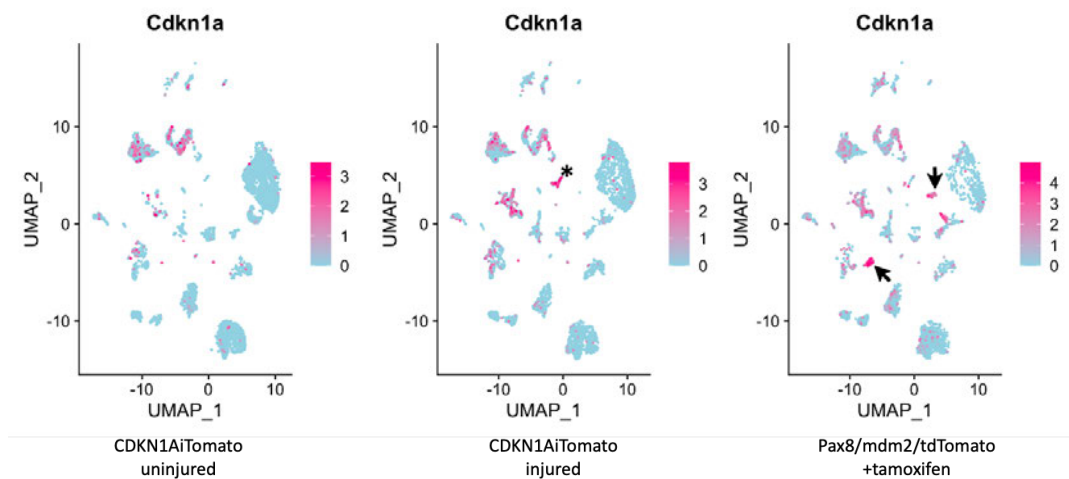


Figure 57 – *Cdkn1a* expression in whole kidney sorts

Cdkn1a expression across mouse groups. CDKN1AiTomato uninjured: baseline CDKN1AiTomato mouse treated with tamoxifen 200mg/Kg x2 48 and 72 hours prior to cell sorting. CDKN1AiTomato injured mouse: CDKN1AiTomato mouse subjected to unilateral IRI 7 days prior to cell sorting of the injured kidney, treated with tamoxifen 200mg/Kg x2 48 and 72 hours prior to sorting. Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with Tamoxifen 200mg/Kg x3 7 days prior to cell sorting. Young female mice, aged <6 months of age. Whole kidney digest, sorted on live/dead only. Combined 10X and Parse data. **Senescent populations annotated by black arrow. *denotes a neutrophil population in the injured mouse.**

Figure 58 shows data from three additional mice that underwent population-based sorting. The same senescent populations were identified in the induced transgenic mouse, but not at baseline nor post-injury. As expected with the CD45- gating approach, the *Cdkn1a* expressing neutrophil population was no longer identified in the injured mouse.

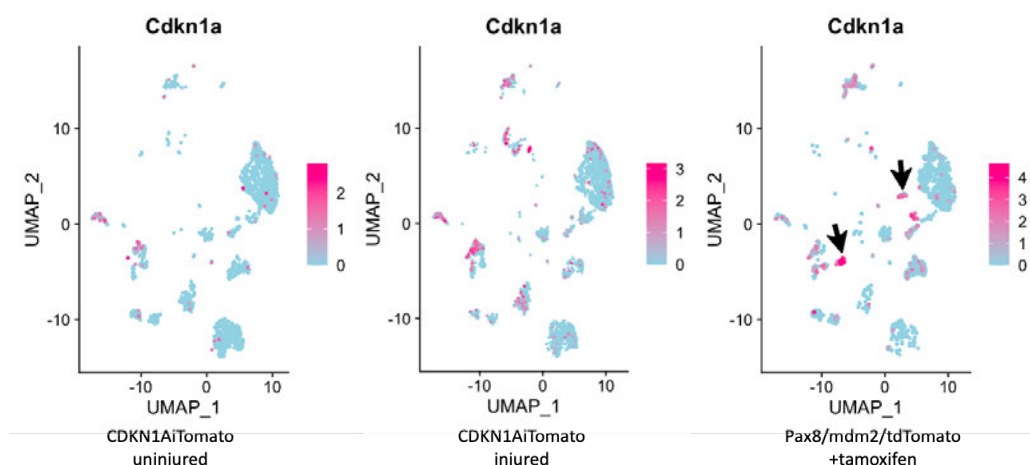


Figure 58 -Cdkn1a expression in population-based sorts

Cdkn1a expression across mouse groups. CDKN1AiTomato uninjured: young male baseline CDKN1AiTomato mouse treated with tamoxifen 200mg/Kg x2 48 and 72 hours prior to cell sorting. CDKN1AiTomato injured mouse: young female CDKN1AiTomato mouse subjected to unilateral IRI 7 days prior to cell sorting of the injured kidney, treated with tamoxifen 200mg/Kg x2 48 and 72 hours prior to sorting. Young male Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with Tamoxifen 200mg/Kg x3 7 days prior to cell sorting. JacalinLTL+tdTomato+ and CD90+ cells sorted. 10X and Parse sequencing data. **Senescent populations annotated by black arrow.**

Figure 59 shows expression of *Cdkn1a* across mouse groups with the highest levels of *Cdkn1a* expression seen in the senescent cell populations as was expected.

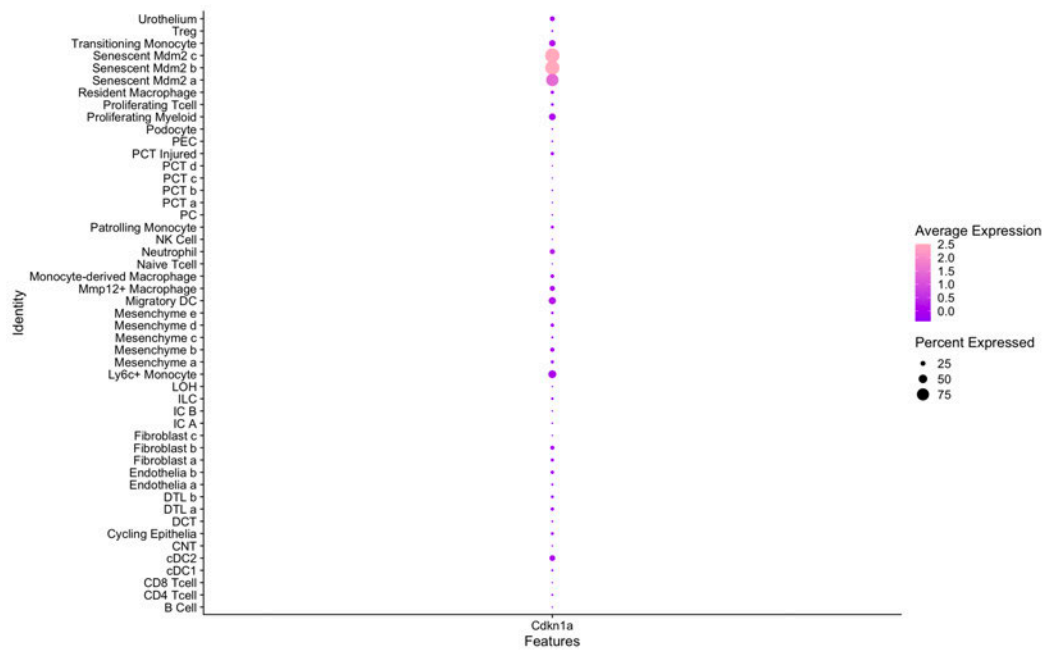


Figure 59 - Expression of *Cdkn1a* across all cells

Cdkn1a expression per cell type across mouse groups. n=10 in total, young aged <6 months, mixed genders. Mouse groups: CDKN1AiTomato uninjured, CDKN1AiTomato injured, Pax8/mdm2/tdTomato n=2 per group assessed at day 7 post injury/induction. Four additional mice were included within this dataset from a day 42 timepoint: CDKN1A injured, CDKN1A baseline uninjured, Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} and a C57BL6J mouse. 10X and Parse sequencing data.

***Pax8* and *Cdkn1a* expression**

Figure 60 demonstrates co-expression of *Cdkn1a* and *Pax8* in senescent cell populations in an induced transgenic mouse.

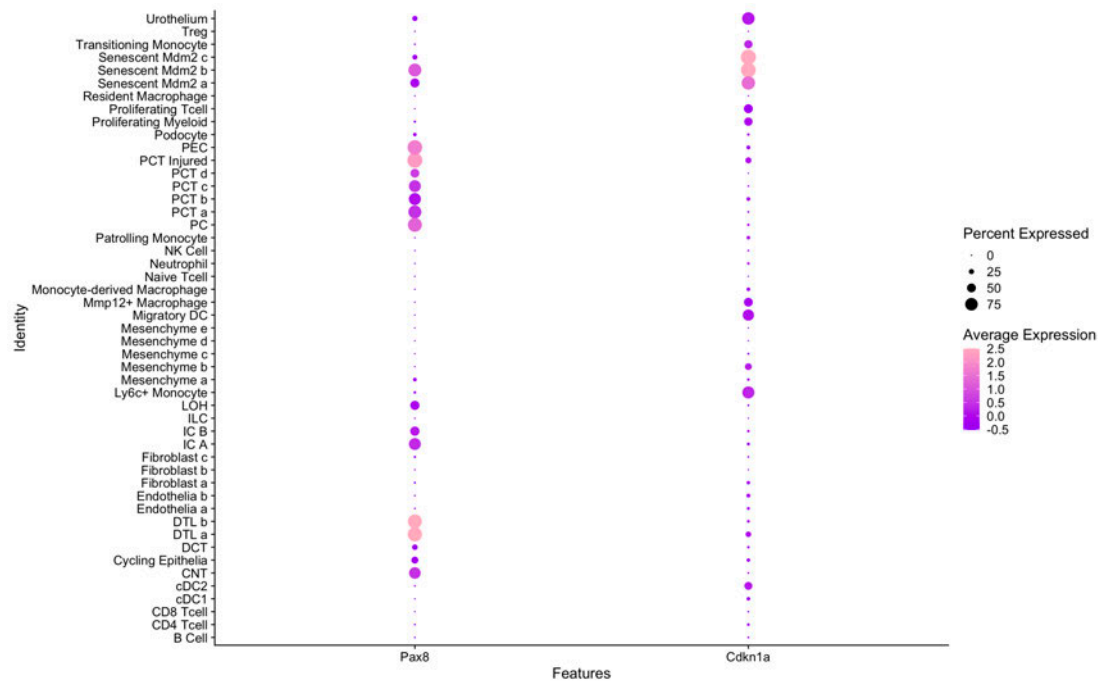


Figure 60 - *Pax8* and *Cdkn1a* expression by cell type in an induced *Pax8/mdm2/tdTomato* mouse

Pax8 and *Cdkn1a* expression in a whole kidney sort from a *Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL}* mouse treated with tamoxifen 200mg/Kg x3 7 days prior to cell sorting. Young female mouse, aged <6 months. 10X and Parse sequencing data.

***Cdkn1a* and *Mki67* expression**

Figure 61 and Figure 62 show the senescent cell populations identified did not concomitantly express *Mki67* and they were transcriptomically distinct from cycling epithelia. This is consistent with the immunofluorescence data which showed distinct p21^{cip1} and Ki67 positive populations.

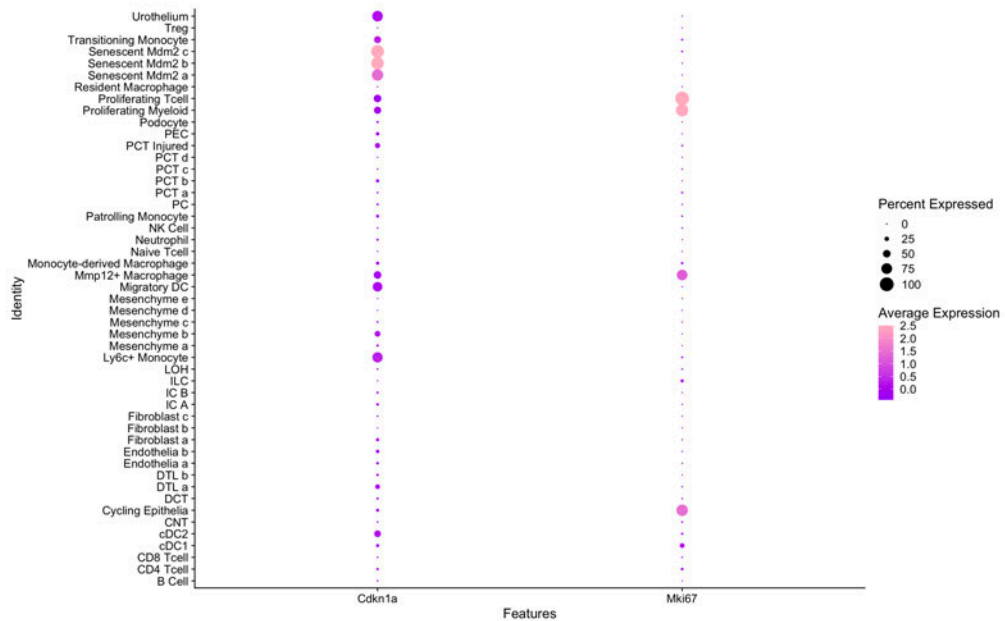


Figure 61 -*Cdkn1a* and *Mki67* expression in an induced Pax8 mouse.

Cdkn1a and *Mki67* expression in a Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with tamoxifen 200mg/Kg x3 7 days prior to cull. Young female mouse aged <6 months.

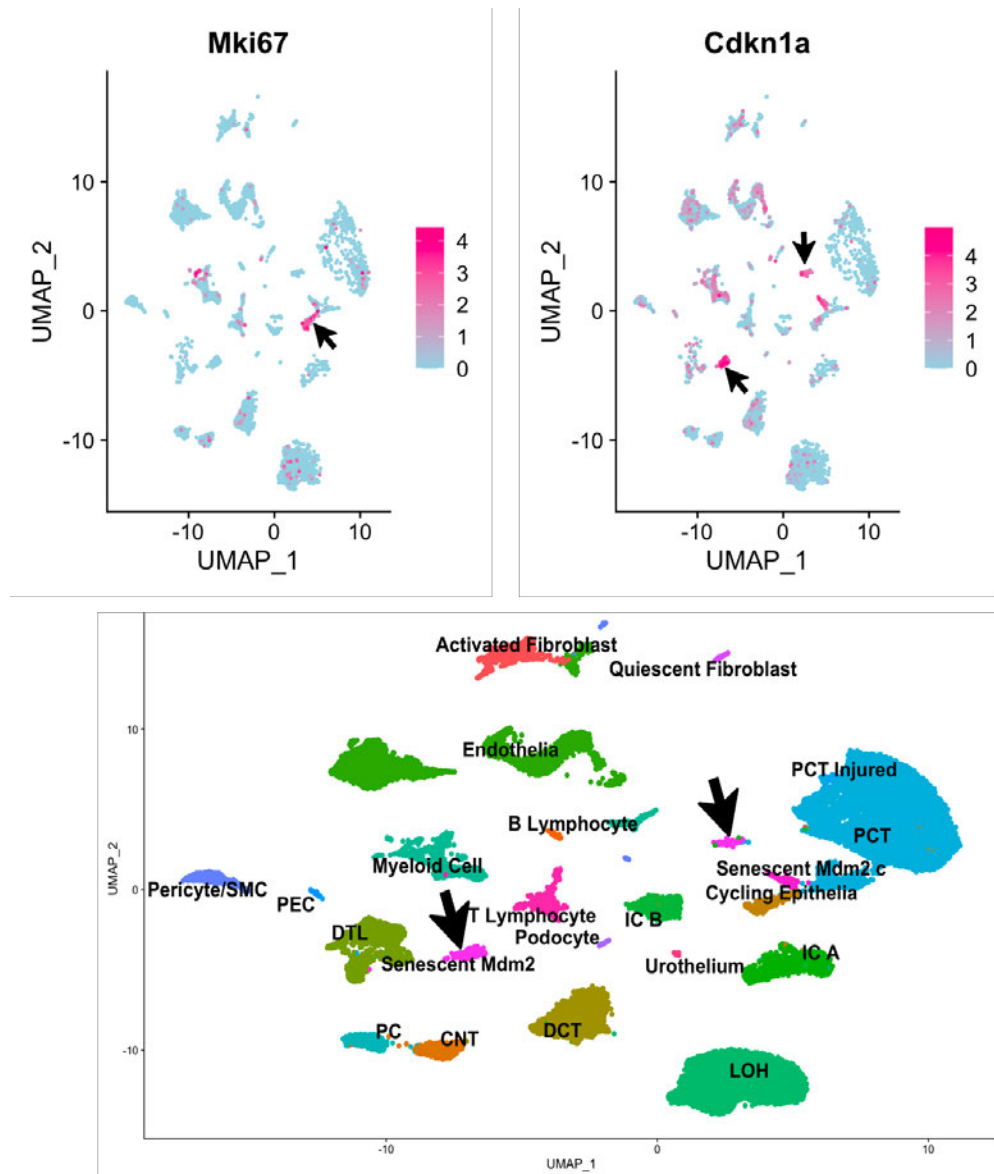


Figure 62 -*Cdkn1a* and *Mki67* expression UMAP

Cdkn1a and *Mki67* expression in an induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with tamoxifen 200mg/Kg x3 7 days prior to cell sorting. Whole kidney sort. Young female mouse. **Populations of interest are identified by a black arrow.** UMAP of all cell clusters is shown for reference.

SASP factors by scRNA sequencing

As shown in Figure 63, by differential gene expression analysis, the senescent cell populations identified in the induced Pax8CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse did not express classical SASP markers previously described in the literature including *IL6*, *Il1b*, *Ccl2* and *Tdffb1* but did express *Csf1* and *Gdf-15*. Figure 63 demonstrates that the highest expression of *Tgfb1* was seen in monocytes and macrophages.

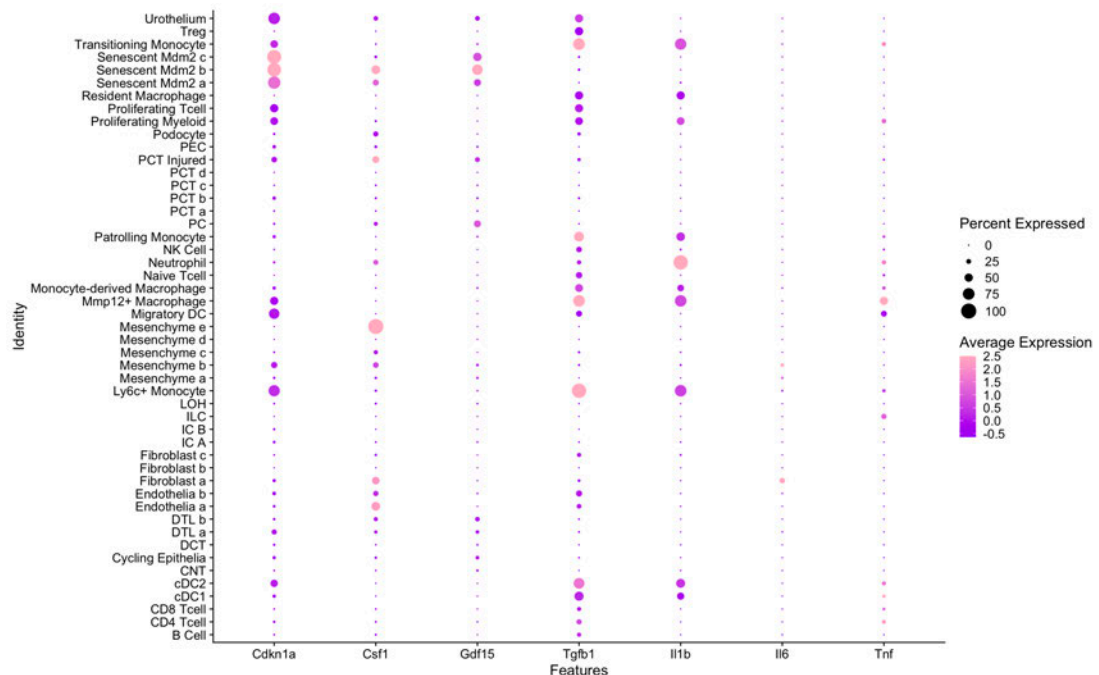


Figure 63 - SASP factors in an induced Pax8/mdm2/tdTomato mouse

Gene expression by cell cluster in an induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse. n=1. Young female mouse <6months. Whole kidney sort. *Csf1* and *Gdf15* are expressed more highly in senescent clusters than *Tgfb1*, *Tnf*, *Il1β* and *IL6*. 10X and Parse sequencing data.

In a widely recognised published database of key SASP factors termed the ‘SASP Atlas’, GDF15 was reported as being highly secreted. This occurred in different cell types (epithelial cells and fibroblasts) and in different modes of senescence induction; irradiation, oncogene-induced and drug-induced (Basisty, Kale et al. 2020).

***Mdm2* expression in senescent epithelia**

As shown in Figure 64, *Mdm2* was highly expressed in the senescent cell populations. As shown by the differential gene analysis lists included in Appendix B, it was the top differentially upregulated gene in both senescent populations.

In the *mdm2* fl/fl mouse, in cells which undergo recombination, a portion of the *Mdm2* gene between exons 5 and 6 is excised (Grier, Yan et al. 2002). This leads to the translation of a truncated dysfunctional MDM2 protein. MDM2 negatively regulates p53 either by binding to p53 and directly inhibiting its function or by targeting p53 for degradation (Vousden and Prives 2009, Moyer, Larsson et al. 2017). The deletion of exons 5 and 6 leads to a loss of most of the p53 binding region. Therefore, this truncated MDM2 protein can no longer negatively regulate p53. The levels of p53 therefore rise. However, within the promoter region of the *Mdm2* gene, which is located within intron 1 and not excised, there is a p53 binding domain (Lalonde, Ouimet et al. 2012, Moyer, Wasylshen et al. 2020). As the relationship is autoregulatory, higher levels of p53 lead to *Mdm2* upregulation. As shown below in Figure 65, genes related to the p53 signalling pathway were highly enriched in senescent epithelia.

The alignment for scRNA sequencing was upstream of exons 5 and 6. Hence scRNA sequencing detected both truncated and full-length transcripts and in cells which have undergone recombination, it was therefore still possible to detect high levels of upregulated truncated *Mdm2* transcript which occurred in response to upregulation of p53.

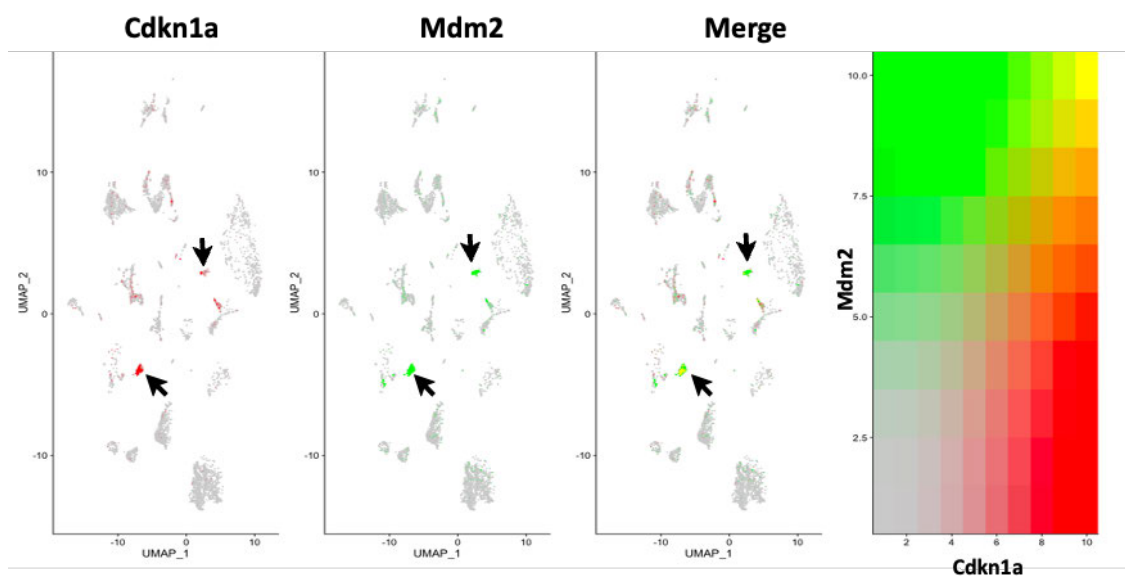


Figure 64-*Cdkn1a* and *Mdm2* expression

Cdkn1a and *Mdm2* expression in a Pax8Cre-ER^{T2}/*mdm2*^{flx/flx}/tdTomato^{LSL} mouse. Young, female <6months. Tamoxifen 200mg/Kg x3 7 days prior to cell sorting. Whole kidney sort. 10X and Parse sequencing data.

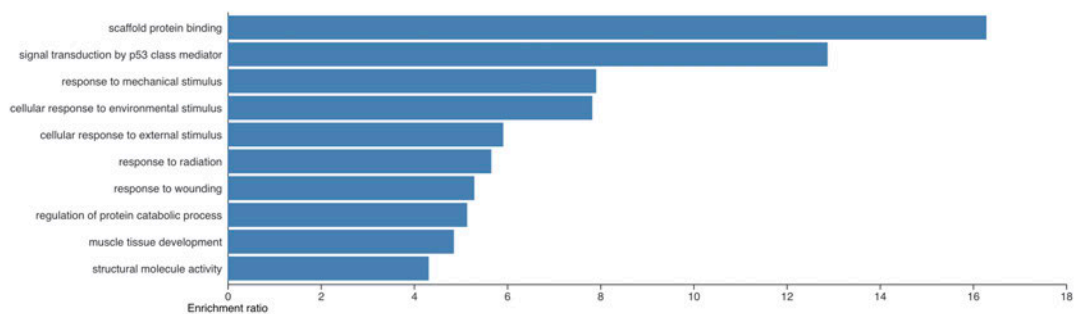


Figure 65 - Enrichment of p53 binding pathways

WebGestalt (WEB-based Gene SeT AnlaLysis Toolkit) enrichment analysis of pathways upregulated in Senescent *Mdm2* b.

The differential gene analysis lists for both senescent cell populations are found in Appendix B. After discussion with Maximillian Reck, I have included the top 200 upregulated genes per senescent population. In addition to *Cdkn1a*, genes which have previously been associated with senescence (O'Sullivan, Mylonas et al. 2022) such as *S100a6* (encodes S100A6) and *S100a11* (encodes S100A11), both recognised to regulate p21^{cip1} (Foertsch, Teichmann et al. 2013, Song, Xu et al. 2020) were upregulated. *Gdf15* (encodes GDF15; a recognised major SASP protein) was upregulated (Basisty, Kale et al. 2020).

Cryab and *Cd24* were also upregulated. Both have been identified in other single cell sequencing datasets and are suggested to be viable senolytic targets (Limbad, Doi et al. 2022, Doolittle, Saul et al. 2023). Senescent cells are recognised to upregulate anti-apoptotic genes (Zhu, Tchkonina et al. 2015). *Bcl2l1* was up-regulated as was *Nrg1* (NRG-1 β) which has been shown to prevent apoptosis in stressed cardiomyocytes (Kuramochi, Cote et al. 2004).

tdTomato mRNA expression vs Cdkn1a expression by single cell RNA sequencing

Figure 66 shows widespread tdTomato mRNA expression was observed in all mice and was detected in epithelial and non-epithelial cells in whole kidney sorts.

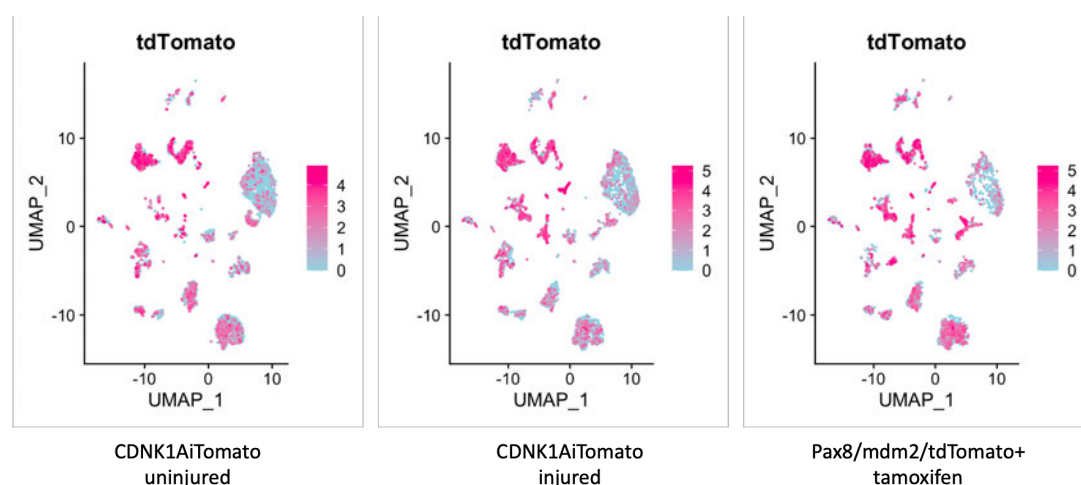


Figure 66 - tdTomato expression in whole kidney sorts

tdTomato expression by single cell RNA sequencing across mouse groups in whole kidney sorts where cells were sorted on live/dead only and not by specific cell populations. n=1 per group. Young female mice aged <6months, CDKN1AiTomato mice were treated with tamoxifen 200mg/Kg x2 48 and 72 hours prior to cull and the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse was treated with tamoxifen 200mg/Kg x3 7 days prior to cull. 10X and Parse sequencing data.

Figure 67 shows an expected depletion of endothelial cells and leucocytes which was due to the selection of CD31 and CD45 negative cells prior to epithelial cell gating with jacalin and LTL. In the remaining epithelial (jacalin and LTL+) and CD90+ cell populations, tdTomato mRNA expression was widespread.

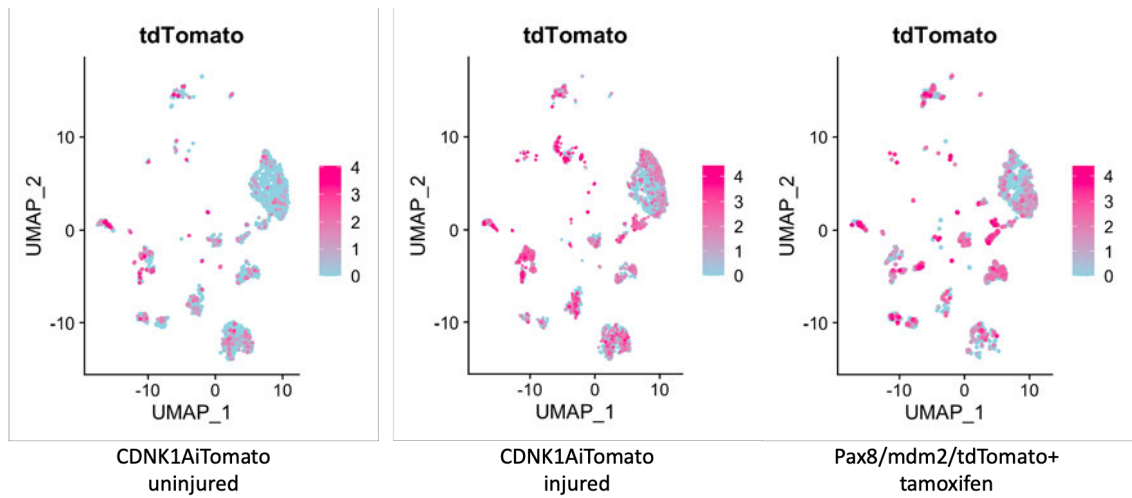


Figure 67 - tdTomato expression in epithelial cell population and CD90+ cell population only sorts

tdTomato expression by single cell RNA sequencing in mice that underwent population-based sorting excluding CD31+ cells (endothelial) and CD45+ cells (immune cells) and were sorted as jacalinLTL+/tdTomato+ cells (tdTomato positive epithelial cells) and CD90+ cells. 10X and Parse sequencing data.

tdTomato transcript appeared more widely expressed than *Cdkn1a* in both the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse and the CDKN1AiTomato mouse as shown in Figure 68 and Figure 69 respectively. tdTomato mRNA is also observed in non-Pax8 expressing cells as shown in Figure 68.

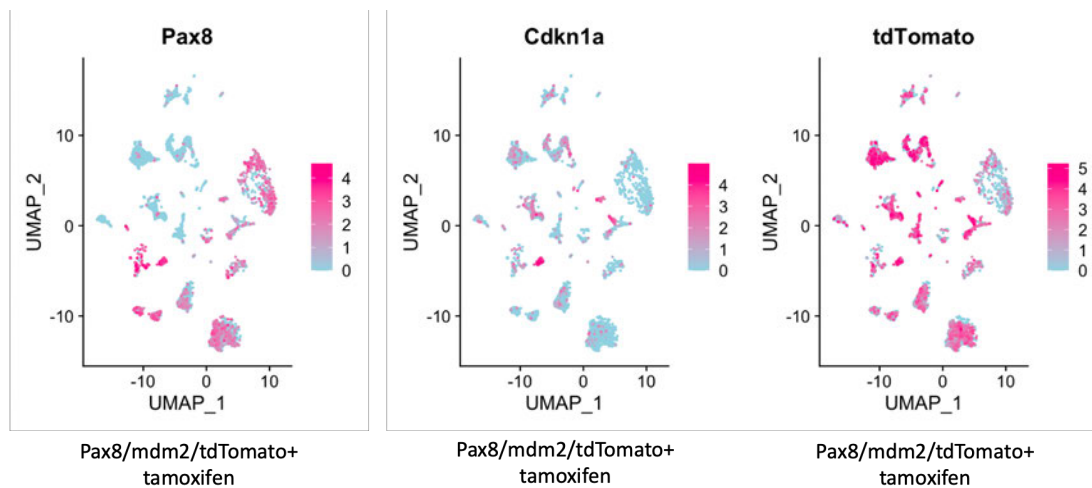


Figure 68 - Pax8, Cdkn1a and tdTomato expression in an induced Pax8/mdm2/tdTomato mouse

tdTomato expression by single cell RNA sequencing in an induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse in whole kidney sorts where cells are sorted on live/dead only and not by cell populations. n=1. Young female mouse aged <6 months. Tamoxifen 200mg/Kg x3 given 7 days prior to sorting. 10X and Parse sequencing data.

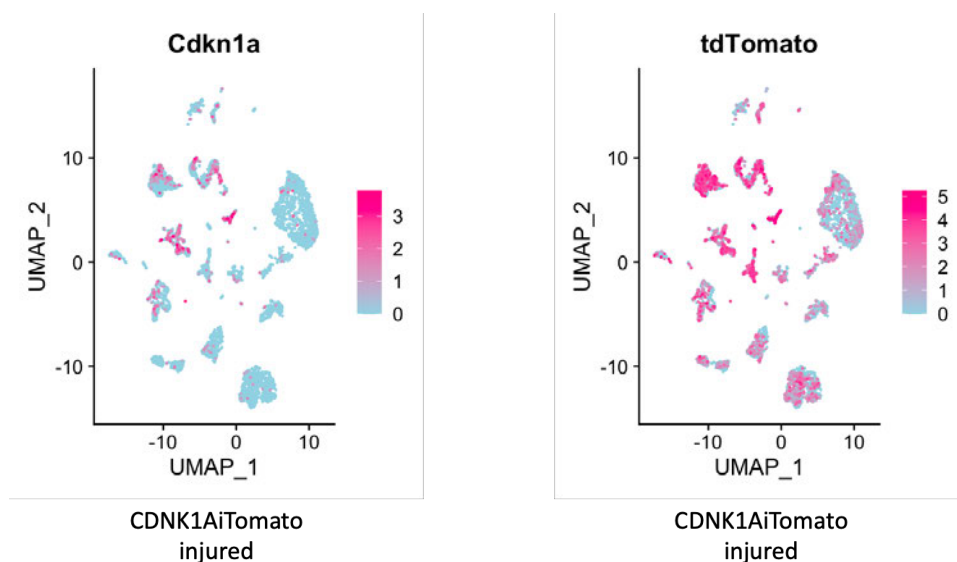


Figure 69 - tdTomato vs Cdkn1a expression in an injured CDKN1AiTomato mouse treated with tamoxifen

Cdkn1a and tdTomato expression by single cell RNA sequencing in an injured CDKN1AiTomato mouse. n=1. Young female mouse aged <6 months. IRI 7 days prior to cell sorting. Tamoxifen 200mg/Kg x2 given day 4 and day 5 after injury, thus 48 and 72 hours prior to cell sorting. 10X and Parse sequencing data.

***Cdkn1a* expression by qPCR in tdTomato positive sorted cells**

Figure 70 shows that *Cdkn1a* expression was significantly higher in tdTomato positive sorted cells from induced transgenic mice when compared to epithelial cells which were considered tdTomato negative at FACS.

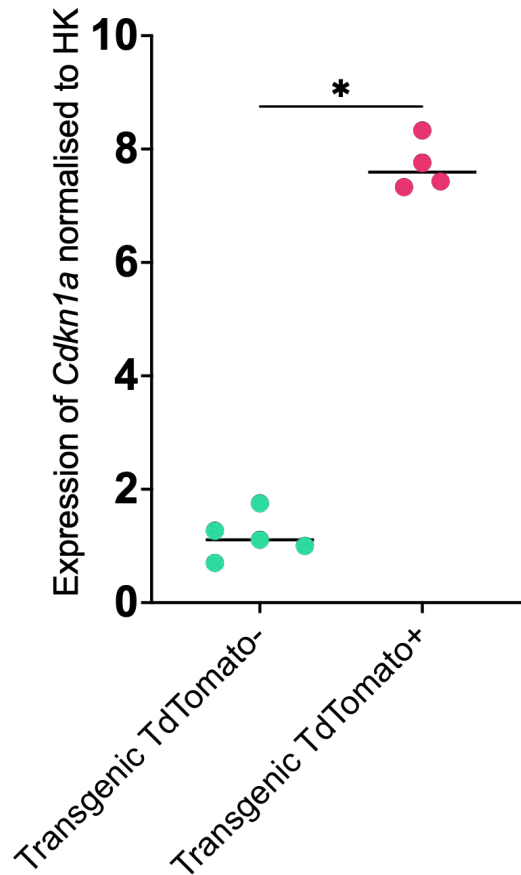


Figure 70 -*Cdkn1a* expression in tdTomato positive cells by qPCR

Expression of *Cdkn1a* relative to housekeeper gene (*Hprt*) in sorted cells. Transgenic tdTomato+: tdTomato positive cells sorted from an induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse. Transgenic tdTomato- cells are epithelial cells- (JacalinLTL+ but not positive for tdTomato) sorted from an induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse. n=4-5 per group. Young female mice aged <6months treated with tamoxifen 200mg/Kg x2 7 days prior to cell sorting. Error bars are mean ± SEM. Mann-Whitney. *p<0.05 (p=0.015).

tdTomato mRNA expression by in-situ hybridisation

In the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse the LoxP sites flank exons 5 and 6 of the native *mdm2* gene (Grier, Yan et al. 2002). Therefore, should recombination occur, this transgenic strain of mouse is still capable of producing a truncated protein which lacks the key p53 binding domain. Unless specifically targeting a genetic sequence contained within those Lox-P binding sites, it would be possible to detect a shortened *mdm2* transcript by ISH. This would give rise to unreliable interpretation of the relationship between both *Cdkn1a* and *mdm2*, and tdTomato and *mdm2*.

To counteract this potential confounder, I used an *in-situ* hybridisation probe which specifically binds to a genetic region contained within exons 5 and 6 of the *mdm2* gene. Thus, detection of *mdm2* by this specific probe means that exons 5 and 6 remain intact and recombination has not occurred. In the images presented below, I refer to this detected transcript as '*mdm2* full length'.

Figure 71 shows that tdTomato mRNA is easily observed across both the renal cortex and the medulla by *in-situ* hybridisation.

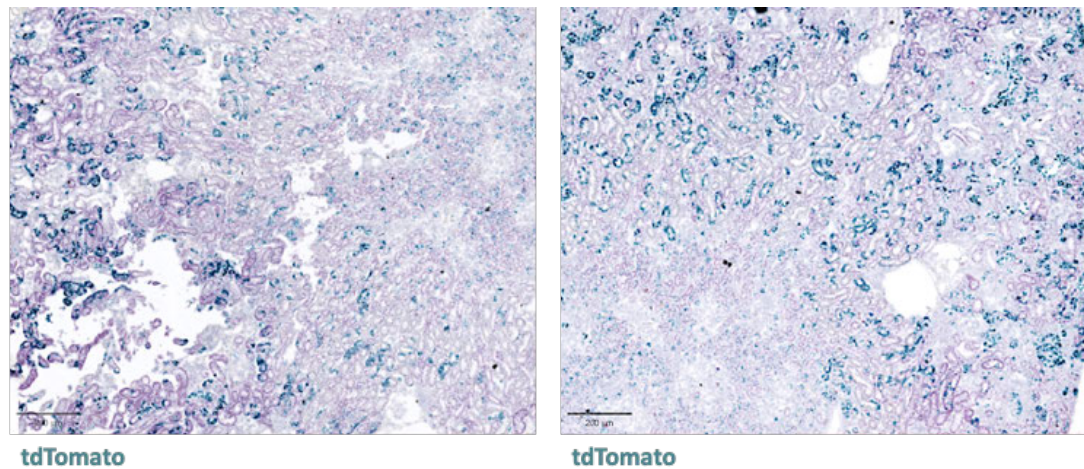


Figure 71 -tdTomato mRNA transcripts in an induced Pax8/mdm2/tdTomato mouse.

tdTomato mRNA by *in-situ* hybridisation. tdTomato mRNA appears in teal and appears visible in the renal cortex and medulla. n=2. Young female mice aged <6 months. Treated with tamoxifen 200mg/Kg x2 7 days prior to cull.

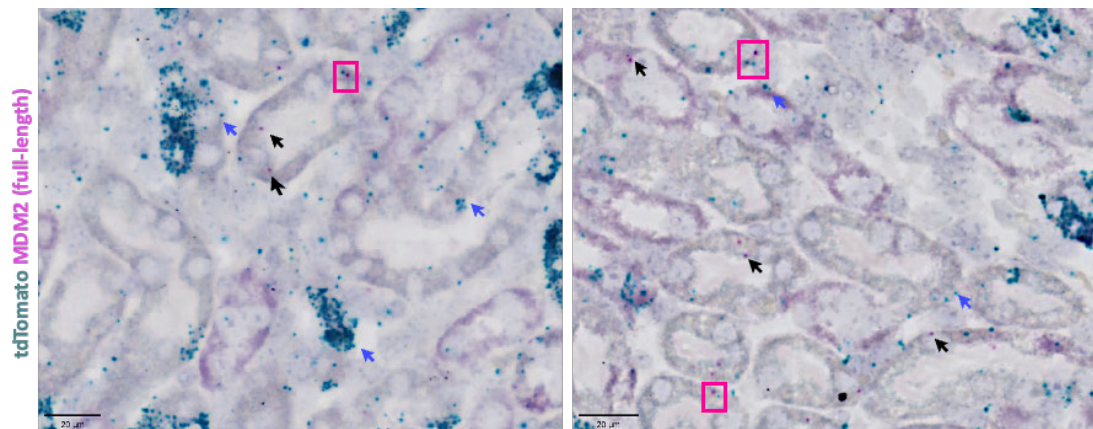


Figure 72 -tdTomato and full length mdm2 transcripts in an induced Pax8/mdm2/tdTomato mouse.

tdTomato and full length mdm2 transcripts by *in-situ* hybridisation. n=2. Young female mice aged <6 months. Tamoxifen 200mg/Kg x2 7 days prior to cull. Full length mdm2 appears in dark pink and some examples are highlighted with black arrows. tdTomato appears in teal and is highlighted by blue arrows. The pink boxes show both transcripts detected within the same cell.

The two images at higher magnification presented in Figure 72 are taken from two different mice. These images show that tdTomato mRNA expression was easily visible

in renal tubules and appeared to be expressed at a high level. The majority of cells which expressed tdTomato did not appear to concomitantly express full length *mdm2* and *vice-versa* where full-length *mdm2* transcripts were seen, these cells did not express tdTomato. There were some instances where both transcripts were detected within the same cell and these cells are highlighted with a pink box. Where both transcripts co-localised, it appeared to be in cells with a single copy of the tdTomato transcript suggesting that these were not cells with a highly active CAG promoter and high tdTomato expression. The disconnect in this expression pattern is discussed in the discussion to this chapter, and in the final discussion in chapter 6.

Owing to the background variability within this imaging dataset, which is apparent from the images presented here, I was unable to proceed with computerised quantification using QUPath and so caution is required in the interpretation.

tdTomato protein expression by immunofluorescence

Figure 73 shows that tdTomato fluorescence was restricted to the kidney and not seen in other organs in an induced transgenic mouse.

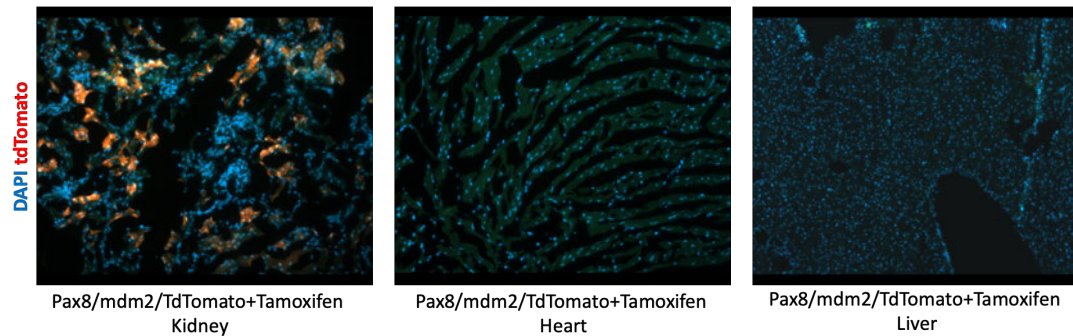


Figure 73 -tdTomato epifluorescence across organs

tdTomato expression in an induced Pax8/mdm2/tdTomato mouse. Young female, aged <6 months of age. Tamoxifen 200mg/Kg x3 7 days prior to cull. Frozen samples stained only with DAPI.

Discussion

The Pax8-CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse is a novel transgenic mouse strain allowing conditional induction of renal epithelial-restricted senescence.

Expression of classical markers of senescence; p21^{cip1} and *Cdkn1a*

I observed a spread of cortical p21^{cip1} nuclear positivity (1.05% to 29% p21^{cip1} positive nuclei of all nuclei measured per representative area of renal cortex). This may represent inherent biological variability within a transgenic system. It is unlikely that all mice will manifest an identical level of recombination. Mice may differ in their ability to metabolise tamoxifen to its active metabolite 4-OHT and will hence appear to respond differently to the same dose of tamoxifen. Whilst care was taken to dose mice consistently with the correct weight-adjusted dose, it remains possible that some received different amounts of tamoxifen.

The single cell RNA sequencing data demonstrated two populations of senescent epithelia in induced Pax8-CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice. These have been annotated Senescent Mdm2 'a' and 'b'. As shown in Figure 56, Senescent Mdm2 a appeared to originate from the proximal tubule. This population expressed classical proximal tubular markers such as *Slc5a2* and *Slc13a3* (Park, Shrestha et al. 2018, Lu, Liao et al. 2021). Senescent population Mdm2 b appeared to originate from the thin descending loop of Henle and expressed markers such as *Aqp1* and *Bst1* (Park, Shrestha et al. 2018, Lu, Liao et al. 2021).

It is not clear why both injured CDKN1AⁱTomato mice did not have any obvious senescent populations identified. It is worth noting that both were female, and it is recognised that female mice display greater tolerance of IRI (Park, Kim et al. 2004). This is one possibility for the absence of any senescent population. It would be of significant interest, if a shared population of senescent cells did exist between these two mouse groups because they have undergone different modes senescence

induction and as discussed, the mode of induction is recognised to markedly influence the behaviour of senescent cells (Coppe, Desprez et al. 2010).

To investigate this in more detail, I have completed an experiment in male mice, comparing induction with IRI vs transgenic induction with group sizes which will allow statistical comparison at a single cell level at different timepoints and as shown below in Figure 74, mouse weights in injured vs non-injured kidneys by day 14 and day 42 post injury were significantly different. Reduced kidney weight post IRI is well recognised and thought to be due to ischaemia induced tubular atrophy. (Lee, Jang et al. 2022). These kidneys therefore are likely to have undergone sufficient damage to allow a fair comparison between both methods of senescence induction; injury vs loss of *mdm2*. I used male mice to mitigate any confounding effect conferred by gender on response to injury. This also provides a mixed gender dataset of significant size to generate statistically robust analyses in both genders. Further analysis of this dataset by scRNA sequencing is underway, but the results are not available to me at the time of writing this thesis.

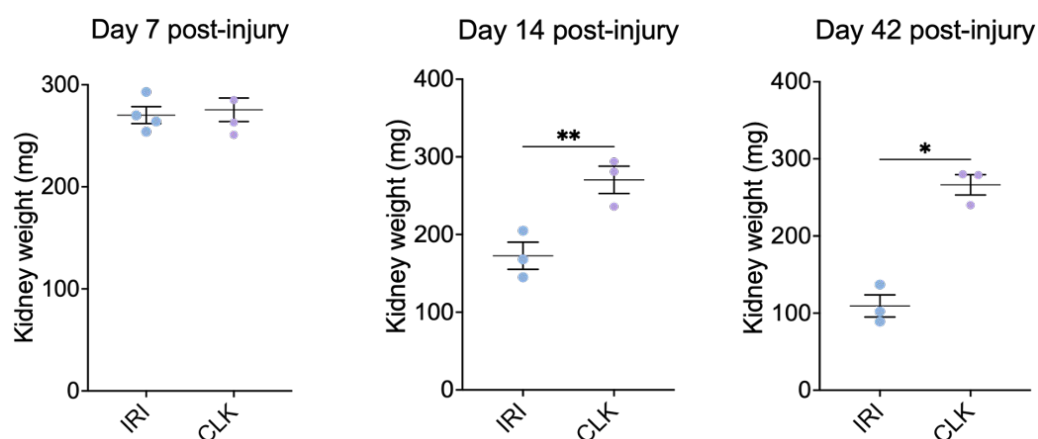


Figure 74 -Kidney weights in male mice subjected to ischaemia reperfusion over time

Contralateral and IRI kidney weights in male mice over time. n=3-4 per group. Young male CDKN1AiTomato mice aged <6 months of age. Unpaired t-test. *p<0.05, **p<0.01. Error bars are mean $\pm$ SEM.

