Unravelling the mechanisms of Senescence-Associated Stemness in 3D Kidney Structures

Towards regenerative therapies for the elderly who suffer from kidney dysfunction

Written research report

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1. Plain language summary

Veroudering wordt gekenmerkt door een geleidelijke afname van fysiologische functies en een verminderd vermogen van de organen om te herstellen na schade. De nieren zijn op latere leeftijd bijzonder kwetsbaar voor de gevolgen van schade. Wanneer de nieren niet meer in staat zijn afvalstoffen uit het lichaam te verwijderen, is er sprake van acuut nierfalen (AKI). Een van de meest voorkomende oorzaken van acuut nierfalen is het gebruik van bepaalde medicijnen, zoals cisplatine, een veel voorgeschreven vorm van chemotherapie. Men spreekt van chronisch nierfalen (CKD) wanneer de nieren ook niet meer in staat zijn om te herstellen. Gezien er buiten dialyse en niertransplantatie geen therapeutische behandelingen beschikbaar zijn, is onderzoek naar nieuwe behandelingen van groot belang. Ook is het belangrijk dat er meer onderzoek wordt gedaan naar waarom de nieren achteruitgaan bij veroudering, ter preventie van chronisch nierfalen.

Tijdens veroudering hopen zich senescente cellen op in de nieren, ofwel ‘beschadigde’ lichaamscellen die niet meer kunnen delen. De ophoping van deze senescente cellen belemmert op den duur de functie van de nier en intrinsiek herstel bij nierschade. Senescente cellen scheiden SASP-moleculen uit die ontsteking stimuleren, maar ook op andere manieren omliggend weefsel beïnvloeden. Hoewel tijdelijke uitscheiding van deze moleculen gunstig is voor herstel, doordat ze bijvoorbeeld het immuunsysteem aanwakkeren, kan de chronische aanwezigheid van deze moleculen schadelijk zijn voor andere cellen in het omringende nierweefsel. In organen zoals de lever was al bekend dat deze SASP-moleculen de functionele staat, ofwel differentiatie, van levercellen kunnen doen terugdringen. Zo zouden ze herstel bij leverschade kunnen belemmeren. Het was echter nog onduidelijk of dit ook geldt voor de functionele staat van niercellen.

Het huidige onderzoek toont aan dat SASP-moleculen, afkomstig van niercellen die beschadigd zijn door cisplatine, de functionele staat van andere niercellen zou kunnen doen terugdringen. Deze bevindingen zijn gedaan in zogenaamde mini 3D-niertjes, die in het lab gekweekt zijn met behulp van menselijke gereprogrammeerde stamcellen. In aanwezigheid van SASP-moleculen blijken niercellen, tijdens de ontwikkeling van de mini 3D-niertjes, in een voorstadium te blijven hangen. In deze 3D-niertjes komen normaal gesproken ook podocyten voor, die kunnen worden gezien als de filtertjes van de nier. Deze podocyten blijken zich ook minder goed kunnen ontwikkelen in aanwezigheid van SASP-moleculen, wat de achterliggende reden zou kunnen zijn waarom de filterfunctie van de nier achteruitgaat, in aanwezigheid van senescente cellen.

Van recent-ontwikkelde medicijnen, zoals FOXO4-DRI is al bekend dat deze de senescente cellen kunnen verwijderen uit het nierweefsel. Hiervan wordt de therapeutische werking bij acuut nierfalen nog onderzocht in muizen. Uit toekomstig onderzoek moet nog blijken of zulke medicijnen de schadelijke gevolgen door de SASP-moleculen, mogelijk kunnen verminderen. Met name op de ontwikkeling en het herstel van podocyten. Zulke medicijnen bieden hoe dan ook veelbelovende hoop voor toekomstige behandelingen om vroegtijdig chronisch nierfalen te voorkomen, bijvoorbeeld bij ouderen die eerder chemotherapeutische behandeling met cisplatine zijn ondergaan.

2. Abstract

During aging, senescent cells accumulate in the kidney, impairing function and intrinsic repair. These senescent cells develop a distinct, heterogeneous Senescence-Associated Secretory Phenotype (SASP), releasing among pro-inflammatory cytokines and proteases. While transient SASP secretion is thought to be beneficial for regeneration after injury, chronic presence of SASP molecules may be harmful to cells in the surrounding tissue. Moreover, the accumulation of senescent cells in the kidney has been linked to the progression of Chronic Kidney Disease.
In organs like the liver, SASP molecules have been found to promote stemness and impair cell differentiation. In this manner, SASP molecules could potentially prevent regeneration from fully taking place. Nevertheless, it remains unclear through which mechanisms the SASP impairs regeneration in the kidney, and whether this occurs through a similar mechanism of promoting stemness and hindering differentiation. This study shows that the SASP, secreted by cisplatin-induced senescent renal proximal tubular epithelial cells (RPTECs), hinders the full differentiation of iPSC-derived 3D renal structures, keeping cells in a progenitor-like state. These effects seem to be dependent on the activation of the NF-κB pathway in the cells recipient of the SASP. Specifically, renal progenitor cells exposed to the SASP maintain high expression of CD44 and express less of NPHS1, a marker for podocytes. At the same time, the differentiation of cells expressing UMOD, a marker of the Loop of Henle, is not affected. Ultimately, emerging senescence-targeting approaches could mitigate the detrimental effects of the SASP in regeneration, and hold promise in preventing the onset of CKD in elderly patients post-cisplatin cancer treatment.