Medullary p21^{cip1} expression

I did not observe a significant difference in medullary levels of p21^{cip1}. One explanation for this is that the renal medulla predominantly comprises the loop of Henle and its supporting structures and the collecting ducts. Given that these tubular epithelial cells of the loop of Henle do not express the highest levels of *Pax8*, it would be reasonable to expect to see less cre-mediated loss of *mdm2* and hence less p21^{cip1} in cells not expressing the promoter of interest (*Pax8*).

I observed high levels of *Pax8* expression in the descending limb of the loop of Henle and notably, one of the senescent cell populations observed in the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse appeared to originate from the thin descending limb. A significant portion of this anatomical structure (thin descending limb) remains within the renal cortex, hence why senescent cells belonging to this population might still be observed at a protein level by p21^{cip1} in the renal cortex.

It remains possible that with higher group numbers I might have observed a difference, or a trend towards a difference. The levels of p21^{cip1} medullary nuclear positivity in the induced transgenic mouse group were $5.3 \pm 2.5\%$ vs $1.84 \pm 0.9\%$ in wild-type mice treated with tamoxifen, vs $1.4 \pm 0.8\%$ in transgenic mice treated with vehicle.

I observed low levels of Ki67 expression in general and I observed a clear distinction between cells that express p21^{cip1} and Ki67. This is supported by the scRNA sequencing data which demonstrated clearly that the epithelial senescent cell populations identified did not co-express *Mki67*.

SASP factors

An interesting finding observed in the data presented in this chapter is that the senescent cell populations seen in the Pax8-CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse did not appear to upregulate 'typical' SASP genes. However, despite this lack of 'typical' SASP, induction of senescence in the Pax8-CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse resulted in immune cell recruitment.

The expression of *Csf1* and *Gdf15* are of interest. CSF1 is a potent macrophage attractant chemokine (Pixley and Stanley 2004). Up-regulation of *Csf1* is of interest for several reasons. Firstly, because SASP-mediated immune cell recruitment is known to contribute to tissue dysfunction and paracrine senescence (Bird, Muller et al. 2018). One of the key questions of this PhD was to define the role of senescent cells in propagating tissue dysfunction and fibrosis and to ascertain if this is a direct senescent cell to mesenchymal cell effect or if this involves an intermediary cell type i.e., senescent cell to immune cell.

Secondly, there is an emerging literature on immune-mediated senescent cell clearance with macrophage and ensuing cytotoxic T-cell clearance recognised to be vital components of this clearance process. (Sturmlechner, Zhang et al. 2021)

Growth differentiation factor 15 (GDF-15) is a stress response cytokine that recognised to effect macrophage polarisation (Zhang and Dong 2022), and is strongly associated with ageing and chronic disease (Fuchs, Trollor et al. 2013, Wollert, Kempf et al. 2017). GDF-15 is also strongly associated with senescence, (Wiklund, Bennet et al. 2010, Wan and Fu 2023) and is described as one of the core SASP proteins in humans (Basisty, Kale et al. 2020).

Immune cell recruitment in conjunction with the induction of cellular senescence is a topic which will be discussed in the following results chapters; both with respect to the development of fibrosis, and senescent cell clearance.

It is vital to state that the analysis of SASP factors presented within this thesis was derived solely using scRNA sequencing. This observed intracellular expression does not necessarily denote secretion and therefore 'action' of these factors. Therefore, to make a more complete assessment of the SASP, it will be vital to correlate key gene expression observed by scRNA sequencing at a secreted protein level; i.e. by immunohistochemistry.

Mdm2 expression in senescent epithelia

Mdm2 was highly expressed by senescent epithelial cell clusters. Whilst *Trp53* itself was not differentially expressed, associated genes such as *Trp53inp1* and *Trpbbp1* were and as shown in Figure 65, p53 binding pathways were highly enriched. *Trp53inp1* encodes Trp53 inducible nuclear protein 1, a recognised target of p53 (Shahbazi, Lock et al. 2013). *Trpbbp1*, encodes Trp53 binding protein 1, a key tumour suppressor protein and DNA repair protein which binds to p53 (Cuella-Martin, Oliveira et al. 2016). The relationship between p53 and MDM2 is autoregulatory and as discussed, p53 strongly upregulates *Mdm2*. The truncated *Mdm2* transcript retains a p53 promoter region (Moyer, Wasylshen et al. 2020) and the alignment for the *Mdm2* gene was upstream of the excised exon5/6 region, hence a the truncated transcript was still detected and the reason for the high expression of this transcript is explained by p53 upregulation. *Mdm2* has been identified as a major p53 target gene in other sequencing datasets which have used the *mdm2* fl/fl mouse (Moyer, Wasylshen et al. 2020).

tdTomato expression by flow cytometry

I also observed a spread of tdTomato expression (9.4% to 50% of proximal and distal renal epithelial cells) at flow cytometry. This may be explained in part by the same mechanisms as those described above in relation to the range of p21^{cip1} expression.

An important caveat to these data is that when imputing the genotype of these mice, I did not specify if they were heterozygous or homozygous for the tdTomato

construct. The rationale behind this omission was that from very early rounds of cross breeding this Pax8-CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} strain, I knew that heterozygous mice robustly expressed tdTomato as I had consistently observed this at flow cytometry and thus, I could use either heterozygous or homozygous mice for the purposes of my experimental questions. However, this would be a potential explanation as to why some mice seemingly have much higher levels of tdTomato expression. This is not the case for the spread of p21^{cip1} expression as all mice used were known to be homozygous for LoxP flanking of *mdm2*.

I have been purposefully rigorous with my gating for what I have considered a tdTomato positive cell. I appreciate that the FMOs were consistent and would have allowed me to gate more liberally but as these cells were sorted for the purposes of downstream analysis looking for markers of senescence, I wanted to be sure that I sorted cells which I was confident expressed tdTomato. A more liberal sorting approach might have resulted in sorted cells appearing to be weakly fluorescent and not truly having recombined and therefore being non-recombined, non-senescent healthy epithelia. This would have confounded further analysis as would have introduced a healthy background population.

The disconnect between cre-mediated excision of *mdm2* vs cre-mediated tdTomato mRNA expression

The recombination efficiency of the *Pax8*CreER^{T2}/*mdm2*^{flox/flox}/tdTomato^{LSL} mouse is not known and despite several experimental attempts across different experimental modalities, I was unable to define this. The data generated remain conflicted. The single cell RNA sequencing data showed that tdTomato mRNA was expressed in cells not expressing *Pax8*. The reasons for this are not clear.

As discussed earlier, the *Rosa26* locus is active in most cells, and is not subject to gene silencing. One possible explanation therefore for widespread tdTomato expression in absence of cre-recombination would be a scenario where the entire CAG promoter, STOP codon mRNA and tdTomato mRNA were transcribed at baseline. In such a scenario, this transcribed section of mRNA would not be translated to protein as this full transcript encodes a STOP codon which would halt translation. Following recombination this STOP codon would be lost and a truncated version of the transcript would exist, minus the STOP codon, which would allow translation of the tdTomato protein. The tdTomato sequences used at scRNA sequencing are aligned to the mRNA of the actual tdTomato sequence, and not to any other part of the construct i.e., not to the LoxP sites or the STOP codon.

We directly approached the senior author of the original publication of the Ai14Ho mouse, Professor Hongkui Zeng. Through direct correspondence with her she explicitly stated that “in the absence of cre-mediated recombination, the mRNA transcripts are truncated within the STOP cassette and do not contain the tdTomato sequence”. This is clearly in contrast to what we observed at the single cell level.

The interpretation of the *in-situ* hybridisation data is challenging because the assay requires further optimisation to allow robust quantification. However, it was clear from the images generated that tdTomato was again widely detected. Through direct correspondence with the company who provided me with this probe (ACD-Bio), I

confirmed that the binding sites for the tdTomato probe are between base pairs 13981 and 14161. When I correlated this with the genetic sequence of the tdTomato construct, published in the original paper (Madisen, Zwingman et al. 2010) and confirmed by personal correspondence with the senior author, these base pairs that the *in-situ* probe binds to are entirely contained within the tdTomato mRNA sequence of the construct.

Therefore, *in-situ hybridisation* and scRNA sequencing are potentially subject to the same detection issue regarding tdTomato mRNA; that if transcription is occurring in the absence of cre-mediated recombination (and only halted at the point of translation), then the transcription of tdTomato mRNA does not represent a meaningful assessment of recombination.

However, as discussed, the Rosa26 locus is active in almost all cells. Therefore, were this to be the case, transcription would be occurring in virtually all cells. This is not what is observed. There are clearly cells within the kidney and indeed outside of the kidney that do not contain the tdTomato transcript.

Once cre-mediated recombination occurs, the CAG promoter is constitutively active. Therefore, a final potential explanation for the data would be a scenario where temporary expression of the promoter of interest e.g., *Cdkn1a* led to recombination. This would explain cells which had not undergone senescence but had instead temporarily expressed *Cdkn1a* as being marked as tdTomato positive and this would provide some explanation in the CDKN1AiTomato mouse. This is less easy to reconcile in the setting of the Pax8CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse as *Pax8* is confined almost exclusively to renal epithelia and tdTomato mRNA expression was clearly seen in non-epithelial cells.

The relationship between tdTomato expression by protein and induction of senescence

tdTomato expression as assessed by flow cytometry is that of the tdTomato protein and therefore represents true tdTomato labelled cells. When I extracted RNA and looked for *Cdkn1a* by qPCR in these cells I knew were expressing tdTomato, I found an approximately 8-fold higher level of *Cdkn1a* in tdTomato positive vs negative cells.

Whilst this is suggestive that the individual genetic elements (loss of *mdm2* and expression of tdTomato) recombine at a similar level, these results should be interpreted with caution. Although the group sizes allow for statistical comparison, they are small; $n = 4-5$. I routinely sorted cell numbers of minimum 100,000 per population but it is recognised that cells undergoing sorting encounter stress and show reduced viability which is likely to impact on the yield of RNA (Nishimoto, Newkirk et al. 2007). I observed consistently low RNA yields, not always sufficient to allow conversion to cDNA. This significantly reduced my group sizes, and therefore the statistical power, and when I did have sufficient RNA to investigate this sorted cell population by qPCR, the low yield of RNA meant that I was restricted in the number of individual genes I could assess. Given the option of higher RNA yields, I would have extended this panel of genes to include other markers of senescence.

One finding which is at odds with this significantly higher level of *Cdkn1a* seen in sorted cells, is that when cells expressing tdTomato by flow cytometry were assessed by scRNA sequencing, the vast majority of the tdTomato positive sorted cell population did not express any markers of senescence. The same three senescent populations are seen in the Pax8CreER^{T2}/mdm2^{flox/flox}/tdTomato^{L^{SL}} mouse with sorting of live cells only vs tdTomato positive cells and sorting a specific population of tdTomato positive cells did not result in an enriched senescent cell population.

From the data generated by FACS, by immunofluorescence and by *in-situ* hybridisation, the most likely explanation for the current discord in expression levels

is that the tdTomato construct recombines at a much higher efficiency than the *mdm2* LoxP sites.

This would offer a potential explanation as to why when sorting tdTomato positive cells, many of these have not undergone recombination mediated loss of *mdm2* and are therefore not senescent.

Furthermore, the data I have presented showing p21^{cip1} upregulation in the cortex at day 7 have predominantly been derived from mice I also examined by flow cytometry, and I knew to express tdTomato. Whilst the induction of cortical p21^{cip1} in transgenic mice treated with tamoxifen was statistically significant when compared to all other control groups, there were three transgenic mice contained within this dataset that exhibited relatively low levels of cortical p21^{cip1} positive nuclei (1.05%, 1.3% and 3.7% respectively). For two of these mice, those with levels of 1.3% and 3.7%, the levels of tdTomato expression at flow cytometry were 50.9% tdTomato PTECs and 17.2% tdTomato proximal and distal tubular epithelial cells respectively. This would support a more efficient recombination of the tdTomato construct vs the loss of *mdm2* but clearly substantially bigger group sizes are required to make a valid statistical comparison.

Looking to a similar liver model which used the cholangiocyte promoter K19; (K19/*mdm2*^{flox/flox}/tdTom^{LSL}) around 40% of cholangiocytes expressed tdTomato 2 days after tamoxifen induction. Cholangiocyte p21^{cip1} expression was higher at just under 60%. Some cells expressed p21^{cip1} in the absence of tdTomato, and these were suggested to be secondary senescent cells (Ferreira-Gonzalez, Lu et al. 2018).

Future experiments

From the data generated, a further strategy to define individual recombination efficiencies would be to pursue a protein-based approach. For example, running a dual p21^{cip1} and tdTomato immunofluorescence with group sizes adequate to

perform robust statistical analysis. This would still have the caveat that both antigens might differ in their ease of detection, but it would mitigate the potential transcription to translation factor posed by the single cell and *in-situ* data.

Another option would be to look with scRNA sequencing and *in-situ* hybridisation at uninduced full transgenic Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice. If tdTomato is transcribed at baseline and translation only halted in the absence of a STOP codon then tdTomato transcript should be detected in uninduced transgenic mice.

To investigate if a truncated tdTomato transcript is made at baseline containing the tdTomato sequence but not the LoxP-STOP-LoxP sequence I would use an *in-situ* hybridisation probe which is custom made to bind within the LoxP-STOP-LoxP region of the tdTomato gene. In cells which have undergone recombination, I would not expect to find this region detected but I would expect *Cdkn1a* transcript.

Conclusions

Tamoxifen treatment in Pax8-CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice resulted in significant cortical nuclear p21^{cip1} expression. The majority of cells which expressed p21^{cip1} did not concomitantly express Ki67. scRNA sequencing data identified two populations of senescent epithelia in induced Pax8-CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice. Transgenic induction led to immune cell recruitment. Further work is required to confirm the accuracy of tdTomato labelling of cells which have undergone cre mediated *Mdm2* excision.

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Chapter 4: Renal fibrosis and immune cell recruitment at an early timepoint after transgenic senescence induction

Introduction

A key aim of this PhD was to determine if epithelial senescence was sufficient to induce fibrosis in the absence of injury and ageing.

The data in the previous results chapter demonstrated that tamoxifen treatment in transgenic, but not wild-type mice, led to a significant increase in cortical nuclear p21^{cip1} expression, an absence of cell proliferation, and distinct populations of senescent epithelia identified by scRNA sequencing.

To test if such transgenic induction by tamoxifen was sufficient to induce fibrosis in the absence of injury in young female mice, I used the experimental design outlined below in Figure 75.

Experimental design

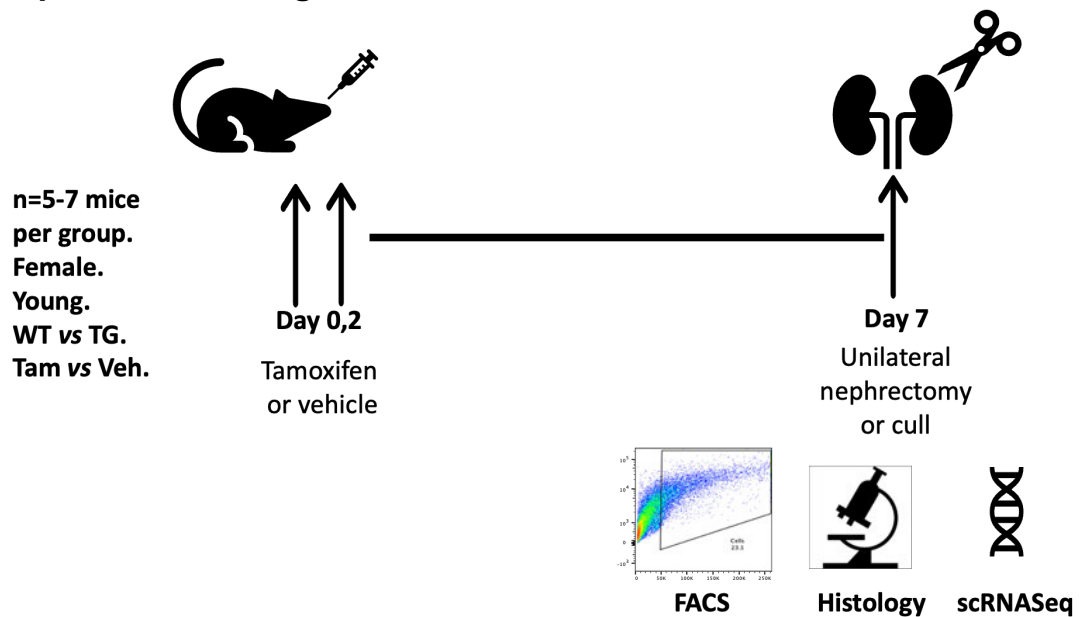


Figure 75 -Experimental schematic to assess fibrosis

Experimental design used to test if transgenic induction at an early timepoint was sufficient to induce fibrosis. Mice were grouped into four experimental groups: WT+vehicle, WT+tamoxifen, TG+tamoxifen, TG+vehicle. At day 7 mice underwent unilateral nephrectomy or cull. Tissue was taken for histological analysis, for flow cytometry and cell sorting followed by downstream scRNA sequencing analysis.

Female mice, under 6 months of age received a standard dose of tamoxifen 200mg/Kg twice by oral gavage on alternate days. Fibrosis was assessed using immunohistochemistry and scRNA sequencing data was used to identify genes associated with inflammation and fibrosis.

Chapter aims and hypotheses

Aims:

- 1) To describe the effect of transgenic induction of renal epithelial senescence on the initiation of fibrosis at an early timepoint in the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse.
- 2) To describe the effect of transgenic induction of renal epithelial senescence on immune cell recruitment at an early timepoint in the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse.

Hypothesis:

- 1) Transgenic induction of renal epithelial senescence *via* loss of MDM2 in Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice would be sufficient to induce immune cell recruitment and renal fibrosis at an early timepoint in the absence of any other renal insult.

Results

Cortical fibrosis at an early timepoint

Transgenic mice treated with tamoxifen demonstrated significantly higher levels of cortical fibrosis by day 7.

Figure 76 shows collagen I expression by immunofluorescence and shows that collagen I expression was three times higher in transgenic mice treated with tamoxifen vs all other control groups: $3.5 \pm 0.5\%$ vs $1.1 \pm 0.3\%$ vs $1.04 \pm 0.3\%$ vs $1.0 \pm 0.2\%$ (TG+tamoxifen, WT+tamoxifen, WT+vehicle, TG+vehicle respectively).

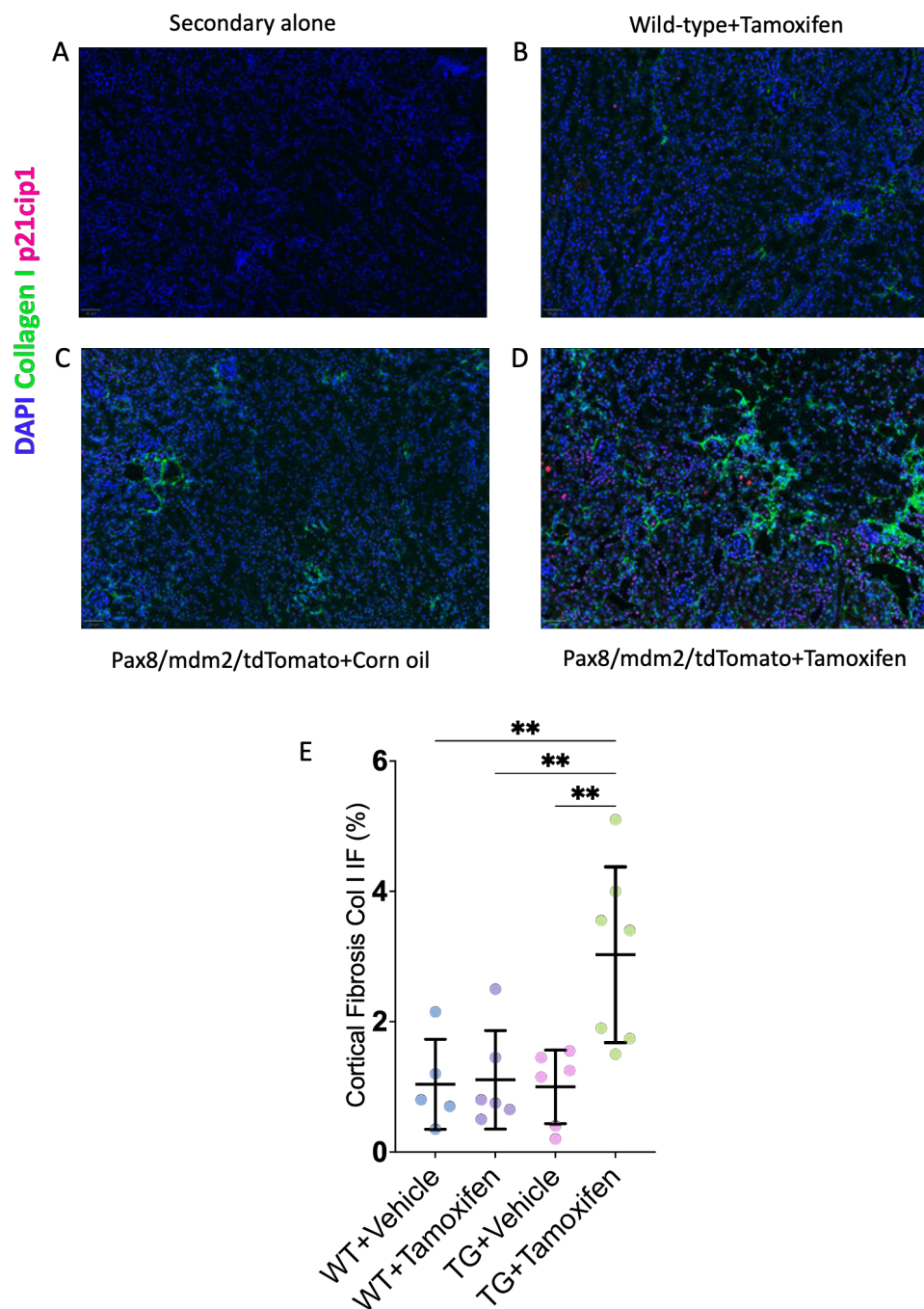
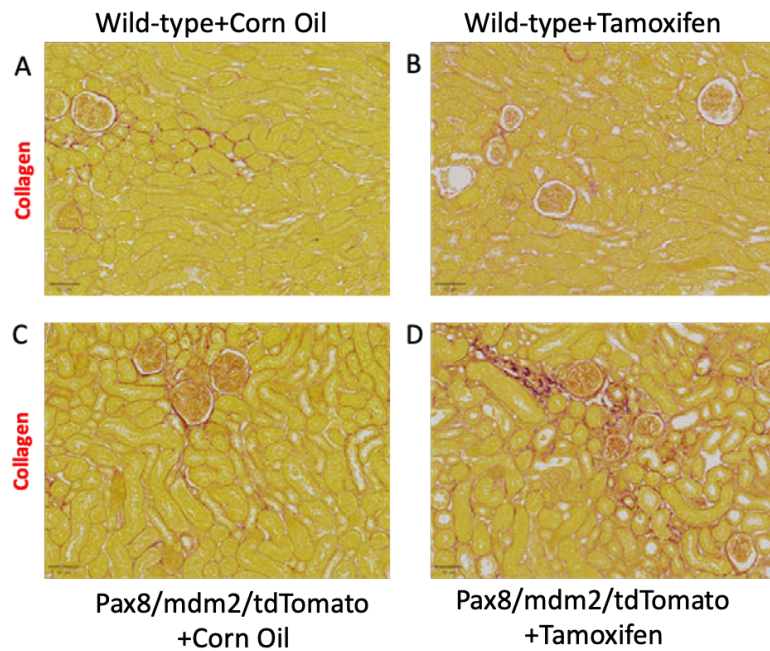


Figure 76 -Cortical collagen I expression at an early timepoint

Immunofluorescence images showing collagen I expression in the renal cortex. A) Secondary alone control. B) Wild type +tamoxifen. C) Pax8/mdm2/tdTomato+corn oil. D) Pax8/mdm2/tdTomato+ tamoxifen. Dual immunofluorescence staining, collagen I appears green, p21^{cip1} appears deep pink. Tamoxifen 200mg/Kg x2. Young female mice. E) Cortical collagen I expression across groups. n=5-7 per group. Tamoxifen 200mg/Kg x2. One-way ANOVA with Tukey's multiple comparisons test. All error bars are mean ± SEM, **p<0.01.

I used total collagen as a second marker of fibrosis. Figure 77 shows that cortical fibrosis when assessed by PiSR staining for total collagen was also significantly higher in transgenic mice treated with tamoxifen when compared to wild-type mice treated with tamoxifen or corn oil. There was a trend to higher levels of cortical fibrosis when compared to transgenic mice treated with corn oil ($p=0.06$).



E Cortical Fibrosis by PiSR Day 7

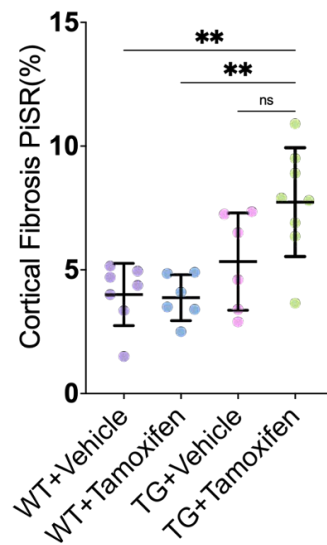


Figure 77 -Cortical fibrosis by PiSR at an early timepoint

PiSR images demonstrating total collagen staining in the renal cortex 7 days after treatment with tamoxifen or vehicle. A) Wild-type+corn oil. B) Wild-type+tamoxifen. C) Pax8/mdm2/tdTomato+corn oil. D) Pax8/mdm2/tdTomato+tamoxifen. Collagen appears red. Tamoxifen 200mg/Kg x2. E) Cortical collagen expression across mouse groups 7 days after treatment with tamoxifen vs vehicle. n=6-8 per group, female mice aged <6 months treated with tamoxifen 200mg/Kg x2. One-way ANOVA with Tukey's multiple comparisons test. All error bars are mean $\pm$ SEM, **p<0.01.

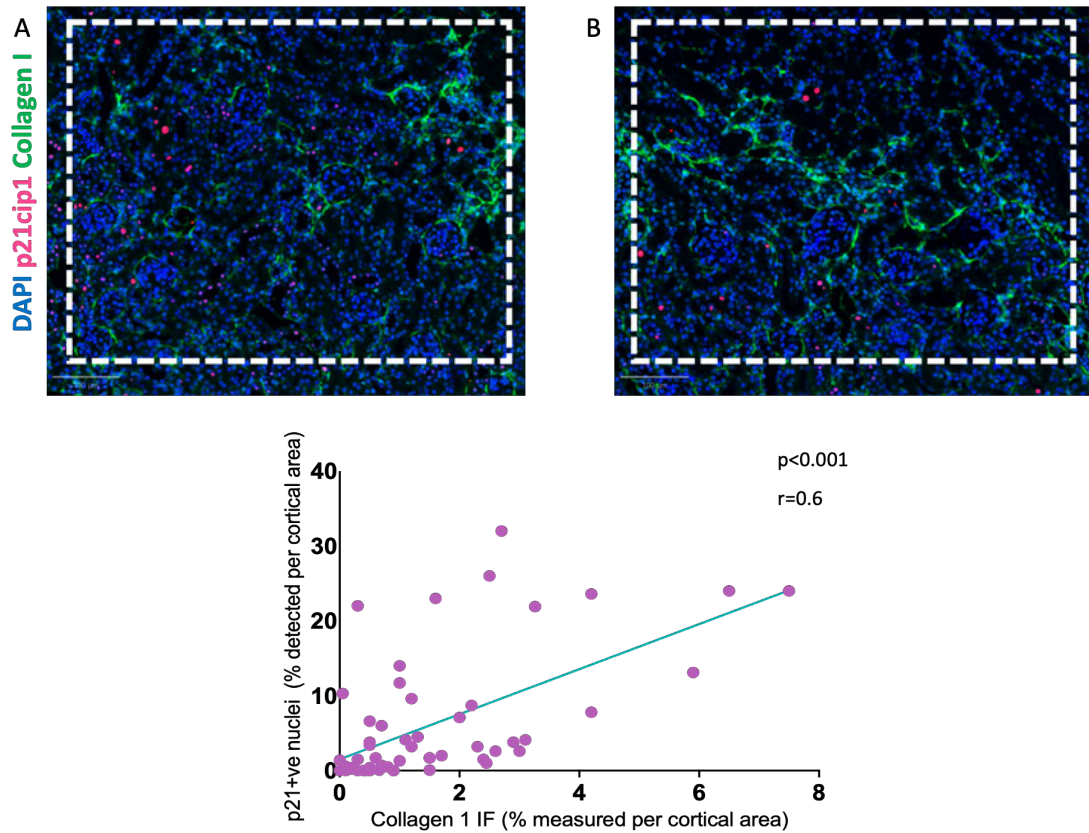


Figure 78 -Correlating cortical p21^{cip1} and collagen I

Correlation of cortical nuclear p21^{cip1} staining and interstitial collagen I immunofluorescence 7 days after treatment with tamoxifen or vehicle. Dual p21^{cip1} and collagen I expression in the cortex of an induced transgenic mouse. Two sections of cortex were analysed per mouse. (A) and (B) are two representative areas in an induced transgenic mouse. Within the white box there is the area of cortex which has been analysed for **both** p21^{cip1} nuclear staining **and** collagen I interstitial staining. n=27 mice in total, two cortical areas plotted per mouse. Young female mice, mixed genotypes aged <6 months of age treated with tamoxifen 200mg/Kg x2 or corn oil. The degree of correlation was determined by correlation analysis by Spearman r. Spearman correlation co-efficient $r=0.6$, $p<0.001$.

Figure 78 shows that cortical p21^{cip1} expression and collagen I expression by immunofluorescence were correlated.

Cortical Fibrosis by PiSR Day 7

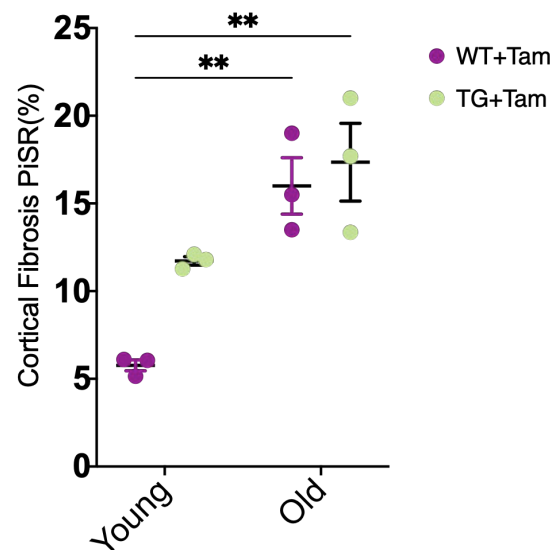


Figure 79 - Cortical fibrosis in young and old mice

Cortical fibrosis in young and old mice at an early timepoint of 7 days after treatment with tamoxifen assessed by total collagen staining by PiSR. 2-way ANOVA with Tukey's multiple comparisons test. All error bars are mean $\pm$ SEM. $n=3$. Female mice. Young mice were <6 months of age. Old mice were >18 months of age. All mice were treated with tamoxifen 200mg/Kg x2.

Figure 79 demonstrates that in older mice, there was no significant difference in the levels of cortical fibrosis when comparing induced transgenic mice with tamoxifen-treated wild-type controls. As expected, age was highly significant for the development of fibrosis ($p=0.0004$). Though there is no significant difference between tamoxifen treated young wild type and transgenic mice, there is a trend to significance with a p value of 0.06 and the effect of genotype overall is significant with a p value of 0.02. This analysis was performed on slides which were stained at a different time to those which generated the data shown in Figure 77. This explains why the numbers of mice presented are different and why I have not pooled all results for young mice. The numbers of young mice analysed in this particular staining run were small and with higher numbers the comparison between young transgenic vs wild-type mice treated with tamoxifen may have reached statistical significance.

Medullary fibrosis at an early timepoint

Figure 80 and Figure 81 show that there was no significant difference in medullary collagen I or total collagen expression between induced transgenic mice and control mice.

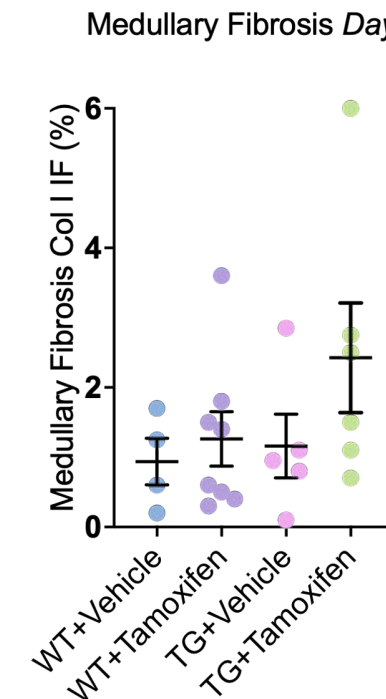
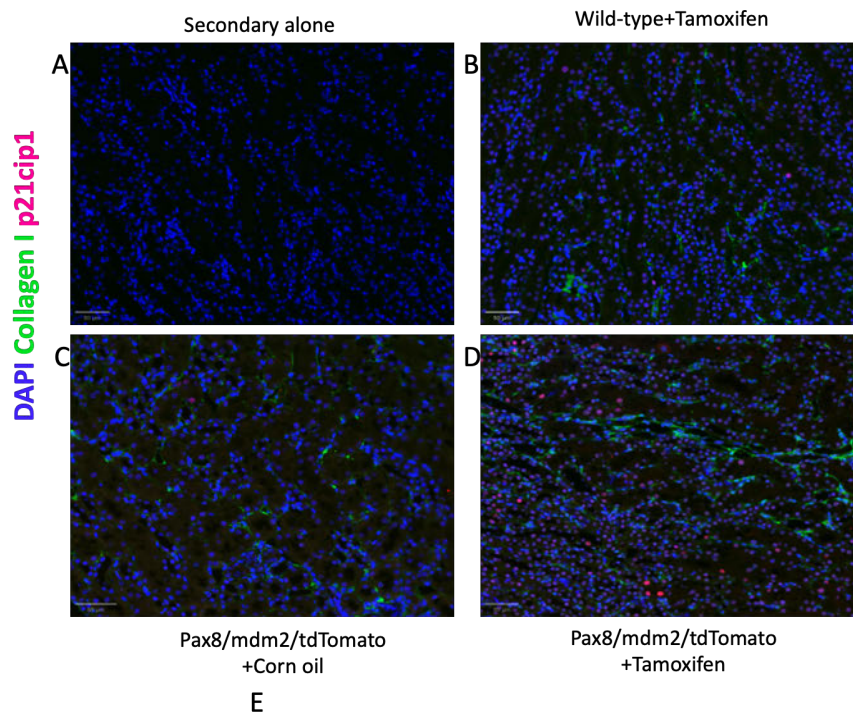
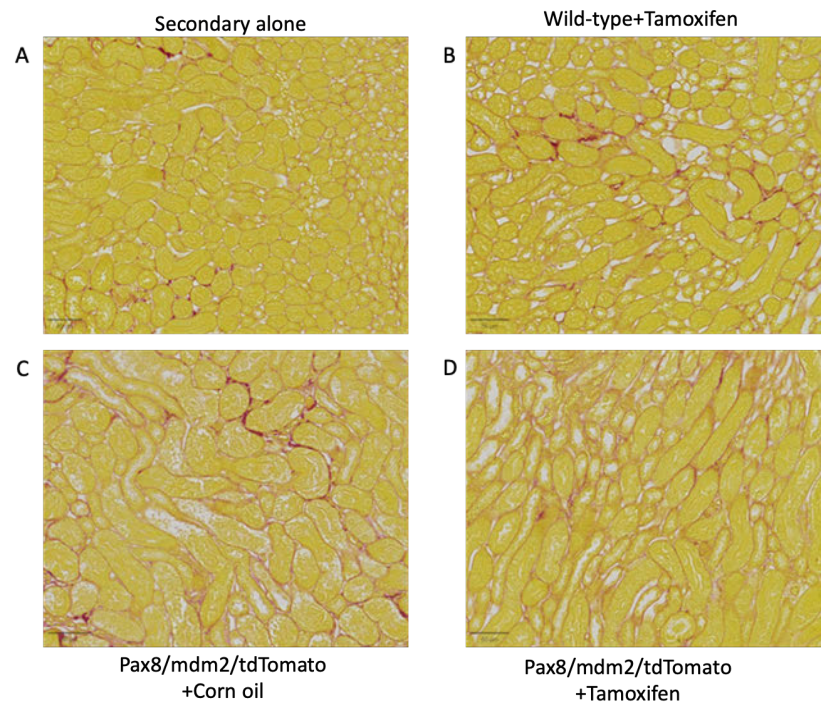


Figure 80 - Medullary fibrosis at an early timepoint: collagen I

Collagen I expression in the medulla. A) Secondary alone control. B) WT treated with tamoxifen. C) Transgenic mouse treated with corn oil. D) Transgenic mouse treated with tamoxifen. This is a dual stain where collagen I appears green and p21^{cip1} appears dark pink. Tamoxifen 200mg/Kg x2. Young female mice aged <6 months. E) Medullary collagen I expression 7 days after treatment with tamoxifen vs vehicle. One-way ANOVA with Tukey's multiple comparisons test. All error bars are mean $\pm$ SEM. n=4-8. Young female mice aged <6 months of age treated with tamoxifen 200mg/Kg x2



E Medullary Fibrosis Day 7

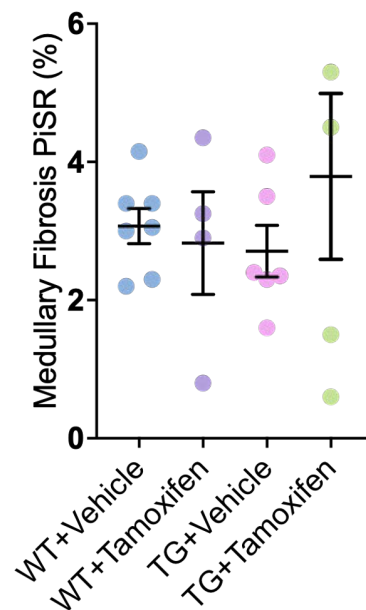


Figure 81 -Medullary fibrosis an early timepoint: total collagen

Collagen expression in the renal medulla. A) WT treated with corn oil. B) WT treated with tamoxifen. C) Transgenic mouse treated with corn oil. D) Transgenic mouse treated with tamoxifen. Collagen appears red. Tamoxifen 200mg/Kg x2. E) Medullary collagen expression 7 days after treatment with tamoxifen vs vehicle. One-way ANOVA with Tukey's multiple comparisons test. All error bars are mean $\pm$ SEM. n=4-8. Young female mice aged <6 months of age treated with tamoxifen 200mg/Kg x2.

scRNA sequencing fibrosis data

The scRNA sequencing dataset described below is the same dataset as discussed in the previous results chapter. The experimental design is outlined in Figure 27 and as discussed, this dataset included induced transgenic mice, injured CDKN1AiTomato post-IRI mice and baseline uninjured control mice. In the overall cell clustering for the entire dataset, an ‘activated fibroblast’ cell population was identified as shown in Figure 82. This population expressed markers such as *Col1a1*, *Col3a1*, *Pdgfra*, and *Pdgfrb*.

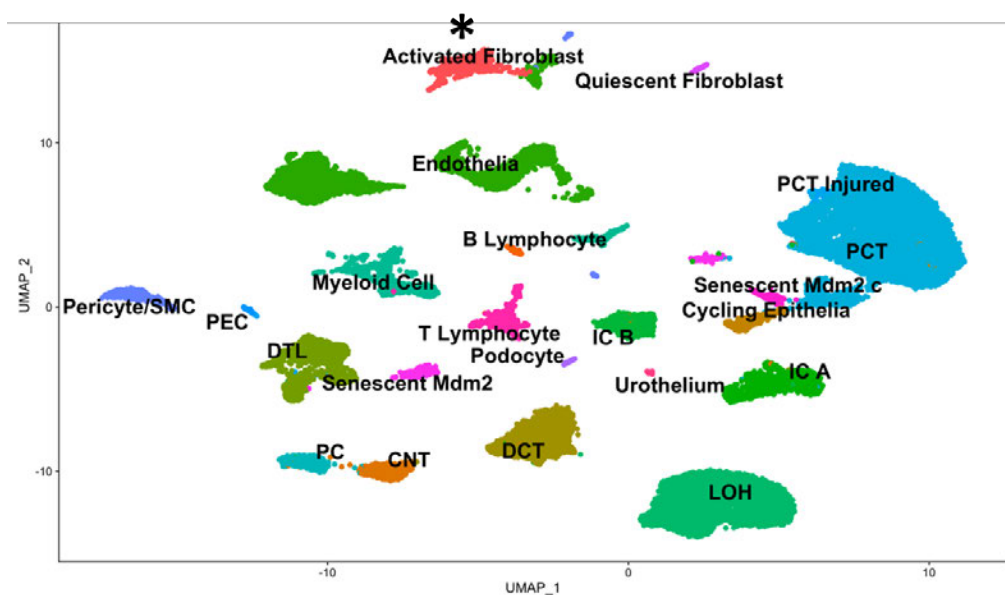


Figure 82 -UMAP showing activated fibroblast population

Cell clusters identified in mouse kidney sorts. Sorted by population based sorting or whole kidney sorts. Mixed gender. n=10 total, aged <6 months. Broad mouse groups: CDKN1AiTomato uninjured, CDKN1AiTomato injured, and induced Pax8/mdm2/tdTomato. n=2 per group, culled and sorted at day 7 post injury/post induction. Tamoxifen 200mg/Kg x2 given 48 and 72 hours prior to sorting (and thus day 4 and 5 post injury) in the CDKN1AiTomato mice and tamoxifen 200mg/Kg x3 7 days prior to sorting in the Pax8/mdm2/tdTomato mice. Contained within this UMAP are data derived from four additional mice culled at day 42 post injury/induction. n=4. Young mice, aged <6 months, mixed genders. 10X and Parse data.

Figure 83, Figure 84 and Figure 85 show this population of activated fibroblasts across mouse groups. To allow as fair a comparison as possible between UMAPs, the total cell count in each has been normalised to 4000 cells per mouse.

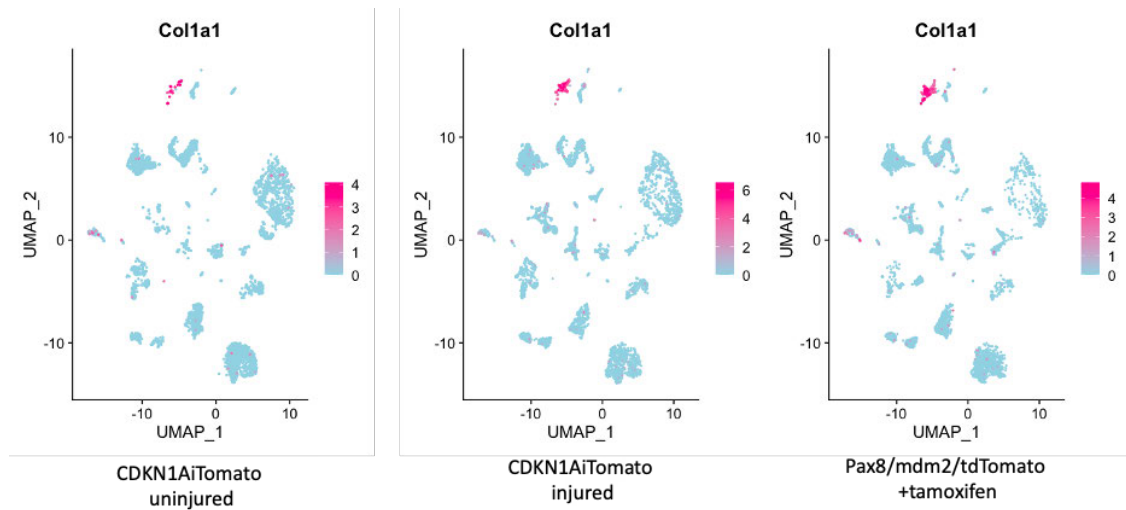


Figure 83 - *Col1a1* expression by scRNA sequencing

Col1a1 expression. CDKN1AiTomato mouse uninjured, CDKN1AiTomato mouse 7 days after IRI both treated with tamoxifen 200mg/Kg x2 48-72 hours prior to cull and a Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse culled 7 days after tamoxifen 200mg/Kg x3. Young female mice, aged <6 months. Cell count normalised to 4000 cells per mouse. 10X and Parse data.

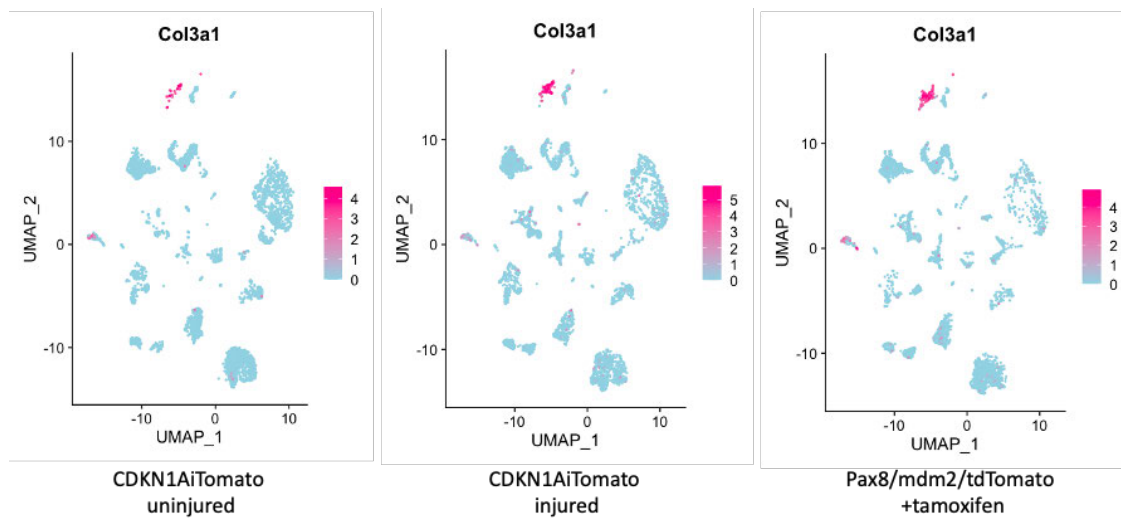


Figure 84 - Col3a1 expression by scRNA sequencing across mouse groups

Col3a1 expression. CDKN1AiTomato mouse uninjured, CDKN1AiTomato mouse 7 days after IRI both treated with tamoxifen 200mg/Kg x2 48-72 hours prior to cull and a Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse culled 7 days after tamoxifen 200mg/Kg x3. Young female mice, aged <6 months. Cell count normalised to 4000 cells per mouse. 10X and Parse data.

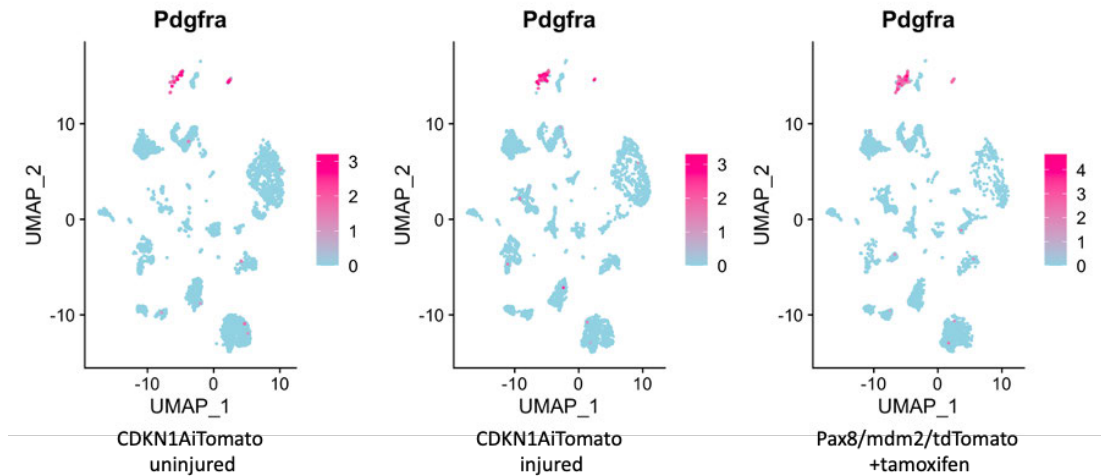


Figure 85 -*Pdgfra* expression across mouse groups

Pdgfra expression. CDKN1AiTomato mouse uninjured, CDKN1AiTomato mouse 7 days after IRI both treated with Tamoxifen 200mg/Kg x2 48-72 hours prior to cull and a Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse culled 7 days after tamoxifen 200mg/Kg x3. Young female mice, aged <6 months. Cell count normalised to 4000 cells per mouse.

It is not clear from these data alone whether this activated fibroblast population has been directly activated by the populations of senescent cells we observed in induced transgenic mice, or rather, activated by recruited immune cells. To test this, we intend to perform ligand receptor analyses between all three populations and test any relevant interaction at a protein level. Additionally, we intend to perform trajectory analyses on the senescent populations. Thus, allowing a further analysis of the transcriptomic changes observed in these cells over time.

Figure 86 shows that as a proportion of the total cell number analysed per mouse, the activated fibroblast population was largest in the induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse and accounted for 2.5% of all cells analysed from that mouse. Overall, the cell numbers analysed in each mouse were similar; 4405 CDKN1AiTomato uninjured, 5970 CDKN1AiTomato post-IRI, and 5102 in the induced Pa8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL}.

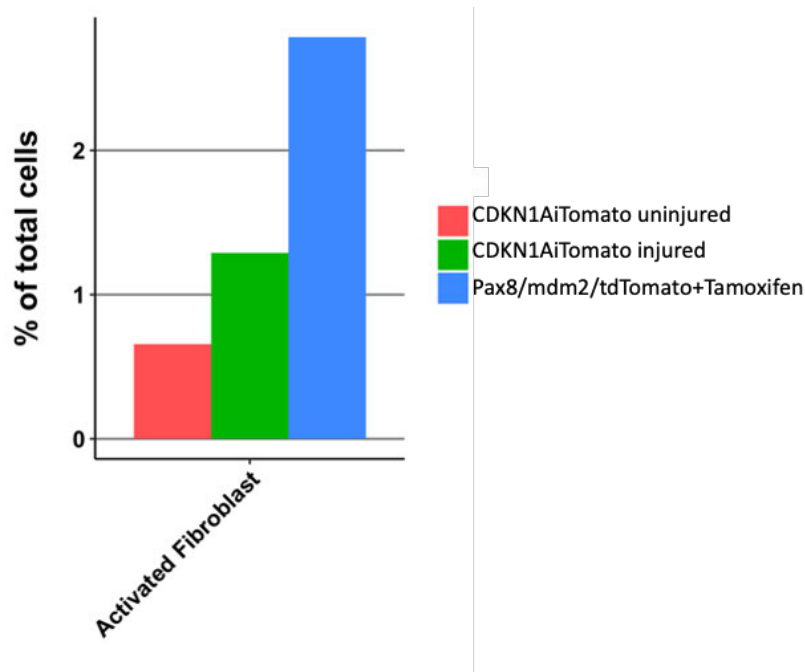


Figure 86- Activated fibroblast population

A population of activated fibroblasts. n=1 per group. CDKN1AiTomato mouse uninjured, CDKN1AiTomato mouse 7 days after IRI both treated with Tamoxifen 200mg/Kg x2 48-72 hours prior to cull and a Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse culled 7 days after tamoxifen 200mg/Kg x3. Young female mice, aged <6 months. 10X and Parse data.

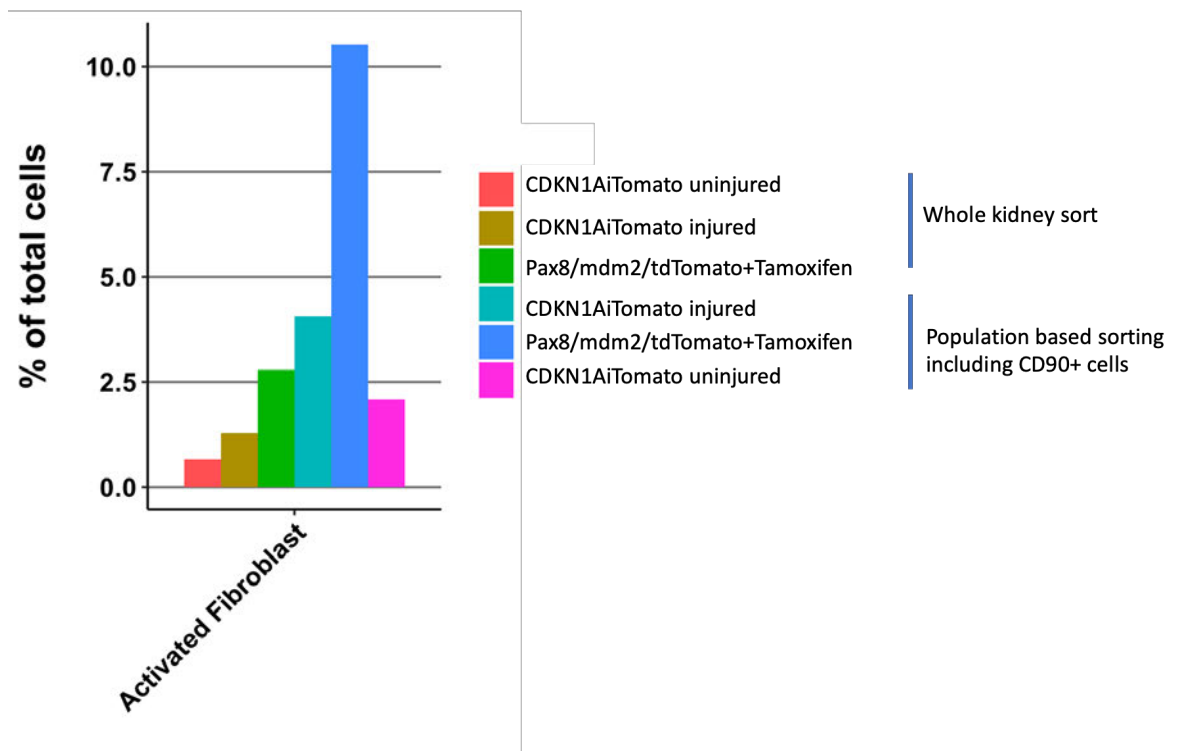


Figure 87 -Activated fibroblasts whole kidney and CD90+ sorting

A population of activated fibroblasts across mouse groups. n=2 per experimental group. Mice have undergone either whole kidney sort or population-based sorting where a population of CD90+ cells was sorted. Experimental groups: CDKN1AiTomato mouse uninjured, CDKN1AiTomato mouse 7 days after IRI both treated with Tamoxifen 200mg/Kg x2 48-72 hours prior to cull and a Pax8/mdm2/tdTomato mouse culled 7 days after tamoxifen 200mg/Kg x3. Young mice, aged <6 months. Mixed gender. The uninjured CDKN1AiTomato and the Pax8cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice that underwent population-based sorting were both male. 10x and Parse data.

Figure 87 shows that the activated fibroblast population was higher in mice who underwent population-based sorting (gating strategy shown in Figure 48) as would be expected as I sorted CD90+ cells. Again, this population appeared highest overall in the induced transgenic mouse.

Immune cell recruitment in induced transgenic mice

A key question of this PhD was to answer if the induction of epithelial senescence led to fibrosis. Although fibrosis was significantly higher at a protein level in induced transgenic mice, classical pro-fibrotic SASP factors such as *Tgfb1* and *Tgfb2* were not highly expressed by senescent epithelia in the scRNA sequencing dataset. As shown in Figure 63, *Tgfb1* was most highly expressed by immune cells, raising the possibility that immune cells were key to the generation of fibrosis in induced transgenic mice. Figure 88 shows that senescent epithelia appeared to express high levels of *Csf1* and this was confirmed by differential gene analysis (Appendix C).

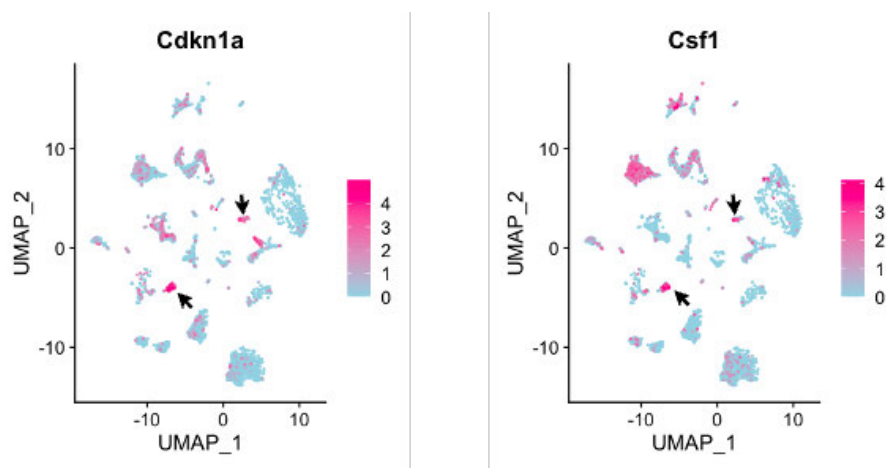


Figure 88 -*Csf1* expression by senescent epithelia in an induced *Pax8/mdm2/tdTomato* mouse

Csf1 and *Cdkn1a* expression in a *Pax8/mdm2/tdTomato* culled 7 days after tamoxifen 200mg/Kg x3. Young female mouse, aged <6 months. Whole kidney sorts. **Senescent epithelial populations are highlighted by black arrows.** 10X and Parse data.

Figure 89 and Figure 90 both demonstrate a population of cells which expressed recognised macrophage markers such as *Aif1* (encodes Iba1) *Adgre1* (encodes F4/80).

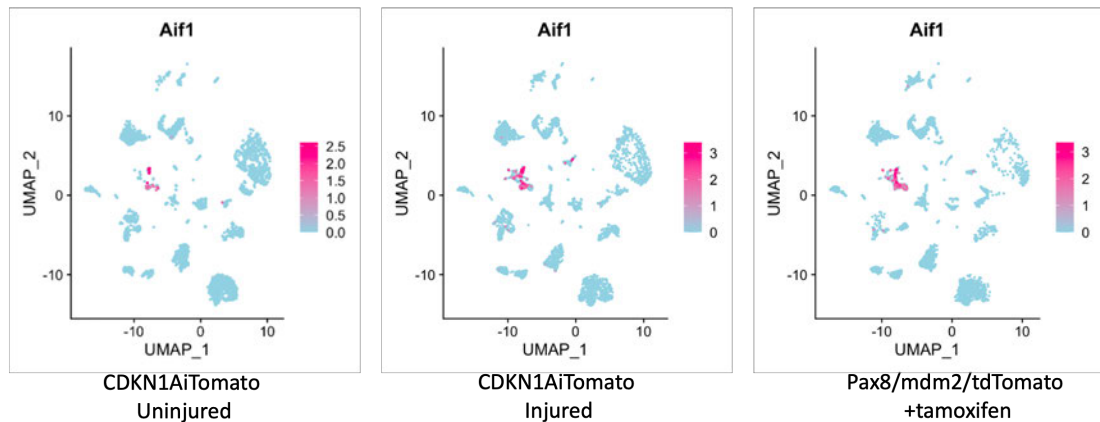


Figure 89 – *Aif1* expression across mouse groups

Aif1 expression. CDKN1AiTomato mouse uninjured, CDKN1AiTomato mouse 7 days after IRI both treated with Tamoxifen 200mg/Kg x2 48-72 hours prior to cull and a Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse culled 7 days after tamoxifen 200mg/Kg x3. Young female mice, aged <6 months. Cell count normalised to 4000. 10X and Parse data.

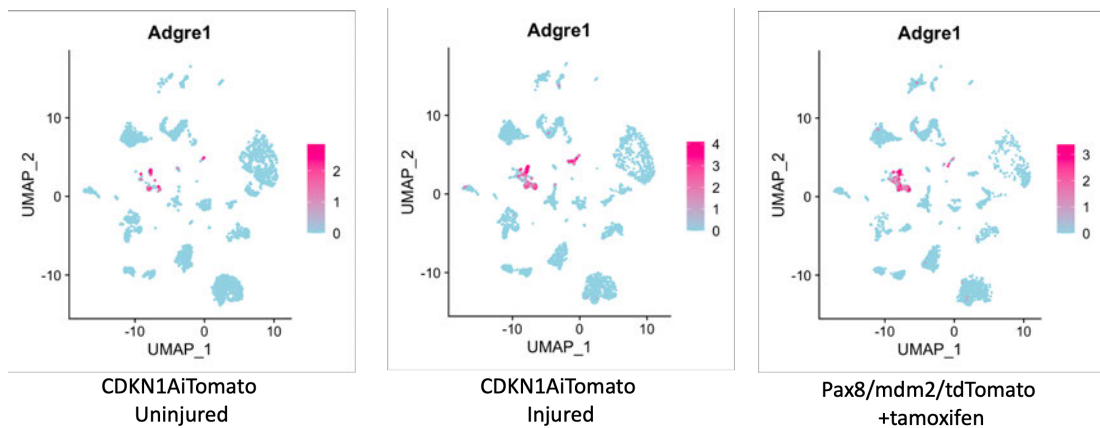


Figure 90 -F4/80 expression across mouse groups

Adgre1 expression. CDKN1AiTomato mouse uninjured, CDKN1AiTomato mouse 7 days after IRI both treated with Tamoxifen 200mg/Kg x2 48-72 hours prior to cull and a Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse culled 7 days after Tamoxifen 200mg/Kg x3. Young female mice, aged <6 months. Cell count normalised to 4000. 10X and Parse data.

Figure 91 shows co-expression of *Mki67*, *Adgre1* and *Aif1* in a population of proliferating myeloid cells in an induced transgenic mouse.

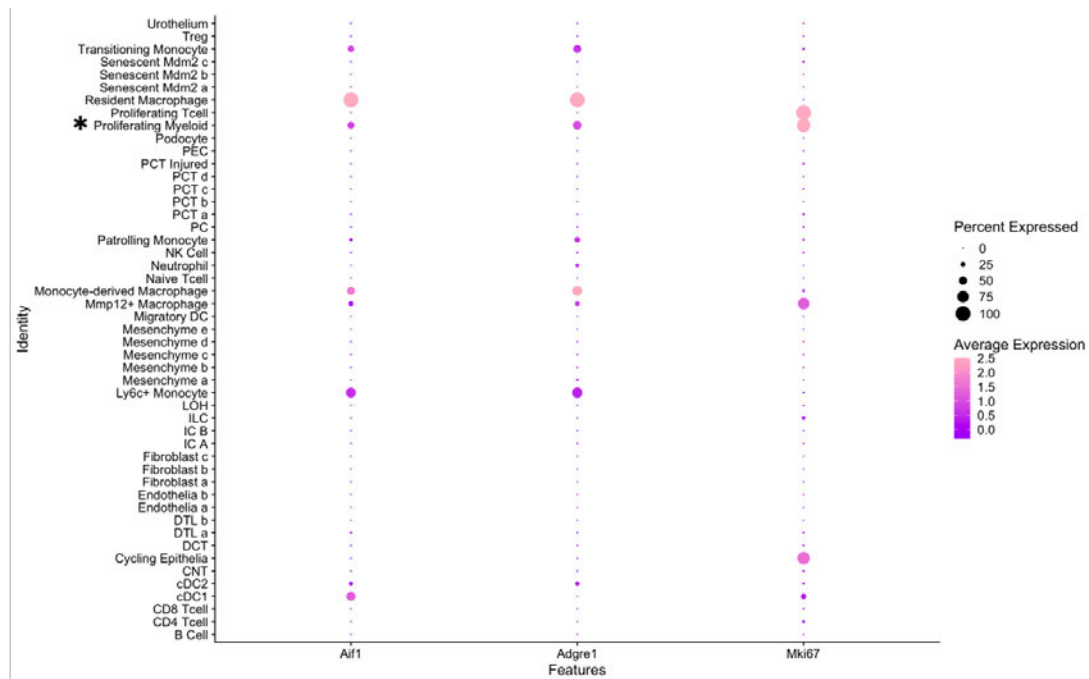


Figure 91 - Co-expression of *Mki67*, *Aif1* and *Adgre1* in an induced transgenic mouse

Mki67, *Adgre1*, *Aif1* co-expression in an induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with tamoxifen 200mg/Kg x3 7 days prior to cull. Young female mouse <6 months. Population highlighted by *. 10X and Parse data.

Figure 92 shows that that as a proportion of the total cell number, myeloid cells were higher in an induced transgenic mouse compared to baseline, though not as high as the level seen after IRI.

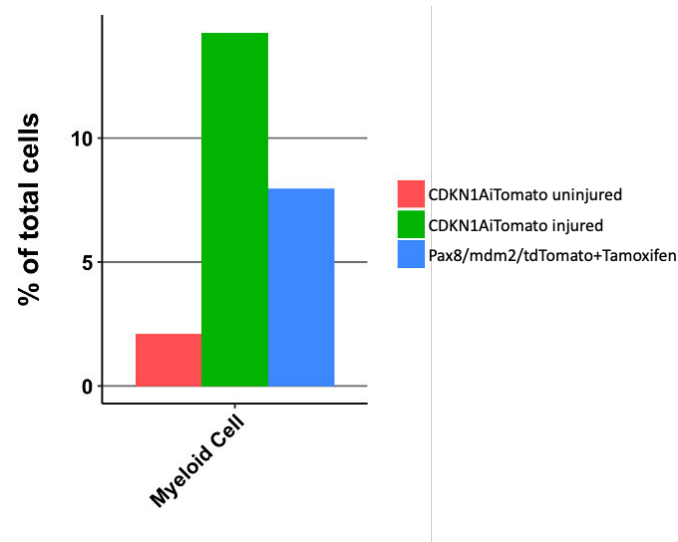


Figure 92 -Myeloid Cell population

Myeloid cell population in different mice. n=1 per group. CDKN1AiTomato mouse uninjured, CDKN1AiTomato mouse 7 days after IRI both treated with Tamoxifen 200mg/Kg x2 48-72 hours prior to cull and a Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse culled 7 days after tamoxifen 200mg/Kg x3. Young female mice, aged <6 months. 10X and Parse data.

Despite there not being any obvious senescent cell populations after injury, Figure 93 shows that there was a population of injured PTECs in mice that had undergone IRI.

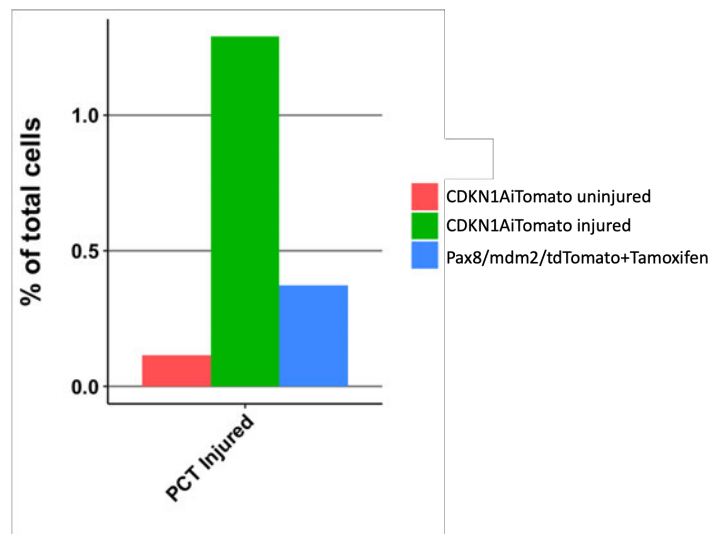


Figure 93 -Injured PTEC population

A population of injured PTECs in the CDKN1AiTomato mouse post-IRI. 10X and Parse data.

Immune cell recruitment at FACS

The experiments presented in this chapter were carried out alongside work by Dr Katie Mylonas and Ross Campbell who have an interest in senescent cell and immune cell interactions.

As discussed, it will be important to confirm if the upregulation of genes suggesting immune cell recruitment observed by scRNA sequencing is also observed at a protein level. This could be achieved by immunohistochemistry, looking for immune cell markers and work is underway to look at this.

Flow cytometry offers another way of looking at protein expression by cell surface markers. Figure 92 shows that higher numbers of immune cells were observed with higher doses of tamoxifen in transgenic mice. There were significantly higher numbers of recruited macrophages observed in induced transgenic vs wild-type mice. The wild-type mice included in Figure 94 A, B and D also received tamoxifen (200mg/Kg x2). Therefore, the increase in CD45 and F4/80 expressing cells

demonstrated in Figure 94 reflects transgenic induction and not tamoxifen administration as this increased immune cell population was not observed in tamoxifen treated wild-type mice.

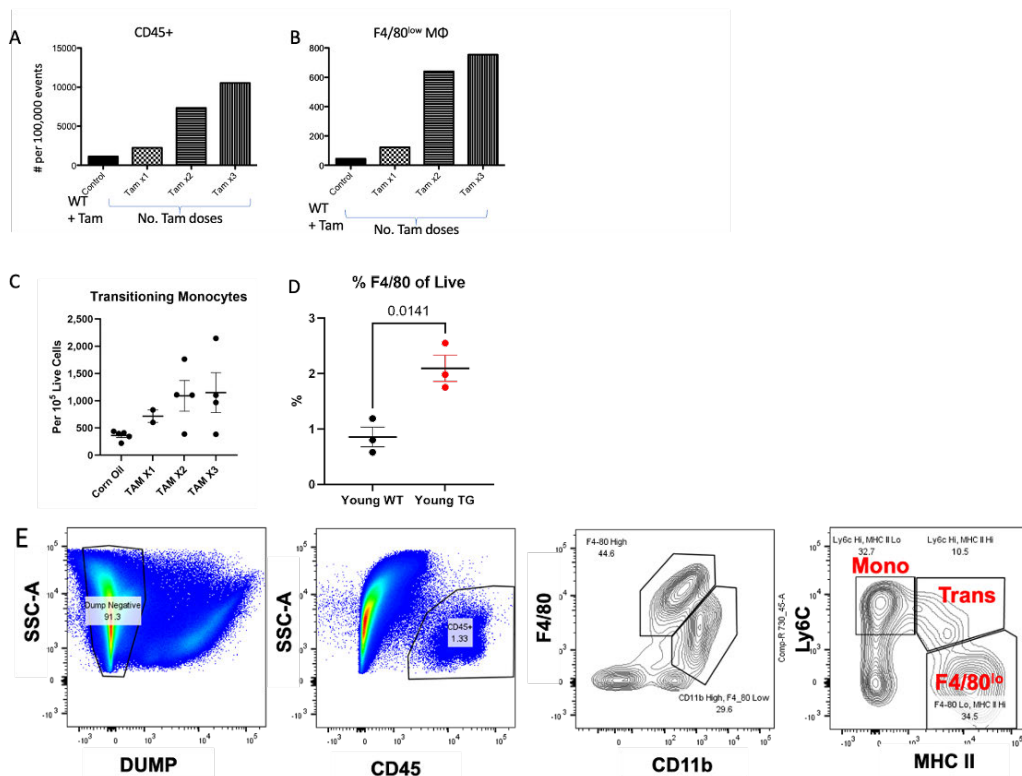


Figure 94 -Immune cells at flow cytometry. Figure provided by Ross Campbell and Dr Katharine Mylonas, University of Edinburgh. Unpublished.

A) Increased CD45+ cells at flow cytometry with increased tamoxifen B) Increased F4/80^{lo}/Cd11b macrophages. C) Transitioning monocytes increased with increased tamoxifen D) F4/80 positive cells were significantly higher by flow cytometry in induced transgenic mice vs young WT mice. E) Gating strategy used to identify transitioning monocytes. n=2-5, young female mice aged <6 months of age. TAMx1: Tamoxifen 200mg/Kg x1. TAMx2: Tamoxifen 200mg/Kg x2. TAMx3: Tamoxifen 200mg/Kg x3, given by oral gavage on alternate days.

In other models of murine injury such as UUO, expression of *Ly6c2*, *Ccr2*, and *Chil3* has been shown to be associated with a population of inflammatory monocytes. (Conway, O'Sullivan et al. 2020). Using the genetic markers identified by Conway et al, this population was noted in the context of injury and genetic induction as shown in Figure 95.

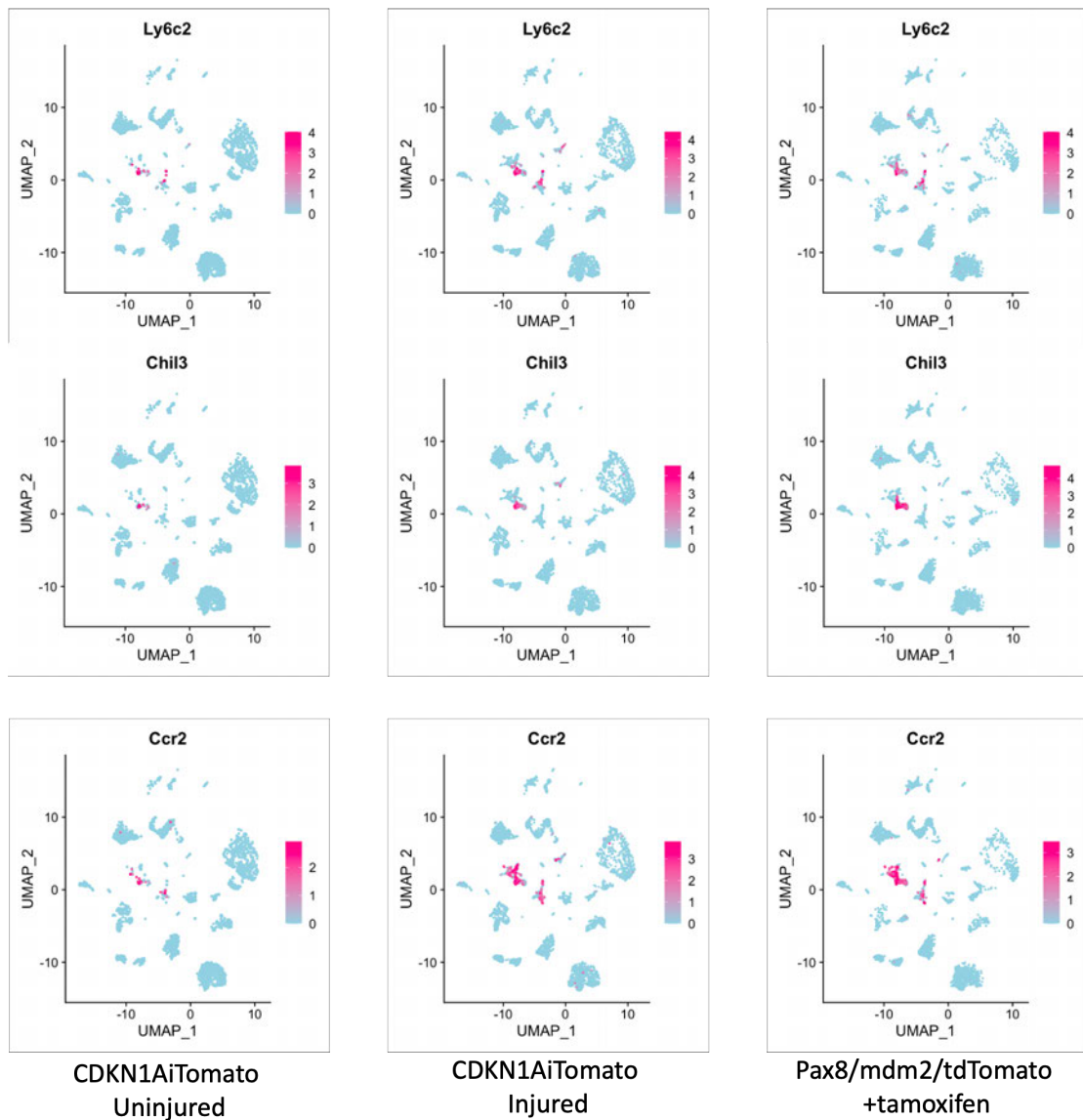


Figure 95 - A population of inflammatory *Ly6c*⁺ monocytes

Ccr2, *Chil3*, *Ly6c2* expression. n=1 per group. CDKN1AiTomato mouse uninjured, CDKN1AiTomato mouse 7 days after IRI both treated with Tamoxifen 200mg/Kg x2 48-72 hours prior to cull and a Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse culled 7 days after Tamoxifen 200mg/Kg x3. Young female mice, aged <6 months. Cell count normalised to 4000 cells per mouse. 10X and Parse data.

Whilst macrophage polarisation is an extensive topic within its own right, as shown below in the induced transgenic Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse, in addition to ‘pro-inflammatory’ type macrophages, I observed distinct populations which expressed M1 markers such as *Fcgr1* (encodes CD16) (van den Bosch, Caliskan et al. 2017) and also different populations which expressed M2 markers such as *Mrc1* (encodes CD206) (Tsuchiya, Suzuki et al. 2019)

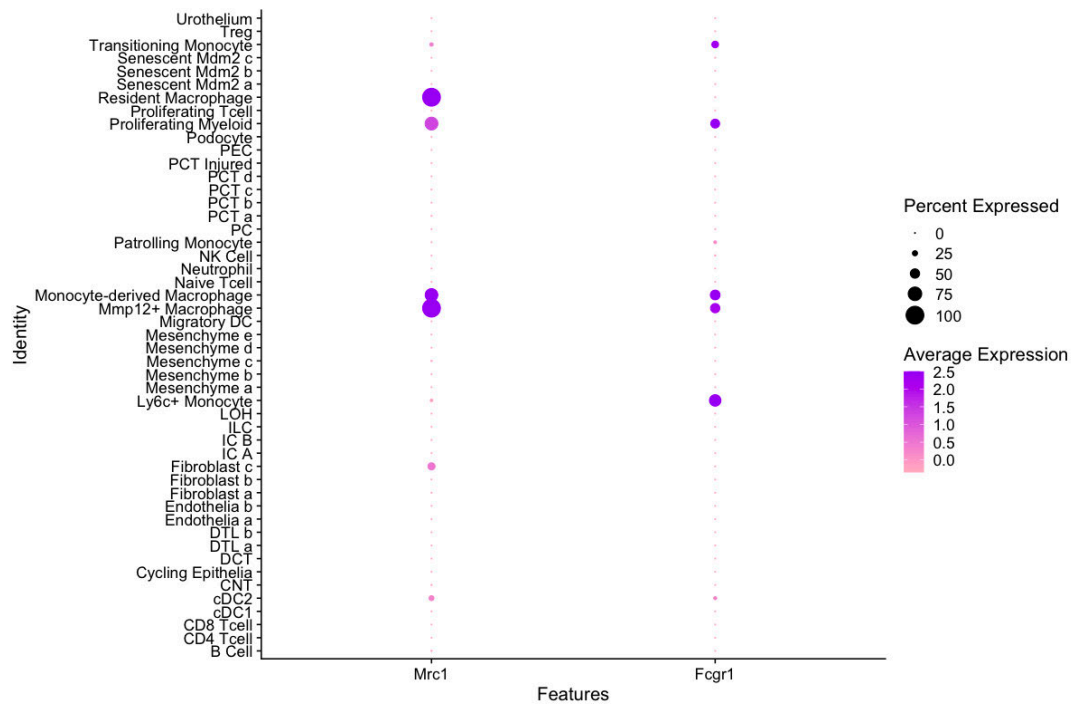


Figure 96 -Macrophage populations in an induced transgenic mouse

Mrc1 and *Fcgr1* co-expression in an induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse treated with tamoxifen 200mg/Kg x3 7 days prior to cull. Young female mouse <6 months. Population highlighted by *. 10X and Parse data.

Discussion

Transgenic induction results in the initiation of fibrosis at an early timepoint

These results demonstrate that transgenic induction in young female Pax8CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice was sufficient to induce cortical fibrosis in the absence of any other renal insult.

Collagen I expression was three times higher in induced transgenic mice compared to all respective control groups. The data for total collagen by PiSR staining ranged from 3.8 ± 0.3 % in wild type mice treated with tamoxifen to 7.7 ± 0.7 % in transgenic mice treated with tamoxifen ($p=0.0019$). With respect to any direct anti-fibrotic effect of tamoxifen, both vehicle treated groups had higher levels of cortical fibrosis than wild-type mice treated with tamoxifen: 4 ± 0.5 % in wild-type mice and 5.3 ± 0.8 % in transgenic mice. Though these values were higher, they were not significant when compared to wild-type mice treated with tamoxifen with $p=0.89$ and $p=0.4$ respectively: WT+vehicle, TG+vehicle. Although there was no significant difference in total collagen expression between transgenic mice who received tamoxifen vs those who received vehicle, there was a trend to significance with a p value of 0.06.

Dual staining of p21^{cip1} and collagen I demonstrated that nuclear p21^{cip1} expression correlated with interstitial collagen I in the surrounding cortical tissue. These data suggest that senescent cells promote local fibrosis. In a liver model of p21^{cip1} driven senescence, α -SMA positive cells were found in close proximity to senescent cells and mice who had undergone transgenic induction with tamoxifen had higher levels of total collagen measured by PiSR (Ferreira-Gonzalez, Lu et al. 2018). Collagen I correlated strongly with p16^{INK4A} and p21^{cip1} expression in mice that had undergone irradiation and ischaemia reperfusion injury (Mylonas, O'Sullivan et al. 2021). In lung biopsies from humans with IPF, p16 positive epithelial cells were found close to fibroblastic foci (Schafer, White et al. 2017).

To look at the expression of p21^{cip1} I performed immunofluorescence only. Because p21^{cip1} and Collagen 1 could be co-stained and visualised together by immunofluorescence (using separate secondary antibodies), I was able to comment on spatial correlation. Using only one method of identification at a protein level for p21^{cip1} is a limitation of this project because it would be powerful to perform an analysis of p21^{cip1} expression and all collagen (a wider marker of fibrosis than Collagen 1). I looked at all collagen expression using PiSR staining only. To perform a correlation analysis, I would repeat my p21^{cip1} analysis using an immunohistochemistry assay. This would allow a more reliable analysis than comparing two different methods of staining (immunofluorescence and immunohistochemistry) which I am limited by with my current data.

It would be interesting to confirm if there is a correlation between senescent cells and immune cell infiltration and this work is currently being undertaken by Ross Campbell, a fellow PhD student in our group.

As was the case with medullary p21^{cip1} expression, there was no significant difference in levels of medullary fibrosis between groups. Induced transgenic mice had the highest levels of fibrosis overall, though this did not reach significance. It remains possible with a larger dataset that a significant effect would be observed in the medulla. One explanation for this is that p21^{cip1} senescent cells promote local fibrosis (either through recruitment of immune cells and subsequent myofibroblast activation, or through direct myofibroblast activation). This local fibrosis effect is supported by the correlation data presented in Figure 78. As discussed in chapter 3, I did not observe significant medullary senescence and perhaps this is why I also did not observe significant medullary fibrosis.

scRNA sequencing data demonstrated a population of fibroblasts expressing genes which are associated with activation and collagen secretion such as *Col1a1*, *Col3a1* and *Pdgfra*. This activated fibroblast population was largest in the context of

transgenic induction. It has been demonstrated in scRNA sequencing datasets that expression of *Col1a1* peaked at day 2 post IRI and had fallen by day 14 (Kirita, Wu et al. 2020). Therefore, had I looked at an earlier timepoint, I may have found a larger activated fibroblast population in the post IRI mice.

scRNA sequencing data also demonstrated a recruited immune cell population in the context of transgenic induction and injury. This was supported by data from flow cytometry. It is not possible to determine if fibroblast activation occurred through a direct senescent epithelial to mesenchymal cell interaction, or *via* recruited immune cells. However, as discussed in the previous results chapter, expression of classical markers associated with myofibroblast activation such as *Tgfb1* was not observed in the senescent epithelial populations. *Tgfb1* expression appeared to be limited to immune cells.

One caveat when comparing induced transgenic mice and injured mice in the scRNA sequencing dataset is that as discussed, the post IRI mice did not appear to have a defined population of senescent epithelial cells. It remains possible that there was an operative technical failure in causing the injury, though the recruitment of an obvious immune cell population goes against this. Additionally, both injured mice appeared not to have any defined senescent populations and it seems unlikely that such an operative failure would occur twice. Furthermore, a population of injured PTECs was identified in this mouse, suggesting that injury had occurred as expression of genes associated with this population such as *Havcr1* and *Vcam1* was not seen in the baseline healthy kidney. Whilst these injured mice appeared to have a smaller activated fibroblast population when compared to induced transgenic mice, this population was still notably higher than at baseline at day 7. Unfortunately, I used the entire IRI kidney in both injured mice for scRNA sequencing. This therefore prevented me from analysing these kidneys at a histological level for senescence, fibrosis and inflammation. These protein read outs are vital and they will be available in a larger dataset of male mice.

Future experiments

From the data generated thus far, my hypothesis is that senescent epithelial cells recruit leukocytes *via* secreted cytokines and chemokines and these recruited immune cells induce myofibroblast activation. In murine acute liver injury, it has been demonstrated that macrophage derived TGF β 1 is key in the transmission of paracrine senescence. Prevention of leucocyte recruitment by CCL2 inhibition or macrophage ablation by clodronate reduced *Tgfb1* expression by 87% and resulted in significantly less paracrine senescence. Transgenic myeloid-specific *Tgfb1* deletion resulted in improved regeneration (Bird, Muller et al. 2018). In p21^{cip1} driven senescence *via* loss of Mdm2, large infiltrates of F4/80 positive macrophages were observed after senescence induction. (Ferreira-Gonzalez, Lu et al. 2018) In transgenic mice that over-expressed p21^{cip1}, macrophage recruitment was apparent early after senescence induction and was prevented by CXCL-14 blockade. (Sturmlechner, Zhang et al. 2021)

To investigate this hypothesis further, I would look for a spatial correlation between senescent cells and immune cells at a protein level, for example by immunohistochemistry. Should cytokines such as CSF1 be identified as candidate SASP markers in a larger scRNA sequencing dataset, it would be important to confirm their expression at a protein level and to consider the option of blockade. To investigate if recruited immune cells secondary to senescence induction are key to myofibroblast activation, I would block this recruitment, or deplete leucocytes in induced transgenic mice. I would expect to see a reduction in fibrosis despite significant levels of induced senescence. If blocking immune cell recruitment or depleting immune cells made no difference to fibrosis, that would suggest an alternative hypothesis, such as direct activation of myofibroblasts by senescent epithelia.

The relationship between senescent epithelia and fibroblasts could also be tested *in vitro*. This would involve culturing epithelial cells from transgenic mice followed by induction with the active metabolite of tamoxifen (4-OHT) *in vitro*. I was able to

culture epithelial cells from wild type and transgenic mice. Tamoxifen (4OHT) treatment *in-vitro* resulted in tdTomato expression as shown below in Figure 97. Due to time constraints, I was not able to optimise antibody staining or RNA extraction; both of which are required to determine if these tdTomato expressing cells also express markers of senescence.

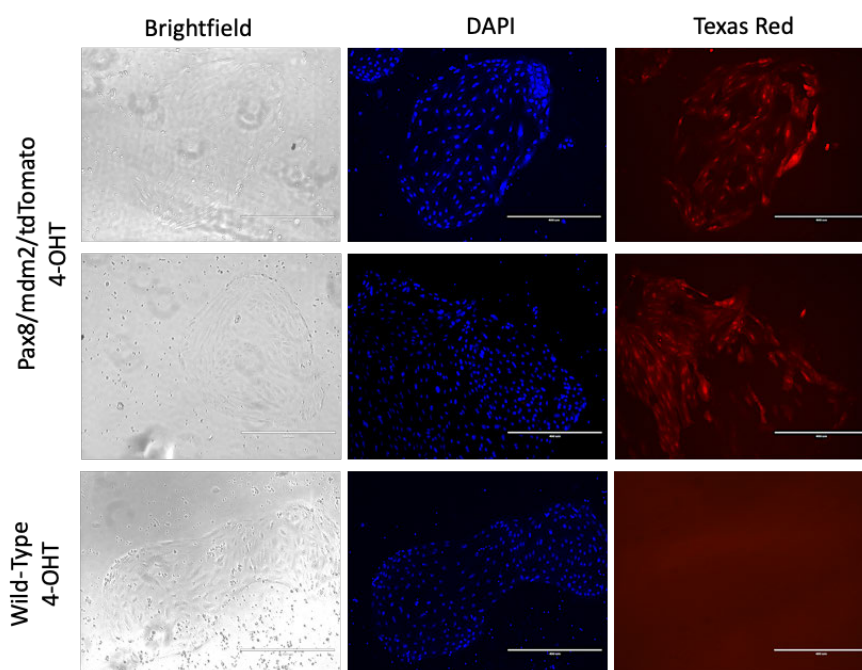


Figure 97 – *In vitro* cell culture

tdTomato expression *in vitro*. Jacalin/LTL+ epithelial cells were sorted from transgenic mice and wild-type mice. Cells were cultured with 4-OHT. Cells were fixed day 10 after 4-OHT treatment, stained with DAPI and imaged on the EVOS FL microscope.

Assuming senescence was induced successfully *in vitro*, these epithelia could be co-cultured with fibroblasts. If the activation of fibroblasts requires recruited immune cells, then it should not occur *in vitro*.

Conclusions

Transgenic induction in young female Pax8Cre-ERT²/mdm2^{flox/flox}/tdTomato^{LSL} mice was sufficient to induce cortical fibrosis and immune cell recruitment at both an mRNA and protein level at an early timepoint.

Chapter 5: The evolution of senescent cells *in vivo* over time

Introduction

Immune cell recruitment to sites of senescence is well recognised (Krizhanovsky, Yon et al. 2008, Munoz-Espin, Canamero et al. 2013, Storer, Mas et al. 2013, Sturmlechner, Zhang et al. 2021). In these contexts, immune cells are thought to play a role in the timely clearance of senescent cells.

These examples contrast with the persistence of senescent cells at the sites of injury and in age (Baker, Childs et al. 2016, Mylonas, O'Sullivan et al. 2021). The accumulation of senescent cells with age is not fully understood. It is thought in part to be due to the accruing of cellular dysfunction over time (van Deursen 2014) and in part to immunosenescence. The concept of immune cell senescence is well described (Liu, Liang et al. 2023). Whether these aged and exhausted cells are less capable of clearing senescent cells is not known. Similarly, why senescent cells persist post-injury when there is a concomitant immune cell recruitment is not clear.

The data presented in the previous two results chapters demonstrated that tamoxifen treatment in Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice led to renal epithelial cortical p21^{cip1} expression, immune cell recruitment and fibrosis at an early timepoint. This system allowed me to test the hypothesis that senescent cells persist in young immunocompetent mice, in the absence of confounding biological processes such as ageing or injury.

To investigate whether the increased p21^{cip1} expression and fibrosis I observed at an early timepoint persisted, or progressed or improved, I used the experimental design shown below in Figure 98.

Experimental design

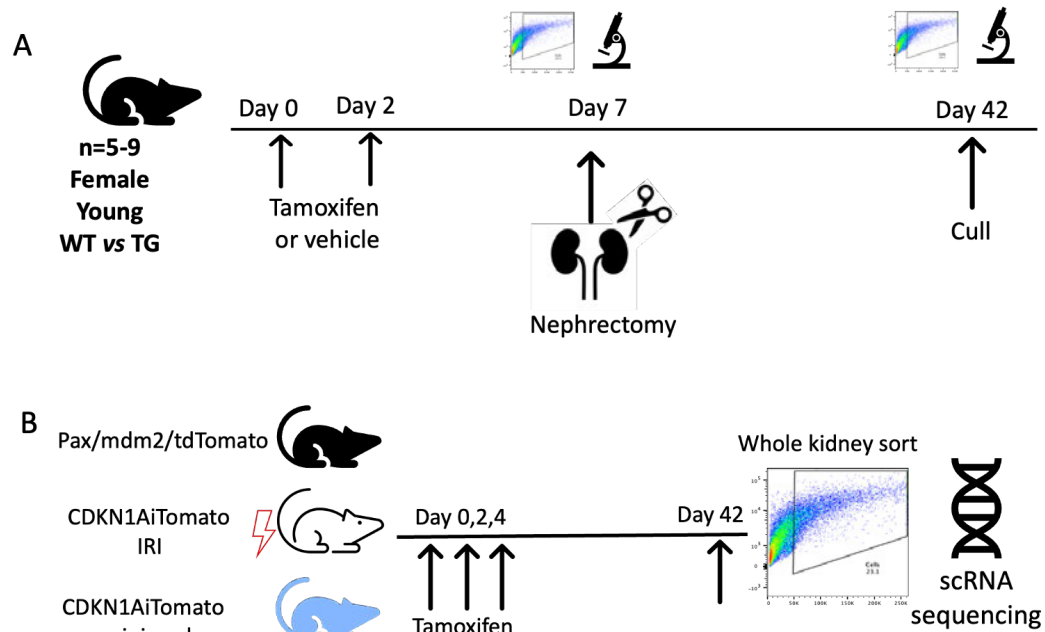


Figure 98 -Experimental schematic for late timepoint assessment

A) Young female mice aged <6 months were grouped into four experimental groups: WT+tam, WT+veh, TG+tam, TG+veh. Mice underwent unilateral nephrectomy 7 days after the first tamoxifen or vehicle treatment. Mice were culled at 42 days post treatment, 35 days post nephrectomy. Mice received tamoxifen 200mg/Kg x2 by oral gavage on alternate days. n=5-9 per group. B) 4 young female mice in total, n=1 per group. C57B6 and Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice received tamoxifen 200mg/Kg x3 on alternate days by oral gavage and were culled 42 days after the first tamoxifen dose. CDKN1AiTomato mice received tamoxifen 200mg/Kg x2 day 4 and 5 post injury and were culled 42 days after injury.

Chapter aims and hypotheses

Aims:

1) To describe the findings observed at a late timepoint after transgenic induction

PaxCre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice

Hypotheses:

1) p21^{cip1} expression would remain significantly elevated by day 42 in induced transgenic mice.

2) Levels of fibrosis would progress.

Results

Cortical p21^{cip1} expression falls by day 42 in induced transgenic mice

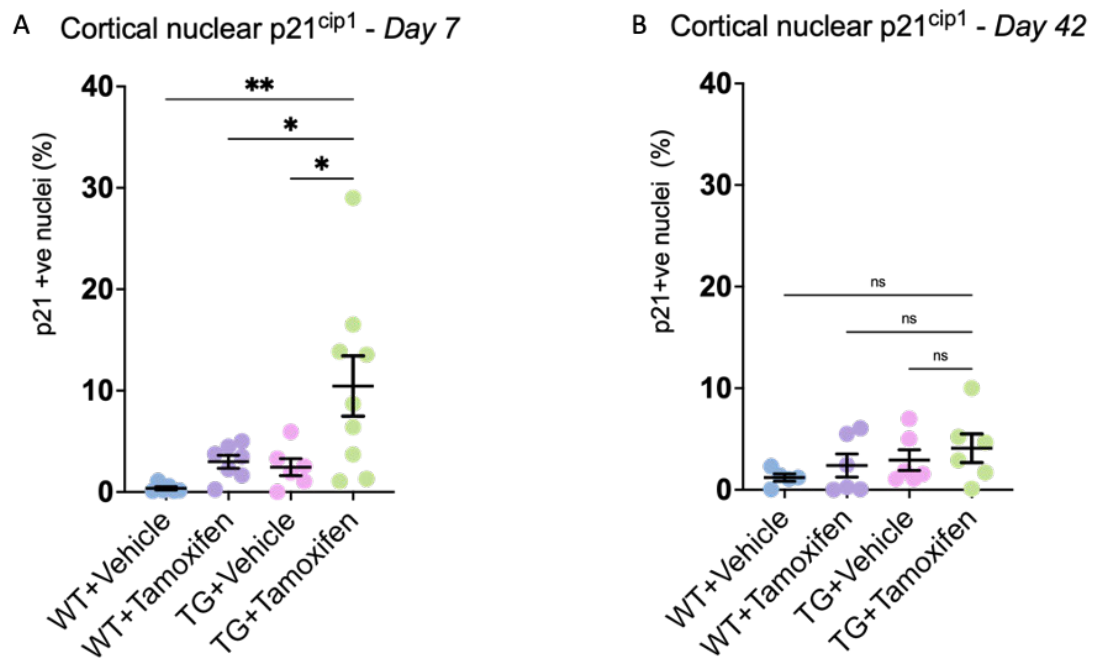


Figure 99 -Cortical nuclear p21^{cip1} staining over time

A) Cortical nuclear p21^{cip1} expression at day 7 and (B) day 42. p21^{cip1} positive nuclei are expressed as a percentage of all nuclei in a representative area of renal cortex. Three cortical readings have been taken and averaged per mouse. n=5-9 per group. Female mice, <6 months. All mice were treated with tamoxifen 200mg/Kg x2 and underwent unilateral nephrectomy 7 days after tamoxifen treatment and were culled 42 days after the first tamoxifen dose. One way ANOVA with Tukey's multiple comparisons. All error bars are mean $\pm$ SEM *p<0.05, **p<0.01.

Figure 99 shows that p21^{cip1} expression in the cortex fell by day 42 and there was no longer a significant difference between experimental groups. The mean level of cortical nuclear p21^{cip1} expression in induced transgenic mice fell from $10.45 \pm 2.9\%$ to $4.09 \pm 1.4\%$. With these data alone, it is not possible to confirm whether this fall in senescence represents clearance of senescent cells or a re-entry into a replicative cycle. One way to test this would be to look for tdTomato expression at day 42. At the time of writing this thesis, I did not have sufficient data to reliably confirm the recombination efficiency of both transgenic elements in this mouse; i.e. to confirm whether loss of *Mdm2* and *tdTomato* occurred at a similar frequency. However, if these are subsequently proven to recombine at a similar rate, then I would expect if

senescent cells are cleared to also observe vastly reduced tdTomato expression by day 42. If however, senescent cells have reverted back to a replicative cycle and have lost therefore lost the expression of the cell cycle arrest protein p21^{cip1}, I would still expect to see obvious tdTomato expression by day 42. The immune cell recruitment observed in induced transgenic mice suggests immune mediated clearance, but further work is required to prove this. I have outlined the various approaches I plan to take to look at this further in the discussion of this results chapter and in my general discussion.

Figure 100 (A) shows that there is a fall in cortical p21^{cip1} expression over time in transgenic mice treated with tamoxifen, though this is not significant. There was no change in the level of cortical p21^{cip1} expression in the control groups. Figure 100 (B) shows representative histological appearances in p21^{cip1} and collagen I expression in mice at day 7 and at day 42. These are side by side images taken from two different time points in the same mouse and show that tubular epithelial p21^{cip1} expression by immunofluorescence was noticeably less by day 42.

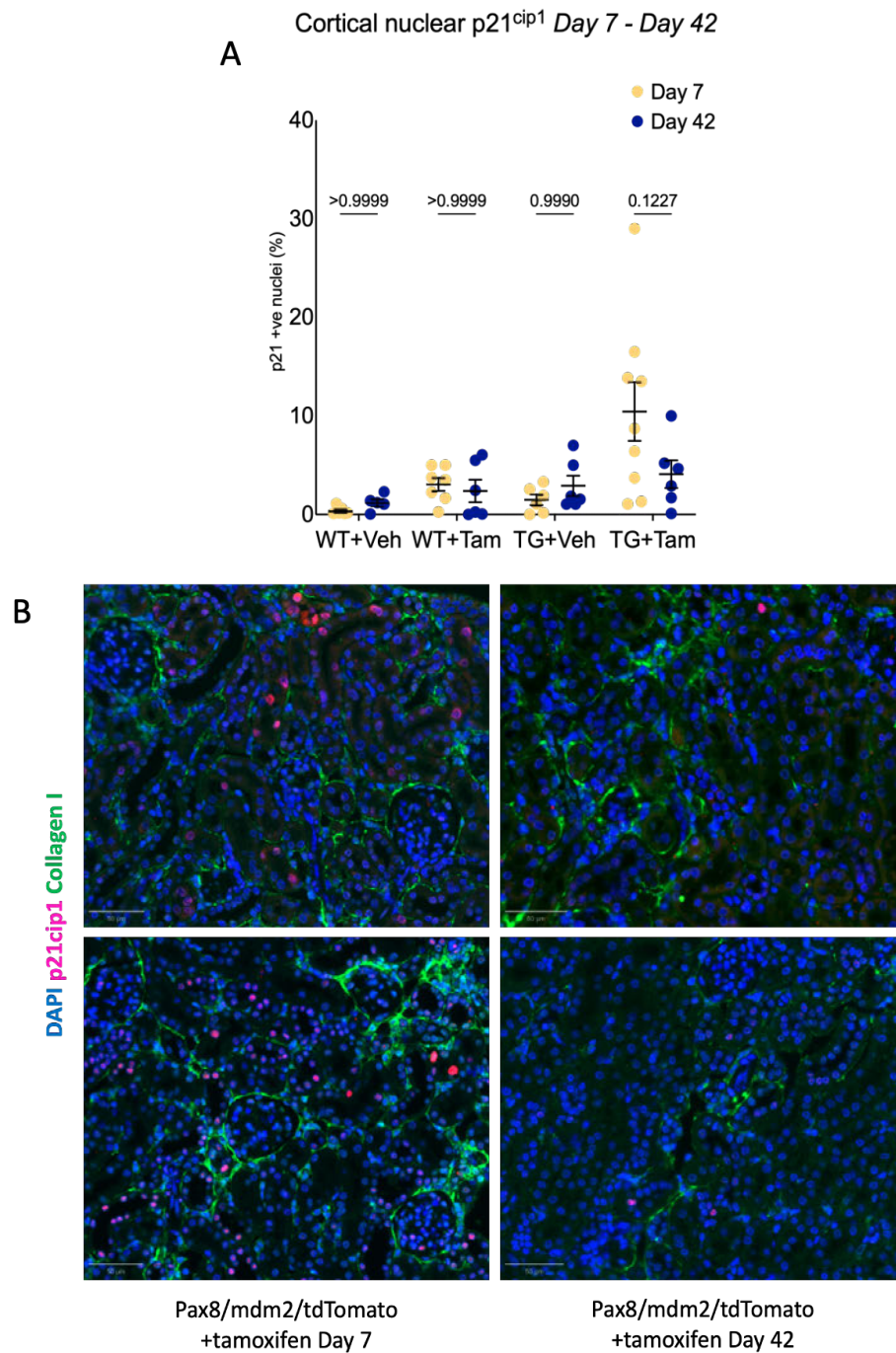


Figure 100 - Day 7 and day 42 cortical p21^{cip1} staining in all mice

A) Cortical nuclear p21^{cip1} staining at day 7 and day 42. n=5-9. p21^{cip1} positive nuclei are expressed as a percentage of all nuclei in a representative area of renal cortex. Three cortical readings have been taken and averaged per individual mouse. Female mice, <6 months. 2-way ANOVA. Tukey's multiple comparisons. All error bars are mean $\pm$ SEM. B) Immunofluorescence images in young female Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice at two different time points. The images presented on the left are from day 7 post tamoxifen, and on the right day 42. n=2. Tamoxifen 200mg/Kg x2. p21^{cip1} appears dark pink and appears less by day 42 in both mice. Collagen I appears green.

The data presented in Figure 99 (B) and Figure 100 day 42 timepoint are from mice that underwent unilateral nephrectomy at day 7 after tamoxifen treatment. These data confirmed that there was no rise in p21^{cip1} in the contralateral kidney in young female mice. Figure 101 shows paired day 7 and day 42 timepoints in nephrectomised mice. The reason that the numbers are not identical to those presented in Figure 100 is due to mouse death or paired tissue not being available to me at the time of the analysis.

Figure 101 shows that p21^{cip1} expression fell in four out of six induced transgenic mice. Of note, these four mice had higher levels of p21^{cip1} expression at day 7 than the two mice in which levels of p21^{cip1} rose.

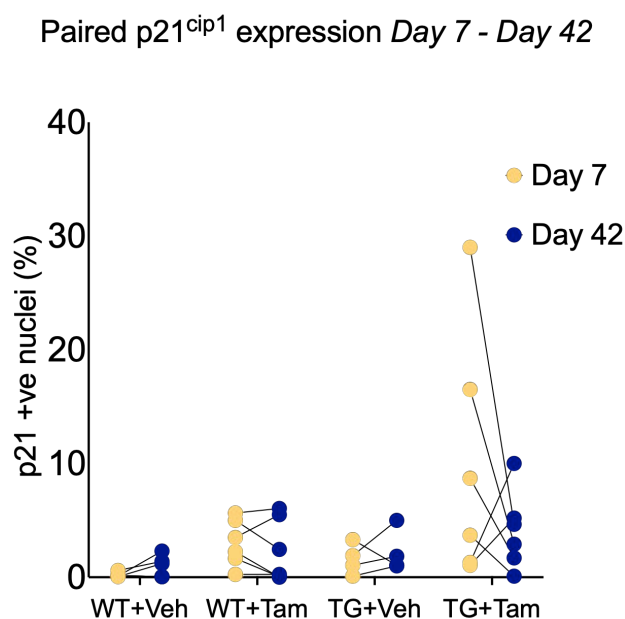


Figure 101 - Day 7 and day 42 cortical p21^{cip1} expression in the same mouse over time

Cortical nuclear p21^{cip1} expression in the same mouse at day 7 and day 42. p21^{cip1} positive nuclei are expressed as a percentage of all nuclei in a representative area of renal cortex. Three cortical readings have been taken and averaged per individual mouse. n=4-6 per group. Female mice, <6 months. Multiple unpaired t-tests, Holm-Šidák method.

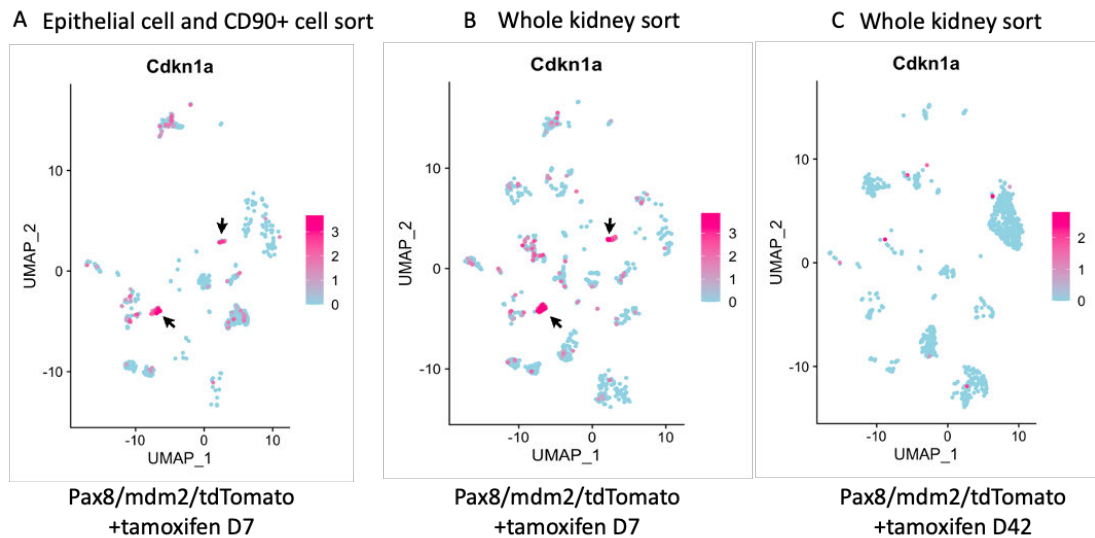


Figure 102 -Cdkn1a expression in transgenic mice at different time points

Cdkn1a expression at day 7 and day 42. Cell count normalised to 1000 cells per mouse. n=1 per group, young female mice. Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} treated with tamoxifen 200mg/kg x3. A) Population based sort for tdTomato+ and CD90+ cells. Cells sorted 7 days after tamoxifen. B) Whole kidney sort. Cells sorted 7 days after tamoxifen. C) Whole kidney sort. Cells sorted 42 days after tamoxifen. Tamoxifen 200mg/Kg x3. **Parse sequencing data only.**

The day 42 timepoint did not include any 10X sequencing data. Therefore, to allow a fair comparison, the plots shown in Figure 102 show Parse sequencing data only. I have removed all 10X data from the plotted day 7 timepoints in Figure 102 (A) and (B). The cell count has been normalised to 1000 cells per mouse.

The plots presented in Figure 102 (A) and (B) are taken from two different mice and demonstrate that distinct populations of senescent epithelia were identified early after treatment with tamoxifen with both cell sorting approaches. By day 42, these populations were no longer present. These populations are indicated in Figure 102 (A) and (B) by black arrows. As can be seen in Figure 102 (C), these populations were no longer present. Only whole kidney sorts were performed at the later timepoint. Population-based sorting did not enrich for senescent epithelia and whole kidney sorts had the added advantage of capturing immune cells. The data presented in Figure 102 are consistent with the reduction in p21^{cip1} expression shown in Figure 99 and Figure 100.

Cortical fibrosis persists but does not progress from day 7 to day 42

Figure 103 and Figure 104 show that at day 42 cortical collagen I and total collagen expression remained significantly higher in transgenic mice treated with tamoxifen compared to wild-type mice treated with tamoxifen and wild-type mice treated with corn oil.

With respect to total collagen expression, as shown in Figure 104 and Figure 105 there was no significant difference between transgenic mice treated with tamoxifen and transgenic mice treated with corn oil. The observed difference between these groups however was less by week 6 with a p value of 0.2 compared to 0.06 at day 7.

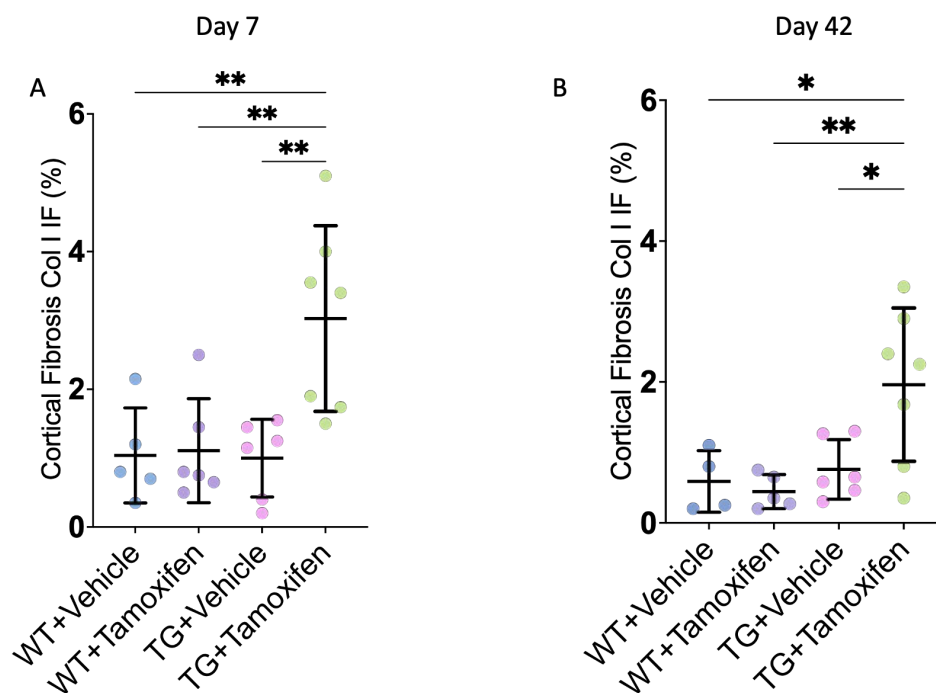


Figure 103 -Cortical fibrosis over time: collagen I immunofluorescence

Cortical collagen I immunofluorescence at day 7 (A) and day 42 (B). n=4-7. Young female mice aged <6 months. Tamoxifen 200mg/Kg x2. One way ANOVA with Tukey's multiple comparisons. All error bars are mean ± SEM *p<0.05, **p<0.01.

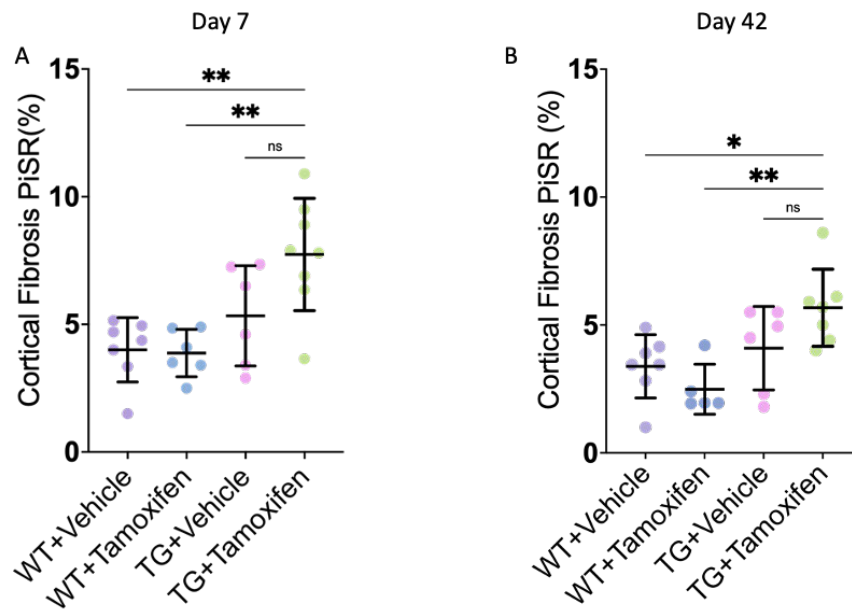


Figure 104 -Cortical fibrosis over time: PiSR

Cortical fibrosis as assessed by total collagen by PiSR at day 7 (A) and day 42 (B). n=4-7. Young female mice aged <6 months. Tamoxifen 200mg/Kg x2. One way ANOVA with Tukey's multiple comparisons. All error bars are mean $\pm$ SEM *p<0.05, **p<0.01.

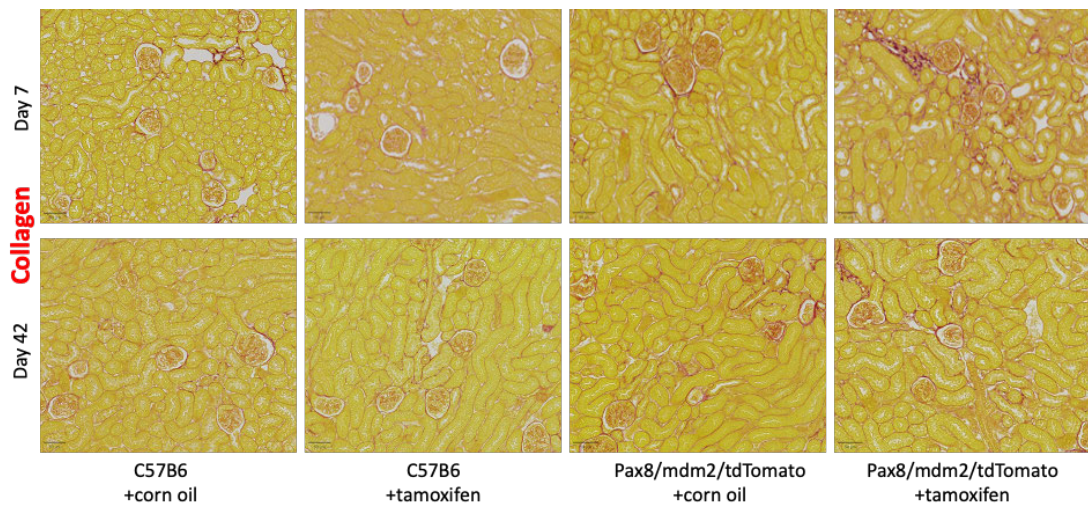


Figure 105 -Total collagen by PiSR across groups

Cortical PiSR staining at day 7 and day 42 in nephrectomised mice. Young, female mice <6 months. Tamoxifen 200mg/Kg x2.

Figure 106 shows that there was no significant difference in collagen I or total collagen expression over time in any of the groups.

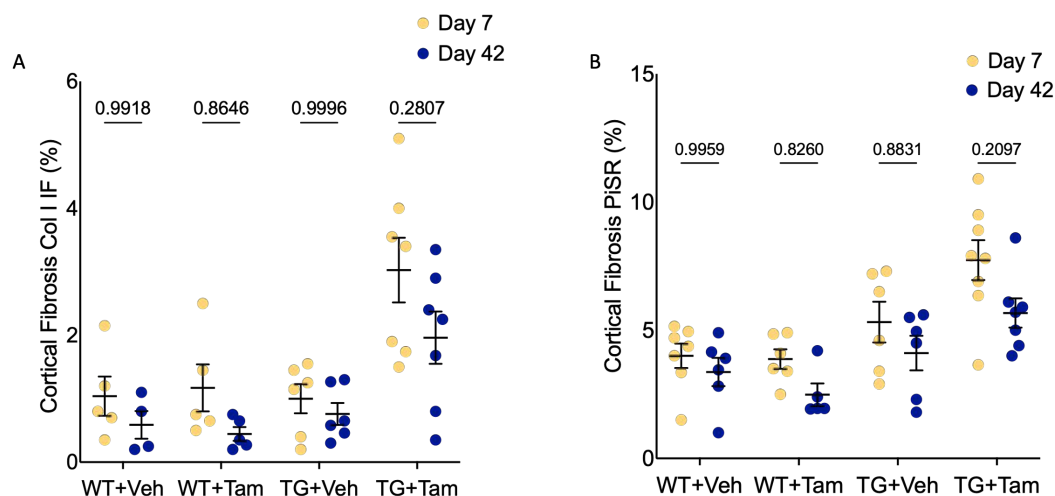


Figure 106 -Cortical fibrosis over time

Cortical fibrosis as assessed by collagen I immunofluorescence (A) and total collagen (B). n=4-8. Young female mice aged <6 months. Tamoxifen 200mg/Kg x2. 2-way ANOVA. Tukey's multiple comparisons. All error bars are mean ± SEM.

The mean levels of collagen I and total collagen expression appeared to fall across all groups. In Figure 106 this appeared more marked in the wild-type and transgenic mice treated with tamoxifen. Therefore, I analysed individual experimental groups separately as shown in Figure 107 and Figure 108. This apparent reduction in collagen I expression remained non-significant in all groups with the exception of wild-type mice treated with tamoxifen ($p=0.0476$).

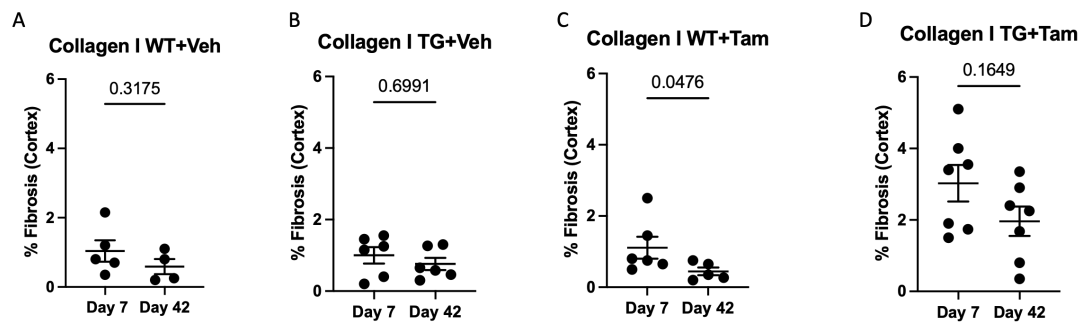


Figure 107 -Collagen I by individual group

Cortical collagen I expression over time in (A) Wild-Type+Vehicle, (B) Transgenic+Vehicle, (C) Wild-Type+Tamoxifen and (D) Transgenic+Tamoxifen. $n=4-7$. Young female mice. Tamoxifen 200mg/Kg x2. Mann-Whitney. All error bars are mean $\pm$ SEM.

With respect to total collagen expression, again there was a significant reduction in wild-type mice treated with tamoxifen ($p=0.0476$), and a trend to significant in transgenic mice treated with tamoxifen ($p=0.054$).

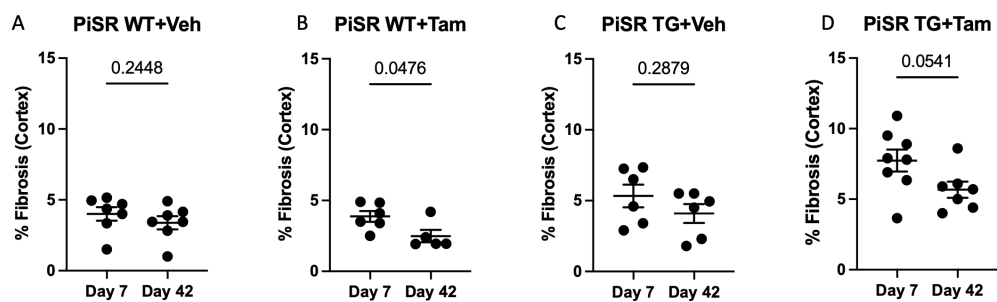


Figure 108 -PiSR by individual group

Cortical collagen expression over time in (A) Wild-Type+Vehicle, (B) Transgenic+Vehicle, (C) Wild-type+Tamoxifen and (D) Transgenic+Tamoxifen. $n=5-8$. Young female mice. Tamoxifen 200mg/Kg x2. Mann-Whitney. All error bars are mean $\pm$ SEM

Figure 109 shows that there was no change over time in expression of medullary collagen I or total collagen in any group.

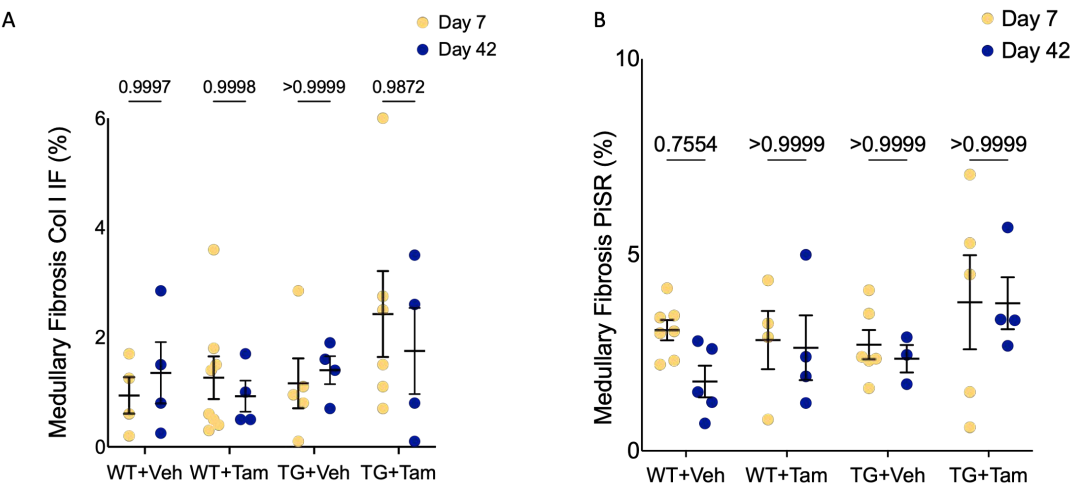


Figure 109 -Medullary fibrosis over time

Medullary collagen I expression (A) and total collagen expression (B). n=4-7. Young female mice aged <6 months. Tamoxifen 200mg/Kg x2. 2-way ANOVA. Tukey's multiple comparisons. All error bars are mean $\pm$ SEM.

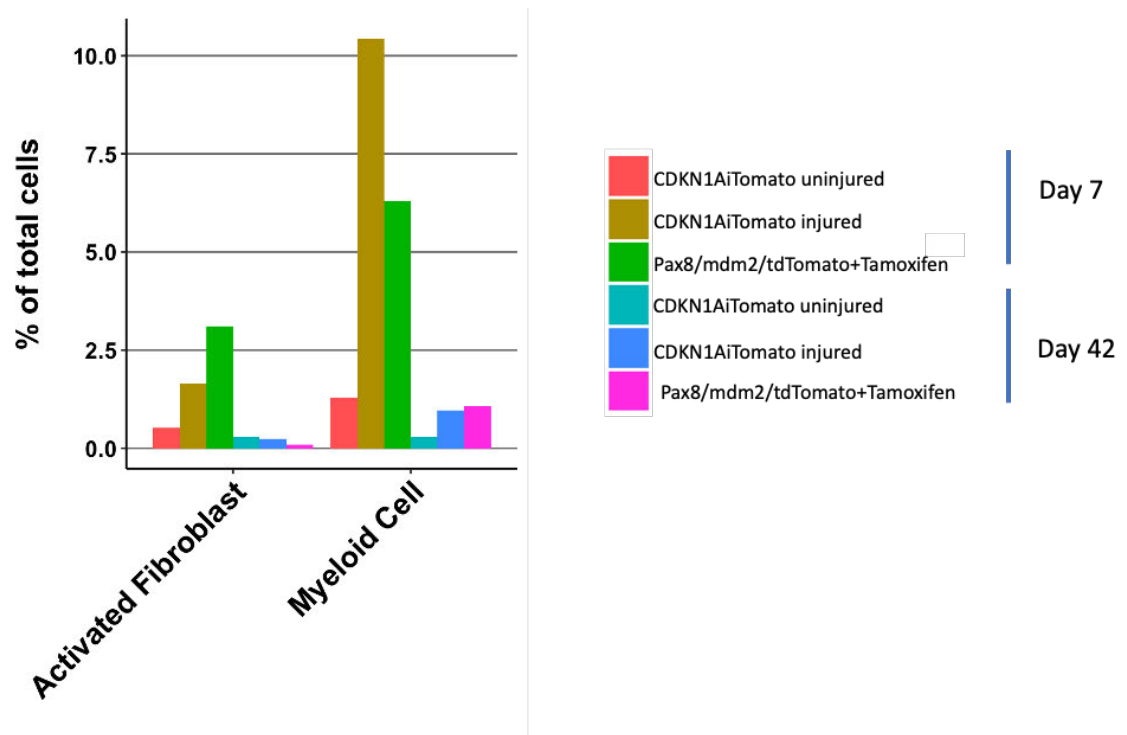


Figure 110 - Fibroblast and myeloid cell populations at day 42

Activated fibroblast and myeloid cell populations at day 7 and day 42. n=6 total, 1 mouse per experimental group per timepoint. Whole kidney sorts. Young female mice. Tamoxifen 200mg/Kg x3. **Parse sequencing data only.**

Figure 110 shows that as an overall percentage of all cells sorted per mouse both the activated fibroblast and myeloid cell populations appeared smaller by day 42.

tdTomato fluorescence remains apparent at day 42

Figure 111 and Figure 112 show that a tdTomato positive population remained apparent at flow cytometry at day 42. As discussed, a change in tamoxifen dosing did not significantly impact on tdTomato expression and therefore the data in Figure 112 (A) includes all doses.

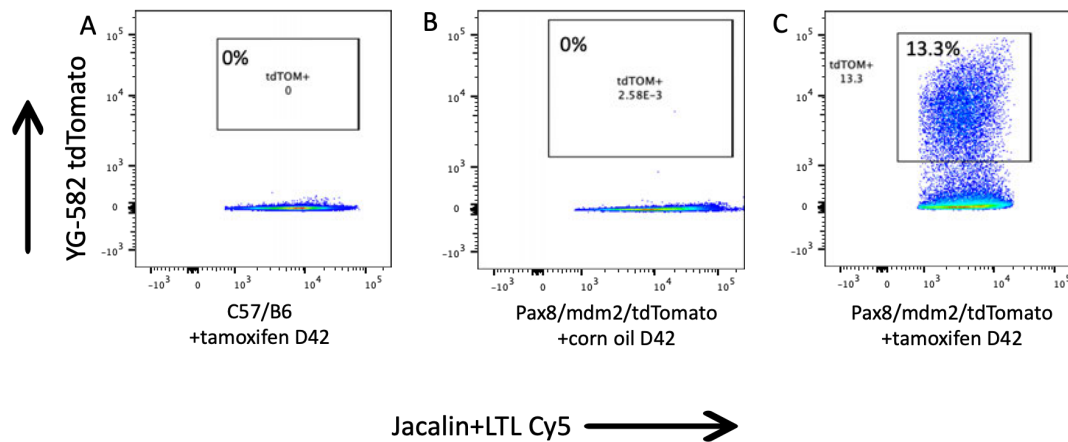


Figure 111 - Flow cytometry tdTomato population at day 42

A) Wild-type mouse treated with tamoxifen 200mg/Kg x2. B) Pax8/mdm2/tdTomato mouse treated with corn oil. C) Pax8/mdm2/tdTomato mouse treated with tamoxifen 200mg/Kg x2. Young female mice.

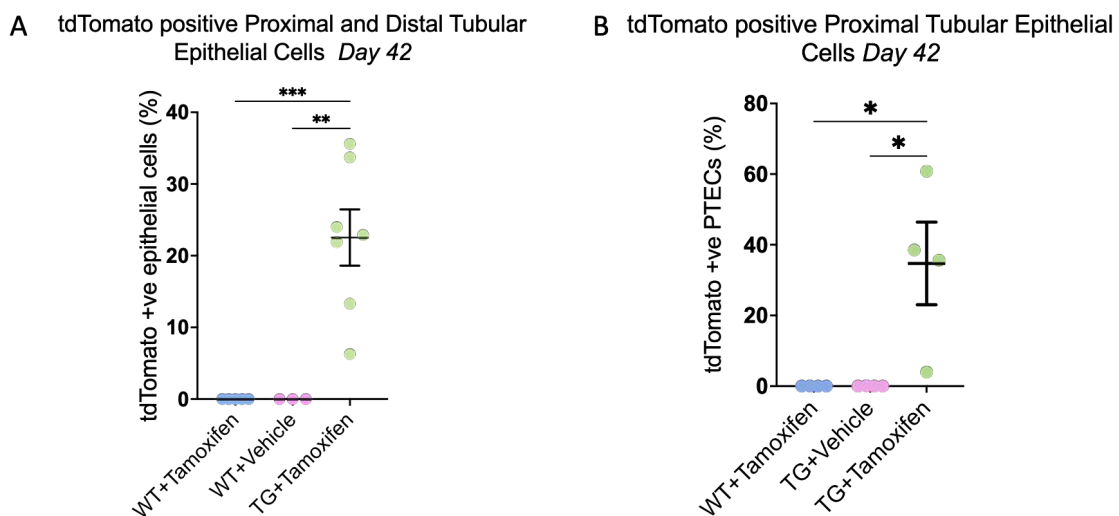


Figure 112 - tdTomato expression across experimental groups

A) tdTomato positive proximal and distal tubular epithelial cells (gated on JacalinLTL and tdTomato positivity). B) tdTomato positive proximal tubular epithelial cells (gated on LTL and tdTomato positivity) n=4-5. Young female mice. One-way ANOVA. All error bars are mean $\pm$ SEM. *p<0.05, ****p<0.001.

Figure 113 shows that whilst tdTomato expression was still apparent by day 42, in some mice the expression levels were lower. The lower levels of day 42 tdTomato expression shown in Figure 113 ranged from 3.97% to 13.3% of epithelial cells.

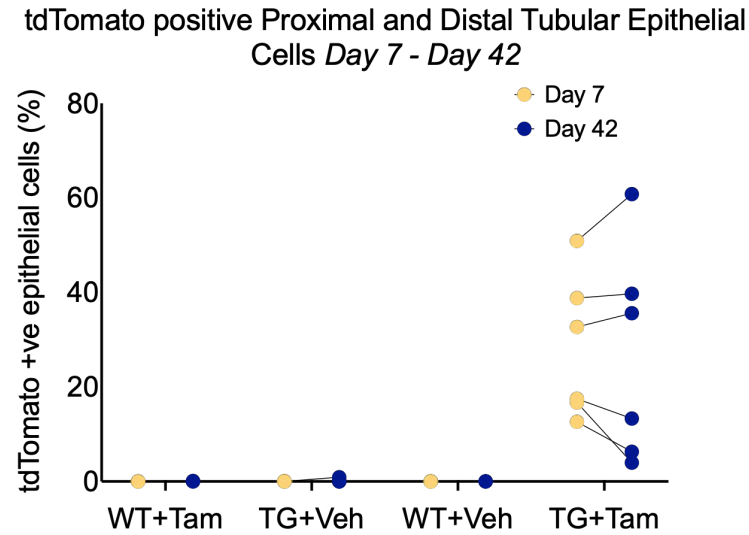


Figure 113 -tdTomato expression over time

tdTomato expression at flow cytometry in the same mouse at day 7 and day 42. n=3-6 per group. Female mice, <6 months. Tamoxifen 200mg/Kg x2. Multiple unpaired t-tests, Holm-Šídák method.

Figure 114 shows that transcription of tdTomato was still apparent at day 42, though appeared less obvious than at day 7.

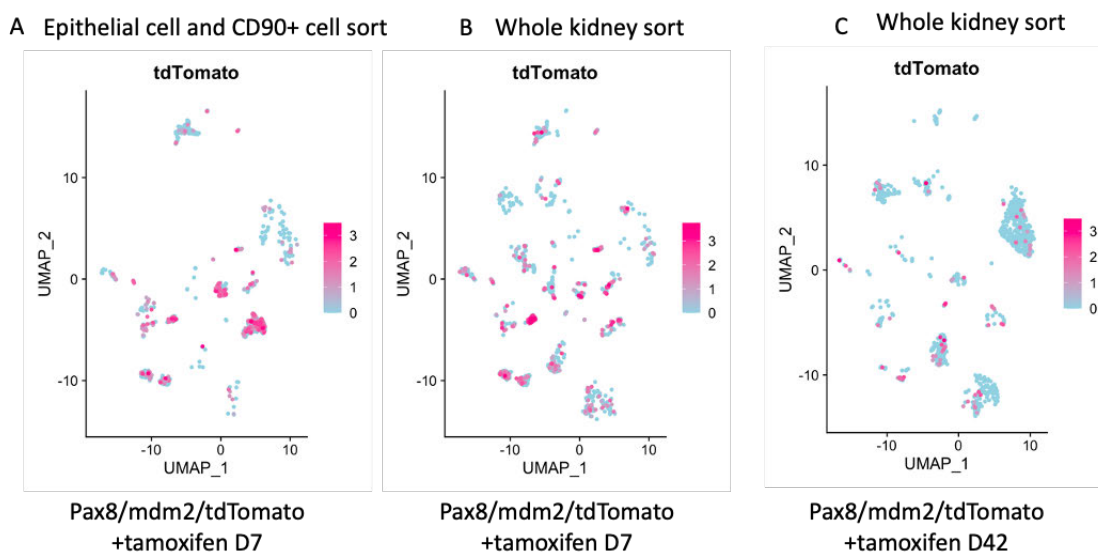


Figure 114 -tdTomato expression at different timepoints

tdTomato expression at day 7 and day 42. Cell count normalised to 1000 cells per mouse. n=1 per group, young female mice. Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} treated with tamoxifen 200mg/kg x3. A) Population based sort for tdTomato+ and CD90+ cells. Cells sorted 7 days after tamoxifen. B) Whole kidney sort. Cells sorted 7 days after tamoxifen. C) Whole kidney sort. Cells sorted 42 days after tamoxifen. **Parse sequencing data only.**

Discussion

p21^{cip1} expression falls by day 42

Cortical nuclear p21^{cip1} expression fell by day 42 in induced transgenic mice from $10.45 \pm 2.97\%$ to $4.09 \pm 1.04\%$. Whilst this did not reach significance, there was a trend to significance with a p value of 0.12 comparing day 7 and day 42 induced transgenic mice vs $p \geq 0.999$ in all other experimental groups.

The fall in p21^{cip1} expression was unexpected. In both irradiation and IRI, renal expression of *Cdkn1a* remained significantly elevated at a later timepoint; 16 weeks for irradiation, 5 weeks for IRI (Mylonas, O'Sullivan et al. 2021). In a liver model of senescence induced by loss of MDM2, p16^{INK4A} positive cholangiocytes and hepatocytes were noted at 90 days post induction (Ferreira-Gonzalez, Lu et al. 2018).

As discussed in the previous results chapter (and shown in Figure 63), *Csf1* was expressed by senescent epithelia. Expression levels were around 3.5 times higher in senescent epithelia than in any other cell as shown in the differential gene analysis list (Appendix B). Immune cell recruitment was noted after transgenic induction. It was recently demonstrated in a transgenic model of p21^{cip1} over expression that macrophages were recruited *via* CXCL14 (Sturmlechner, Zhang et al. 2021). By day 12 post induction, there was a significant reduction in the number of senescent cells and senescent cells were noted to be surrounded by CD8+ T cells. A concomitant increase in dying tdTomato+ hepatocytes led the authors of this paper (Sturmlechner, Zhang et al. 2021) to conclude that senescent cells underwent cytotoxic T cell clearance.

Cortical fibrosis persists but does not progress

As shown in Figure 106 to Figure 108, there appeared to be a small reduction in collagen I and total collagen expression across all groups by day 42. When analysed as an entire dataset by 2-way ANOVA, no group had a significant reduction in either marker of fibrosis. When analysed individually as single experimental groups, wild-

type mice treated with tamoxifen had a small but significant reduction in both collagen I and total collagen over time. Transgenic mice treated with tamoxifen showed a trend to significance with respect to total collagen expression ($p=0.054$).

Regarding the observed reduction across all groups, the day 42 analysis was performed in mice that underwent unilateral nephrectomy 5 weeks earlier. It is recognised that in the context of nephrectomy, the contralateral kidney undergoes a compensatory increase in size and function (Kikuchi, Chou et al. 2023). It is possible that although there was no significant change in kidney weight over time as shown in Figure 115 that there was expansion of the renal parenchyma and as I measured interstitial staining as a % of representative areas, and expansion of the other structures in those areas may have affected that.

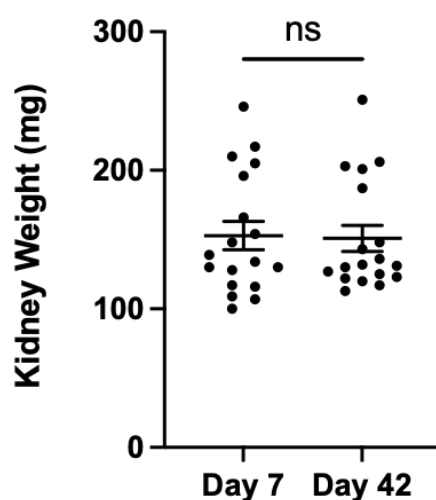


Figure 115 - Kidney weight day 7 vs day 42

Kidney weight at unilateral nephrectomy vs cull. $n=18$. Young female mice aged <6 months. All experimental groups (WT+veh, WT+tam, TG+veh, TG+tam). Paired t-test. Error bars mean $\pm$ SEM.

It was recently demonstrated that peroxisome proliferator-activated receptor α (PPAR α) was up-regulated after unilateral nephrectomy and was strongly associated with the compensatory growth of proximal tubules (Kikuchi, Chou et al. 2023). PPAR α has been implicated in the reduction of fibrosis in several murine models. PPAR α deficient mice exhibited higher levels of liver fibrosis in a murine fatty liver mode,

and PPAR α agonism led to less activated macrophages and less fibrosis (Pawlak, Lefebvre et al. 2015). PPAR α knockout mice developed more serious pulmonary fibrosis in response to bleomycin (Lakatos, Thatcher et al. 2007).

It is possible that nephrectomy led to the upregulation of an anti-fibrotic compound e.g. PPAR α in the contralateral kidney. The observed reduction in fibrosis however was very small and I think it is unlikely there was any direct anti-fibrotic effect. This experiment has been repeated in non-nephrectomised mice and will be analysed for the same markers of fibrosis in due course. The mice that were used for the scRNA sequencing experiment did not undergo unilateral nephrectomy. Therefore, it was not possible for me to look for up-regulation of *Ppara* in the 'contralateral kidneys' of these mice, though this would have been interesting.

Overall, the reduction in fibrosis was non-significant when the entire dataset was analysed. When I looked specifically at individual groups, wild-type mice who received tamoxifen had a small but significant reduction in total collagen expression ($3.8 \pm 0.37\%$ to $2.4 \pm 0.43\%$) and collagen I expression ($1.1 \pm 0.3\%$ to $0.44 \pm 0.1\%$). There was a trend to significance in reduction of total collagen expression in transgenic mice who received tamoxifen ($p=0.0541$). It is recognised that tamoxifen has anti-fibrotic effects in female mice which extended beyond a washout period of 14 days between cessation of the drug and assessment of tissue (Falke, Broekhuizen et al. 2017). Longer washout periods have not been assessed and it is possible that tamoxifen itself had an anti-fibrotic effect. It will be important to take this into consideration when analysing male tissue in the near future.

Given that wild-type tamoxifen treated mice also demonstrated a reduction in markers of fibrosis, care must be taken when interpreting any fall in induced transgenic mice. However, despite collagen I and collagen expression being significantly higher in this group, fibrosis did not progress. The data I have generated demonstrated that induction of senescence led to the initiation of fibrosis. Whether this is a direct epithelial to mesenchymal effect, or *via* recruited leukocytes (or both)

remains unclear. However, the fact that after induction fibrosis progression appears to have been halted is interesting. One explanation for this is that senescent epithelia have been cleared and hence pro-fibrotic stimuli are no longer present by day 42. It will be important to investigate senescent cell clearance with larger experimental groups.

In a p21^{cip1} driven cholangiocyte senescence, fibrosis persisted to 21 days, and gut fibrosis to 90 days (Ferreira-Gonzalez, Lu et al. 2018). This study included male and female mice. A direct comparison of an early vs late timepoint was not published. In models of renal senescence induced by injury or irradiation, significant fibrosis was evident 35 days post-injury. Again, no direct comparison was made between day 35 and an early timepoint and so it is not possible to say if fibrosis progressed over this period. It is worth noting that in both of these examples senescent cells also persisted. (Ferreira-Gonzalez, Lu et al. 2018, Mylonas, O'Sullivan et al. 2021).

The fact that neither p21^{cip1} nor markers of fibrosis rose in any experimental group suggests that unilateral nephrectomy *per se* was not injurious to the remaining contralateral kidney.

tdTomato expression remains apparent by day 42

tdTomato positive populations were still apparent by day 42 at flow cytometry and tdTomato mRNA was detected 42 days after transgenic induction, though the overall levels of transcription appeared less.

The most likely explanation for this is a disconnect in recombination efficiencies between loss of Mdm2 and expression of tdTomato. Defining the recombination efficiency of both elements will be key to answering this. I have discussed the various possible methods of investigating this further in the chapter 3 discussion and in the general discussion.

Future experiments

To investigate potential immune cell clearance, I would look for an association between immune cells and senescent cells at a protein level. As discussed, it would be important to confirm macrophage recruitment at a protein level. Sturmlechner *et al* (2021) observed a switch in macrophage polarisation 4 days after senescence induction, with subsequent CD8+ T cell recruitment. The authors suggested that a p21^{cip1} growth arrest led to a time period during which the cell could repair, and if this period was exceeded, stressed cells expressing p21^{cip1} underwent immune cell clearance.

If immune cells are key to the clearance of senescent cells, then blocking their recruitment to sites of senescence should impair such clearance. As discussed in the previous results chapter, this could be achieved by blocking key SASP components. Unlike Sturmlechner *et al* (2021) I did not observe increased expression of *Cxcl14* by senescent cells. However, they reported data from senescent hepatocytes and it is recognised that the SASP varies by cell type (Coppe, Desprez *et al.* 2010).

NK cells have also been implicated in senescent cell clearance *via* an interaction between the NK activator receptor NKG2D and ligands on the surface of stressed cells such as MICA and ULBP1-6 (Iannello and Raulet 2013). Mice lacking the NKG2 receptor had significantly higher SA- β -Gal expression compared to control mice in a murine liver injury model (Sagiv, Burton *et al.* 2016). As shown below in Figure 116, in the context of transgenic induction, a population of NK cells was identified, and they expressed *Klrk1* (encodes NKG2). However, the senescent epithelial populations identified in the induced transgenic mice did not express *Ulbp1*. Again, a larger dataset will allow this to be investigated more definitively. I have not looked by immunohistochemistry for NK cells and it would be interesting to look for any spatial association between senescent cells and NK cells at a protein level.

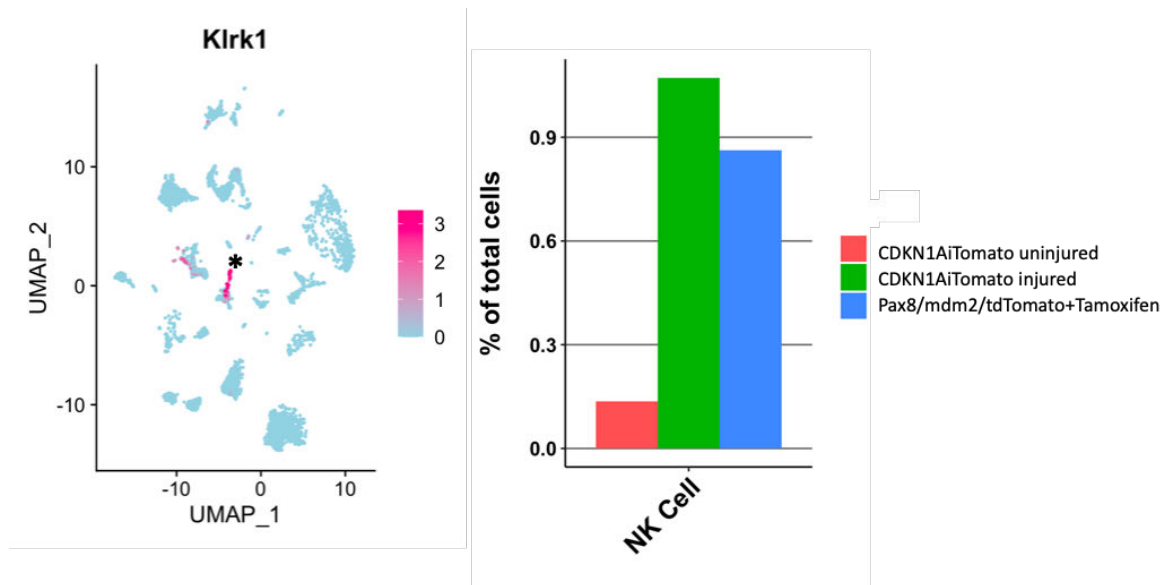


Figure 116 -NK cell population

NK cell population. n=3 total, 1 mouse per experimental group. Whole kidney sorts. Young female mice. Pax8Cre-ERT²/mdm2^{flox/flox}/tdTomato^{LSL}; tamoxifen 200mg/Kg x3 7 days prior to cell sorting. CDKN1AiTomato mice; tamoxifen 200mg/Kg x2 48-72 hours prior to cell sorting. A population of NK cells expressing the activated NK receptor NKG2 is highlighted by *.

A further option for investigating immune cell mediated clearance of senescent cells would be to induce senescence in immunodeficient mice deficient for the immune cell of interest; for example, CD8+ T cell deficient mice (Ilatovskaya, Pitts et al. 2019). If immune cell mediated clearance is key, senescent cells should persist when the immune cell (s) suspected to mediate such clearance are no longer present.

It is interesting to note that the most marked reduction in p21^{cip1} expression was observed in mice that had higher day 7 levels of p21^{cip1}. If the hypothesis that senescent cells in this model have induced their own clearance through leucocyte recruitment, then it is possible that higher levels of p21^{cip1} led to more robust immune cell recruitment, and ultimately more efficient senescent cell clearance.

Conclusions

p21^{cip1} and *Cdkn1a* expression fell by 42 days post induction. Levels of fibrosis were also lower and there was no suggestion of a persistent immune cell population at this later timepoint. Further work with a larger dataset is underway to confirm if the apparent clearance of senescent epithelia is significant. Immune cell mediated clearance of senescent cells is recognised (Krizhanovsky, Yon et al. 2008, Sagiv, Burton et al. 2016, Sturmlechner, Zhang et al. 2021) and at present, I think this is the most likely explanation for the changes observed by day 42.

Chapter 6: General discussion

Summary of findings

The key question I set out to answer during this PhD was if the induction of renal epithelial senescence, without any other renal insult, was sufficient to induce fibrosis in young mice.

Using a conditional transgenic model of renal epithelial restricted *Mdm2* loss, I observed significant $p21^{cip1}$ expression in induced transgenic Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice. In line with international guidelines on senescent cell identification (Childs, Durik et al. 2015, Gorgoulis, Adams et al. 2019), an absence of Ki67 expression confirmed that $p21^{cip1}$ expressing cells were not proliferating. The scRNA sequencing data showed that distinct populations of senescent epithelia existed in induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice.

At an early timepoint of seven days after the first dose of tamoxifen, I observed a significant increase in cortical fibrosis in transgenic, but not wild-type mice. This was associated with immune cell recruitment, demonstrated by flow cytometry and scRNA sequencing. These data show that epithelial senescence alone was sufficient to induce fibrosis in young immunocompetent female mice. Fibrosis persisted but did not progress. There was a suggestion at both an mRNA and protein level that senescent epithelia were cleared by day 42.

Defining senescence in this model

It is recommended that senescent cells are defined using a three-step approach (Gorgoulis, Adams et al. 2019) as shown below in Figure 117.

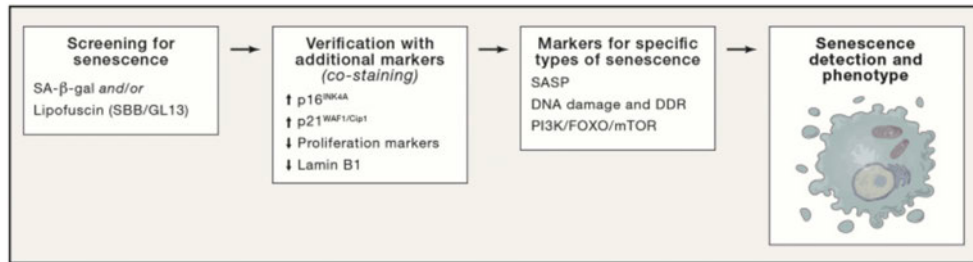


Figure 117 - A three step approach to defining senescence (Gorgoulis, Adams et al. 2019)

The data I have presented were from *in vivo* studies and the use of fixed tissue prevented SA-β-gal staining. I demonstrated that p21^{cip1} expressing cells did not express Ki67. Senescent populations identified by single cell sequencing were highly enriched for genes associated with senescence and the SASP.

The differential gene analysis lists for both senescent cell populations are found in the Appendix B. In addition to *Cdkn1a*, genes which have previously been associated with senescence (O'Sullivan, Mylonas et al. 2022) such as *S100a6* (encodes S100A6) and *S100a11* (encodes S100A11), both recognised to regulate p21^{cip1} (Foertsch, Teichmann et al. 2013, Song, Xu et al. 2020) were upregulated.

Cryab and *Cd24* were also upregulated. Both have been identified in other single cell sequencing datasets and are suggested to be viable senolytic targets (Limbad, Doi et al. 2022, Doolittle, Saul et al. 2023). Senescent cells are recognised to upregulate anti-apoptotic genes (Zhu, Tchkonina et al. 2015). *Bcl2l1* was up-regulated as was *Nrg1* (NRG-1β) which has been shown to prevent apoptosis in stressed cardiomyocytes (Kuramochi, Cote et al. 2004).]

As discussed, *Mdm2* upregulation seen in senescent epithelia is consistent with its activation by p53. MDM2 is a negative regulator of p53. Therefore, in the context of

cre-mediated excision of part of the *Mdm2* gene and the translation of a non-functional truncated MDM2 protein, levels of p53 rise. *Mdm2* deletion occurs in exons 5 and 6. This leaves an intact p53-binding promoter region in the truncated *Mdm2* gene (Grier, Yan et al. 2002). Thus, p53 mediated upregulation can still occur in cells with truncated *Mdm2*. The relationship is known to be autoregulatory (Nag, Qin et al. 2013). As expected, senescent epithelia were highly enriched for *Trp53* related genes. *Mdm2* has been identified as a major p53 target gene in other datasets (Moyer, Wasylshen et al. 2020) which used this *Mdm2*^{fl/fl} mouse, and this is also what we observed.

It is possible that some senescent epithelia retained full length *Mdm2* and instead were induced to senesce by neighbouring cells. Paracrine senescence is well described (Ferreira-Gonzalez, Man et al. 2022). NOTCH signalling has been implicated in the propagation of paracrine senescence (Teo, Rattanavirotkul et al. 2019). Senescent epithelia upregulated *Notch3*. By immunofluorescence, I observed 'groups' of senescent nuclei in close proximity. For future work, I would look for p21^{cip1} and tdTomato co-expression. Primary senescent cells in this model should express both tdTomato and p21^{cip1}, whereas I would expect secondary senescent cells to express p21^{cip1}/other markers of senescence but not tdTomato.

Taken together, the histology and sequencing data suggest that transgenic induction in young female Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice resulted in epithelial senescence which was pro-fibrotic at an early timepoint. Senescent epithelia which persist after renal injury (vs transgenic induction) have also been demonstrated to be pro-fibrotic (O'Sullivan, Mylonas et al. 2022). In this Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} model, there is a suggestion that senescent epithelia are cleared over time and this differs to the persistence observed in the context of a concomitant renal insult (O'Sullivan, Mylonas et al. 2022). Whilst senescent cell clearance is described (Kang, Yevsa et al. 2011, Sagiv, Burton et al. 2016, Sturmlechner, Zhang et al. 2021), I am not aware of a published model of epithelial

senescence reporting both early fibrosis and subsequent immune-mediated senescent cell clearance. Sagiv *et al* (2016) looked at mesenchymal senescence, and whilst Sturmlechner *et al* (2021) and Kang *et al* (2011) published models of hepatic epithelial senescence, neither study reported fibrosis data. Conversely, there are lots of examples of senescence induced fibrosis (Schafer, White *et al.* 2017, Bird, Muller *et al.* 2018, Mylonas, O'Sullivan *et al.* 2021, O'Sullivan, Mylonas *et al.* 2022). In these contexts, however, senescent epithelia persisted. Whilst further work is required to confirm that senescent cells are indeed cleared in induced Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice, it would be really interesting to understand if others have also observed this early fibrosis followed by immune cell mediated clearance.

SASP Factors

Senescent epithelia significantly up-regulated SASP markers such as *Gdf15* and *Csf1*. Tamoxifen treatment in transgenic mice resulted in evidence of recruited immune cells at both a protein and mRNA level which was not observed in wild-type controls.

I did not find evidence of classical SASP markers such as *Tgfb1*, *Il1b*, *Tnf*, or *Il6*. Firstly, my data were from *in vivo* work. Much of the published literature which defined and characterised the actions of SASP components was based on *in vitro* studies (Coppe, Desprez *et al.* 2010, Acosta, Banito *et al.* 2013, Basisty, Kale *et al.* 2020, Yasuda, Koiwa *et al.* 2021). Studies which induced senescence *in vitro* used stimuli (e.g., oncogene expression, irradiation or cytotoxic drug treatment) which differed to the mode of induction I used. It is recognised the SASP varies by cell type and the senescence inducer (Coppe, Desprez *et al.* 2010). *In vivo* studies which have identified key SASP components have analysed whole tissue by using RT-qPCR or bulk RNA Sequencing (Schafer, White *et al.* 2017). It is possible that factors such as *Tgfb1* were being produced by recruited leukocytes and this would not be apparent from whole tissue bulk analysis. In murine acute liver injury, it was macrophage derived *Tgfb1* that was demonstrated to be key to inducing paracrine senescence (Bird, Muller *et al.* 2018).

This is consistent with the expression pattern I observed at scRNA sequencing where *Tgfb1*, *Il1b* and *Il6* expression was limited to immune cells.

It is important to note this expression pattern was observed in two mice and hence has been repeated with larger experimental groups. The sequencing data will be available shortly. I also performed RT-qPCR on sorted tdTomato positive epithelial cells which was consistent with a lack of *Tgfb1* or *Il1b*, however these data require validation with larger group sizes to be definitive.

Gdf15

The upregulation of *Gdf15* is interesting. As seen in the differential gene expression list (Appendix B) *Gdf15* expression was 15 times higher in 'Senescent Mdm2 b' compared to all other cells.

GDF15 is a well-recognised SASP protein (Coppe, Desprez et al. 2010, Acosta, Banito et al. 2013). It belongs to the TGF β superfamily (Lawton, Bonaldo et al. 1997). Though the biology is not fully understood, it has been associated with inflammation in both murine and human disease (Johnen, Lin et al. 2007, Abulizi, Loganathan et al. 2017, Wu, Jiang et al. 2018). It is a stress response cytokine and as such is upregulated by inflammatory proteins including IL1 β , TNF, IL2 and MCSF1 (Tsai, Husaini et al. 2018). GDF15 is strongly induced by p53 (Li, Wong et al. 2000), consistent with our observation of *Trp53* target genes being highly differentially expressed by senescent epithelia.

GDF15 expression strongly correlates with disease. It has been identified as a key biomarker of chronological age in humans (Basisty, Kale et al. 2020, Schafer, Zhang et al. 2020). Peripheral blood levels predicted adverse outcomes in patients undergoing cardiac surgery for aortic stenosis (Schafer, Zhang et al. 2020) and high levels of GDF15 have been associated with chronic obstructive pulmonary disease (COPD) exacerbations (Husebo, Gronseth et al. 2017). In a studies of all-cause

mortality in a general population, high levels of GDF15 conferred a 2.6-fold increased risk of death, after adjustment for smoking status, chronological age and BMI. Further adjustment for telomere length, CRP and IL6 levels did not weaken the association. The measured level of circulating GDF15 correlated with both mortality risk and survival time (Wiklund, Bennet et al. 2010).

Whether GDF15 directly causes tissue damage is not understood. GDF15 expression was significantly higher in lung tissue from patients with IPF vs healthy aged-matched controls. Mice that received an anti-GDF15 antibody had significantly less fibrosis after bleomycin injury at both a protein and mRNA level. GDF15 neutralisation had no effect on *Ckd1a* or *Ccl2* expression. Human lung fibroblasts cultured with recombinant GDF15 up-regulated α -SMA, *Col1a1* and *Col3a1*. Signalling was thought to be *via* the TGF β receptor and α -SMA expression fell to baseline with concomitant GDF15 and TGF β blockade (Radwanska, Cottage et al. 2022). Much like TGF β 1 (Kulkarni, Ward et al. 1995), GDF15 has been reported as having anti as well as pro-inflammatory effects (Zhang and Dong 2022). Its effect (protective vs deleterious) has been described as likely relating to dose, tissue environment and cell type (Al-Mudares, Reddick et al. 2020).

Csf1

As discussed, *Csf1* was also differentially expressed by senescent epithelia. CSF1 has been implicated in immune cell recruitment and immunosurveillance. In a murine model of liver cancer, restoration of p53 in tumour cells resulted in expression of *Csf1*, *Mcp1* and *Cxcl1* with subsequent macrophage and NK cell recruitment (Xue, Zender et al. 2007). CSF1 is known to promote myeloid cell proliferation (Stanley and Chitu 2014).

The data presented within this thesis show that transgenic induction in Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice resulted in early cortical fibrosis. As there was no concomitant renal insult, this implies that SASP components are potential drivers of

this process. The observed immune cell recruitment offers a potential explanation for both fibrosis and senescent cell clearance. Confirming if SASP factors clearly recognised to recruit immune cells such as *Csf1* are upregulated in a larger dataset will be informative and will determine future experiments.

Limitations

Recombination efficiency

One of the biggest limitations of this work was my inability to define the individual recombination efficiencies of both floxed constructs. This meant I was not confident in how faithful tdTomato was as a marker of senescent cells. tdTomato mRNA was clearly detected in non-epithelial and non-senescent cells at single cell RNA sequencing. The expression pattern was very similar in Pax8Cre-ERT²/mdm2^{flox/flox}/tdTomato^{LSL} mice and CDKN1AiTomato mice (9 mice in total). The Ai14Ho mouse was used to generate both strains. The alignment was to a sequence in the tdTomato region of the gene (i.e., not to the STOP codon or the CAG promoter). We asked the senior author of the original Ai14 Ho publication on two separate occasions if the gene is transcribed at baseline and she was quite clear that unless recombination occurs, transcription is truncated in the STOP codon.

The tdTomato probe I used for *in-situ* hybridisation also binds to mRNA in the tdTomato region. Both modalities are therefore subject to the same potential issue. If the gene is transcribed at baseline, the mRNA will be detected with scRNA sequencing and *in-situ* hybridisation even in the absence of recombination.

With the knowledge of the data I have at present, I would look for co-expression of markers of senescence and tdTomato at a protein level, i.e., a dual p21^{cip1} and tdTomato immunofluorescence stain. That was the first approach I took early in this PhD. On small staining runs used to optimise protocols, I observed tdTomato being expressed in non-p21^{cip1} positive cells (though less marked than the sequencing data). Hence, I pursued alternative approaches with *in-situ* hybridisation and scRNA

sequencing. It would be important to re-investigate this at a protein level with numbers allowing statistical analysis.

I would also test if tdTomato positive cells expressed markers of senescence *in vitro*. I was able to culture epithelial cells from wild type and transgenic mice. Tamoxifen (4OHT) treatment *in vitro* resulted in tdTomato expression as shown in Figure 97. Due to time constraints, I was not able to optimise antibody staining or RNA extraction. If tdTomato reliably marked senescent cells, I would expect TdTomato positive cells not to proliferate. To test this, I would look for the absence of Ki67 or downregulation of *Mki67*. I would also expect co-expression with senescence markers e.g., p21^{cip1} and by qPCR I would look for up-regulation of *Cdkn1a* and other associated features of senescence e.g., downregulation of *Lmn1*.

Understanding if tdTomato truly labels senescent cells would allow me to identify secondary senescent cells. This is important to further understanding the heterogeneity of senescence as primary and secondary senescent cells are recognised as being functionally distinct (Teo, Rattanavirotkul et al. 2019).

I observed a reduction in nuclear p21^{cip1} expression by day 42. Whilst it is generally accepted that senescence is a form of permanent growth arrest (Gorgoulis, Adams et al. 2019), there are examples of p21^{cip1} expressing cells re-entering a replicative cycle (Hsu, Altschuler et al. 2019). If tdTomato reliably fate labelled senescent cells, then I would expect to see an equivalent reduction in tdTomato expression at a later timepoint if p21^{cip1} expressing cells were cleared.

tdTomato fate labelling would also potentially allow me to test the hypothesis that senescent cells undergo immune cell mediated clearance. For example, if senescent epithelia were phagocytosed by macrophages, I might expect to see a CD45+/tdTomato+ population at flow cytometry.

scRNA sequencing dataset

In the mice used to generate the scRNA sequencing dataset, I used the entire injured kidney for cell sorting and sequencing. This was with the intention of maximising my cell yield but was an oversight as it prevented histological analysis for markers of senescence and fibrosis.

The mouse numbers for the single cell dataset are small. We expected sorting for tdTomato positive and CD90+ cells to enrich for senescent and activated fibroblasts. Population-based sorting made no difference to enrichment. The marked immune cell recruitment is interesting and, as discussed, potentially important from both a fibrosis and senescent cell clearance point of view. Excluding CD45+ cells meant that immune cells could only be studied at scRNA sequencing in single mice making definitive comparisons impossible. Dr Katie Mylonas and Ross Campbell who are part of our research group both have an interest in immune cell and senescent cell interactions. It will be important to confirm if senescent epithelia are spatially correlated with recruited immune cells at a protein level. Work is already underway to look at this.

The single cell experiment has been repeated in male mice to include a minimum of 3 mice per experimental group and two modes of senescence induction: loss of *Mdm2* vs injury (IRI). An additional timepoint of 2 weeks post injury has been added. If the results are different to the current smaller scRNA dataset, consideration as to whether this simply reflects a gender-based effect will be required. Should a gender effect be suspected, I would repeat a larger experiment in female mice of equal group sizes. I have never looked at day 14 and given the current suggestion from the data I have; it will be interesting to see if senescent epithelia persist at this timepoint. If they are observed at day 14 and not at day 42 then the next logical step would be to pursue an intermediate timepoint e.g., day 28.

Future work

I would continue to investigate if tdTomato labels senescent cells. This is important for my own research and for any other group who choose to use the Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse for future experiments.

Having used male mice and a more severe injury, we expect to find a population of senescent epithelia in injured mice. Senescent cells are heterogenous, and it is likely that post-injury senescent cells will differ to those induced by loss of *Mdm2*. It will be interesting to identify key differences between both populations. One scenario which would be fascinating would be if post-injury senescent epithelia persist, and 'induced genetic' (*Mdm2* loss) senescent cells are cleared. This would provide an experimental model in which we could investigate senescent cell clearance in a highly detailed manner.

If this larger dataset confirms that senescence induction in Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice results in early leukocyte recruitment and fibrosis followed by senescent cell clearance I would confirm that at a protein level, looking for markers of senescence, immune cell and myofibroblast expression, in addition to collagen quantification. As discussed, I would generate a list of candidate SASP markers suspected to be key to immune cell recruitment. I would consider blocking factors individually *in vivo* in induced transgenic mice, for example using antibody blockade. I would also consider immune cell depletion or generating an immunodeficient Pax8Cre-ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse strain. If senescent cells persist in immunodeficiency, this suggests immune cells are important for clearance. Both depletion and immunodeficiency would allow individual immune cell classes to be targeted.

Ligands expressed on the surface of senescent cells are likely to be key to immune cell recognition. There are two options test the importance of such a ligand of interest. Firstly, to use a cre-lox recombinase approach. This would involve identifying a mouse strain with the floxed ligand of choice and crossing this mouse a Pax8Cre-

ER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mouse to generate a mouse in which recombined cells which undergo loss of *Mdm2* are also unable to express the ligand of interest. Alternatively, I would cross mice deficient in the ligand with Pax8CreER^{T2}/mdm2^{flox/flox}/tdTomato^{LSL} mice. The cre-recombinase option is preferable because it tests the importance of senescent cell restricted expression of the ligand and removes any confounding effect from lack of expression in other cell types.

The very high upregulation of *Gdf15* is striking and is a possible explanation for how senescent cells may have directly induced fibrosis. To test this, I would block GDF15, with an anti-GDF15 antibody in mice that had undergone senescence induction.

Final conclusions

The work presented in this thesis demonstrates that senescent epithelia induced by the loss of *Mdm2* were pro-fibrotic in young female mice at an early timepoint. This was in the absence of any other renal insult. Induction of senescence appeared to cause immune cell recruitment, with possible subsequent clearance of senescent cells themselves. If this is confirmed, it suggests there are mechanisms in a healthy kidney which allow detection and clearance of senescence and prevent on-going fibrosis. Renal fibrosis is currently untreatable. Thus, understanding how pathways which promote physiologic clearance of pro-fibrotic senescent epithelia are lost with chronic injury or ageing is of potential therapeutic importance.

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Cellular Senescence and Senotherapies in the Kidney: Current Evidence and Future Directions

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Cellular senescence refers to a cellular phenotype characterized by an altered transcriptome, pro-inflammatory secretome, and generally irreversible growth arrest. Acutely senescent cells are widely recognized as performing key physiological functions *in vivo* promoting normal organogenesis, successful wound repair, and cancer defense. In contrast, the accumulation of chronically senescent cells in response to aging, cell stress, genotoxic damage, and other injurious stimuli is increasingly recognized as an important contributor to organ dysfunction, tissue fibrosis, and the more generalized aging phenotype. In this review, we summarize our current knowledge of the role of senescent cells in promoting progressive fibrosis and dysfunction with a particular focus on the kidney and reference to other organ systems. Specific differences between healthy and senescent cells are reviewed along with a summary of several experimental pharmacological approaches to deplete or manipulate senescent cells to preserve organ integrity and function with aging and after injury. Finally, key questions for future research and clinical translation are discussed.

Keywords: senescence, kidney, fibrosis, regeneration, aging, pharmacokinetics, pharmacotherapeutic approaches

INTRODUCTION

Cellular senescence is characterized by a typically irreversible cell cycle arrest coupled with widespread alterations at a transcriptional, metabolic and secretory level along with modified cellular morphology and chromatin organization (Sturmlechner et al., 2017; Docherty et al., 2019; Gorgoulis et al., 2019). Senescence is induced as a physiological component of development or in response to multiple insults including genotoxic injury, oncogene activation, cellular stress, mitochondrial dysfunction, lack of nutrients, or hypoxia (Figure 1).

The term senescence derives from the Latin "Senex" or "old" and was first used by Hayflick and Moorhead in 1961 following their seminal observation that human diploid cells have a finite capacity to replicate *in vitro* (Hayflick and Moorhead, 1961). Hayflick himself attributed his discovery to aging at the cellular level and the description in their paper is now recognized as replicative senescence occurring due to critical telomere shortening.

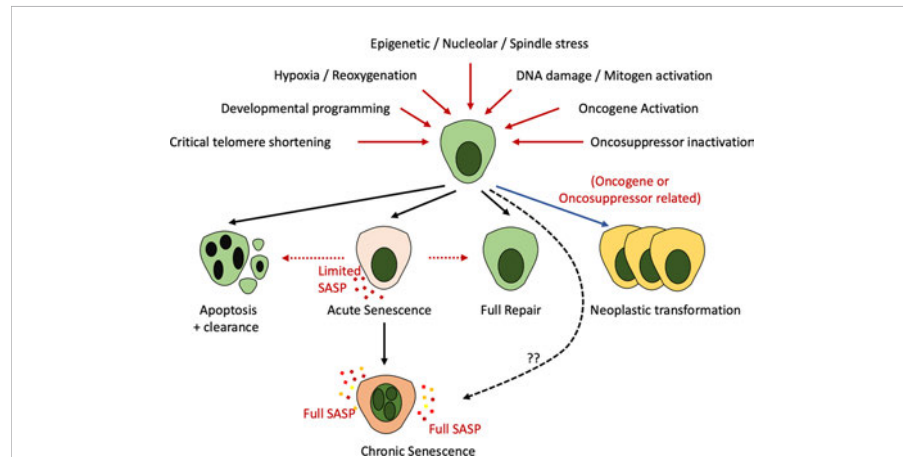


FIGURE 1 | Recognized stimuli to cell fate decisions including apoptosis, senescence, repair, or neoplastic transformation. Multiple developmental, stress-associated, or DNA damage induced cues (red lines) can result in apoptosis, senescence, neoplastic transformation (recognized only in the context of DNA damage—blue line) or full repair. Existing evidence indicates that cells enter “acute” senescence in response to these cues which may lead to repair, clearance, or evolution to the transcriptionally altered “chronic” senescent cell. Whether altered injury cues may generate a cell with immediate properties of “chronic” senescence remains unknown (dotted line).

The association between aging and senescence is now well established (Campisi, 2013; O’Sullivan et al., 2017), while accumulating evidence has demonstrated that senescent cells also have important physiological and pathophysiological roles in a number of other biological processes including embryonic development (Munoz Espin et al., 2013; Storer et al., 2013), tumor suppression (Serrano et al., 1997), wound healing (Jun and Lau, 2010), and tissue repair *in vivo* (Krizhanovsky et al., 2008). Of note, recent experiments depleting senescent cells in models of aging *in vivo* have been shown to postpone the onset of age related diseases and extend healthy lifespan, igniting clinical, and research interest and inspiring the development of targeted senolytic drugs to eliminate senescent cells associated with age and disease (Baker et al., 2011; Baker et al., 2016; Xu et al., 2018).

In this review, we examine our current understanding of the physiological and pathological roles of cellular senescence, with a focus on the kidney and reference to other organ systems where appropriate. We discuss the genetic and pharmacological approaches that have been used to manipulate senescent cell numbers and the potential impact these therapies may have on human health in the future.

THE INFLUENCE OF INJURY TYPE AND TIMING ON SENESCENCE OUTCOMES

Cellular senescence is a complex, diverse, and dynamic process. It can be triggered by a wide variety of stressors in many different cell

types. There is also accumulating evidence that part of the heterogeneity seen in senescent cells reflects temporal changes in their transcriptome (Hernandez Segura et al., 2017) and phenotype and resultant influence this has on their environment and clearance patterns (van Deursen, 2014; Herranz and Gil, 2018). Current evidence indicates that chronic senescence evolves from acutely senescent cells in the absence of immune mediated or programmed cell death and clearance.

“Acute senescence” appears to have a physiological role limiting fibrosis in response to injury *via* fibroblast senescence induction, in successful embryonic organogenesis and tissue homeostasis (Krizhanovsky et al., 2008; Jun and Lau, 2010; Munoz Espin et al., 2013; Demaria et al., 2014). In these tightly controlled processes, the senescent cells appear to be a key component in healthy wounding and are subsequently removed by leukocytes including macrophages and Natural Killer cells in a timely manner (van Deursen, 2014).

In “chronic senescence”, the senescent cells persist and accumulate within affected organs. This can be triggered by a number of insults including critical telomere shortening as a result of repeated cell division (d’Adda di Fagnana et al., 2003), DNA damage (Rodier et al., 2009), oncogenic mutations (Aird et al., 2013), and metabolic stress in response to insults such as free radical release, hypoxia, and oxidative stress (Campisi and d’Adda di Fagnana, 2007). Cellular senescence thus provides a mechanism that prevents the undesirable proliferation of damaged cells, however, in contrast to their elimination through cell death mechanisms such as apoptosis, senescent

cells remain viable, and continue to be metabolically active. Cell death and senescence can be triggered by the same stressors and we do not yet have a full understanding of what determines each cell's fate (Herranz and Gil, 2018). Furthermore, whether particular injury stimuli can induce senescent cells with immediate features of chronic senescence remains unproven *in vivo*. The documented changes seen with acute and chronic senescence, including in the kidney, are summarized in **Figure 2**.

IDENTIFICATION OF SENESCENT CELLS

The characterization of senescent cells *in vivo* remains challenging, in part because we have not yet identified a single marker that is specific to senescent cells. The signaling events that trigger a cell to become senescent vary depending on the senescence inducing stimuli with multiple pathways resulting in the induction of cyclin dependent kinase inhibitors P16^{INK4A} and P21^{CIP1}, leading to cell cycle arrest by enforcing the G1/S checkpoint. Increased senescence associated β galactosidase (SA β GAL), another important distinguishing characteristic of senescent cells, reflects the enhanced lysosomal content of senescent cells, though SA β GAL does not itself appear to be

necessary for the senescence to occur (Hernandez Segura et al., 2018). Importantly, the presence of any of these markers alone is insufficient to confirm senescence and false positives can occur. For example, P16^{INK4A} is not found in all senescent cells (Hernandez Segura et al., 2017) and can be expressed in some non senescent cells (Sharpless and Sherr, 2015). In addition, macrophages can express both P16^{INK4A} and SA β GAL (Hall et al., 2017). **Table 1** summarizes several of the positive and negative markers associated with senescence in the kidney. A recent publication from the International Cell Senescence Association has provided a consensus definition and three step multi marker workflow for detecting senescent cells (Gorgoulis et al., 2019). This approach takes account of the fact that while there are many potential markers of senescence, their specificity is context specific, and will be influenced by variety of factors including cell type and organismal developmental stage.

The first step is to screen for senescence, by assessing SA β GAL activity. SA β GAL, however, cannot be used for paraffin embedded tissue sections or in live cells, limiting its use. An alternative screening tool is to quantify lipofuscin, an emerging indicator of senescence which can be visualised in lysosomes *via* histochemical staining (Georgakopoulou et al., 2013; Evangelou et al., 2017).

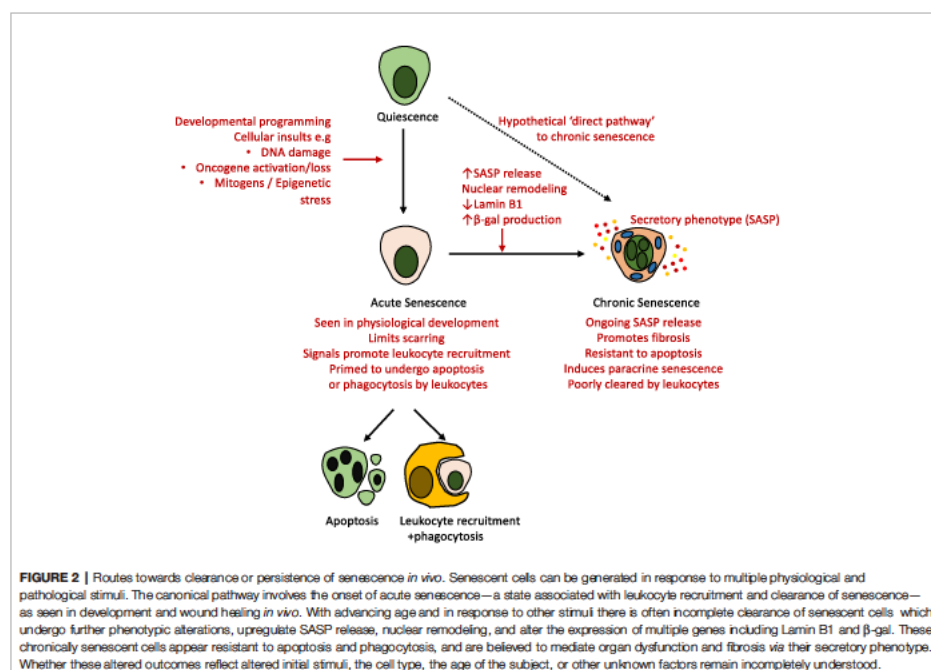


TABLE 1 | Tissue markers associated with senescence in the kidney.

Marker	Function	Expression levels in senescence/aging
P16INK4A	Cyclin-dependent kinase inhibitor 2A – blocks cell cycle at G1/S checkpoint	Increased after irradiation in the kidney (Freund et al., 2012), increased in tubular epithelial cells (Luo C. et al., 2018)
P19ARF	Cyclin-dependent kinase inhibitor 2A (alternate reading frame)– blocks cell cycle at G1/S checkpoint	Increased in the aging rodent kidney (Krishnamurthy et al., 2004)
P21CIP1	Cyclin-dependent kinase inhibitor 1A – blocks cell cycle at G1/S checkpoint	Increased. Seen in Human IgAN (Liu et al., 2012) and Rat Diabetic Nephropathy (Kitada et al., 2014)
P27KIP1	Cyclin-dependent kinase inhibitor 1B – blocks cell cycle at G1/S checkpoint	Higher levels of P27KIP1 linked to progression of diabetic nephropathy (Awazu et al., 2003)
KI67	Nuclear protein associated with cellular proliferation	Absent in senescent cells. Reduced with aging (Meik et al., 2009)
SA-beta-gal	Lysosomal beta-galactosidase	Increased with aging in the kidney (Baar et al., 2017)
LMNB1	Lamin-B1. Nuclear Lamina protein.	Decreased in human fibroblasts and in irradiated murine kidneys (Freund et al., 2012)
Gamma-H2AX	Component of DNA strand break repair machinery	Combination of high Gamma-H2AX+absent KI67 used as marker of DNA damage induced senescence in kidney transplants (Braun et al., 2012)
SAHF	Senescence associated heterochromatin foci	Reported with altered DNA packaging in senescent cells in the aging kidney (Lin et al., 2016)

The next step is to co-stain for additional markers of senescence. This includes P16^{INK4A} and P21^{CIP1}, which are increased in senescence as well as the absence of proliferation markers and lamin-B1 (LMNB1), a major component of nuclear lamina (Shimi et al., 2011; Shah et al., 2013).

The third step is to identify senescence markers that would be expected to be altered in the specific biological context being studied. This includes evidence of DNA damage, which is associated with the cellular DNA damage response (DDR) (Munoz-Espin and Serrano, 2014) as well as FOXO, a transcription factor, and phosphoinositide 3-kinase (PI3K), an intracellular signal transducer, both of which enable senescent cells to resist apoptosis (and hence, are potential targets for therapies to deplete senescent cells, discussed further below) (Hernandez-Segura et al., 2018). Finally, the secretome of senescent cells, collectively termed the senescence-associated secretory phenotype (SASP), can be quantified.

THE SENESCENCE-ASSOCIATED SECRETORY PHENOTYPE

The senescence-associated secretory phenotype (SASP) includes a wide range of pro-inflammatory cytokines, chemokines, growth factors, and proteases which act in an autocrine and paracrine fashion on neighboring cells and are critical to understanding the impact senescent cells have on the function of tissues and organs (Coppe et al., 2008; Coppe et al., 2010; Laberge et al., 2012). Exploring these individually is out with the scope of this review, but excellent summaries are available in the current literature (Wang W. J. et al., 2017).

Established SASP components include but are not limited to IL-1 β , IL-6, IL-8, GRO α , TGF β 1, and WNT16B (Yang et al., 2006; Coppe et al., 2008; Kuilman et al., 2008; Binet et al., 2009; Ito et al., 2017). Studies examining the transcriptome of senescent cells (Hernandez-Segura et al., 2017) and proteomic analysis of the supernatant secreted from senescent cells (Basisty et al., 2020) have identified a much larger pool

of potential SASP makers and demonstrated that there is significant heterogeneity in the SASP composition depending on the senescence inducer and cell type involved. Furthermore, single cell RNA-sequencing studies, where senescence has been induced in a single cell type *via* the same mechanism, has demonstrated significant variability exists in SASP expression in cells from different lineages (Wiley et al., 2017).

The SASP, to a large extent, is initiated through NF- κ B, which itself is triggered by the DDR (Salminen et al., 2012). The Mammalian target of Rapamycin (mTOR) protein also has an important role regulating SASP production by promoting the activation of NF- κ B *via* IL-1A translation (Laberge et al., 2015) and by inhibiting the breakdown of numerous SASP components by inhibiting the RNA-binding protein ZFP36L1 (Herranz et al., 2015).

The SASP mediates many of the beneficial and deleterious effects of senescent cells including inducing senescence in neighboring cells (Acosta et al., 2013). It contributes to a pro-inflammatory microenvironment that can activate immune cells and lead to the subsequent elimination of senescent cells (Munoz-Espin and Serrano, 2014). Why developmental and some forms of acute senescence are rapidly and completely cleared by senescent cell death and phagocytosis, while chronic senescence appears resistant to immune clearance remains an important unanswered question.

Emerging evidence indicates that extracellular vesicles (EVs) are also important mediators by which senescent cells impact on their microenvironment (Borghesan et al., 2019). EVs, including exosomes and microvesicles, are membrane bound structures that contain proteins, lipids, and RNA (Sagini et al., 2018). They have a crucial role in maintaining cellular homeostasis by excreting harmful DNA and inhibition of exosome secretion has been shown initiate DDR in both senescent and non-senescent cells (Takahashi et al., 2017). There is an increase in EVs secreted from senescent cells (Jeon et al., 2019) and they can induce paracrine senescence in neighboring cells (Borghesan et al., 2019).

METABOLIC ALTERATIONS IN SENESENCE

Although “senescent” may be interpreted as “inert”, senescent cells retain an active but altered metabolism. Impaired mitochondrial oxidative phosphorylation is associated with both aging and senescence in nematode worms, experimental mice and man (Boffoli et al., 1994; Ishii et al., 1998; Trifunovic et al., 2004). It is recognized that signaling mechanisms exist by which mitochondrial cues can alter gene expression in the nucleus including Sirtuin-1 (Sirt1) and mammalian target of rapamycin (mTOR) expression, pathways implicated in cell survival and senescence induction (Kwon et al., 2019). One such signaling pathway is reactive oxygen species generation, itself a source of DNA, protein, and lipid damage and hence a potential senescence trigger (Shiloh, 2003).

Senescent cells also contain more glycogen granules—implicating a role for glycogenesis in the induction of senescence with inhibitors of glycogen synthase kinase 3 (GSK3) including TGFβ1 release inducing senescence and glycogen accumulation (Seo et al., 2008; Byun et al., 2012). Protein synthesis and proteostasis are also key requirements for cellular health. Metabolic regulators GSK3, AMP-activated protein kinase (AMPK), and mTOR all modulate protein degradation and synthesis—and in senescence altered activation of these pathways results in abnormally enhanced protein synthesis (Mazucanti et al., 2015).

Finally, autophagy also plays a role in organelle quality control, and inhibition of GSK3 can impair autophagy and increase ROS generation (Byun et al., 2012). Of the three key signaling kinases, GSK3 and AMPK are positive regulators, with mTOR a negative regulator of the metabolic features of senescence.

PHYSIOLOGICAL ROLES OF SENESENCE

Developmental Senescence

Senescent cells have been identified in the developing embryos of multiple species including humans (Munoz-Espin et al., 2013), birds (Nacher et al., 2006), amphibians (Davaapil et al., 2017), and fish (Villiard et al., 2017) indicating the role of senescence in embryogenesis is highly conserved. In mice, senescent cells have been found in the apical ectodermal ridge of the developing limb, the mesonephros, the neural tube, and the endolymphatic sac of the inner ear (Munoz-Espin et al., 2013; Storer et al., 2013) with cells being identified on the basis of SA-β-GAL and P21^{CIP1} expression and SASP mediators. Notably, other markers of senescence such as P16^{INK4A} and markers of DNA damage were absent. Senescence is followed by macrophage infiltration, which clear the senescent cells as part of tightly controlled cellular process. It appears in every embryo, demonstrating that this is normal programmed behaviour and not a response to injury (Rhinn et al., 2019). The role of senescent cells appears to be in the fine tuning of organogenesis; P21^{CIP1} knockout mice with altered ability to induce senescence remain viable with a

compensatory increase in apoptosis, while exhibiting detectable abnormalities in their kidneys, limbs, and vagina (Munoz-Espin et al., 2013; Storer et al., 2013).

A role for senescence in the timing of parturition has also been suggested (Cha and Aronoff, 2017; Menon et al., 2017). In pregnant mice with a conditional deletion of p53 in uterine tissues, half went into preterm labor (compared to none in controls) with increased markers of senescence including P21^{CIP1} in post implantation decidual cells, i.e., cells of the pregnant endometrium that form the maternal placenta (Hirota et al., 2010). Intriguingly, the same group demonstrated that this premature decidual senescence occurred as a result of increased mTOR complex 1 signaling and that the administration of the mTOR inhibitor Rapamycin reduced the incidence preterm birth (Hirota et al., 2011). In addition, it has been hypothesised that signaling from senescent cells in the foetal membrane to maternal uterine tissues can drive parturition (Menon et al., 2017).

Wound Healing

Acute senescence is part of the regenerative response following injury. Murine studies have demonstrated that senescent endothelial cells and fibroblasts appear early in response to cutaneous injury and promote wound healing by secreting platelet-derived growth factor AA (PDGF-AA), a SASP mediator that induces myofibroblast differentiation. Senescent cell depletion delayed but did not prevent wound healing (Demaria et al., 2014). Other studies have demonstrated that senescent fibroblasts accumulate and act to limit fibrosis in the granulation tissue of healing skin wounds (Jun and Lau, 2010) and in the heart post myocardial infarction (Zhu et al., 2013). Senescent hepatic stellate cells also appear to limit fibrosis in the liver following injury (Krizhanovsky et al., 2008), however, when their subsequent Natural Killer cell-mediated depletion was impaired, their accumulation lead to increased fibrosis (Sagiv et al., 2016).

Cancer Defense

Cell division is essential for growth and repair but tight physiological control is essential to prevent malignancy. Oncogene activation can induce senescence and act as a barrier to neoplastic transformation (Hinds and Pietruska, 2017). Genes related to cellular senescence are frequently mutated in cancer (Zhao et al., 2016). In studies of P21^{CIP1}-deficient mice with alterations in their ability to undergo senescence, an increased susceptibility to multiple cancers was observed (Martin-Caballero et al., 2001). In humans, melanocytic nevi (moles), frequently harbor the oncogenic BRAF^{V600E/K} mutation, but only rarely progress into malignant melanoma as the BRAF^{V600E/K} mutation induces senescence with both P16^{INK4A} and SA-β-Gal evident in lesions (Michaloglou et al., 2005). Calcitriol, an active form of vitamin D, has anti-proliferative activity against a number of cancers including renal cancer. *In vitro* studies have attributed this effect to calcitriol inducing senescence with increased expression of P16^{INK4A} (Shen et al., 2017).

The paracrine effects of the SASP also play a key role. In addition to reinforcing growth arrest in neighboring cells, the

SASP drives the recruitment and activation of immune cells, which then eliminate damaged cells. This process has been termed "senescent surveillance". Murine pre-malignant senescent hepatocytes secrete chemokines and cytokines that drives the immune mediated clearance of pre-malignant cells. Impaired removal of senescent hepatocytes, promoted the development murine hepatocellular cancer (Kang et al., 2011).

However, the SASP can also have a pro-tumorigenic impact on pre-malignant cells and on the tumor microenvironment. Senescent human fibroblasts stimulate both pre-malignant and malignant epithelial cells to form tumors in mice (Krtolica et al., 2001). In addition, studies have demonstrated that the SASP from human senescent cells, particularly IL-6 and IL-8, induces an epithelial-to-mesenchyme transition (Coppe et al., 2008), which is strongly associated with tumor progression and subsequent tissue invasion (Ye and Weinberg, 2015). In a study using a mouse model where senescence can be induced in osteoblasts using tamoxifen, the authors found that this increased cancer spread from breast to bone by creating a favourable microenvironment for malignant cells. Notably, tumor burden was significantly reduced by the administration of IL-6 neutralising antibodies (Luo et al., 2016).

SENESCENCE IN THE KIDNEY

Why is Senescence Relevant in Kidney Disease?

Older kidneys exhibit decreased function and increased susceptibility to acute kidney injury (AKI) (Ishani et al., 2009; Coca, 2010; Ferenbach et al., 2011; Clements et al., 2013; O'Sullivan et al., 2017). For many years, this was accepted as a fully recoverable entity; with serum parameters of kidney function returning to normal post-injury. We now understand from epidemiological data with both adequate statistical power and length of follow-up, that survivors of AKI have a significantly increased risk of developing chronic kidney disease (CKD). (Ishani et al., 2009; Coca et al., 2012; Ferenbach and Bonventre, 2015) CKD is generally progressive, even in circumstances where the original renal injury is no longer

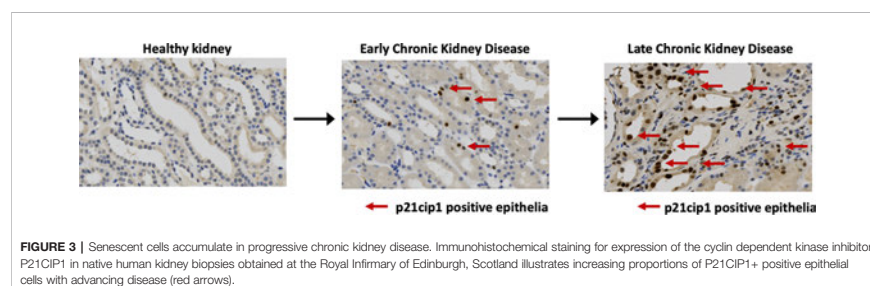
present or has been treated and is incurable; with a common progression pathway of multiple renal diseases being accumulating fibrosis.

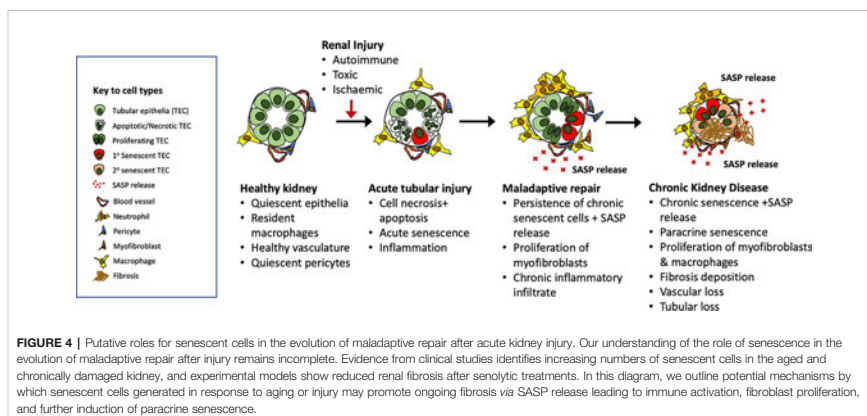
An association between aging and increasing numbers of senescent cells has been noted in multiple organs including the kidney (Sis et al., 2007; Melk et al., 2009; Liu et al., 2012). A question which is the focus of intense ongoing research relates to whether these increased numbers of senescent cells merely represent a biomarker of aging or previous injury, or whether senescent cells actively promote maladaptive repair, organ fibrosis, and dysfunction, and hence represent a novel therapeutic target.

Senescent cells fulfil several criteria which make them novel candidate drivers of kidney disease. Namely, they are found in the context of tissue injury, are resistant to apoptosis and persist within the kidney secreting pro-inflammatory and pro-fibrotic signals which may contribute to progressive damage. (Melk et al., 2005; Sis et al., 2007; Braun et al., 2012; Liu et al., 2012) While key questions remain unanswered in the kidney, several recent papers using transgenic or pharmacological depletion of senescent cells in murine models support a causal role for senescence in progressive renal fibrosis (Baker et al., 2016; Baar et al., 2017; Kim et al., 2019).

Senescent cells accumulate in the kidney in three broad settings: with age, with any insult causing acute kidney injury, including post-transplantation, and in chronic kidney disease. In each case, higher levels of senescent cells associate with worsened kidney function and outcome (Figure 3).

The tubular epithelium is implicated as the primary location for renal senescence. This is demonstrated by both human and murine data: In Baker et al's seminal study of ablation of senescent cells in INK-ATTAC transgenic mice, they demonstrated senescence occurring in proximal tubules with increasing age (Baker et al., 2016). In a series of human renal transplant biopsies, all biopsy specimens showed P16^{INK4A} staining in the nuclei of distal tubules and collecting duct but staining was also present in podocytes, parietal epithelium of glomeruli, vascular smooth muscle cells, and interstitial cells (Melk et al., 2005). Furthermore, a series of patients with glomerular disease showed P16^{INK4A} staining in a subset of glomerular, tubular, and interstitial cell nuclei; however,





senescent tubular epithelial cells were the key difference between disease and control kidneys, present in 80% of cases compared with 21% in healthy living donor control kidneys (Sis et al., 2007). Additionally, a biopsy series of patients with IgA nephropathy demonstrated increased P21^{CIP1} and P16^{INK4A} protein expression which was confined to tubules (Liu et al., 2012).

As discussed earlier in this review and elsewhere, there is an increasing appreciation of the prevalence of maladaptive repair in the aftermath of kidney aging and injury (Ferenbach and Bonventre, 2015) leading to fibrosis within the kidney. Based on the available evidence in the kidney and beyond we propose that in the aftermath of renal injury, chronically senescent renal epithelial cells mediate progressive fibrosis, vascular rarefaction and organ dysfunction *via* SASP release (Figure 4).

Pathological Roles of Senescence Beyond the Kidney

In contrast to the tightly controlled and beneficial roles outlined earlier in this review, the accumulation of senescent cells in aging is now known to be detrimental, with their clearance extending median healthspan in mice by an average of 40% (equating to > 30 years in humans) (Xu et al., 2018).

From an evolutionary perspective healthy aging does not undergo selection pressure, i.e., traits of senescence conferring improved health to the point of reproduction undergo positive selection (cancer defense, embryogenesis, parturition), with no selection for potential detrimental late life effects. Thus, while induction of senescent cells may improve early wound repair, conversely their SASP release may contribute to tissue dysfunction in aging and pathological conditions; post-injury and in disease.

There are now striking examples across tissues and diseases of senescent cell clearance being beneficial. Much of our early

understanding of senescent cell removal derives from transgenic approaches which have used components of the cyclin-dependent kinase 2A gene (P16^{INK4A}) to drive concomitant expression of a "suicide gene" activated upon administration of a drug or dietary compound, thus conferring selective vulnerability upon P16^{INK4A} expressing cells to pharmacological clearance (Baker et al., 2011; Baker et al., 2016; Chang et al., 2016). Although informative, the results of studies based exclusively upon such transgenes may not fully recapitulate the spectra of senescent cells seen *in vivo*, unless all such cells express P16^{INK4A}, which seems unlikely.

Senescence and Fibrosis

As described in detail above, the SASP comprises pro-fibrotic and pro-inflammatory molecules. Thus, it seems plausible, and indeed likely, that part of the deleterious effect of SC accumulation is mediated *via* tissue fibrosis. The means by which SCs exert a pro-fibrotic effect within an organ are yet to be fully defined. However, there are clear examples of this effect already in published literature.

Cellular senescence has been shown to be important in the pathogenesis of idiopathic pulmonary fibrosis; a progressive fibrotic lung disease with devastating consequences on both mortality and morbidity. (Faner et al., 2012; Hashimoto et al., 2016) Senescent cell depletion (either by transgenic means, or by pharmacological depletion by dasatinib and quercetin) resulted in healthier, less fibrotic lungs in mice.

An association between cellular senescence and resultant fibrosis has also been shown in the liver, where transgenic induction of senescence significantly exaggerates fibrosis in the context of acute injury. (Ferreira-Gonzalez et al., 2018) Similarly, in the kidney, transgenic depletion of SCs in the INK-ATTAC model resulted in significantly less glomerulosclerosis (Baker et al., 2016).

MODULATION OF SENESCENCE IN HEALTH AND DISEASE

Animal Models of Senescent Cell Deficiency or Depletion in the Kidney

Our understanding of senescent cell accumulation and clearance in renal disease remains limited. To date, there are three key experimental strategies which have been used to test the manipulation or removal of senescent cells. These are: transgenic mice with alterations in global senescence induction pathways, transgenic approaches to deplete senescent cells, and pharmacological targeting of senescent cells and/or their secretome (Megyesi et al., 2001; Wolstein et al., 2010; Baker et al., 2011; Munoz-Espin et al., 2013; Baker et al., 2016; Chang et al., 2016; Baar et al., 2017; Xu et al., 2018; Kim et al., 2019).

Studies in Mice With Transgenically Modified Senescence Induction Pathways

Over the course of the last 20 years, several studies of kidney injury and disease have been published using transgenic mice with alterations in senescence induction pathways (summarized in **Table 2**); predominantly global P16^{INK4A} and P21^{CIP1} knockout models. These models have used several models of

experimental renal injury; including ischaemia reperfusion injury (IRI), unilateral ureteric obstruction (UUO), diabetic nephropathy (DN), and post transplantation with mixed results; with an approximately even split between benefit and harm (Al-Douhaji et al., 1999; Megyesi et al., 2001; Wolf et al., 2005; Wolstein et al., 2010; Braun et al., 2012; Megyesi et al., 2015).

P16^{INK4A} knockout mice exposed to experimental renal injury show increased epithelial proliferation and functional recovery after IRI but worsened fibrosis in UUO models. This implies that senescent cells can behave differently in response to altered injury cues with their generation both time and population determining their subsequent influence on tissue repair and fibrosis (Wolstein et al., 2010; Lee et al., 2012).

Transgenic Approaches to Deplete Senescent Cells

An alternative experimental approach to inhibiting the induction of senescence is to selectively deplete cells only after they have become senescent. As senescent cells are programmed to survive stress signals that would lead to cell death in normal proliferating cells; published transgenic approaches thus far use a senescence promoter; typically, the promoter for the P16^{INK4A} gene, to drive

TABLE 2 | Transgenic murine models of altered senescence induction and their outcomes in the kidney.

Reference	Renal model	Modulation of senescence	Renal Disease outcome	Effect of any Intervention
Baker et al. (2016)	Natural aging In INK-ATTAC mice	INK-ATTAC +AP20187 or vehicle administration to deplete P16 ^{INK4A} cells	↓Glomerulosclerosis ↓β-gal positivity	↓Glomerulosclerosis ↓β-gal positivity
Baar et al. (2017)	Natural aging P16-3MR mice and fast aging Xpd TTD/TTD mice	FOXO4-DRI agent causes p53 nuclear exclusion. Ganciclovir Rx to P16-3MR mice causes P16 ^{INK4A} restricted cell death	↑Serum Urea ↓Lmn1 expression ↑SASP expression (both Xpd ^{TTD/TTD} and aged P16-3MR)	FOXO4-DRI or GCV to P16-3MR admin: ↓Serum Urea ↓Lmn1 expression ↓SASP expression (both Xpd ^{TTD/TTD} and aged P16-3MR)
Munoz-Espin et al. (2013)	Nephrogenesis	WT vs P21 ^{CIP1} KO mice with deficient growth arrest in nephrogenesis	↓β-gal positivity in P21 ^{CIP1} KO mice utero. ↑K67 expression but ↓Apoptosis maintains development	Use of P13K inhibitor augments developmental senescence in WT mice
Wolstein et al. (2010)	UUO	WT vs P16 ^{INK4A} KO mice with impaired cell cycle arrest	UUO induces β-gal positivity, apoptosis and collagen deposition in WT mice	↓β-gal positivity ↓Apoptosis ↑Collagen, ↑proliferation after UUO in P16 ^{INK4A} KO
Megyesi et al. (2001)	Renal IRI	WT vs P21 ^{CIP1} KO mice with impaired cell cycle arrest	WT mice show tubular injury and raised blood urea levels after IRI	↑proliferation ↓Renal function ↑Mortality in P21 ^{CIP1} KO
Megyesi et al. (2015)	Renal UUO and IRI	WT vs P21 ^{CIP1} KO vs P21 ^{CIP1} KO+KAP2-driven P21 ^{CIP1}	WT mice show renal fibrosis after both UUO and IRI	UUO: ↓fibrosis in P21 ^{CIP1} KO vs WT, ↑fibrosis in P21 ^{CIP1} KO kidneys with KAP2 promotor driven P21 ^{CIP1} vs P21 ^{CIP1} KO. IRI: ↑TGFβ1 in P21 ^{CIP1} KO kidneys with KAP2 promotor driven P21 ^{CIP1} vs P21 ^{CIP1} KO.
Lee et al. (2012)	Renal IRI	WT vs P16 ^{INK4A} /p19ARF Double KO mice with impaired cell cycle arrest	WT mice show marked P16 ^{INK4A} and p19ARF induction 28d after IRI, with apoptosis and reduced tubular density	P16 ^{INK4A} and p19ARF deficient mice show improved epithelial and microvascular repair, with increased myeloid cell recruitment
Al-Douhaji et al. (1999) Wolf et al. (2005)	Diabetic Nephropathy	WT vs P21 ^{CIP1} KO WT vs p27KIP1 KO	WT mice develop albuminuria and glomerular hypertrophy	Both p27KIP1 KO and P21 ^{CIP1} KO mice were protected from proteinuria and glomerular expansion
Braun et al. (2012)	Renal Transplant	P16 ^{INK4A} KO mice with impaired cell cycle arrest	WT mice develop interstitial fibrosis and tubular atrophy	P16 ^{INK4A} KO mice develop less atrophy and fibrosis after Tx

In each case ↑ ↓ markers were used to indicate that the p value was less than 0.05.

the expression of a concomitant suicide gene which can subsequently be activated by administration of a drug or dietary compound.

In the 2016 study by Baker et al. (2016), senescent cell depletion was achieved by using P16^{INK4a} promoter-driven expression of FK506-binding-protein-Caspase8; a transmembrane receptor coupled to an intracellular caspase pathway which was activated upon administration of AP20817 (AP). (Baker et al., 2016) Successful elimination of senescent cells in the kidney was confirmed by SA- β -Gal staining with a 75% reduction in the numbers of senescent cells observed by transmission electron microscopy.

The kidneys of mice who underwent senescent cell depletion showed significantly less glomerulosclerosis, and lower blood urea levels. Of note, despite improved glomerular appearance; senescent cells were located primarily in the tubular epithelium and not in the glomeruli. Interestingly, AP-treated mice showed decreased levels of angiotensin receptor 1a (atr1a), both at transcript and protein level. In humans, angiotensin receptor blockers attenuate age and disease-related glomerulosclerosis under normotensive conditions and are used to control proteinuria; leading to the theory that over-activation of the local renin-angiotensin-aldosterone system (RAAS) drives glomerulosclerosis. (Ferder et al., 2003; Paul et al., 2006) The reduction of receptor expression in mice with lower numbers of senescent tubular epithelial cells implicates a senescence driven activation of the renal RAAS as potentially contributory to the blood pressure changes seen with aging and in human CKD.

A similar approach was subsequently used by Baar et al. using the P16:3MR mouse model, (Baar et al., 2017) where the P16^{INK4a} promoter drives the expression of a viral thymidine kinase, conferring selective sensitivity to the effects of the antiviral drug Ganciclovir. Mice who underwent senescent cell depletion by means of Ganciclovir treatment had lower levels of plasma serum urea and creatinine.

Though powerful in terms of informing future direction of study, transgenic depletion and the global knockout (KO) models described above must be interpreted in the knowledge that a generic P16^{INK4a} (or P21^{CIP1}) promoter driven genetic KO is neither specific to senescent cells, nor captures all potential senescent cells, i.e., some non-senescent cells express P16^{INK4a} and some senescent cells do not express P16^{INK4a}.

Both these transgenic models (INK-ATTAC and P16:3MR) have been used to study disease in other organs and have demonstrated benefit post depletion in several models of disease including bone marrow irradiation, tau-mediated neurodegeneration, and bleomycin induced pulmonary fibrosis (Chang et al., 2016; Schafer et al., 2017; Bussian et al., 2018).

Pharmacological Depletion of Senescent Cells

Studies of murine models with defects in senescence induction and transgenic methods of senescent cell depletion have made major contributions to our understanding of the role played by senescence in aging and disease. In order to translate studies from the lab to the clinic there is a requirement for

pharmacological agents capable of manipulating the survival and/or function of senescent cells.

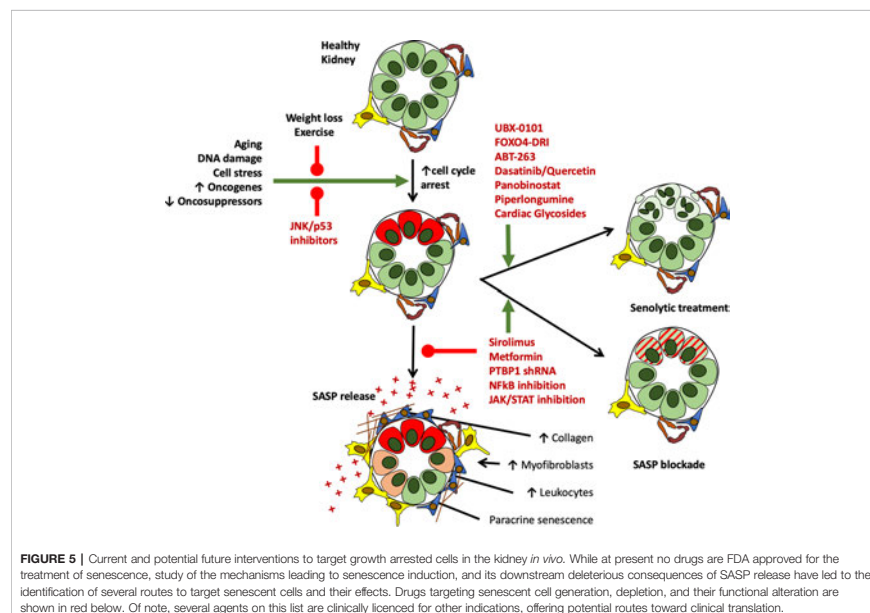
Chronically senescent cells are typically able to resist apoptosis. They have commonly survived a significant cell stress and entered into permanent growth arrest and have thus often up-regulated anti-apoptotic pathways (Childs et al., 2014; Zhu et al., 2015; Soto-Gamez et al., 2019). By blocking or interfering with these pathways it has proved possible to induce senescent cell apoptosis (Baar et al., 2017). Though still in its relative infancy, this is an approach with both marked experimental value and enormous translational potential at both an organ and whole animal level. Experimental pharmacological depletion of senescent cells has been shown to improve outcomes in organs other than the kidney across several models of disease and has been shown to not only improve median lifespan, but also reduced late life morbidity (Xu et al., 2018; Hickson et al., 2019). Several agents have been shown to have efficacy in modulating senescent cell number and/or function in an experimental setting. Published literature thus far suggests that different senolytic agents are efficacious against different cell types (Xu et al., 2018). Common agents which have been used in a variety of *in vivo* and *in vitro* models include FOXO4-DRI, ABT-263 (Navitoclax), and the combination of dasatinib and quercetin (D+Q) (Chang et al., 2016; Baar et al., 2017; Schafer et al., 2017; Bussian et al., 2018; Xu et al., 2018). These and other agents under investigation are discussed below (Figure 5).

FOXO4-DRI

While at present, little data exist which report specifically on renal outcomes after pharmacological depletion, in 2017, Baar et al. identified the FOXO4 protein as an important regulator of p53 activity in senescent cells. The p53 pathway is an important controller of apoptosis and senescence and as such is a potential target for senolytic treatment. FOXO4 was hypothesised to bind with p53 and thus prevent p53 mediated apoptosis. By designing an interfering peptide "FOXO4-DRI", which competes with endogenous FOXO4 for p53 binding, they demonstrated that selective apoptosis could be achieved in both aging and chemotherapy-induced senescent cells. Fast-aging mice (a murine model of the human autosomal dominant disease trichothiodystrophy; in which mice are deficient in DNA repair) and mice with Doxorubicin-induced senescence post-chemotherapy that were treated with FOXO4-DRI were less frail, more responsive and had significantly improved "healthspan" compared to mice who had not undergone senescent cell clearance (Baar et al., 2017).

In keeping with expected age-related decline in kidney function; fast-aging mice had higher urea levels when compared to wild-type counterparts and when assessed specifically for senescence, kidneys from fast-aging mice were demonstrated to have significantly higher levels of SA- β -Gal and loss of LMNB1. (Freund et al., 2012) Again, senescent cells were primarily located in the tubular epithelium. (Baker et al., 2016) Staining for IL-6, a known SASP component, was also increased.

Treatment with FOXO4-DRI significantly reduced the percentage of tubular cells lacking in LMNB1 and resulted in lower blood urea levels and lower IL-6 expression. As the



mechanism of this approach is to induce cell death, it is reassuring to note that there was no significant change in overall kidney weight in the treated group; thus, averting concern of widespread tissue death and loss of tissue architecture. Indeed, this maintenance of weight (in the presence of confirmed cell loss) suggests regeneration. This is a potentially similar phenomenon to that seen in bone marrow, where depletion of senescent cells improves marrow clonogenicity (Chang et al., 2016).

ABT-263 (Navitoclax)

As aforementioned, senescent cells are able to evade apoptosis (Zhu et al., 2015). One route to achieving this is through the up-regulation of pro-survival proteins; Bcl-2, Bcl-w, and Bcl-xL. These proteins exert their anti-apoptotic effects by direct binding actions that regulate mitochondrial outer membrane permeabilisation (MOMP). ABT-263 (and related compounds ABT-737 and ABT-199) is a Bcl-2/w/xL inhibitor (Tse et al., 2008) which attaches to the BH3-binding groove on these molecules, thus preventing the binding of pro-apoptotic proteins. These proteins therefore remain in an active (unbound) state and are capable of mitochondrial membrane permeabilisation and apoptosis (Kale et al., 2018).

Sub-lethal total body irradiation induces bone marrow aging in mice and has been shown to induce haematopoietic stem cell (HSC) senescence. Treatment with ABT-263 reduces haematopoietic senescent stem cell number. Although Chang et al. showed that ABT-263 was efficacious against senescent renal epithelial cells, the impact of ABT-263 treatment on renal function and fibrosis remains unknown (Chang et al., 2016).

HSCs subjected to senescence induction by irradiation are phenotypically similar those found in aged mice. Treatment of irradiated mice or naturally aged mice was shown to rejuvenate irradiated marrow such that when transplanted into a healthy recipient (i.e., non-irradiated) both clonogenicity and long-term engraftment were improved, potentially *via* an improvement in the stem cell microenvironment (Chang et al., 2016).

Similarly, in both murine models of neurodegenerative disease and atherosclerosis, treatment with ABT-263 resulted in an attenuated disease phenotype with significantly less tau phosphorylation and reduced atherosclerotic burden. (Childs et al., 2016; Bussian et al., 2018) ABT-263 can lead to a thrombocytopenia (Kaefer et al., 2014) which has limited its prolonged use as an antineoplastic agent. Whether a short period of treatment would be sufficient to deplete senescent cells and impact meaningfully on subsequent clinical outcomes in man remains to be determined.

Additionally, within this family (i.e., the BH3 mimetics), there exist differences in the efficacy of senolysis between specific drugs and this remains of therapeutic interest. Both senescent cells, malignant cells, undifferentiated stem cells, and megakaryocytes show selectivity in response to different members of this class of drug, related to varying expression of different members of the Bcl-2 family. For example, venetoclax (ABT-199); a selective Bcl-2 inhibitor which is used in the treatment of multiple myeloma has been shown to be effective in the setting of high Bcl-2 expression with concomitant low Bcl-XL expression and cells which co-express Bcl-2 and Bcl-XL to similar levels are resistant to venetoclax and require additional treatment with a Bcl-XL inhibitor (Punnoose et al., 2016).

Inhibition of Bcl-XL and Bcl-W *in vitro* and *in vivo* has been shown to induce apoptosis of senescent cells, whereas Bcl-2 inhibition alone had no significant senolytic effect. This suggests that ABT-263 or ABT-737 may be better therapeutic choices in terms of senolytics. (Yosef et al., 2016) It is worth noting, as mentioned above, Bcl-XL is a key pro-survival protein involved in the maintenance of platelets; thus explaining the resultant thrombocytopenia observed with ABT-263 and not seen with ABT-199 (Kile, 2014).

Dasatinib and Quercetin

Transplantation of senescent cells into healthy young mice has been shown to induce physical dysfunction, and earlier death. Depletion of senescent cells in this context with combination of dasatinib and quercetin (D+Q) attenuated the deterioration in walking speed, hanging endurance, and grip strength with similar beneficial effects on frailty in naturally aged mice (Xu et al., 2018).

With respect to mechanism of action; D+Q are proposed to have multiple intracellular targets. (Paez-Ribes et al., 2019) Dasatinib is a tyrosine kinase inhibitor which inhibits Ephrin B (EFNB)-dependent suppression of apoptosis, and has been shown to preferentially decrease viability and increase cell death in senescent compared to non-senescent human pre-adipocytes. (Zhu et al., 2015) Quercetin is a naturally occurring flavonoid which inhibits phosphoinositide 3-kinase (PI3K) and has been shown to induce cell death in senescent human umbilical vein endothelial cells (HUVECs) (Zhu et al., 2015).

Old mice (24–27 months) treated intermittently with D+Q had a 36% increase in median lifespan, with no reduction in physical function in that time (Xu et al., 2018). A single administration of D+Q in aged mice was sufficient to improve cardiovascular function and also reduced the expression of P16^{INK4A} and prevalence of SA- β -Gal positive cells after localized limb irradiation (Zhu et al., 2015).

Senescent cell depletion with D+Q has also been shown to be of benefit in a murine model of bleomycin-induced pulmonary fibrosis, with treated mice demonstrating an improvement across several markers of lung, and whole animal health; including reduced pulmonary resistance (as measured by enhanced pause, an indirect measurement of lung compliance in mice), preserved body weight, and improved exercise capacity. (Schafer et al., 2017) Despite improvements in lung function following senescent cell clearance, quantified

reductions in lung fibrosis histopathology however did not reach statistical significance.

Mice exposed to six months of high-fat diet (HFD) have been shown to develop increased renal cellular senescence and increased fibrosis. Prolonged (ten week) treatment with oral Quercetin reduced several markers of senescence including SA- β -Gal staining, whole kidney expression of p16, p19, and p53, and expression of classic SASP factors CCL2 and IL-1 α . However, there was no significant difference in p21 (CKD1a) expression between groups; i.e., no increased p21 expression in the context of HFD and no significant change post-treatment with quercetin. Mice treated with quercetin were found to have significantly less tubular epithelial cell apoptosis and had similar levels of creatinine and microalbuminuria to wild type littermate controls (vs HFD mice treated with vehicle). Quercetin was also shown to reduce the renal cortical hypoxia (as measured by magnetic resonance imaging, MRI) found in HFD mice. With respect to fibrosis; mice treated with quercetin had less interstitial fibrosis as quantified by Masson's trichrome staining, but no significant difference in collagen-1 expression as measured by immunofluorescence. (Kim et al., 2019) In another study using D+Q in obese mice, senolytic therapy led to an improvement in microalbuminuria and in increased expression of Wilms tumor protein (Wt-1), which is a measure of podocyte integrity and function (Palmer et al., 2019).

To date, there have been three studies of senolytics published in humans, with several more on-going. Thus far, two trials have shown D+Q to be safe and well-tolerated in the setting of idiopathic pulmonary fibrosis and in diabetes (Hickson et al., 2019; Justice et al., 2019). Furthermore, D+Q were effective in reducing senescent cell number in adipose tissue thus suggesting that the senolytic effects observed in mice are reproducible in humans (Hickson et al., 2019). An additional single arm study, used Dasatinib alone in patients with systemic sclerosis associated interstitial lung disease (Martyanov et al., 2017). A *post hoc* re-examination of the results from 12 patients found a decrease in the skin expression of SASP markers with treatment that correlated with clinical improvement (Martyanov et al., 2019).

Piperlongumine and Panobinostat

Piperlongumine is a biologically active component of the *Piper Longum* pepper plant with reported senolytic properties (Bezerra et al., 2013). It exhibits wide-ranging biological actions including anti-cancer, anti-inflammatory, and senolytic properties, even at very small dose, making this a potentially important compound in terms of limiting toxicity to healthy cells (Bezerra et al., 2013). Piperlongumine regulates cell signaling *via* receptor tyrosine kinase (Raf-1) and extracellular signal-regulated kinases ERK1/2. It has been shown to have moderate senolytic activity against senescent human fibroblasts *in vitro* (Wang et al., 2016).

Panobinostat is a histone deacetylase inhibitor (HDACi) has been shown to have senolytic activity in the setting of Cisplatin-induced senescence in non-small cell lung cancer (NSCLC), and in head and neck squamous cell carcinoma (HNSCC) cell lines (Samaraweera et al., 2017). HDACis have been shown to mediate reduction of Bcl-xl activity and thus in part, this may explain the

senolytic effect of panobinostat (Duan et al., 2005; Samaraweera et al., 2017).

Cardiac Glycosides

Most recently, the cardiac glycosides digoxin, proscillaridin A, and ouabain have all been shown to exert senolytic activity when tested against primary lung adenocarcinoma and fibroblast cell lines induced to senescence by Bleomycin *in vitro* (Triana-Martinez et al., 2019). Digoxin proved to be an effective senolytic in the context of *ex vivo* senescent chondrocytes from osteoarthritic donors. One proposed mechanism of action is inhibition of the Na⁺/K⁺-ATPase pump and subsequent imbalance in cellular sodium and potassium, with senescent cells being primed by an already slight increase in depolarisation of the plasma membrane; thus, lowering the threshold for cell death when exposed to digoxin.

Oubain has also been shown to be a broad spectrum senolytic, effective against murine hepatic senescent cells and in adamantinomatous craniopharyngioma; a pituitary paediatric tumor in which clusters of β -catenin + pre-neoplastic senescence cells positively influence tumor progression in a paracrine manner (Gonzalez-Meljem et al., 2017; Guerrero et al., 2019).

The senolytic effect of cardiac glycosides remains to be fully determined. However, transcriptomic analysis performed by Guerrero et al. revealed that ouabain and digoxin activate the expression of genes of the pro-apoptotic BCL2 family. Among those, NOXA is induced by the JNK, GSK-3 β , and P38 pathways, and partially mediates the senolytic effects of CGs (Guerrero et al., 2019).

Inhibition of G2/M Cell Cycle Arrest

As described, most senescence-inducing stimuli result in the induction of the cyclin-dependent kinase inhibitors p16^{INK4a} and/or p21^{CIP1} which enhance check-point activity, inducing cell-cycle arrest at the G1/S cell-cycle checkpoint with induction of the senescent phenotype. However, important work by the Bonventre group (Yang et al., 2010) has identified that accumulation of cells arrested at the later G2/M check point in the aftermath of kidney injury results not only in senescence, but triggers a pro-fibrotic secretory phenotype. This arrest is mediated *via* inhibition of cdc25c/cdc2 by p53 or by activation of the Chk1 and Chk2 proteins. Mimicking results seen in transgenic mice with reduced P16^{INK4A} and P21^{CIP1} leading to reduced G1/S cell cycle arrest—they employed JNK, histone deacetylase, and p53 inhibitors to reduce G2/M growth arrest and reduce renal fibrosis after ischaemic injury (Yang et al., 2010; Jenkins et al., 2014; Luo Q. et al., 2018).

Replicative senescence, and senescence induced by the DNA-damage response pathway (DDR) can also be induced at the G2/M checkpoint. (Mao et al., 2012; Jullien et al., 2013) Delineating the differences and commonality between G1/S and G2/M arrested cells, and the interplay between both checkpoints, will be of interest in the future and has potential implications for pharmacological inhibition of senescence; for example inhibition of movement of the cell

through G1/S may reduce the number of arrested cells as G2/M.

Pharmacological Targeting of the SASP — “Senostatics”

The SASP is a pro-inflammatory response that activates and reinforces senescence, modulates fibrosis and promotes regeneration (Acosta et al., 2008; Coppe et al., 2008). As such, it is an important facet of senescent cells in terms of their ability to influence whole organ or whole animal effects and it is therefore a potential therapeutic target for drugs (termed “senostatics”) which rather than killing senescent cells, instead impede their ability to release deleterious SASP molecules.

However, there are several important caveats to this approach. Firstly, the SASP is context dependent and dynamic (Coppe et al., 2008) and an approach to block or manipulate individual components of the SASP would require consideration of this. Secondly, the SASP comprises a vast array of secreted molecules and at present it is not clear which are pathogenic or how they induce damage. Finally, targeting the SASP mandates that treatment would likely be continuous as SASP production would continue unless senescent cells themselves were removed. In order to speed clinical translation of potential drugs, potential repurposing of existing clinically licenced agents merits attention.

NF- κ B Inhibition

The NF- κ B pathway is central to inflammation and has been proposed as a master regulator of the SASP (Chien et al., 2011). A cross-species study, which used motif mapping in promoters of genes upregulated with aging, suggested that NF- κ B is the transcription factor most associated with aging (Adler et al., 2007).

While the full effect of NF- κ B inhibition remains untested, it seems likely that with such a central role in cell signaling and inflammation, inhibition is likely to be context and cell dependent but has been proposed as a potential senolytic target (Salminen et al., 2012).

Drugs which have been considered for this purpose include antioxidants and inhibitors of the NF- κ B pathway (Nelson et al., 2012; Tilstra et al., 2012). Genetic reduction of NF- κ B in a fast aging mouse was shown to delay the onset of several age-related pathologies.

Likewise, pharmacological inhibition of NF- κ B signaling can be achieved by using an inhibitor of the NF- κ B activating kinase IKK. As is the case with genetic depletion, treated mice showed a delay in the onset of the majority of aging symptoms (Tilstra et al., 2012).

Furthermore, calorie restriction (CR) is widely recognized to extend longevity and in mice reduces senescent cell burden (Colman et al., 2008; Anderson et al., 2009; List et al., 2016). The beneficial effects of CR are not fully known but are in part attributed to suppression of NF- κ B. CR inhibits NF- κ B signaling at the level of the IKK complex. Short term CR resulted in a decrease in NF- κ B activity in the kidneys of aged rats (Jung et al., 2009). Thus, CR; a known mediator of improved healthspan and a known senolytic approach appears to function at least in part through NF- κ B inhibition.

JAK/STAT Inhibition

The SASP is rich in pro-inflammatory cytokines and chemokines. The JAK/STAT pathway plays an important role in regulating cytokine production (Yu et al., 2009; Meyer and Levine, 2014; Xu et al., 2015). Consistent with this, old rats have increased numbers of senescent pre-adipocytes and have higher levels of activated JAK1 and JAK2 (Xu et al., 2015). Treatment of senescent pre-adipocytes and HUVECs *in vitro* with JAK1 or JAK2 inhibitors significantly suppressed mRNA levels of key SASP components, including IL-6, IL-8, MMP12, and MMP3 (with little effect on control non-senescent cells). Similarly, *in vivo* old mice treated with Ruxolitinib (selective JAK1/2 inhibitor) resulted in reduced levels of cytokine expression otherwise normally associated with age, including IL-6 and CXCL-1 (Xu et al., 2015).

PTRP1 Inhibition

A recent study undertook an RNAi-based approach to identify transcriptional factors mediating the SASP profile of senescent cells. Using this approach, PTRP1 was identified as a factor mediating the inflammatory effects of the SASP with blockade inhibiting the pro-inflammatory and tumorigenic properties of senescent cells in the liver (Georgilis et al., 2018).

mTOR Inhibition

The mammalian target of rapamycin (mTOR), a serine/threonine kinase that plays central roles in various biological processes and has been shown to be important in some forms of senescence (Antonoli et al., 2019). Indeed, sirolimus (also known as rapamycin); a drug well known to nephrologists, has been shown experimentally to inhibit the senescence phenotype (Gu et al., 2016; Lesniewski et al., 2017; Wang R. et al., 2017). Given the importance of senescent cells in wound healing (Demaria et al., 2014), it is interesting to consider whether a previously unrecognized effect on senescence inhibition may have contributed to the impaired wound healing associated with clinical sirolimus use, though this remains to be proven.

Metformin

Similarly, metformin; another well-known drug with over 60 years' worth of clinical use is now being considered as an encouraging senostatic candidate. Metformin targets multiple intracellular signaling pathways closely associated with the development of aging, such as inflammation, cellular survival, stress defence, autophagy, and protein synthesis. It stimulates adenosine monophosphate-activated protein kinase (AMPK); a pathway known to limit cellular stress and protect from oxidative stress (Fang et al., 2018; Piskovatska et al., 2019) and inhibits NF- κ B pathway (Kanigur Sultuybek et al., 2019).

Blockade of Individual SASP Components

An alternative option is to consider manipulating individual SASP components. TGF β is a well-recognized SASP component and a known driver of fibrosis (Acosta et al., 2013; Meng et al., 2016). Clinically relevant TGF β blockers are available (Neuzillet et al., 2015) and inhibition of TGF β signaling with Galunisertib has been shown to be effective in animal models of biliary disease

and acute liver injury by blocking paracrine senescence leading to subsequent hepatocyte proliferation and improved liver function (Bird et al., 2018; Ferreira-Gonzalez et al., 2018). Connective tissue growth factor (CTGF, also known as CCN2) is another pro-fibrotic SASP mediator (Valentijn et al., 2018). In a phase 2 trial in patients with idiopathic pulmonary fibrosis, pamrevlumab, a fully recombinant human monoclonal antibody against CTGF, attenuated the decline in lung function and reduced the proportion of patients with disease progression compared to placebo (Richeldi et al., 2020).

Future Directions

Fundamental questions remain surrounding the pathways regulating senescence, their roles in tissue aging and dysfunction, and their potential therapeutic use.

How Does the Site and Timing of Senescence Influence Outcomes?

Our understanding of senescent cells *in vivo* remains incomplete. Global knockout models and generic P16^{INK4A} driven approaches have been informative, but have caveats as described. Further use of inhibitors, and novel transgenic tools; for example, transgenic models allowing conditional induction of senescence in specific cell lineages at specific times in the presence/absence of tissue injury (Bird et al., 2018; Ferreira-Gonzalez et al., 2018) have the potential to enhance our understanding further. Such models should allow a deeper characterization of the pathways to senescence at single cell level; i.e., from "early" to "deep" senescence and permit further delineation of the effect of senescence on organ function and fibrosis in the absence of other general processes such as age or injury.

Can We Have the "Good" Features of Senescence Without the "Bad"?

As described, senescence is important for wound healing (Krizhanovsky et al., 2008; Jun and Lau, 2010; Demaria et al., 2014). With respect to renal injury, it is likely that optimal recovery requires the induction of senescent cells (most likely of different cell lineages) at key time-points. Thus, a delicate balance is required to promote adaptive rather than maladaptive repair. The specific identity and roles of these cells in renal repair versus development of fibrosis remain unknown at present and it is imperative that we understand this to deliver optimal senolytic therapies.

CONCLUSIONS

Over the last five years depletion of senescent cells in animal models has demonstrated striking results across multiple tissues, organs and at whole animal level and this continues to inspire the development and refinement of therapy. However, as yet, their efficacy and safety has not been extensively tested in humans.

Multiple early stage clinical trials are underway and early reports are encouraging (Hickson et al., 2019; Justice et al., 2019).

These include NCT02848131, a trial which tests dasatinib and quercetin as senolytics in diabetic patients with chronic kidney disease, NCT04063124 which will test the same agents in the modulation of progression of Alzheimer's disease and NCT04313634 comparing dasatinib, quercetin and fisetin on senescence levels and bone mineral density in elderly women. There are also trials on-going looking at the effects of known senolytics/senostatics on frailty and aging; in particular fisetin (NCT0367524) and metformin (NCT02915198, NCT03451006) whose results will clearly be of interest to the field in general. Additionally, the mdm2/p53 inhibiting peptide UBX0101 (NCT04129944) is under ongoing investigation as a local treatment for symptomatic osteoarthritis of the knee.

An important question relates to how the essential roles and/or beneficial effects of senescence in development, tissue repair and the opposition of malignancy can be retained while their negating their apparently deleterious actions on tissue fibrosis and dysfunction. It is to be hoped that in the next five years many of these questions will be answered. Current and future studies will undoubtedly broaden our knowledge of the key pathogenic mechanisms of senescent cells and hence permit refined therapies to deplete senescent cells in a targeted manner. It

may also be possible to target the metabolic alterations present with senescence to deliver selective therapies to limit senescent cell survival. Finally, the presence of clinically licenced compounds with the ability to restrain the deleterious effects of senescence *via* inhibition of SASP release may allow these to be the first agents to transition from experimental models to clinical use.

AUTHOR CONTRIBUTIONS

MD planned the study and wrote and revised the text. DB planned the study and wrote the text. JH reviewed and revised the text. DF planned the study, designed the figures, and reviewed and revised the text.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cellular Senescence in the Kidney

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ABSTRACT

Senescent cells have undergone permanent growth arrest, adopt an altered secretory phenotype, and accumulate in the kidney and other organs with ageing and injury. Senescence has diverse physiologic roles and experimental studies support its importance in nephrogenesis, successful tissue repair, and in opposing malignant transformation. However, recent murine studies have shown that depletion of chronically senescent cells extends healthy lifespan and delays age-associated disease—implicating senescence and the senescence-associated secretory phenotype as drivers of organ dysfunction. Great interest is therefore focused on the manipulation of senescence as a novel therapeutic target in kidney disease. In this review, we examine current knowledge and areas of ongoing uncertainty regarding senescence in the human kidney and experimental models. We summarize evidence supporting the role of senescence in normal kidney development and homeostasis but also senescence-induced maladaptive repair, renal fibrosis, and transplant failure. Recent studies using senescent cell manipulation and depletion as novel therapies to treat renal disease are discussed, and we explore unanswered questions for future research.

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Global demographics are changing, with the proportion of individuals over the age of 65 in the United States predicted to double within the next 25 years.¹ Older kidneys exhibit decreased function, altered homeostasis, and increased susceptibility to pathologies including AKI, maladaptive repair, and the subsequent development of CKD.^{2,3} In recent years, cellular senescence has emerged as an important driver of aging and age-related disease in multiple organs including the kidney. This review examines our current understanding of renal senescence, along with examples from other organ systems where this highlights areas of potential research and translational relevance to kidney homeostasis and disease.

WHAT IS SENESCENCE?

“Senescence” derives from the Latin “senex” meaning “old” and was originally used by biologists interchangeably with the term “aging” to describe the decline in organism function with time. A seminal description by Leonard Hayflick in 1961 of what is now recognized as replicative senescence due to progressive telomere shortening with prolonged cell culture *in vitro* helped shape our modern understanding of the term. Senescence is now recognized as both a response to prolonged culture or cell stress *in vitro* and as an important cellular stress and aging response *in vivo*.⁴ Although telomere shortening is accepted as sufficient

to induce growth arrest and senescence *in vitro*, the key importance of telomere shortening in human aging is less well established, and is not the focus of this review.⁵ Although the association between aging and senescence is well recognized,^{6,7} only recently have experiments depleting senescent cells in murine models been shown to postpone the onset of age-related diseases and extend healthy lifespan, reigniting clinical and research interest.^{8–10}

Initiation of Senescence

Today, the term “cellular senescence” is used to describe irreversible proliferative arrest with associated changes in chromatin organization, gene transcription, and protein secretion either *in vitro* or *in vivo*.^{11,12} Senescence can occur in multiple contexts across tissue and organ lifespan including physiologically appropriate “acute senescence” responses during injury, normal organogenesis, tissue homeostasis, and repair.¹³ In these settings senescence appears to limit fibrosis before senescent cells are removed by apoptosis or immune clearance.¹⁴ This form of senescence seems a tightly controlled, scheduled process.

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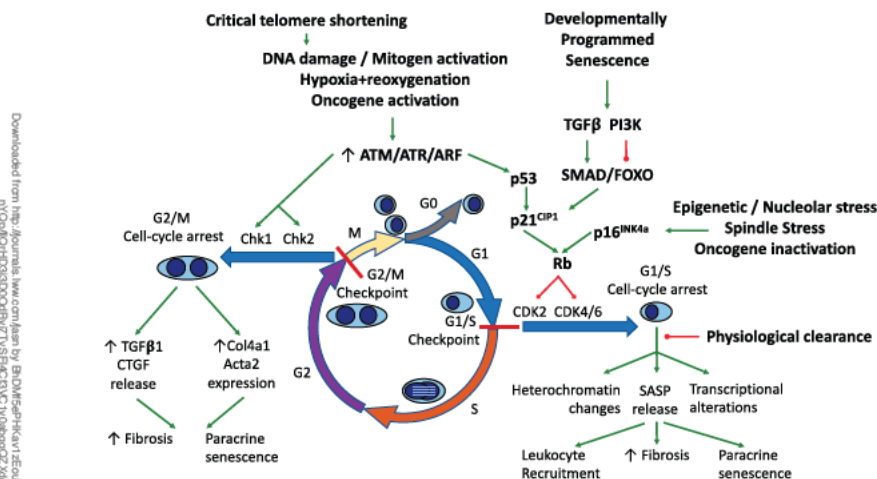


Figure 1. Pathways to cellular senescence in eukaryotic cells. Multiple discrete cellular insults act via distinct signaling mechanisms to induce cell-cycle arrest in the kidney at either the G1/S (via inhibition of cdk2 and/or cdk4/6) or G2/M checkpoints (via Chk1/2 activation or cdc2/25c inhibition). Inactivation of oncogenes and spindle/epigenetic/nucleolar stress trigger activation of the cyclin-dependent kinase inhibitor p16^{ink4a}. Telomere shortening, DNA damage, mitogen or oncogene activation, and hypoxia/reoxygenation also result in G1/S cell-cycle arrest, via a pathway dependent on p53 and p21^{CIP1} activation. In contrast to this, developmental senescence appears to induce p21^{CIP1} by pathways mediated by TGFβ/PI3K and independent of p53. ATM/ATR/ARF, Ataxia-Telangiectasia Mutated/Ataxia Telangiectasia and Rad3-related protein/p14 Alternate Reading Frame (human).

In contrast, chronically senescent cells accumulate in organs in response to a variety of stressors including DNA damage or critical telomere shortening leading to exposure of DNA ends,¹⁵ oncogenic mutations, metabolic stress, mitochondrial dysfunction, and inflammation.^{16,17} These insults trigger cell cycle inhibition and permanent cell cycle arrest via pathways either dependent on or independent of the DNA damage response (summarized in Figure 1). These cells persist within affected organs and are increasingly recognized as drivers of disease progression rather than bystanders.

Most senescence inducing stimuli result in the induction of the cyclin dependent kinase inhibitors p16^{ink4a} and/or p21^{CIP1} which enhance checkpoint activity, inducing cell cycle arrest at the G1/S cell cycle checkpoint with induction of the senescent phenotype.^{11,12} Studies of experimental renal disease in mice lacking p16^{ink4a} and/or

p21^{CIP1} show increased cellular proliferation but also increased mortality after ischemia/reperfusion injury, suggesting that close regulation of the cell cycle is necessary for postinjury repair.^{18,19} Work with several experimental models of fibrotic kidney disease identified that accumulation of cells arrested at the later G2/M checkpoint (via inhibition of cdc25c/cdc2 by p53 or activation of the Chk1 and Chk2 proteins) not only results in senescence but triggers a profibrotic secretory phenotype.^{20–22} Inhibiting this checkpoint using JNK, histone deacetylase, or p53 inhibitors results in reduced numbers of G2/M arrested cells and reduced fibrosis in a model of unilateral renal ischemia.²⁰ Delineating the differences and commonality between G1/S and G2/M arrested cells will be of interest in the future. Inhibition of movement of the cell through G1/S may, for example, reduce the number of cells that are arrested at G2/M.

Defining and Detecting Senescent Cells

Whereas the physical and functional alterations seen with senescence *in vitro* are well characterized, identifying and characterizing senescent cells *in vivo* remains challenging.²³ This is due in part to the lack of any single feature uniquely identifying senescence, and difficulties in assaying multiple attributes in individual cells within a tissue.^{11,12,14} Important markers include senescence associated β galactosidase (SA β gal), p16^{ink4a}, and p21^{CIP1}; however, the presence/absence of one or more of these factors is insufficient to confirm senescence. The Van Deursen group has proposed that quantifying tissue senescence should comprise three approaches: staining of cell cycle arrest markers, quantifying the effects of senescence associated protein release, and excluding coexisting cellular proliferation.¹² The generation of transgenic mice expressing genes driven by promoters

cloned from the Cdkn2a locus which encodes p16^{ink4a} (INK ATTAC, p16 [LUC], and p16 3MR mice) represents a major contribution to this field.^{24–26} Recent advances in single cell sequencing should facilitate the identification and characterization of senescence on an individual cell basis for the first time.

The Senescence-Associated Secretory Phenotype

Despite exiting the cell cycle, senescent cells remain metabolically active, releasing a modified secretome into local tissues which can significantly affect neighboring cells and ultimately tissue function. This secretion of cytokines, chemokines, growth factors, and proteases is termed the senescence associated secretory phenotype (SASP).^{27–29} SASP components include IL 1 β , IL 6, IL 8, TGF β 1, and WNT16B and these can act in a paracrine and autocrine fashion leading to persistence and propagation of senescence within tissues.^{14,30–33}

Proinflammatory cytokines and chemokines are relatively conserved SASP components across multiple organ injuries.²⁷ However, SASP composition may differ depending on the mode of senescence induction and biologic context. Although direct evidence in the kidney is lacking, studies in other organ systems provide a framework for future studies in experimental renal disease. In repair of cutaneous wounds *in vivo*, the secretion of CYR61 (CCN1) is reported to be a key stimulus of fibroblast senescence, whereas SASP derived PDGF AA is required for successful wound healing.^{24,34} In contrast, immune clearance of oncogene induced senescent cells in the liver is promoted by CCL2 and chronically senescent cells are reported to induce IL 6, plasminogen activator inhibitor 1 (PAI 1), and other diverse factors.^{10,35} The composition of the SASP reflects both the origin of the cell and the initiating stimuli, often initiated by NF κ B and p38 mitogen activated protein kinase signaling.³⁶

The SASP provides an important mechanism through which senescent cells affect organ function *via* induction of paracrine senescence. Recent research shows that transplanting senescent

preadipocytes intraperitoneally into young mice results in a 50 fold increase in total body senescent cell number compared with controls.⁸ Furthermore, administration of serum from patients with chronic obstructive pulmonary disease (COPD) induces senescence in bronchial epithelial cells *in vitro*, suggesting that circulating factors may promote systemic senescence.³⁷

PHYSIOLOGIC FUNCTIONS OF SENESCENT CELLS

Embryogenesis/Development

Developmental senescence is a highly ordered and conserved cellular process observed in multiple species including tetrapods and fish.³⁸ The mesonephros and endolymphatic sac of the human embryo contain senescent cells which affect tissue remodeling and cell balance.¹³ Murine studies provide evidence that TGF β and PI3K signaling activate p21^{cip1} induced senescence *in vitro*.^{13,39} Studies in mice show induction of SA β gal in the mesonephros simultaneous with falling proliferation markers immediately before involution of mesonephric tubules.¹³ Further studies show that growth arrest requires p21^{cip1} induction independent of p53, with mice lacking p21^{cip1} exhibiting delayed mesonephric regression but developing functional kidneys with increased apoptosis restoring appropriate cell number.¹³

Postinjury Repair

Acute senescence is known to be important in successful scar formation, with murine studies showing that senescent cell depletion after skin wounding *in vivo* results in delayed wound closure although eventual healing still occurs.²⁴ Induction of fibroblast senescence controls the fibrotic pathways required for normal healing,³⁴ whereas activation of hepatic stellate cell senescence limits liver fibrosis in a murine injury model.⁴⁰ Interestingly, p16^{ink4a} knockout mice exposed to experimental renal injury show increased epithelial proliferation and functional recovery after ischemia/reperfusion injury but worsened fibrosis in unilateral ureteric obstruction models, suggesting

that senescent cells are required at specific timepoints and in specific populations in response to different injuries for optimal repair.^{19,41}

Cancer Defense

Senescence induction by oncogene activation is recognized as an important physiologic mechanism preventing neoplastic transformation. Although data in human renal cancer are limited, one study showed that lowered expression of senescence markers in human renal cell carcinoma associates with worsening prognosis, whereas the antiproliferative effects of calcitriol on human renal cancer cells *in vitro* have been attributed to induction of senescence.^{42,43} Oncogene activation in murine hepatocytes drives secretion of chemokines and cytokines resulting in immune mediated clearance of these premalignant cells. Impaired removal of senescent hepatocytes results in subsequent development of hepatocellular carcinoma³⁵ introducing the concept of “senescent surveillance” as a defensive process, protecting against malignant transformation by inducing cell cycle arrest. The components of the SASP act to reinforce the growth arrest in neighboring cells, protecting an area of tissue from cancerous change.^{13,39} Animal models where senescence induction is blocked demonstrate increased susceptibility to several cancers.^{44,45}

SENESCENCE IN RENAL DISEASE

Tubular epithelial cells are frequently implicated in renal senescence, although other cell types can exhibit positive staining for markers of cell cycle arrest. In a series of human renal transplant biopsies, all biopsy specimens showed p16^{ink4a} staining in the nuclei of distal tubules and collecting duct but staining was also present in podocytes, parietal epithelium of glomeruli, vascular smooth muscle cells, and interstitial cells.⁴⁶ Furthermore, a series of patients with glomerular disease showed p16^{ink4a} staining in a subset of glomerular, tubular, and interstitial cell nuclei; however, senescent tubular epithelial cells were

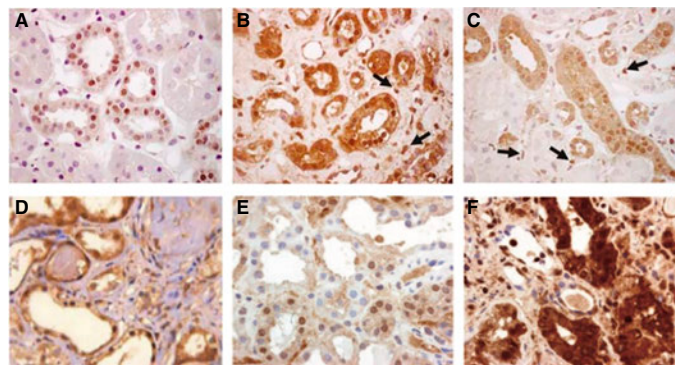


Figure 2. Renal disease increases renal expression of the cyclin-dependent kinase inhibitor p16^{ink4a}. (A) Normal kidney biopsy specimen from a 42-year-old living related donor with p16^{ink4a} staining in some tubular epithelial cell nuclei.⁴⁷ (B) Membranous nephropathy from a 64-year-old man demonstrating cytoplasmic and nuclear p16^{ink4a} in tubular epithelium and interstitial nuclei (arrows).⁴⁷ (C) FSGS biopsy specimen from a 48-year-old man, showing increased p16^{ink4a} staining in some tubules and interstitial cell nuclei (arrows).⁴⁷ (D) Grade 5 IgA nephropathy, with p16^{ink4a} staining demonstrating increased cytoplasmic and nuclear staining.⁴⁸ (E) Implantation biopsy specimen from a 45-year-old donor with expected low levels of p16^{ink4a}-positive staining.⁴⁶ (F) Repeat biopsy specimen of the same kidney 7 years post-transplantation demonstrating intense nuclear and cytoplasmic staining⁴⁶ (note that the anti-p16^{ink4a} [F12] antibody from Santa Cruz Biotechnology used in all of these images is no longer available).

the key difference between disease and control kidneys, present in 80% of cases compared with 21% in normal kidneys.⁴⁷ Additionally, a biopsy series of patients with IgA nephropathy demonstrated increased p21^{cip1} and p16^{ink4a} protein expression which was confined to tubules.⁴⁸

Experimental data support the importance of the tubular epithelium. p16^{ink4a} knockout mice have increased senescence and subsequent fibrosis in tubular and interstitial cells after unilateral ureteric obstruction, most prominently in the collecting duct.⁴¹ After ischemia reperfusion injury, nuclear p21^{cip1} was localized to both distal and proximal nephron, including collecting ducts, with no glomerular staining found.¹⁸ In Baker *et al.*'s²⁶ seminal 2016 study of ablation of senescent cells in INK ATTAC transgenic mice demonstrated senescence occurring in proximal tubules with increasing age. The importance of the epithelial cell is supported by *in vitro* data which demonstrate that a variety of injuries can induce renal proximal tubular epithelial cells into a senescent phenotype.⁴⁹

CKD

Senescent cells are known to be present at increased levels in many renal diseases (Figure 2), and it appears likely that senescence plays a role in the maladaptive repair which contributes to many cases of progressive renal fibrosis after injury (Figure 3). Senescence, as indicated by p16^{ink4a} expressing cells, increases in patients with glomerular disease as compared with age-matched controls.⁴⁶ In IgA nephropathy, shorter telomere lengths are found in mononuclear cells extracted from urinary sediment collections versus healthy controls, and a significant inverse correlation is shown between telomere length and serum creatinine.⁵⁰ Recent bioinformatic analysis of both murine experimental renal injury and human renal transplantation identifies significant upregulation of Cdkn1a (p21^{cip1}) in the aftermath of injury and in human transplanted kidneys undergoing AKI to CKD transition.⁵¹ Finally, senescence may be a factor in progression of diabetic nephropathy (DN). Levels of p16^{ink4a} and SA β gal increase in renal epithelial cells in biopsy specimens from patients with DN.⁵² A p21^{cip1}/p27^{kip1} bigenic knockout mouse model of DN

shows reduced proteinuria, glomerular hypertrophy, and tubule interstitial damage, implicating senescence induction as contributing to disease progression.^{53,54}

Autosomal Dominant Polycystic Kidney Disease

In contrast to the above, autosomal dominant polycystic kidney disease (ADPKD) is an example of senescence attenuating disease progression. Levels of p21^{cip1} are reduced in kidneys from human patients with ADPKD and in animal models of PKD.⁵⁵ Treatment with the cyclin dependent kinase (CDK) inhibitor roscovitine restores p21^{cip1} levels, increases SA β gal staining, decreases tubular cell proliferation, and attenuates disease progression in a murine model of ADPKD.^{55,56} This suggests that a failure to induce senescence in ADPKD may contribute to cystic progression.

Kidney Transplantation

Human studies have correlated levels of pre-transplant senescent cells in the kidney with the subsequent development of interstitial fibrosis, tubular atrophy, and

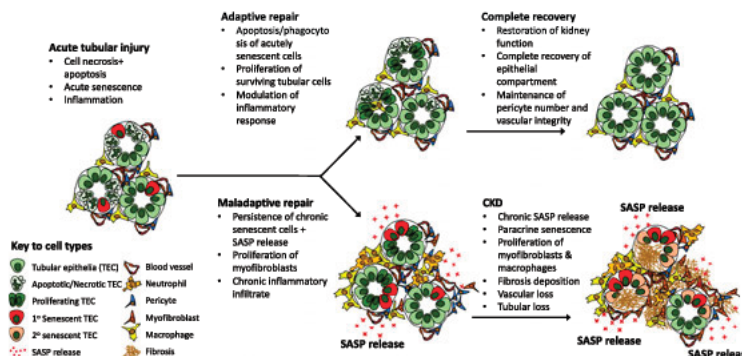


Figure 3. The potential tissue effects of pathologic growth arrest in maladaptive repair. This flowchart illustrates the putative steps required for repair after AKI. In the case of adaptive repair, acute senescence is induced but then cleared in a tightly controlled manner, with normal tissue structure being restored by proliferation of resident cells. In maladaptive repair, it is recognized that there is an accumulation of both tissue fibrosis and secondary senescent cells. Recent studies indicate that pharmacologic/genetic targeting of senescent cells may protect against progressive fibrosis in murine models of aging.^{26,71}

chronic allograft nephropathy.^{46,47,57} Supporting this, elevated mRNA expression of p21^{cip1} and p16^{ink4a} in pre transplant biopsy specimens correlates with poor outcomes in both experimental animals and man.^{58,59} In the setting of renal transplantation, immune activation and reduced immune clearance due to immunosuppression may contribute to senescent cell accumulation.^{60,61} The interaction between senescent cells and the post transplant immune milieu is likely complex, because allograft rejection is associated with increased senescence and glomerular, tubular, and interstitial cells from rejected grafts express elevated levels of p16^{ink4a} and p27^{kip1}, correlating with the grade of chronic allograft nephropathy.⁶²

Senescence may explain some of the effects of age on graft outcome. In humans, increased numbers of senescent cells in an older donor kidney reduce the regenerative capacity of that transplanted kidney in response to injury.⁶³ Grafts from older human donors are more vulnerable to ischemic injury and more likely to develop delayed graft function post transplant.^{64,65} Murine work supports a role for senescence in induction in graft outcome, with p16^{ink4a}

KO mice developing less fibrosis when receiving kidneys transplanted from p16^{ink4a} KO mice and having significantly higher graft survival, less tubulointerstitial damage, and higher tubular epithelial cell proliferation scores.⁶⁶

Immunosenescence and Senescent Cell Clearance

Although accumulation of senescent cells with advancing age and with renal disease is well documented, whether this reflects (1) increased senescence induction, (2) altered SASP release preventing immune clearance, or (3) a primary failure within the immune system itself remains largely unexplored. Although no direct studies exist on the role of the immune system in senescent cell clearance from the adult kidney, SASP release is immunostimulatory³⁵ and studies within the murine liver, uterus, and endometrium have reported key roles for monocytes, macrophages, CD4⁺ T lymphocytes, and natural killer cells in the physiologic clearance of senescent cells.^{35,67,68} Studies using senolytic agents show that senescent cell ablation results in reduced levels of inflammation in mice²⁶ and in cells removed from inflamed human joints.⁶⁹ Complementary

work has shown that, after irradiation, the generation of senescent hematopoietic stem cells results in immune dysfunction, with selective depletion of these cells improving both stem cell and overall immune function in mice.⁷⁰ Whether senescence within the kidney or the immune system interrupts the physiologic clearance of senescent cells and promotes renal disease remains an important unanswered question.

THERAPEUTIC TARGETING OF SENESCENCE IN THE KIDNEY

Animal Models of Senescent Cell Deficiency or Depletion in the Kidney

Our understanding of senescent cell accumulation and clearance in renal disease remains incomplete. Important lessons have been learned using wild type and transgenic mice to test the effect of defective senescent cell induction on renal development and disease.^{13,18,19,41,53,54} Recent seminal studies demonstrate the safety and efficacy of senescent cell depletion *in vivo*, using transgenic mice with selective sensitivity to pharmacologic agents,^{26,70} or wild type mice treated with

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Table 1. Experimental models of senescent cell deficiency/induction/depletion and their effects on renal outcomes

Reference	Renal Model	Modulation of Senescence	Renal Disease Outcome	Effect of Intervention
Baker <i>et al.</i> ²⁶	Natural aging in INK-ATTAC mice	INK-ATTAC +AP20187 or vehicle administration to deplete p16 ^{INK4a} cells	↓Glomerulosclerosis ↓β-gal positivity	↓Glomerulosclerosis ↓β-gal positivity
Barar <i>et al.</i> ⁷¹	Natural-aging p16-3MR mice and fast-aging Xpd TTD/TTD mice	FOXO4-DRI agent causes p53 nuclear exclusion. Ganciclovir treatment to p163MR mice causes p16 ^{INK4a} -positive restricted cell death	↑Serum urea ↓Lumbl1 expression ↑SASP expression (both Xpd ^{TTD/TTD} and aged p16-3MR)	FOXO4-DRI or GCV to p16-3MR admin ↓Serum urea ↓Lumbl1 expression ↓SASP expression (both Xpd ^{TTD/TTD} and aged p16-3MR)
Muñoz-Espín <i>et al.</i> ¹³	Nephrogenesis	WT versus P21 ^{Cip1} KO mice with deficient growth arrest in nephrogenesis	↓β-gal positivity in P21 ^{Cip1} KO mice <i>in utero</i> ↑Ki67 expression but apoptosis maintains development	Use of PI3K inhibitor augments developmental senescence in WT mice
Wolstein <i>et al.</i> ⁴¹	UUO	WT versus p16 ^{INK4a} KO mice with impaired cell-cycle arrest	UUO induces β-gal positivity, apoptosis, and collagen deposition in WT mice	↓β-gal positivity ↓Apoptosis ↓Collagen
Magyesi <i>et al.</i> ¹⁸	Renal IRI	WT versus P21 ^{Cip1} KO mice with impaired cell-cycle arrest	WT mice show tubular injury and raised blood urea levels after IRI	↑Proliferation after UUO in p16 ^{INK4a} KO ↑Proliferation ↑Renal function
Lee <i>et al.</i> ⁷⁹	Renal IRI	WT versus p16 ^{INK4a} /p19 ^{ARF} double KO mice with impaired cell-cycle arrest	WT mice show marked p16 ^{INK4a} and p19 ^{ARF} induction 28 d after IRI, with apoptosis and reduced tubular density	↑Mortality in P21 ^{Cip1} KO p16 ^{INK4a} - and p19 ^{ARF} -deficient mice show increased epithelial and microvascular repair, with increased myeloid cell recruitment
AL-Douhaji <i>et al.</i> ⁵³	Diabetic	WT versus P21 ^{Cip1} KO	WT mice develop albuminuria and glomerular hypertrophy	Both p27 ^{Kip1} KO and p21 ^{Cip1} KO mice were protected from proteinuria and glomerular expansion
Wolf <i>et al.</i> ⁵⁴	Nephropathy	WT versus p27 ^{Kip1} KO		
Braun <i>et al.</i> ⁶⁶	Renal transplant	p16 ^{INK4a} KO mice with impaired cell-cycle arrest	WT mice develop interstitial fibrosis and tubular atrophy	p16 ^{INK4a} KO mice develop less atrophy and fibrosis after Tx

Transgenic and genetic brook trout have been used to study the effect of (1) deficiencies in the induction of senescence or (2) depletion of established senescent cells. Several of these models are summarized in Table 1. Abbreviations used in the experiments include: viral disease induced, vi; bacterial infection, bi; ureteric obstruction, ur; ischemia-reperfusion injury, ri; Transant, TGA; genetic cross, WT, d-type KO, interline, inter; renal failure, rfa; disease outcomes, TTD/TD, trichothiodystrophy/trichothiodystrophy; scavenger cells, sc; leukocytes, lc.

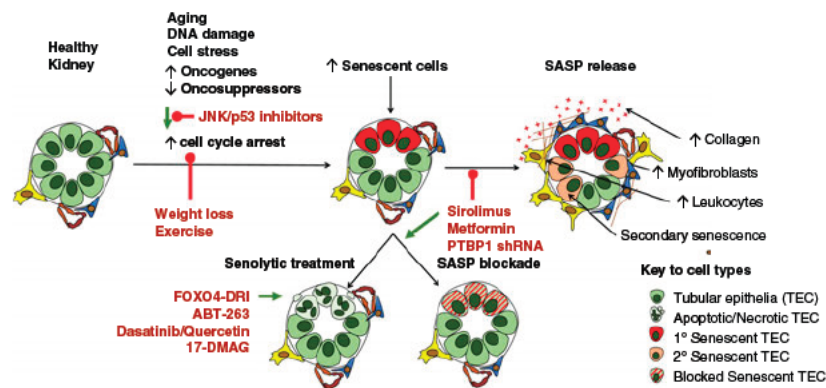


Figure 4. Current and potential future interventions to target growth-arrested cells in the kidney *in vivo*. Multiple points in the generation of senescent cells are under investigation as potential windows to alter their accumulation and/or pathogenic effects. Interventions are being trialed to prevent senescent cell formation (e.g., weight loss, exercise), promote senescent cell apoptosis (e.g., FOXO4-DRI, ABT-263), inhibit SASP release (e.g., sirolimus, metformin), or utilize the metabolic activity of senescent cells to activate compounds (e.g., galacto-oligosaccharide-conjugated drugs). shRNA, short hairpin RNA.

drugs targeting antiapoptotic pathways upregulated in senescent cells^{8,70,71} (summarized in Table 1). These studies provide evidence that senescent cell depletion increases healthy lifespan by postponing the onset of several age associated pathologies. Importantly, such benefits have not come at the expense of increased cancer incidence, indicating that depletion of chronically senescent cells differs from models lacking the ability to induce senescence in response to DNA damage.²⁶ Accordingly, there is major interest in the potential to manipulate senescent cell number or behavior to delay the onset and progression of numerous age related diseases, including those of the kidney (Figure 4).

Nonpharmacologic Approaches to Reduce Senescent Cell Accumulation

Increased levels of physical activity and weight loss represent two nonpharmacologic approaches to reduce senescent cell generation. In mice exposed to a high fat diet, regimes of increased exercise reduce expression of senescence markers and SASP release in adipose tissue.⁷² Studies examining circulating leukocytes in both

mice and track and field athletes show that exercise associates with reduced markers of senescence.⁷³ In mice undergoing controlled weight gain, animals switching to reduced calorie intake reduce senescent cell number in white adipose tissue.⁷⁴

Senolytics

The INK ATTAC and p16 3MR transgenic mice use components of the Cdkn2a promoter (responsible for p16^{ink4a} expression) to confer selective sensitivity of p16^{ink4a} expressing cells to pharmacologic depletion.^{24,26,70} Depletion of chronically senescent cells from both strains in aged or irradiated animals benefits multiple organs including the kidney.^{26,70} Although informative, the results of studies based exclusively upon such transgenes may not fully recapitulate the spectra of senescent cells seen *in vivo*, unless all such cells express p16^{ink4a}, which seems unlikely.

Although the field of senolysis is in its infancy, several agents have generated encouraging results in rodent models. One such agent is ABT 263 (Navitoclax), a Bcl 2/w/xL inhibitor which targets a dependence on the Bcl 2 pathway in senescent cells, resulting in widespread

senescent cell apoptosis. Recent studies have used this drug successfully to target irradiation induced senescence *via* the c Jun/Bcl xL/p21^{Cip1} pathway.^{70,75,76} With ABT 263 currently in phase 1 and 2 clinical trials for both hematologic and solid organ malignancies there is potential for early clinical translation in non malignant conditions.^{77,78}

Another combination includes dasatinib (a tyrosine kinase inhibitor) and quercetin (a flavonoid) discovered using a bioinformatics driven approach by Zhu *et al.*⁷⁹ in 2015. Cultured senescent and nonsenescent cells were exposed to compounds known to target senescent cell apoptotic pathways. Dasatinib and quercetin eliminate senescent but not nonsenescent cells in rodent and human cell cultures, freshly isolated human tissue explants, old mice, and mice with accelerated ageing.^{8,79,80} Treating with dasatinib and quercetin alleviated the physical dysfunction and frailty associated with increased senescence. Mice lived longer and performed better in all measures of frailty tested.⁸

Other groups used an assay of SA β gal expression to guide identification of drugs capable of selectively targeting

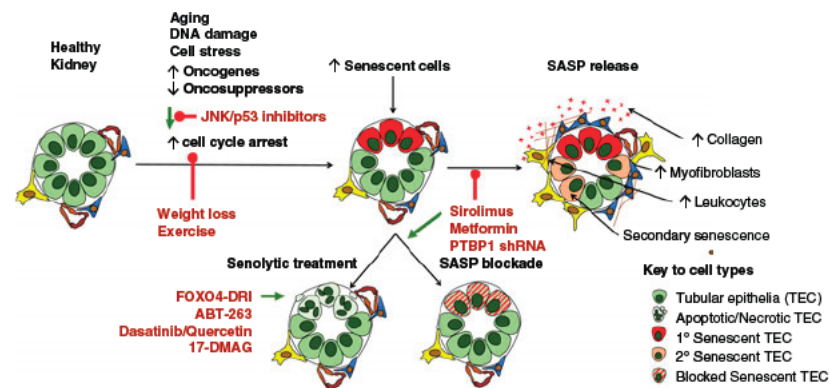


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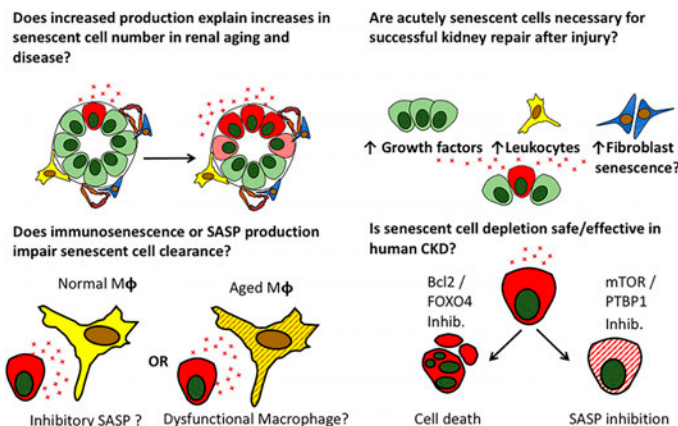


Figure 5. Unanswered questions about the roles and significance of senescent cells in the kidney. Several key questions regarding the role of the senescent cells in the kidney require ongoing research, with the goal of (1) quantifying senescent cell load noninvasively, (2) understanding the need for senescent cells in renal repair after injury, (3) determining the factors preventing senescent cell clearance from the kidney, and (4) testing the efficacy of senescence-modifying interventions in man.

senescence leading to the identification of the HSP90 inhibitor 17DMAG as a compound capable of increasing healthy lifespan and reducing p16^{ink4a} positive cell number in a progeroid mouse model.⁸¹ Similarly, the increased expression of SA β gal present within diverse senescent cells has recently been harnessed to allow selective activation of cytotoxic compounds *in vivo*. This approach was used to target senescent cells effectively in two models of cancer and fibrosis and represents an exciting avenue for further clinical translation.⁸² Important work by Baar *et al.*⁷¹ identified the FOXO4 protein as an important regulator of p53 activity in senescent cells, and, by designing an interfering peptide “FOXO4 DRI,” demonstrated that selective apoptosis could be achieved in both aging and chemotherapy induced senescent cells, with resultant improvement in organismal fitness, including protection of baseline renal structure and function.

Senostatics

Senostatics are drugs that inhibit elements of the senescent phenotype without

removing the cells. Drugs familiar to nephrologists are being examined for this purpose, including metformin and sirolimus (rapamycin), both of which activate autophagy and improve mitochondrial complex I function and can extend lifespan.^{83–85} mTOR signaling is known to be an important pathway in some forms of senescence⁸⁶ and furthermore there is some experimental evidence that sirolimus itself inhibits the senescent cell phenotype.^{87–89} Given the previously mentioned role of senescent cells in wound healing, it is interesting to speculate whether a hitherto unrecognized effect on senescence inhibition may contribute to the impaired wound healing associated with clinical sirolimus use, although this link remains unproven.

Other candidate drugs include inhibitors of I κ B kinase, NF κ B, and the Janus kinase pathway.^{90,91} Such approaches inhibit proinflammatory signaling pathways, such as NF κ B or p38 mitogen activated protein kinase pathways, disrupting SASP production.^{92,93} A recent study undertook an RNAi based approach to identify transcriptional factors mediating the SASP profile of senescent

cells. Using this approach, PTRP1 was identified as a factor mediating the inflammatory effects of the SASP with blockade inhibiting the proinflammatory and tumorigenic properties of senescent cells in the liver—a good example of basic science identifying novel and druggable targets for translation.⁸¹

THE FUTURE

Fundamental questions remain surrounding the pathways regulating senescence, their roles in tissue aging and dysfunction, and their potential therapeutic use (Figure 5).

How Can Senescent Cell Load Be Determined *In Vivo*?

Senescent cell burden in the kidney may be a useful predictor of renal outcomes. Current means of detection are not sufficiently specific and are often impractical because stained tissue is required. Whether increased senescent cell number on renal biopsy predicts progression and functional decline better than existing markers remains untested. Prospective studies

following patients with differing numbers of senescent cells will be required to answer this question. Noninvasive serum or urine biomarkers of senescence would help researchers associate senescent burden with clinical phenotypes, inform basic science research, and build a more complete understanding of their role in disease.

Are Senescent Cells Important in Recovery?

It is likely that optimal recovery post renal injury requires the induction of senescent cells at key timepoints, and a delicate balance is needed to promote adaptive rather than maladaptive repair. The specific identity and roles of these cells in renal repair versus development of fibrosis remain unknown.

Why Do Senescent Cells Accumulate with Age and Disease?

Whether the accumulation seen in multiple organs, including the kidney, with age reflects increased production or reduced immune clearance remains poorly understood. Intriguing studies from the Fogo laboratory show that murine renal fibrosis is reduced in the aftermath of a young bone marrow transplant when compared with an older donor.⁹⁴ The potential for therapies targeting immune system aging and function to affect renal disease merits further study.

Is Depletion of Senescent Cells Safe in Man?

Although agents are being developed to deplete senescent cells, their efficacy and safety in humans remain unproven. Whether minimizing development of senescent cells or reducing total senescent cell burden will have clinical relevance for elderly patients is unknown. It is encouraging that interventions started in relatively elderly mice still positively affect aging pathologies. Four clinical trials are currently underway testing senolytic therapies in man, including one (NCT02848131) testing dasatinib and quercetin as senolytics in patients with diabetes with CKD.

Research into senescence will continue to shed light on these ubiquitous yet mysterious players in the aging and

damaged kidney. With better understanding of their role in damage and repair it may become possible to target them therapeutically, without compromising their essential regulatory functions in healing and cancer suppression.

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DISCLOSURES

J.V.B. is coinventor on KIM-1 patents that are assigned to Partners HealthCare and licensed to Johnson & Johnson, Sekisui, Novartis, Biogen Idec, R&D, and Astute. He is a consultant for Astellas, Novartis, Roche, and Sekisui regarding the safety and efficacy of therapeutics or diagnostics for AKI. He holds equity in MediBeacon, Sentien, and Thrasos, and has grant support from Boehringer Ingelheim and Roche. All other authors report no conflicts of interest.

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KIDNEY DISEASE

Cellular senescence inhibits renal regeneration after injury in mice, with senolytic treatment promoting repair

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The ability of the kidney to regenerate successfully after injury is lost with advancing age, chronic kidney disease, and after irradiation. The factors responsible for this reduced regenerative capacity remain incompletely understood, with increasing interest in a potential role for cellular senescence in determining outcomes after injury. Here, we demonstrated correlations between senescent cell load and functional loss in human aging and chronic kidney diseases including radiation nephropathy. We dissected the causative role of senescence in the augmented fibrosis occurring after injury in aged and irradiated murine kidneys. In vitro studies on human proximal tubular epithelial cells and in vivo mouse studies demonstrated that senescent renal epithelial cells produced multiple components of the senescence-associated secretory phenotype including transforming growth factor β 1, induced fibrosis, and inhibited tubular proliferative capacity after injury. Treatment of aged and irradiated mice with the B cell lymphoma 2/w/xL inhibitor ABT-263 reduced senescent cell numbers and restored a regenerative phenotype in the kidneys with increased tubular proliferation, improved function, and reduced fibrosis after subsequent ischemia-reperfusion injury. Senescent cells are key determinants of renal regenerative capacity in mice and represent emerging treatment targets to protect aging and vulnerable kidneys in man.

INTRODUCTION

Hundreds of millions of people worldwide live with chronic kidney disease (CKD), a condition characterized by progressive kidney fibrosis, renal functional decline, and premature cardiovascular death (1). CKD prevalence is increasing, and aging adults (>65 years old) are known to be at particular risk (2). Aging adults and patients with CKD are also more susceptible to acute kidney injury (AKI), a life-threatening complication that, itself, can accelerate progression of underlying renal fibrosis and loss of function (3). Therefore, incompletely understood factors in the aging and chronically injured kidney promote fibrosis and inhibit renal regeneration. Understanding these processes and modifying them to protect vulnerable aging and injured kidneys represents an unmet research and clinical need.

Senescent cells (SCs) are permanently growth-arrested and phenotypically altered cells, which accumulate in vivo with aging, organ injury, and after genotoxic insults such as ionizing radiation. Senescence is associated with worsened damage and reduced regenerative capacity after tissue injury (4–6). Recent evidence implicated SC accumulation as contributing to aging and age-related diseases in fast-aging and naturally aged mice, with depletion strategies extending healthy life span (7, 8). SCs have also been reported to be present in increased numbers in human and rodent kidneys with aging, CKD, and after transplantation (9–12). Whether SCs actively contribute to the increased fibrosis and reduced regenerative capacity seen in aged and injured kidneys subject to further injury is a topic of major clinical and research interest (13).

SCs are attractive therapeutic targets because they remain metabolically active with constitutive nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) activation driving expression of the senescence-associated secretory phenotype (SASP) with release of cytokines [interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), chemokines [chemokine (C-C motif) ligand CCL2, chemokine (C-X-C motif) ligand CXCL8], and profibrotic transforming growth factor beta- β (TGF- β) (14). ABT-263, a potent B cell lymphoma (Bcl)2/w/xL inhibitor, effectively depletes SCs when administered orally to irradiated or normally aged mice (15). Depletion reduces irradiation-induced premature hematopoietic aging and rejuvenates aged tissue stem cells (15). The efficacy of ABT-263 treatment on kidney regeneration and fibrosis after injury in aged animals and animals with prematurely senescent kidneys [via total body irradiation (TBI)] remains untested.

In this study, we tested the hypothesis that senescent renal epithelia are key determinants of fibrosis and regenerative capacity after renal injury and can be therapeutically targeted. Senescent proximal tubular epithelial cells (PTECs) were identified in naturally aged mice, were also induced by radiation exposure in vitro and in vivo, and were depleted using ABT-263. By doing so, we demonstrate the importance of epithelial senescence in kidney fibrosis, damage response, function, and repair after injury, as well as the impact of their depletion on successful regeneration.

RESULTS

Markers of renal senescence are increased in patients with age and CKD

Using two separate human datasets of renal transcriptomic data available on the Nephroseq repository (16, 17), we determined expression of markers of permanent cell cycle arrest in healthy and diseased kidneys using *CDKN1A* (P21CIP1) and *CDKN2A* (P16INK4A) expression (Fig. 1A). Both *CDKN1A* and *CDKN2A* were increased in diseased kidneys versus healthy controls (Fig. 1B; *CDKN1A*, $P = 5.52 \times 10^{-9}$;

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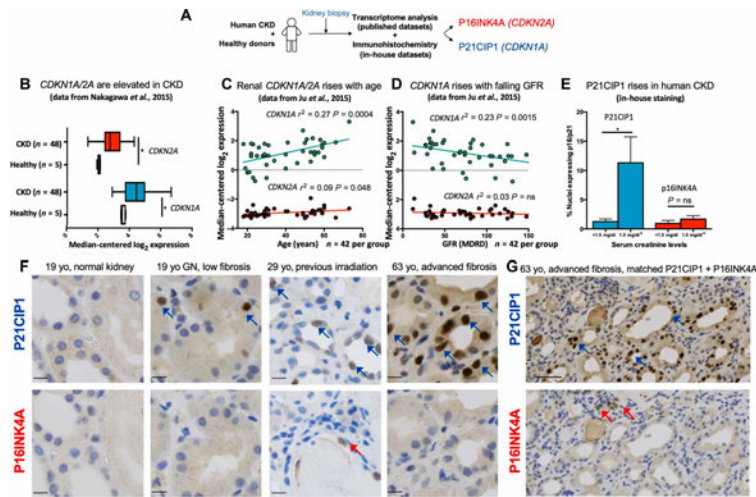


Fig. 1. Markers of cellular senescence increase at transcript and protein levels in human renal disease. Datasets published on Nephroseq were examined for human *CDKN1A* and *CDKN2A* transcript expression, and in-house human biopsy samples were stained for P16INK4A and P21CIP1 protein expression (A). Analysis of two published transcriptomic datasets shows that both *CDKN1A* and *CDKN2A* rise in kidneys with CKD. (B) $n = 48$ patients with CKD; $n = 5$ normal controls (17). In a separate cohort, *CDKN1A* and *CDKN2A* both increase in the kidney with increasing age, $n = 42$, (16) (C). *CDKN1A* alone correlated with advancing renal impairment, $n = 42$, (16) (D). GFR, glomerular filtration rate; MDRD, modification of diet in renal disease. Staining performed in a UKAS-certified histology facility demonstrated P16INK4A and P21CIP1 protein expression in patients with CKD ($n = 10$), (E) ($P < 0.05$; serum creatinine, ≥ 1.5 mg/dl versus < 1.5 mg/dl). Sample images are shown from normal kidneys, early CKD, young patients with radiation nephropathy, and from older patients with CKD (F). Scale bars, 10 μ m. Expression patterns differed between P21CIP1 and P16INK4A with P21CIP1 expressed predominantly in tubular epithelial nuclei, here shown in a patient with advanced renal fibrosis (G). ns, nonsignificant. * $P < 0.05$ by unpaired t test. yo, years old. Scale bar, 50 μ m.

CDKN2A, $P = 1.2 \times 10^{-8}$; based on median centered log₂ expression) and rose progressively with age, with *CDKN1A* expressed more highly (Fig. 1C; $P = 0.004$; *CDKN2A* $P = 0.048$). Only *CDKN1A* was significantly associated with falling kidney function in CKD (measured by estimated renal glomerular filtration rate, eGFR) (Fig. 1D; $P = 0.0015$), validating *CDKN1A* as a marker of both age- and injury-related renal senescence. Multivariate regression demonstrated that age and eGFR were independently associated with changes in *CDKN1A*, whereas the univariable models' associations of age with *CDKN2A* were lost in the multivariable model (table S1). The transcriptome analysis of kidney biopsies in patients with human CKD and healthy donors also identified up-regulation in multiple transcripts of members of the SASP, fibrosis, and BCL family members (fig. S1).

Immunohistochemistry (IHC) staining of a local Edinburgh dataset performed in our United Kingdom Accreditation Service (UKAS)-certified histology laboratory demonstrated that nuclear P21CIP1 staining was induced in diseased kidneys versus healthy controls (Fig. 1E; $P < 0.05$). IHC for P16INK4A and P21CIP1 demonstrated minimal staining in the young healthy kidney but increased P21CIP1 expression was detected in diseased [early glomerulonephritis, radiation nephropathy, and in advanced renal fibrosis (Fig. 1F)]. In each case, P21CIP1 staining appeared confined to tubular epithelia, in contrast to P16INK4A that was expressed at much lower concentrations in epithelial and interstitial cells (Fig. 1, G and H).

ABT-263 specifically targets senescent human renal PTECs

Human renal PTECs (RPTECs) exposed in vitro to 10 gray (Gy) γ -radiation developed reduced total cell number ($P < 0.05$) and increased cell size ($P < 0.05$) compared to nonirradiated controls (Fig. 2, A to C), consistent with induction of senescence. Quantitative polymerase chain reaction (qPCR) analysis at day 7 after irradiation revealed significantly decreased proliferation as measured by *MKI67* (*Ki67*) expression ($P < 0.005$); loss of *LMNB1* (Lamin B1; $P < 0.05$); increased cyclin-dependent kinase inhibitors *CDKN2A* ($P < 0.05$) and *CDKN1A* (P21CIP1; $P < 0.005$); and increases in SASP markers *IL1B* ($P < 0.05$), *TNF* ($P < 0.05$), and *TGFBI* ($P < 0.0005$), *MMP1* (matrix metalloproteinase 1), and neutrophil chemotactic factor, *CXCL8* (Fig. 2D). Because ABT-263 targets BCL-2 family members, *BCL2* (BCL-2), *BCL2L1* (BCL-xL), and *BCL2L2* (BCL-w) were measured and all demonstrated increased mRNA expression in irradiated/senescent RPTECs versus control (Fig. 2D; *BCL2*, $P < 0.01$; *BCL2L1*, $P < 0.01$; and *BCL2L2*, $P < 0.01$). Irradiated RPTECs also increased senescence-associated (SA)- β -galactosidase expression (Fig. 2E, $P < 0.001$). Collectively, these findings indicated that 10-Gy irradiation induces senescence and its associated secretory phenotype in human RPTECs (hRPTECs).

We then tested the specificity of ABT-263 to induce cell death in human senescent RPTECs. ABT-263 selectively killed SCs in a dose-dependent manner, with higher concentrations of ABT-263 (1 and 5 μ M), reducing viable SCs to 2 and 0% (relative to vehicle-treated

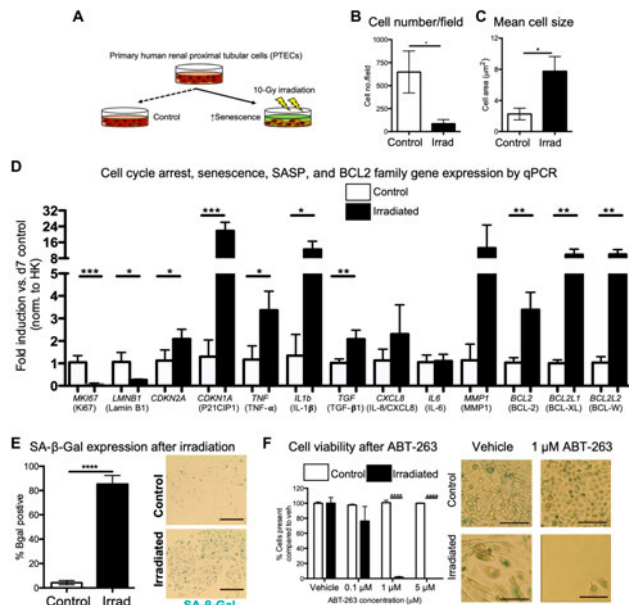


Fig. 2. Senescent human tubular epithelial cells demonstrate selective sensitivity to ABT-263 in vitro. Senescence was induced in human PTECs via 10-Gy γ -irradiation (A). Irradiation resulted in reduced cell number per field (B) and increased average cell size (C). qPCR analysis of markers of senescence, SASP factors, and BCL-2 family members including *MKI67*, *LMNB1*, *CDKN2A*, *CDKN1A*, *TNF*, *IL18*, *TGF β 1*, *BCL2*, *BCL2L1*, and *BCL2L2* in irradiated cells (D). SA- β -galactosidase (SA- β -Gal) staining in irradiated cells (E). Scale bars, 100 μ M. PTECs (control and irradiated) were treated with ABT-263 at various concentrations (0.1, 1, and 5 μ M), and cells per field were counted and analyzed compared to vehicle controls (F). Representative micrographs are shown in the bottom panel. Scale bars, 40 μ M. * P < 0.05, ** P < 0.01, *** P < 0.005, and **** P < 0.001. n = 4 per group. Unpaired t tests used for experimental comparisons in all groups except (F): two-way ANOVA.

SCs) (Fig. 2F). There was no toxicity to nonirradiated cells at even the highest concentrations of ABT-263 (Fig. 2F), which was unexpected because previous studies had found some toxicity at higher concentrations in nonsenescent human renal epithelial cells (15).

Aged mice have higher expression of SASP factors and worsened fibrotic responses after IRI

Baseline expression senescence/fibrosis in kidneys of old and young mice were measured (Fig. 3A). qPCR analysis revealed up-regulation of senescence-associated *Cdkn1a* (*P21CIP1*, P < 0.05), *Cdkn2a* (P < 0.005), and SASP genes (*Il6*, P < 0.05; *Tnf*, P < 0.01; and *Tgfb1*, P < 0.05) in old compared with young kidneys (Fig. 3B). Fibrosis, as measured by collagen I immunofluorescence (IF) was higher in old kidneys (P < 0.01; Fig. 3C), with an equivalent rise in total collagen seen on picrosirius red (PSR) staining (P < 0.05; Fig. 3D). γ H2A.X staining showed evidence of increased DNA damage and DNA damage response (DDR)

in the outer medulla and cortex of old kidneys compared to the young kidneys (Fig. 3E). Lamin B1 antibody IF was performed, confirming that Lamin B1 was lost in kidney tubular nuclei with age (Fig. 3F). To assess the impact of age on renal regeneration after injury, the outcome of unilateral ischemia reperfusion injury (IRI) was tested in old and young mice (Fig. 3G), demonstrating that old mice develop greater fibrosis after identical durations of injury (P < 0.005; Fig. 3H), with IRI resulting in an increase in expression of senescence-associated *Cdkn1a* by qPCR (P < 0.01; Fig. 3I).

Clearance of SC in aged mice protects the kidney after AKI

To test whether preexisting SCs present at increased numbers in old animals promote fibrosis and inhibit regeneration after subsequent AKI, old female mice were treated with ABT-263 to deplete SCs before unilateral IRI surgery (Fig. 4A). Five weeks after, IRI kidneys were harvested and analyzed. Senolytic pretreatment significantly reduced the numbers of SCs present in the uninjured injury (P < 0.05; fig. S2A) and reduced post-IRI fibrosis (P < 0.01; fig. S2, B and C).

Key markers of senescence, SASP release, and *Bcl2* family transcripts were measured by qPCR, compared to old naïve kidneys (with naïve kidney expression shown by the red line, Fig. 4B and fig. S2D). These demonstrated the induction of multiple senescence-associated transcripts, with *Cdkn1a* expression reduced by ABT-263 treatment (Fig. 4B) and *Bcl2l2* (*BCL-W*; P < 0.05) also showing reduction. Posts ischemic kidney weight (versus the contralateral kidney from the same mouse; Fig. 4C) was increased in senolytic-treated compared to control-treated animals, although this remained below healthy weight (shown by red line). Given that IRI leads to tubular loss (18), this implicated SCs in impeding tubular recovery and survival after injury, with senolytic treatment potentially restoring regenerative capacity by increasing proliferative capacity in remaining renal tubules (19). To dissect these processes, further experiments were undertaken.

Kidney injury molecule 1 (*KIM-1/Havcr1*) is up-regulated acutely in damaged proximal tubules in humans (20), and chronic expression in CKD predicts likelihood of progression to end-stage renal disease in man (21). We quantified KIM-1 protein 5 weeks after IRI as a marker of ongoing tubular injury and progressive fibrosis in diseased kidneys. KIM-1 was reduced in ABT-263-treated IRI versus vehicle-treated IRI kidneys (P < 0.01; Fig. 4D), illustrating that senescent tubular cells drive chronic KIM-1 release in diseased kidneys, and their depletion in vivo was safe and opposed ongoing injury.

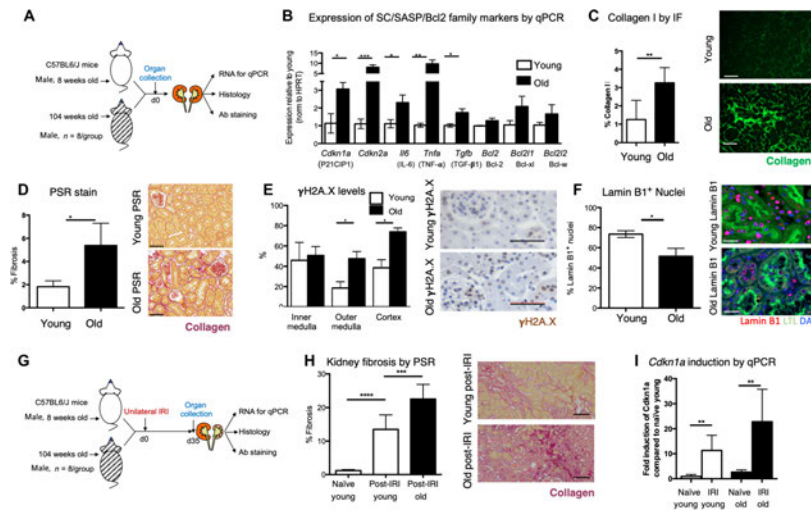


Fig. 3. Aged murine kidneys display more senescence, DDR, and fibrosis than young kidneys and are more susceptible to post-IRI fibrosis. Experimental schema of sample collections from young and old mice (A). Ab, antibody. Whole kidney qPCR on samples from young (6 to 10 weeks) and old mice (>18 months) measuring senescence and SASP markers (B). Markers of tissue fibrosis and senescence-associated DNA damage were quantified using IF for collagen I (C), PSR (D), and yH2A.X IHC (E). Representative micrographs are shown in right panels. Scale bars, 50 μ m. The percentage of Lamin B1⁺ (red) nuclei (DAPI, blue) in kidney tubules (LTL, green) of young versus old kidneys (F) ($n = 3$ to 4 groups). Representative micrographs are shown in right panels. Scale bars, 20 μ m. Old versus young IRI kidneys analyzed for collagen deposition (PSR) (G and H) (comparisons made using one-way ANOVA test with Tukey posttest used). Representative micrographs are shown in the right panel. Scale bars, 100 μ m. Quantitative PCR of *Cdkn1a* in IRI kidneys from old and young mice compared to naïve kidneys illustrates marked up-regulation in both IRI groups (I). All groups are $n = 4$ to 8 with unpaired t tests used for experimental comparisons unless otherwise stated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, and **** $P < 0.001$.

Hematoxylin and eosin (H&E) staining revealed improved renal architecture in ABT-263 versus vehicle-treated mice (fig. S2E).

Kidney sections were then stained with *Lotus tetragonolobus* lectin (LTL), a marker of differentiated renal tubules (22) and the proliferation marker Ki67 (Fig. 4, E and F). IRI induces loss of tubules (18). Proliferating Ki67⁺ LTL⁺ tubules were counted (Fig. 4F, white arrows in Fig. 4E). ABT-263-pretreated old IRI kidneys exhibited significantly increased tubular proliferation compared to control ($P < 0.01$; Fig. 4F). Thus, senescence inhibits epithelial proliferation in response to injury. Because TGF- β 1 is a recognized driver of renal fibrosis after IRI (23–25) and is produced by SC (26, 27), this protein was measured in the kidney. Senolytic treatment reduced TGF- β 1 in diseased kidneys ($P < 0.05$; Fig. 4G), consistent with a role of senescent epithelia in controlling renal TGF- β 1 through direct production $\pm$ recruitment of TGF- β 1-producing cells. TGF- β 1 is thought to drive renal fibrosis in part through mediating macrophage infiltration and switching to a profibrotic CD206⁺ phenotype (25, 28). ABT-263 treatment reduced the overall number of F480⁺ macrophages ($P < 0.05$; fig. S2, G and H) and percentage of F480⁺/CD206⁺ macrophages in the post-IRI diseased kidney ($P < 0.01$; fig. S2I). Therefore, senescence affects macrophage phenotype in post-IRI kidney disease, increasing profibrotic CD206⁺ macrophages. Whereas SA- β -galactosidase was not reduced (fig. S2J), a marked reduction

in yH2A.X foci in the cortex ($P < 0.05$; fig. S2K) and increase in Lamin B1-positive nuclei ($P < 0.01$; fig. S2L) were both noted with ABT-263 treatment.

To establish whether the improvement in multiple markers of senescence and fibrosis translated into enhanced renal functional recovery after IRI, further experiments used a bilateral IRI injury to generate a model of functional kidney injury and impairment (Fig. 5A). Aged mice treated with ABT-263 before bilateral IRI had significantly improved renal function measured by serum cystatin C ($P < 0.01$ at both days 7 and 14; Fig. 5B) and reduced numbers of p21CIP1⁺ SCs before injury ($P < 0.05$; Fig. 5C). This functional protection was accompanied by reduced renal fibrosis by both PSR staining ($P < 0.01$; Fig. 5, D and E) and collagen I analysis by IF ($P < 0.01$; Fig. 5, F and G). Hence, SC depletion improves the subsequent structural and functional regenerative capacity of the naturally aged kidney.

TBI induces cellular senescence and fibrosis in the murine kidney in vivo

To distinguish the specific effects of senescence from those of the general aging phenotype, γ -irradiation was used to induce senescence in the young murine kidney (15). Young C57BL/6 mice underwent 7-Gy TBI with tissue harvest 8 and 16 weeks later (Fig. 6A), with imaging of whole kidneys to determine patterns of DNA damage by

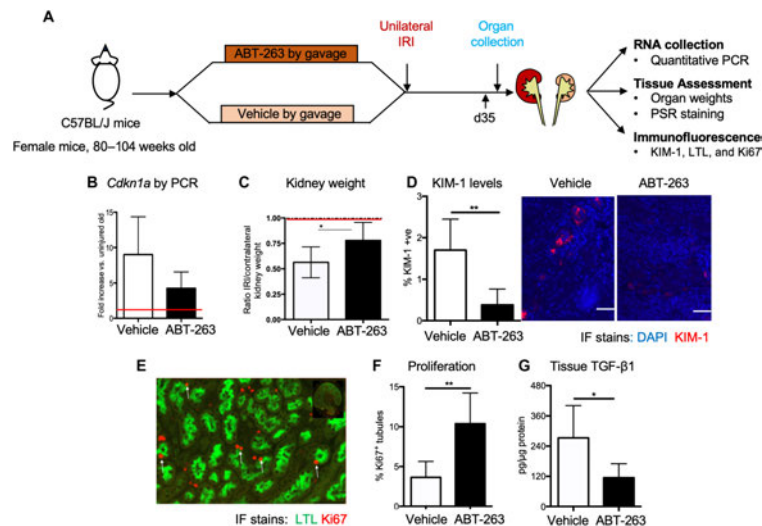


Fig. 4. ABT-263 treatment reduces fibrosis and increases the regenerative capacity of aged murine kidneys after IRI. Diagram detailing ABT-263 treatment regime and unilateral IRI surgery in old female mice (A). qPCR analysis of *Cdkn1a* expression in vehicle and ABT-263-treated mice (B) (red line, expression in old age-matched control tissue). The weight ratios of IRI/CLK kidneys were also calculated in ABT-263-treated and vehicle-treated mice (C) (dashed line represents left/right (l/r) kidney ratio in healthy animal). Kidney injury molecule 1 (KIM-1/*Havcr1*) IF analysis of post-IRI kidneys $\pm$ ABT-263 (D). Representative micrographs are shown. Scale bars, 50 μ m. Kidney sections were costained for proximal tubule marker LTL (green) and the proliferation marker Ki67 (red). Representative micrograph (E) shows the Ki67⁺ nuclei in LTL⁺ tubules (white arrows). Scale bar, 50 μ m. The % LTL⁺ tubules positive for Ki67 was calculated in both groups (F). TGF- β protein abundance was measured in whole IRI kidney lysates by ELISA (G) ($n = 4$ to 5 per group).

γ H2A.X staining (Fig. 6, B and C). qPCR analysis of kidneys showed that the senescence marker *Cdkn1a* (P21CIP1) increased over time after TBI, reaching significance by 16 weeks after TBI ($P < 0.05$; Fig. 6D) with a similar pattern seen for *Cdkn2a*. Analysis of markers of the SASP, fibrosis, and *Bcl2* family (measured by qPCR; Fig. 6E) and the senescence marker SA- β -galactosidase (Fig. 6F) all had higher means at week 16 after irradiation but with a high biological variation.

IF staining demonstrated increased P16INK4A protein expression by 16 weeks after TBI ($P < 0.01$; Fig. 6G). In line with previous observations in the lung after TBI (15), senescence in the kidneys rose modestly by 8 weeks after TBI, reaching significance by 16 weeks, a finding compatible with ongoing SASP activation, driving progressive late senescence distinct from the initial genotoxic insult. Consistent with these findings, TBI drove initiation of the DDR in the diseased kidney, with maximal increases seen in the cortex, mirroring findings in aged kidneys (Fig. 6H). Collagen I increased at 16 weeks after TBI ($P < 0.01$; Fig. 6I) with a similar pattern seen on PSR staining (Fig. 6J).

To test the sensitivity of irradiation-induced SCs to senolytic therapy, mice were treated with ABT-263 before kidney harvest at 20 and 25 weeks after TBI (Fig. S3A). *Cdkn1a* (P21CIP1) mRNA was

significantly decreased with senolytic treatment as measured by qPCR ($P < 0.05$; Fig. S3B). Although no change in *Cdkn2a* transcript levels was detected, P16INK4A protein abundance also fell after ABT-263 treatment (Fig. S3, C and D). *Lmnbl* was found to increase in the post-TBI kidney in ABT-263-treated mice (Fig. S3E) at 20 weeks after TBI. ABT-263 reduced evidence of the DDR in the irradiated kidney, with a reduction in positive γ H2A.X positive nuclei in the cortex of treated kidneys ($P < 0.05$; Fig. S3F). Fibrosis as measured by PSR staining for collagen did not differ between groups, indicating that ABT-263 had no detectable impact on established fibrosis (Fig. S3G).

Irradiation worsens fibrotic responses to subsequent renal injury, which is prevented by ABT-263 pretreatment

To dissect the effect of senescence on subsequent injury responses from generic aging effects, we compared post-IRI kidney disease outcomes in young irradiated mice with increased numbers of SCs ($\pm$ ABT-263, Fig. 7A). PSR staining for collagen showed that previous TBI increased fibrosis in post-IRI kidney disease, which was prevented with senolytic treatment (Fig. S4, A and B). qPCR analysis of the profibrotic factors *Colla1*, *Col3a1*, *Fln* (Fibronectin 1), and *Acta2* (α -SMA) demonstrated that post-IRI fibrosis/matrix deposition

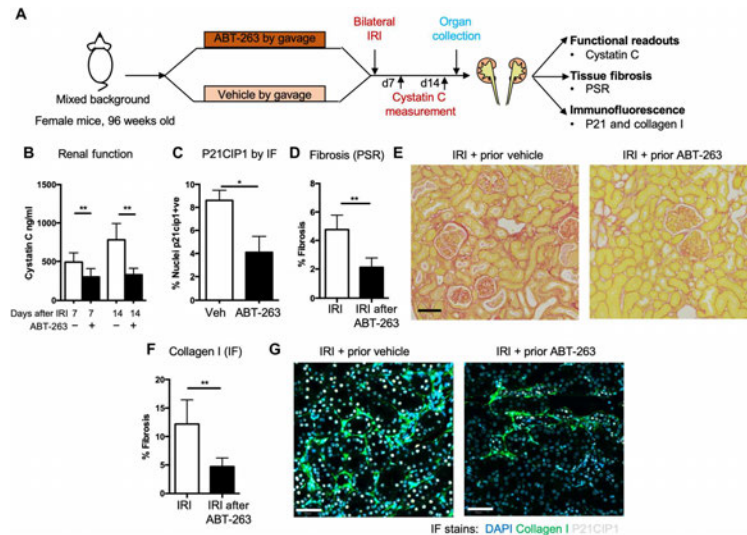


Fig. 5. ABT-263 treatment improves kidney function in the bilateral IRI model of renal injury. Diagram detailing ABT-263 treatment regime and bilateral IRI surgery in old female mice (A). Serum cystatin C is shown at days 7 and 14 after IRI in ABT-263-treated mice (B) ($n = 7$ to 8 per group, Mann-Whitney test). ABT-263 treatment effect on p21CIP1⁺ senescent cells in baseline kidneys (C) (Mann-Whitney test). PSR staining after IRI to evaluate fibrosis in ABT-263-treated animals (D) (Mann-Whitney test, $n = 4$ to 7 per group). (E) Representative micrographs are shown. Scale bar, 50 μ M. Collagen I staining by IF after IRI in ABT-263-treated animals compared to controls (F) (Mann-Whitney test, $n = 4$ to 7 per group). (G) Representative micrographs are shown. Scale bars, 50 μ M. All $n = 4$ to 6 per group unless stated, unpaired t tests used for all comparisons unless stated otherwise. * $P < 0.05$ and ** $P < 0.01$.

was exacerbated by prior TBI, with ABT-263 pretreatment providing protection (fig. S4, C to F). IRI induced renal *Cdkn1a* (P21CIP1) and *Cdkn2a* expression in age-matched control mice (in the young range, ~30 weeks old), showing that senescence is induced in young kidneys after IRI, in agreement with published reports (6, 29). However, these were higher in preirradiated mice ($P < 0.05$) unless pretreated by ABT-263 ($P < 0.01$) (Fig. 7B and fig. S4G). A similar pattern was found in P16INK4A protein abundance (IF; fig. S4H).

SA- β -galactosidase quantification in diseased kidneys after IRI confirmed that senescence was further increased by previous TBI and was reversed with ABT-263 (fig. S4I). qPCR showed that SASP markers (*Il6*, *Tnf*, and *Tgfb1*) also increased in post-IRI kidney disease, were higher with irradiation, and reduced with senolytic treatment (fig. S4, J to L). *Bcl2* was also reduced by ABT-263 treatment in irradiated animals (fig. S4M). There were significantly more Lamin B1-positive tubular nuclei in the kidneys of irradiated mice pretreated with the senolytic before IRI ($P < 0.01$; fig. S4N). γ H2A.X quantification after IRI confirmed that DNA damage and DDR were further increased by previous TBI and were reversed with ABT-263, with maximal changes seen in the renal cortex (fig. S4, O and P).

Post-IRI kidney disease decreases kidney mass because of destruction of tubules without appropriate repair. By weighing the post-IRI and contralateral kidneys, we demonstrated that IRI reduces kidney

weight, with prior irradiation causing greater weight loss. ABT-263 pretreatment led to significantly less weight loss, reflecting improved tubular regenerative capacity ($P < 0.01$; Fig. 7C; dashed line represents healthy weight ratio). We also examined ongoing renal damage by analyzing chronic KIM-1 expression in the diseased kidney after IRI. KIM-1 was elevated after irradiation and lowered by ABT-263 administration ($P < 0.01$; Fig. 7D).

LTL⁺ tubules were costained with Ki67 to measure proliferation (Fig. 7E). ABT-263 treatment increased numbers of healthy LTL⁺ tubules present after IRI (Fig. 7F). Many LTL cells had Ki67⁺/DAPI⁺ (4',6-diamidino-2-phenylindole-positive) nuclei in post-IRI kidney disease, representing proliferating interstitial cells (Fig. 7E, red arrow). The number of Ki67⁺ nuclei associated with LTL⁺ tubules were counted (Fig. 7E, yellow arrows). ABT-263 administration significantly increased Ki67⁺ tubules ($P < 0.05$; Fig. 7G), indicating that senescence impairs tubular regeneration after injury, with senolytic treatment restoring regenerative capacity to previously irradiated kidneys. Dual staining with Ki67 and p21CIP1 demonstrated that these two markers were mutually exclusive, confirming that Ki67 did not reflect the amount of senescence (fig. S4Q).

TGF- β 1 protein, as measured in injured kidney lysates, was highest after irradiation and was reduced with senolytic treatment ($P < 0.05$; Fig. 7H), in agreement with reductions observed in transcript expression

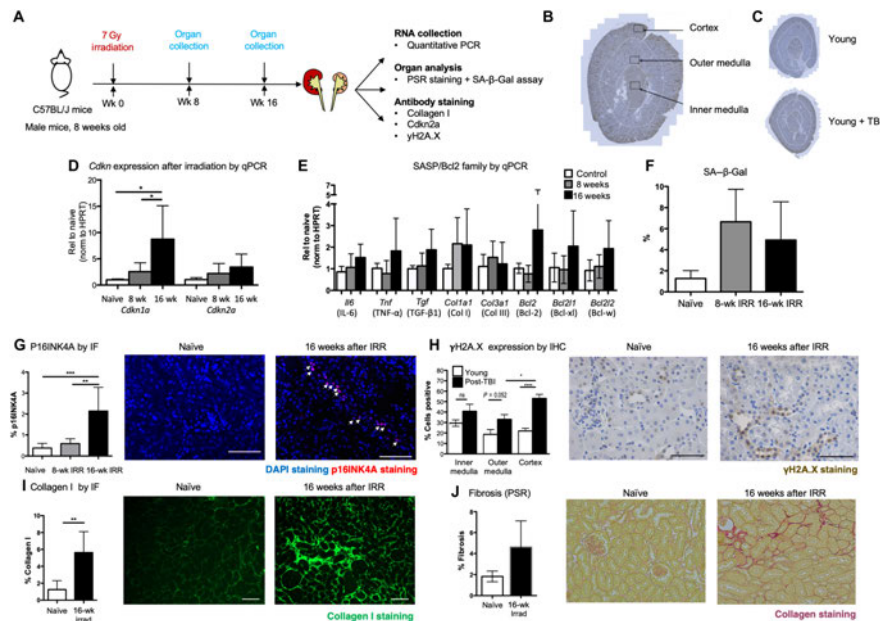


Fig. 6. TBI drives senescence and fibrosis in young murine kidneys. Diagram detailing irradiation of mice and analysis of kidneys 8 and 16 weeks after irradiation (A). IHC and whole kidney imaging were used to identify areas of γH2A.X induction (B and C). qPCR analysis of whole kidneys for *Cdkn1a* (P21CIP1) and *Cdkn2a* after irradiation (D) ($n = 3/6/7$ for age-matched control versus 8 versus 16 weeks after irradiation, comparisons by one-way ANOVA test with Tukey posttest). qPCR whole kidney for various SASP and Bcl-2 family members was carried out (E). SA-β-Gal staining and analysis of kidney sections after irradiation (F). P16INK4A IF on irradiated kidneys at 8 and 16 weeks after injury (G). Representative micrographs are shown. Scale bars, 100 μm. γH2A.X staining of the irradiated kidneys to evaluate DNA damage (H). Representative micrographs are shown. Scale bars, 50 μm. Collagen I IF of irradiated kidneys at 8 and 16 weeks after irradiation (I). Representative micrographs are shown. Scale bars, 100 μm. A similar pattern of PSR staining was seen in naive and post-irradiated kidneys (J). For (E) and (F), $n = 6$ to 8, comparisons by one-way ANOVA test with Tukey posttest. Unpaired t tests used for all comparisons unless otherwise stated. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$.

(fig. S4L) and in aged mice (Fig. 4G), indicating that SCs are a major determinant of renal TGF-β1. Because of the effect of TGF-β1 on macrophages after IRI, we next investigated macrophage numbers and phenotype. F4/80 IHC and *Cd68* qPCR analysis (fig. S5, A to C) confirmed an increase in macrophage influx after IRI post-TBI, compared to nonirradiated kidneys. Senolytic treatment reduced CD206⁺ profibrotic macrophages in the IRI kidney of TBI mice ($P < 0.05$; fig. S5D).

Last, we undertook bilateral IRI studies to establish whether SC depletion restored functional regeneration in the young, irradiated kidney (Fig. 8A). These studies demonstrated that irradiated mice treated with ABT-263 before IRI had improved renal function measured by serum cystatin C ($P < 0.01$ both for days 7 and 35; Fig. 8B) and reduced numbers of p21CIP1⁺ SCs after injury ($P < 0.01$; Fig. 8C). This functional protection was accompanied by reduced renal fibrosis by both PSR staining (Fig. 8, D and E) and collagen I analysis

by IF (Fig. 8, F and G). Dual staining of P16INK4A with collagen I and p21CIP1 with collagen I demonstrated that markers of both arms of the senescence induction pathway correlated with collagen deposition in the immediate surrounding tissue of the kidney (Fig. 8, H and I).

Age-matched young nonirradiated mice were included in all the groups above as additional controls. In all cases, ABT-263 therapy had no impact on these mice compared to vehicle treatment in any parameter studied (fig. S6, A to G); so, for simplicity, they have otherwise been omitted from the presented data. This finding illustrates that, in the absence of increased numbers of preinjury SCs, ABT-263 exerts no other protective effect on non-SCs such as epithelia, mesenchyme, or leukocytes.

The presence of SCs impaired structural and functional recovery after injury and promoted fibrosis in both naturally aged and young irradiated mice. Furthermore, preinjury depletion of SCs improved renal structural and functional regenerative capacity.

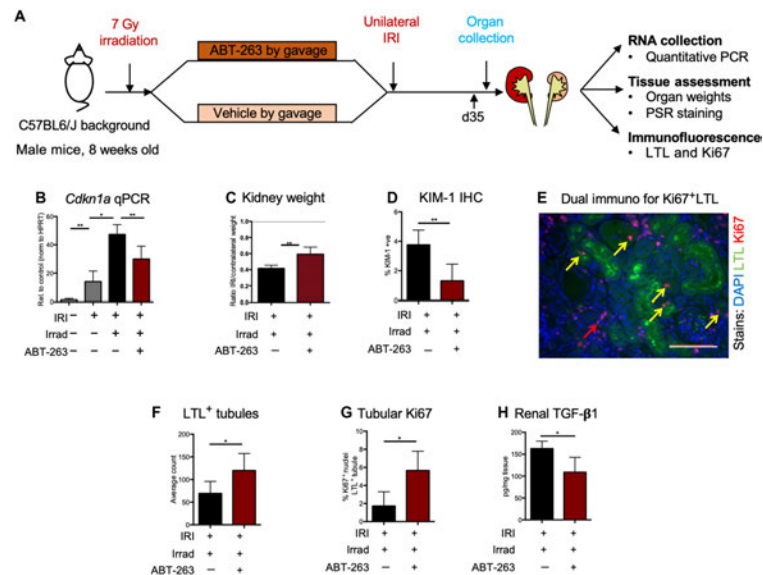


Fig. 7. Irradiation-induced epithelial senescence impairs post-injury renal regeneration and promotes fibrosis in murine kidneys, with ABT-263 pretreatment providing protection. Diagram detailing ABT-263 treatment regime and unilateral IRI surgery in irradiated mice (A). Whole kidney qPCR for *Cdkn1a* in pre-irradiated kidneys after IRI, compared to IRI alone (B); ABT-263 treatment effects on postischemic kidney weight (C) (dashed line represents I/r kidney ratio in healthy animal); and tubular injury marker KIM-1 by IHC (D). Dual IF staining was performed for Ki67 and the tubular marker LTL (E) (yellow arrow, Ki67⁺LTL⁺; red arrow, Ki67⁺LTL⁻). Scale bar, 100 μm. Quantification of LTL⁺ tubules (F), tubular proliferative capacity (G), and renal TGF-β1 protein (H).

DISCUSSION

Aged patients are known to be at elevated risk of AKI and subsequent progressive CKD (3). Several studies have shown correlations between increased senescence within the kidney and progression of CKD or with adverse outcomes after transplantation (9, 30–34). Whether SCs themselves directly influence impaired repair and drive fibrosis after subsequent injury remains a key unanswered question. Determining the safety and efficacy of SC depletion before renal injury would clarify the contribution of SCs to repair and fibrosis and would be of translational importance for aged patients at risk of AKI and CKD.

Whereas aged patients are most susceptible to AKI and CKD, murine models of AKI and CKD are typically undertaken in young healthy animals. In this study, we tested the impact of senescence on renal regenerative capacity by using both naturally aged and young irradiated mice, demonstrating that cellular senescence increased fibrosis and inhibited regeneration after AKI. Pretreatment with the senolytic ABT-263 reduced SC number, augmented epithelial proliferation, and reduced deposition of fibrosis in the aftermath of renal injury in both aged and young irradiated mice, similar to healthy young mice. These findings are consistent with and extend previous interventions with senolytics on the uninjured kidney (7, 8). On the

basis of these data and supporting evidence from the literature that senescent epithelial cell growth arrest suppresses regenerative capacity (35, 36), we propose that SCs in the kidney represent a key determinant of renal regenerative capacity in vivo. These findings mirror those recently reported in other organ systems including the lung and liver (37, 38). SASP factor release has been shown to activate myofibroblasts to secrete matrix with TGF-β1, a prime candidate as a known fibroblast activator and mitogen (39). In this study, TGF-β1 decreased after IRI (RNA and protein) with senolytic pretreatment, implicating senescence as a major regulator of TGF-β1 expression and release.

These data support the hypothesis that chronically senescent cells are deleterious to longer-term renal homeostasis and function. In the models of aging and injury studied, SC depletion preinjury improved subsequent renal regenerative capacity and reduced fibrosis. Further studies would be indicated including assessment of long-term pharmacological treatment of senescence on kidney structure and function and trials of clinically licensed agents capable of suppressing SASP factor release (40). Given the reported importance of senescence in acute wound repair, it will be important to assess the optimal timing of senescent depletion, because the safety and efficacy of depletion in the aftermath of acute injuries such as renal transplantation and postsurgical AKI remain unknown.

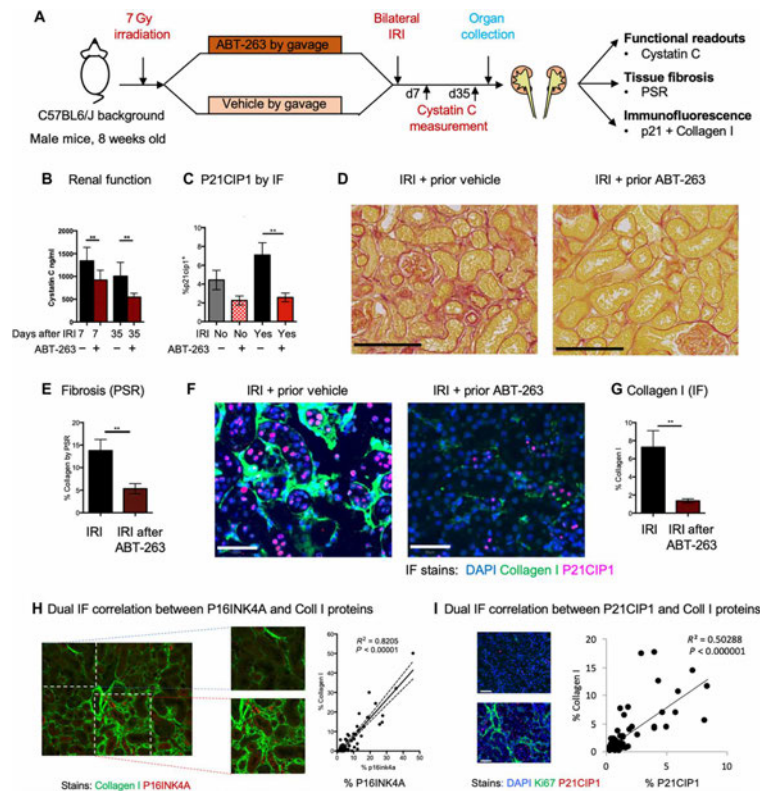


Fig. 8. Depletion of irradiation-induced SCs improves kidney function after subsequent bilateral IRI. Diagram detailing ABT-263 treatment regime and bilateral IRI surgery in irradiated mice (A). Serum cystatin C concentrations at days 7 and 35 after IRI in ABT-263-treated mice (B). ABT-263 treatment effects on p21CIP1⁺ SCs in pre-IRI and post-IRI kidneys (C). PSR staining after IRI shows effects on fibrosis in ABT-263-treated animals (D and E). Representative micrographs are shown. Scale bars, 100 μ m (D). Collagen I staining by IF after IRI shows effects on fibrosis in ABT-263-treated animals (F and G). Representative micrographs are shown. Scale bars, 50 μ m (F). Dual IF staining (P16INK4A, red; collagen I, green) was carried out in irradiated IRI kidneys and age-matched controls, and regression analysis undertaken to determine correlation between areas of positivity of both proteins (H). Further dual IF staining (P21CIP1, red; collagen, green) was carried out in irradiated IRI kidneys and age-matched controls, and regression analysis undertaken to determine correlation between areas of positivity of both proteins (I). Key: IRI, ischemia reperfusion injury; Irrad, total body irradiation. $n = 4$ to 8 per group. Unless otherwise stated, comparisons were made by unpaired t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, and **** $P < 0.001$.

ABT-263 has been found to be selectively toxic to SCs via its actions on Bcl2/w/xL (15), but prolonged dosing has been complicated by thrombocytopenia occurring as an on-target effect of Bcl-2 inhibition (41). The selectivity of ABT-263 for senescent human epithelia in vitro could facilitate its use in carefully selected patients, where the limited duration of dosing required for senolysis minimizes bleeding risk. In the work presented here, ABT-263 was safe and efficacious in SC depletion and preservation of renal regenerative capacity with

no gastrointestinal distress, toxicity, or bleeding problems detected. On the basis of the results reported here, approval has been granted for a preclinical study to quantify the efficacy and safety of ABT-263 administration to ex vivo human kidneys that have been rejected for transplantation in man. Should efficacy and safety in human kidneys be similar to the results in experimental animals, further studies should investigate treating kidneys before transplantation and high-risk patients with CKD before surgical procedures with high risks of AKI.

There are several limitations to this study, which should be addressed in future research. Whereas two distinct modes of SC generation (irradiation and natural aging) were studied, this does not encompass the full range of senescence-generating stimuli present in human aging and kidney disease. Our study focused on the role of SCs present before a further renal injury and did not address the therapeutic efficacy of post-injury SC depletion. Last, our study used ABT-263 as our senolytic agent for all *in vivo* work, and the efficacy of alternative senolytic drugs with different modes of action remains to be determined in both murine models of disease and in human patients. The findings presented here support further studies using alternative senolytic drugs, timings of administration, and modes of senescence induction to establish the breadth of potential benefits for SC depletion.

This work demonstrates cellular senescence to be a key determinant of renal regenerative capacity and a mediator of post-injury fibrosis and function. Furthermore, our data show that senolytic treatment may be efficacious as an intervention in populations with preexisting SCs including the elderly, those with CKD, and previously irradiated patients. With several agents, including ABT-263, entering clinical trials, there is the potential for senolysis to fill an unmet need for those with elevated senescence who are heightened risk of progressive fibrotic renal disease.

MATERIALS AND METHODS

Study design

This study addressed the hypothesis that SCs present in the aged and young, prematurely senescent kidney negatively affect the ability of the kidney to regenerate from injury and that their therapeutic depletion would therefore be protective. A series of controlled laboratory experiments were performed to determine the degree of fibrosis present in the kidney after injury with and without treatment with ABT-263 *in vivo*. All studies were conducted in accordance with ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) (42). On the basis of previous work, a two-sided two-sample test was used to generate group sizes for all studies. All studies were performed at the University of Edinburgh, UK, with data collection performed at prespecified end points unless limited by animal distress as identified by facility staff and/or veterinary surgeons. No available data were excluded from the analysis. Primary and secondary end points were prespecified. Each mouse represented one experimental unit. In all experiments, there was randomized allocation to groups. Drug administration and surgical timing were rigorously rotated across all groups. Tissue was collected by two staff members blinded to the treatment groups, using a coding system for each animal. Semi-blinded staining and image analysis were undertaken with unblinding only being performed after all data acquisition was complete.

Human biopsies

After appropriate ethical approval from the Tissue Governance at the Royal Infirmary of Edinburgh, sections were obtained from renal biopsy tissue from $n = 10$ renal biopsies, selected to encompass a range of renal function spanning from normal to stage IV CKD. Slides were cut and IHC performed for P16INK4A and P21CIP1. Staining was performed in the UKAS-certified laboratory of Edinburgh Royal Infirmary stained on Leica Bond III autostainers using Bond Refine detection kits [peroxidase–3,3'-diaminobenzidine (DAB) detection].

Antibodies to P16INK4A were produced by Cintech, supplied by Roche/Ventana, and used at 1:1 dilution. Antibodies to P21CIP1 were produced by Leica [code NCL-L-WAF1(P21)] and used at 1:20 dilution. Both underwent high-pH antigen retrieval for 20 min. Transcriptomic datasets were obtained from published and publicly available resources from 95 renal transcriptomes from eligible studies in Nephroseq (University of Michigan, Ann Arbor, MI) (16, 17).

Cell culture

hRPTEC [American Type Culture Collection (ATCC)] were maintained in Dulbecco's modified Eagle's medium–F-12 and GlutaMAX-1 supplemented with an human telomerase reverse transcriptase (hTERT) immortalized RPTEC growth kit (ATCC) and Geneticin (50 mg/ml) (Gibco-Life Technologies). Cells were grown at 37°C in 5% CO₂. hRPTEC cells were plated at 2×10^5 cells per well of a six-well plastic culture dish. After overnight culture, these cells were exposed to 10-Gy radiation. Cells were incubated for 7 days with media change every 3 to 4 days. For senolytic treatment with ABT-263, 100,000 PTECs were seeded onto a 12-well plate. The following day, they were exposed to 10-Gy γ radiation. For ABT-263 treatment, on day 5, 0.1, 1, and 5 μ M ABT-263 was added to the culture (replenished every 48 hours) until 12 days after irradiation when the cells were washed with phosphate-buffered saline (PBS). Cells were analyzed on day 14.

Random fields of view within the plate (at $\times 20$ magnification) were captured using light microscopy on an Evos microscope. Scaling was performed using a graticule. Lines were drawn around five random cells per well, with area calculated using ImageJ (National Institutes of Health, USA) (43). For each well, multiple random fields of view were captured for analysis and the results averaged before cell lysis in TRIzol for RNA extraction.

SA- β -galactosidase staining

Staining was performed as per the manufacturer's instructions (Cell Signaling Technology, London, UK). Briefly, samples were fixed using reagents provided before staining (pH 6), in low-CO₂ incubators. RPTECs were imaged after 3 hours of incubation. For kidney, frozen sections were air-dried before staining for 12 hours. Nuclear fast red was used to counterstain slides, before dehydration and mounting. To analyze RPTEC staining, total cells per field were counted and then SA- β -galactosidase⁺ cells (blue) were counted, and % SA- β -galactosidase⁺ was calculated. For kidney sections, fields of view at $\times 10$ magnification were captured. Percentage positivity (areas of blue) was calculated over multiple random fields of view using ImageJ imaging software.

Experimental mice

All mice were on either a C57BL/6 or a mixed genetic background. All experiments comprised male mice, unless otherwise stated. Ages ranged from 6 to 8 weeks for young and 18 to 24 months for old. Mice were bred and maintained at the University of Edinburgh or purchased from Harlan, UK. Paraffin-embedded samples of kidney tissue from P16INK4A wild-type (WT) and P16INK4A knockout mice were sourced from our collaborators P. Krimpenfort at the Netherlands Cancer Institute and A. Melk and R. Schmitt at Hannover Medical School. All animal work was compliant with Institutional Animal Care and Use Committee guidelines, conducted in accordance with the U.K. Government Animals (Scientific Procedures) Act 1986, in conventional barrier unit facilities with conventional bedding, 12:12-hour light:dark cycle, ambient temperature control and access to food and water *ad libitum*, and was approved by the University of Edinburgh Ethical Review Committee.

ABT-263 senolytic treatment

ABT-263 (Chemgood, VA, USA) was prepared in 10% ethanol, 30% polyethylene glycol 400, and 60% Phosal 50 PG. ABT-263 was administered by gavage (50 mg/kg per day) daily for 2 cycles, each of 7 days.

Mice that had undergone TBI were treated with ABT-263 or vehicle at 15 and 18 weeks after TBI, or at an equivalent age for age-matched controls. At week 20, mice underwent unilateral IRI surgery or were culled for tissue analysis. A subset of mice that had undergone TBI were treated with ABT-263 or vehicle at 15, 18, and 35 weeks after TBI. ABT-263 was administered by gavage (50 mg/kg per day) daily for 3 cycles, each of 7 days. At week 37, mice underwent bilateral IRI for kidney function measurement.

Ischemia reperfusion injury

IRI surgery was performed as previously described (44). Anesthesia was induced with 2% isoflurane. Buprenorphine analgesia was administered subcutaneously. A midline laparotomy was performed, and the left renal pedicle was identified and clipped using an atraumatic clamp for 15 min. During the ischemic period, body temperature was maintained using a heating blanket with homeostatic control (Harvard Apparatus) via a rectal temperature probe. After clamp removal, peritoneal closure with 5/0 suture and skin closure with clips were performed before subcutaneous administration of 1 ml of 0.9% saline. The right kidney was uninjured in some surgeries (unilateral IRI) and is referred to as the contralateral kidney. In later experiments, both kidneys were clamped (bilateral IRI). Animals were maintained for 2 to 5 weeks after IRI before tissue harvest. Kidneys were weighed at the time of sacrifice. Tissue was fixed in methacarn (methanol 60%, chloroform 30%, and glacial acetic acid 10%) or snap frozen in liquid nitrogen/dry ice. In later experiments, tissue was also fixed in 4% paraformaldehyde, as this was found to be optimal for p21CIP1 antibody staining, allowing us to carry out IHC for this extra marker, which was not possible in earlier experiments.

Murine irradiation

Young mice were exposed to a sublethal dose (7 Gy) of TBI using a closed cesium-137 γ source. Age-matched control mice were not irradiated but housed in the same facility and conditions. A subset of irradiated mice were culled 8, 16, 20, and 25 weeks after exposure for tissue analysis of radiation-induced renal senescence.

Immunohistochemistry**Picrisirius red**

For PSR staining, sections were deparaffinized and rehydrated before treatment in hematoxylin for 8 min. After washing, these were stained in PSR for 1 hour, before acidified water washing, dehydration, and mounting.

Hematoxylin & eosin

Sections were deparaffinized and rehydrated before staining with H&E, before acidified water washing, dehydration, and mounting.

DAB staining

Tissue was fixed in methacarn overnight, changed to 70% ethanol, and processed. Briefly, paraffin-embedded tissue sections (5 μ M) were deparaffinized and rehydrated. Antigen unmasking was undertaken (see table S2). Endogenous peroxidase was quenched with 3% H₂O₂ (Sigma-Aldrich) for 15 min, before avidin/biotin (Vector Laboratories) and protein blocking (Dako) for 10 min each. Slides were then incubated with antibody overnight at 4°C. Species-appropriate

biotinylated secondary antibodies (1:200) were added and staining developed using the Vector Laboratories ABC kit and DAB.

To investigate the distribution of DNA damage/senescence in young, aged, and irradiated kidneys, before and after IRI, γ H2A.X IHC was carried out. Images of whole kidneys were collected as .CZI files on the Zeiss Axioscan. This allowed visualization of full kidney sections using QuPath (45). Sections were divided into inner medulla, outer medulla, and cortex, and areas within these were analyzed for % γ H2A.X positivity.

Immunofluorescence

For IF staining, paraffin-embedded tissue sections were deparaffinized and rehydrated. Antigen unmasking was undertaken (see table S2). Slides were incubated with protein block for 30 min before addition of the primary antibodies overnight at 4°C. Secondary antibodies are specified in table S2 (1:200, 1:500, or 1:1000 dilution). The slides were mounted with Vectashield with DAPI (Vector Laboratories).

Kidney sections were visualized and captured using the Zeiss Axioskop 2mot+ or on an Axioscan slide scanner. Percentage area of positivity was calculated in ImageJ or in QuPath (45). In light of concerns about the specificity of antibodies to P16INK4A, staining performed in normally aged and post-IRI kidneys from P16INK4A^{-/-} and WT mice confirmed that AHP1488 detected no p19ARF protein in aged or injured kidneys (fig. S7). To measure correlation between SCs and collagen staining, dual IF was carried out for the P21CIP1 or P16INK4A proteins (red fluorescence) and collagen I (green fluorescence) on all relevant kidney sections. Images of these kidneys were collected as CZI files on the Axioscan. This allowed visualization of full kidney sections in the QuPath program. Percentage positivity for p21CIP1 and P16INK4A proteins (red) and collagen I (green) was assessed from three to seven random fields per kidney (ImageJ macro). These values were collated and analyzed together to assess any linear correlation. A scatterplot was generated, a trend line was added, and R^2 and P values were calculated in Microsoft Excel. For Lamin B1 calculations, total tubular (LTL⁺) DAPI⁺ and Lamin B1⁺ nuclei were counted per field, and % Lamin B1⁺ nuclei per tubule was calculated. For LTL/Ki67 dual staining, total LTL⁺ tubules and LTL⁺ tubules containing Ki67⁺ nuclei were counted, and % Ki67⁺ tubules was calculated.

RNA extraction and qPCR

RNA was extracted from human PTECs or murine kidneys using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and reverse transcribed to cDNA (Qiagen Quantitect Reverse Transcription Kit). qPCR was carried out using VWR Perfecta qPCR mastermix with TAQman gene expression assays on a Roche LightCycler 480 using standard protocol.

For hRPTEC cells, the TAQman gene expression assays used were glyceraldehyde-3-phosphate dehydrogenase (Hs02758991_g1 GAPDH), hypoxanthine-guanine phosphoribosyltransferase (Hs02800695 HPRT1), peptidylprolyl isomerase A (Hs04194521 PPIA), MKI67 (Hs04260396_g1, Ki67), Lamin B1 (Hs01059210_m1 LMNB1), *Cdkn2a* (Hs00923894_m1 CDKN2A), P21CIP1 (Hs00355782_m1 CDKN1A), IL-1B (Hs01555410_m1 IL1B), IL-6 (Hs00174131_m1 IL6), TNF (Hs00174128_m1 TNF), TGF (Hs00998133_m1 TGFB1), Cxcl8 (Hs00174103_m1 CXCL8), matrix metalloproteinase 1 (Hs00899658 MMP1), Bcl-2 (Hs04986394_s1 BCL2), Bcl-xl (Hs00236329_m1 BCL2L1), Bcl-w (Hs01573809_g1 BCL2L2), and prob FOXO4 (Hs00172973 FOXO4). mRNA expression was

normalized for GAPDH, HPRT1, and PPIA (average) expression and presented as fold increases over day 7 nonirradiated cells analyzed in parallel.

For murine kidneys, the following TAQman gene expression assays were used: housekeeping genes: HPRT (Mm03024075_m1 Hprt), Gapdh (Mm99999915_g Gapdh), peptidylprolyl isomerase A (Mm0234230 PPia), and phosphoglycerate kinase 1 (Mm00435617_m1 Pgk1); senescence and SASP markers: *Cdkn2a* (Mm00494449_m1 Cdkn2a), P21CIP1 (Mm04205640_g1 Cdkn1a), IL-6 (Mm00446190_m1 IL-6), Tnf (TNF; Mm00443258_m1 Tnf), TGF (Mm01178820_m1 Tgfb1), and Lamin B1 (Mm00521949 Lmnb1); BCL-2 family members: Bcl-2 (Mm0477631 Bcl2), Bcl-xl (Mm00437783 Bcl2l1), and Bcl-w (Mm0043054 Bcl2l2); macrophage marker: CD68 (Mm03047343_m1 Cd68); fibrosis markers: collagen (col) 1a1 (Mm00801666_g1 Col1a1), Col3a1 (Mm01254476_m1 Col3a1), fibronectin (Mm01256744_m1 Fn1), and α -smooth muscle actin (Mm00725412_s1 Acta2), mRNA expression was normalized for HPRT or average house keeping gene (HK) expression in the case of old IRI tissue and presented as fold increases over naive or age-matched control analyzed in parallel.

Enzyme-linked immunosorbent assays

TGF- β 1 ELISA

Protein was extracted from whole kidneys using complete radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Santa Cruz). Briefly, kidney tissue was weighed and then placed in 300 μ l of RIPA buffer with a 5-mm steel ball in a 2-ml Eppendorf tube. The tissue was disrupted by high-speed shaking using a tissue lyser (Qiagen). Samples were placed on ice for 10 min before centrifugation for 20 min at 10,000g at 4°C before supernatant collection. Latent TGF- β 1 was activated using 1 N of HCl for 10 min, neutralization with 1.2 N of NaOH/0.5 M Hepes, and then analyzed on a TGF- β 1 enzyme-linked immunosorbent assay (ELISA) (R&D; DuoSet ELISA kit) according to the manufacturer's instructions.

Cystatin C ELISA for kidney function

Tail vein blood was recovered from mice that had undergone bilateral IRI 7, 14, or 35 days previously in an equal volume of 4% citrate buffer before centrifugation at 5000g for 5 min at 4°C. Two microliters of the clear phase was transferred into 250 μ l of PBS[−] with 0.5% bovine serum albumin. The mouse cystatin C ELISA (R&D; DuoSet ELISA kit) was carried out according to the manufacturer's instructions.

Statistical analysis

Data were analyzed with Microsoft Excel, Prism software (GraphPad), or R version 4. Multivariable linear regression was performed on R using the finalfit package (1.0.2). All values are expressed as mean values with error bars representing SDs. For all in vivo studies, *n* numbers reflect biological replicates. Depending on the results of normality, testing groups were compared using unpaired Student's *t* test, Mann-Whitney, or one/two-way analysis of variance (ANOVA) (Tukey post hoc test) for analysis. *P* < 0.05 denotes statistical significance; **P* < 0.05, ***P* < 0.01, ****P* < 0.005, and *****P* < 0.001.

SUPPLEMENTARY MATERIALS

stm.sciencemag.org/cgi/content/full/13/594/eabb0203/DC1

Fig. S1. Expression of selected cyclin-dependent kinase inhibitors, SASP factors, fibrotic, and Bcl-2 family transcripts detected on microarray analysis in patients with CKD and healthy controls, along with adjusted *q* values and fold change [adapted from Nakagawa *et al.* (17)]. Fig. S2. Pretreatment with ABT-263 in aged murine kidneys results in a reduction of fibrosis, DNA damage and macrophages, and favors a reduction of SC/SASP/BCL2 family members and a preservation of LTL⁺ tubules after injury.

Fig. S3. ABT-263 treatment reduces expression of *Cdkn1a* and *Cdkn2a* transcripts, reduces cortical γ H2A.X expression, and increases Lamin B1 in the murine kidney. Fig. S4. Irradiation-induced epithelial senescence promotes fibrosis and senescence in murine kidneys with ABT-263 treatment protective. Fig. S5. Treatment with ABT-263 alters qPCR and protein markers of macrophage infiltration within the murine kidney. Fig. S6. The efficacy of pretreatment with ABT-263 is lost in young mice with low quantities of pre-IRI SCs. Fig. S7. Bio-Rad antibody AHP1488 anti-*Cdkn2a* detects P16INK4A but not p19ARF protein expression in aged and fibrotic murine kidneys. Table S1. Multivariate analysis of CDKN1A and CDKN2A and their effect on estimated glomerular filtration rate (eGFR) in humans. Table S2. Primary and secondary antibodies used for IHC and IF. Data file S1. Raw data for all figures.

View/request a protocol for this paper from Bio-protocol.

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Appendix B

Senescent Mdm2 b

Gene	Avg_log2FC	P_val_adj
Mdm2	4.954839	6.612758e-303
Gdf15	3.880616	0.000000e+00
Cdkn1a	3.865238	0.000000e+00
S100a6	3.668339	3.366791e-116
Grid2	3.650289	0.000000e+00
Anks1b	3.451744	0.000000e+00
Gclc	3.182484	1.618536e-145
Grip1	3.113602	1.648970e-237
Slc4a11	2.929155	0.000000e+00
Ankrd1	2.841030	0.000000e+00
Tubb4b	2.837349	3.334447e-186
Trp53inp1	2.783694	0.000000e+00
Cryab	2.719334	5.677599e-277
Mgmt	2.703762	0.000000e+00
Nrg1	2.607634	2.202960e-185
Rps27l	2.571663	3.047326e-36
Plk2	2.506171	0.000000e+00
S100a11	2.496982	1.994031e-77
Txnrd1	2.432046	9.177785e-160
Atf3	2.381945	4.703440e-112
Ano3	2.377499	0.000000e+00
Ptpn14	2.364884	6.504279e-189
H3f3b	2.306091	2.758987e-49
Fat1	2.298121	1.222574e-206
Gm35330	2.294853	0.000000e+00
Ccng1	2.247465	1.739268e-291
Krt18	2.244928	0.000000e+00
Ifrd1	2.236454	7.400466e-101
Pvt1	2.233780	4.860459e-238
Mdm4	2.217824	6.743968e-151
Fam129b	2.213172	9.593946e-240
Dscaml1	2.201258	0.000000e+00
Lgals3	2.200723	6.163088e-165
Anxa2	2.188314	1.209879e-159
Dsp	2.179355	2.555940e-238
Akap12	2.163286	4.671611e-183
Hspg1	2.145412	7.034313e-134
Mcam	2.144956	0.000000e+00
Sbsn	2.127121	0.000000e+00

Btg2	2.126044	1.453667e-134
Klhl26	2.068834	0.000000e+00
Tbc1d8	2.059966	0.000000e+00
Krt8	2.051588	5.876616e-152
Rps19	2.007259	7.879040e-23
Cstb	1.983848	9.161936e-66
Serpine2	1.980393	0.000000e+00
Phlda3	1.976999	0.000000e+00
Hbegf	1.953397	5.111613e-140
Garnl3	1.951319	0.000000e+00
Cpt1c	1.942878	0.000000e+00
Arhgef4	1.942300	0.000000e+00
Egfr	1.940840	2.937711e-127
Cox6b2	1.939427	0.000000e+00
Samd12	1.931032	3.009365e-208
Itgb4	1.925083	0.000000e+00
Cdc42bpg	1.901733	3.182354e-171
Wnt7b	1.894771	0.000000e+00
Fosb	1.894656	7.484493e-79
Arc	1.893414	0.000000e+00
Tiam1	1.892994	2.953032e-152
Csf1	1.884059	1.270286e-155
Dglucy	1.882705	7.222115e-177
Perp	1.881417	0.000000e+00
Pacrg	1.881339	7.043233e-162
Sdc4	1.872149	2.254055e-84
Cldn4	1.870738	0.000000e+00
Plxdc2	1.869626	3.549639e-116
Nxn	1.867213	7.317985e-154
Adcy2	1.861369	0.000000e+00
Tpm1	1.855094	6.793827e-137
Sulf2	1.843316	1.649877e-259
Exoc4	1.839414	7.594862e-111
Sik1	1.834591	2.591682e-115
Gria3	1.822692	1.800371e-161
Cd81	1.810136	1.035655e-63
Egr1	1.806868	8.484717e-104
Bmt2	1.800008	3.267437e-169
S100a10	1.798572	6.636513e-90
Svop	1.797041	0.000000e+00
Thbs1	1.790265	6.797376e-54
Itpkc	1.788460	5.515694e-168
2610307P16Rik	1.779325	4.322406e-91
Sqstm1	1.770444	4.222886e-45

Galnt18	1.763664	5.797288e-89
Pltp	1.763387	3.358585e-193
Zmat3	1.755083	0.000000e+00
Rpl19	1.743389	5.813247e-18
Jazf1	1.736701	4.638668e-214
Arap2	1.730108	7.290941e-145
Wdr35	1.725768	2.594028e-135
Sprr1a	1.723802	2.497658e-271
Alox5	1.720754	0.000000e+00
Gm15987	1.709373	0.000000e+00
Efna5	1.709290	9.856352e-95
Tmtc3	1.703664	7.106870e-219
Arap1	1.698099	2.307512e-99
Glrx3	1.695871	7.011672e-115
Baiap2	1.692681	1.959545e-63
Cfap44	1.681498	0.000000e+00
Wdr61	1.674961	1.791169e-136
Csnk1g1	1.674127	3.173521e-102
Mpp7	1.670934	8.509835e-104
Dcxr	1.670106	1.045780e-75
Eda2r	1.655831	0.000000e+00
Bcam	1.653925	1.089615e-138
Cpe	1.645708	0.000000e+00
Pmm1	1.634406	1.607818e-299
Map3k20	1.634205	3.417737e-130
Polk	1.623769	1.321386e-187
Tacstd2	1.615501	1.289773e-206
Crip2	1.610552	4.646208e-97
Psrc1	1.606668	0.000000e+00
Apaf1	1.602561	2.814774e-152
Plxna1	1.590694	1.898199e-135
Adamstsl1	1.588645	5.159368e-94
Susd6	1.587265	4.553213e-96
Ptk7	1.583788	6.727232e-255
Bax	1.583432	1.544721e-72
Ddit4l	1.579669	2.095934e-253
St14	1.578532	1.250939e-148
Vps36	1.559778	8.579860e-138
Dnm3	1.559729	7.843490e-106
Gtse1	1.544524	0.000000e+00
Junb	1.544294	3.255056e-72
Prrg4	1.535972	0.000000e+00
Slc2a9	1.535430	2.739847e-249
Jund	1.532500	6.962041e-50

Dstn	1.528245	4.284185e-55
Ptma	1.507138	5.048331e-42
Ankrd44	1.505525	1.046164e-73
tdTomato	1.504477	4.754894e-75
Ephx1	1.503965	8.087257e-200
Esd	1.503502	5.292442e-32
Mybl1	1.500341	0.000000e+00
Gprc5a	1.485763	0.000000e+00
Cfap43	1.475511	2.769597e-182
Plod2	1.468036	6.948329e-147
Npas3	1.465727	3.998293e-201
Anxa1	1.461299	1.011494e-37
Ahnak	1.451372	9.135620e-81
Rap2b	1.450582	0.000000e+00
Lmna	1.450370	1.614118e-93
Ubc	1.444207	2.372750e-43
Arhgap28	1.442857	7.498308e-186
Slc25a17	1.439073	5.995004e-110
Ikbke	1.438025	5.886891e-211
Fry	1.435864	1.590920e-88
Chst8	1.434496	3.198187e-53
Dgka	1.434327	3.620195e-155
Scel	1.432043	1.379355e-144
Pdgfa	1.425841	3.473903e-191
Gstm1	1.423979	4.531846e-74
Bst1	1.422959	2.891251e-208
Cadm1	1.419326	1.417038e-82
Naaladl2	1.407977	6.713426e-78
Tnfrsf18	1.403481	0.000000e+00
Col4a5	1.399240	2.203120e-49
Wnt4	1.398455	0.000000e+00
Itga3	1.396012	1.204896e-87
Cep170b	1.395873	1.075841e-107
Sh3bgrl2	1.395499	8.387255e-163
Ccn1	1.384254	2.018504e-55
Taldo1	1.381095	2.145104e-36
Eef1a1	1.377631	4.302090e-23
Rhbdl2	1.376827	1.571175e-258
El24	1.368038	2.208254e-101
Crim1	1.367227	4.296421e-62
Tmem43	1.365431	1.754657e-282
1700007K13Rik	1.360959	0.000000e+00
Sfn	1.357172	9.494077e-217
Ptptr	1.346465	1.741162e-190

Fhad1	1.344386	0.000000e+00
Nupr1	1.343660	2.782103e-91
Krt7	1.335789	4.622399e-43
Capg	1.332954	2.453340e-123
Foxj1	1.328435	0.000000e+00
Epcam	1.327943	3.526475e-77
Abcb1b	1.325589	1.952021e-232
Epha2	1.325391	1.534883e-260
Slc12a4	1.321909	6.159005e-190
Gls	1.320013	1.419783e-44
Gclm	1.316410	6.066566e-60
Siva1	1.313111	1.024704e-104
Rbms3	1.312961	2.272190e-98
Susd4	1.312850	2.499452e-146
Rnf169	1.311714	7.254950e-75
Plxnb2	1.311683	1.353431e-76
Tulp4	1.310449	1.444195e-67
Cd24a	1.308337	6.359751e-78
Mak	1.307833	0.000000e+00
Pdcd6ip	1.300580	4.422766e-69
Met	1.299806	5.205065e-54
Bcl2l1	1.298652	2.144883e-58
Sema3c	1.295527	6.365398e-80
Ifi27	1.293568	3.981018e-36
Arhgap23	1.290818	2.667021e-92
Ppl	1.284447	4.404439e-239
Ehf	1.278639	1.037473e-129
Itm2b	1.275173	1.386850e-30
Cd55	1.274758	0.000000e+00
Cobl	1.266567	4.760907e-57
Notch3	1.263054	2.021694e-302
Rb1	1.256127	2.014619e-59

Senescent Mdm2 a

Gene	Avg_log2FC	p_val_adj
Mdm2	4.403012	3.674962e-107
Ano3	3.823738	0.000000e+00
Grip1	3.409450	3.246894e-103
Trp53inp1	3.267110	1.588452e-173
Anks1b	2.922494	6.704867e-144
Dscaml1	2.921630	0.000000e+00
Gria3	2.898394	2.329260e-116
Pvt1	2.892312	3.022440e-128
Gc	2.843876	1.323287e-214
Mgmt	2.776209	9.843770e-162
Dpyd	2.762572	1.852047e-31
Grm8	2.673571	5.107415e-280
Nrg1	2.648212	1.299856e-52
Gm35330	2.539146	0.000000e+00
Egfr	2.450538	1.255590e-65
9230114K14Rik	2.416041	3.068475e-142
Tbc1d8	2.388421	4.454695e-150
Txnrd1	2.360031	3.922939e-49
Abcc4	2.344117	1.179847e-39
Gm42047	2.325060	5.649654e-91
Plcd4	2.302586	9.482906e-231
Dglucy	2.275459	1.462438e-86
Fat1	2.252070	2.218512e-78
Klhl26	2.221260	1.746585e-138
Npas3	2.219302	1.790646e-257
Arhgef4	2.208602	0.000000e+00
Serpine2	2.205435	2.273342e-188
Svop	2.203703	0.000000e+00
Mpp7	2.185718	1.590943e-38
Mdm4	2.169902	7.091687e-47
Alox5	2.153714	0.000000e+00
Lpin1	2.142411	1.001510e-93
Abcc2	2.109177	2.070378e-48
Cpt1c	2.103088	0.000000e+00
Cdkn1a	2.093957	3.343261e-169
Ddr1	2.075262	3.241439e-38
Ugt1a8	2.024637	2.550760e-58
Plxdc2	2.008144	3.544460e-46
Ugt1a6a	2.007235	2.866696e-43
Exoc4	2.004765	2.723950e-52
Ptk2b	1.992320	1.523882e-57

Apaf1	1.982314	1.035748e-95
Eda2r	1.979252	0.000000e+00
Wdr35	1.957764	3.539762e-51
Nectin1	1.955179	2.213801e-65
Ercc5	1.917900	8.363633e-64
Lrmda	1.917162	1.463155e-25
Cdc42bpg	1.886101	9.359613e-66
Sulf2	1.884092	1.584526e-84
Trpc1	1.882064	3.275122e-65
Susd6	1.880818	5.154767e-47
Pltp	1.879592	1.519102e-51
Ncor1	1.873051	9.369735e-23
Igsf9b	1.853708	5.617269e-158
Gm45792	1.851990	1.917440e-72
Dgka	1.848315	1.406591e-78
Plod2	1.843801	4.108846e-60
Zfp385a	1.831282	3.276522e-298
Polk	1.824966	8.915497e-83
Rnf169	1.823554	4.091928e-46
Psrc1	1.808470	0.000000e+00
Agap1	1.797456	2.469644e-47
Scn3b	1.788849	0.000000e+00
Gclc	1.767236	4.065239e-23
Ccng1	1.766318	4.699292e-69
Pcx	1.758494	9.651641e-57
Garnl3	1.751542	9.704203e-99
Csnk1g1	1.748471	2.299353e-42
Tct28	1.741914	4.575020e-27
Fas	1.714966	2.746916e-67
Gtf3c1	1.704072	2.351467e-42
Gas6	1.684530	3.787360e-34
Ugt2b34	1.684230	1.740782e-91
Samd12	1.668950	7.159415e-43
Zmat3	1.667210	1.474603e-104
Cep170b	1.662510	4.473332e-44
Cd55	1.657340	2.345005e-208
Axl	1.653267	2.687624e-117
Trio	1.645522	1.041278e-34
Ppard	1.645321	7.582327e-39
Cpped1	1.635630	9.068324e-36
Eva1c	1.619734	7.298931e-104
Map3k20	1.619584	1.146475e-56
Slc5a11	1.611406	5.436433e-51
Gm19950	1.605246	3.172764e-61

Sh3d19	1.593184	1.205363e-35
Insyn2a	1.589004	8.063959e-99
Man2b1	1.572010	3.591104e-21
Tmem63b	1.570133	1.289562e-45
Abat	1.569143	1.517150e-33
Zfp750	1.563568	1.009116e-190
Gtse1	1.550331	0.000000e+00
Ces2f	1.545624	0.000000e+00
Arhgap21	1.545379	1.897863e-25
Plk2	1.541126	2.218923e-57
Ces2e	1.535614	3.960589e-171
Celf5	1.531960	0.000000e+00
Baiap2	1.515886	5.818780e-39
Prrg4	1.515409	1.496083e-131
Rab40c	1.514046	1.940490e-48
Arap2	1.513588	1.350362e-40
Ncor2	1.511632	1.552047e-20
Nsmce2	1.502969	7.728010e-23
Slc1a1	1.501337	2.827898e-27
Slc25a17	1.500988	6.556600e-39
Nlrc5	1.498421	2.332824e-38
Plxna1	1.495230	1.718373e-35
Gbe1	1.493952	1.207576e-24
Rev1	1.491875	3.898110e-39
AC149090.1	1.490971	5.277872e-09
Tmtc3	1.483348	7.410376e-42
Gm20400	1.469045	1.485238e-49
Cep250	1.460129	3.231441e-33
Ugt1a10	1.460104	0.000000e+00
Mab21l3	1.456807	5.107699e-157
Plcx2	1.450766	1.040353e-22
Azin2	1.438595	2.242527e-60
Ephx1	1.436568	1.522856e-60
Bmp6	1.435676	3.392842e-23
Ccdc17	1.434824	4.178755e-141
Sgsm2	1.426219	3.194706e-36
Mreg	1.418426	4.577654e-211
Ccp110	1.411922	1.014851e-64
Alpl	1.397887	8.530715e-31
Adamstsl1	1.396690	4.532119e-14
Hyal1	1.390194	1.375996e-65
Cnksr3	1.388343	2.424340e-22
Adcy2	1.382221	3.042870e-65
Vcan	1.375176	1.157693e-64

Gsta3	1.364606	9.129178e-39
Zbtb38	1.356374	2.067106e-16
Ttll10	1.354021	4.345629e-25
Pcgf3	1.350124	2.738823e-41
Agpat4	1.348353	2.260872e-39
Sh3bgrl2	1.341178	1.895274e-38
Sesn2	1.330447	5.302605e-56
Bmt2	1.321702	6.496247e-27
Tulp4	1.320720	2.438241e-19
Slc19a2	1.306605	2.122285e-141
Myo9b	1.306127	1.417196e-25
Kcnc3	1.302810	1.102920e-09
4930414F18Rik	1.299604	2.672041e-249
Inka2	1.299147	0.000000e+00
Trim7	1.297871	2.518972e-38
Depdc7	1.297120	7.396473e-62
Mroh7	1.295934	5.449980e-68
Crocc	1.295214	2.434702e-25
Atg9b	1.292432	1.259643e-224
Trim11	1.291369	1.366698e-55
Mast4	1.290139	2.511808e-27
Fads2	1.286020	4.621914e-31
Parp10	1.282657	1.739753e-57
Atg4a	1.282294	8.294673e-13
Abcb1b	1.280393	1.712415e-47
Arhgap35	1.277288	6.761593e-24
Pik3cb	1.274973	1.692532e-21
Tmem19	1.265635	3.229960e-37
Afap1l2	1.262770	6.827362e-36
Ddit4l	1.256773	2.064417e-36
Cnst	1.251637	1.142198e-04
Digap4	1.247751	1.533663e-22
Acad11	1.232022	2.613900e-24
Cdyl	1.231027	3.695669e-27
Frrs1	1.222779	3.231698e-29
Mybl1	1.217184	4.557815e-143
Abca1	1.213940	9.119098e-25
Dzip1	1.213414	7.365398e-78
Tbcl2	1.210728	2.481010e-15
Glr3	1.210026	3.176909e-28
Ulk1	1.206346	2.476876e-31
Dlg5	1.206136	4.864603e-18
Sfmbt1	1.200970	2.185664e-26
Slc4a11	1.197406	4.130325e-44

Gdf15	1.193535	5.014180e-79
Gask1b	1.189691	1.020077e-104
Wdr61	1.187079	2.072679e-27
Bcl2l1	1.184516	6.909163e-17
Slc22a12	1.181338	5.821665e-20
Slc2a9	1.180458	6.313387e-58
Map2k4	1.179599	1.256768e-18
Ccn1	1.172708	3.854154e-08
Acbd5	1.172559	1.270248e-18
Ttc7b	1.158505	6.183912e-16
Ctc1	1.154796	2.355806e-45
Naa40	1.145411	1.387542e-28
Hdac4	1.144499	9.313540e-14
Pm20d1	1.142571	2.245930e-42
Pros1	1.141676	4.355252e-12
Fermt1	1.141357	1.329982e-20
Pard6g	1.139821	1.107738e-69
Dnajc6	1.138454	3.329214e-15
Map3k13	1.137247	6.160977e-16
Slc25a1	1.133839	7.297151e-20
Bin1	1.130771	5.828123e-27
Adgrb1	1.126832	0.000000e+00
Clpb	1.120252	5.423579e-19
Nfia	1.117415	4.264564e-20
Aldh2	1.115149	1.575435e-16
B4gaint3	1.110714	2.059742e-25
Glis3	1.109621	1.708199e-22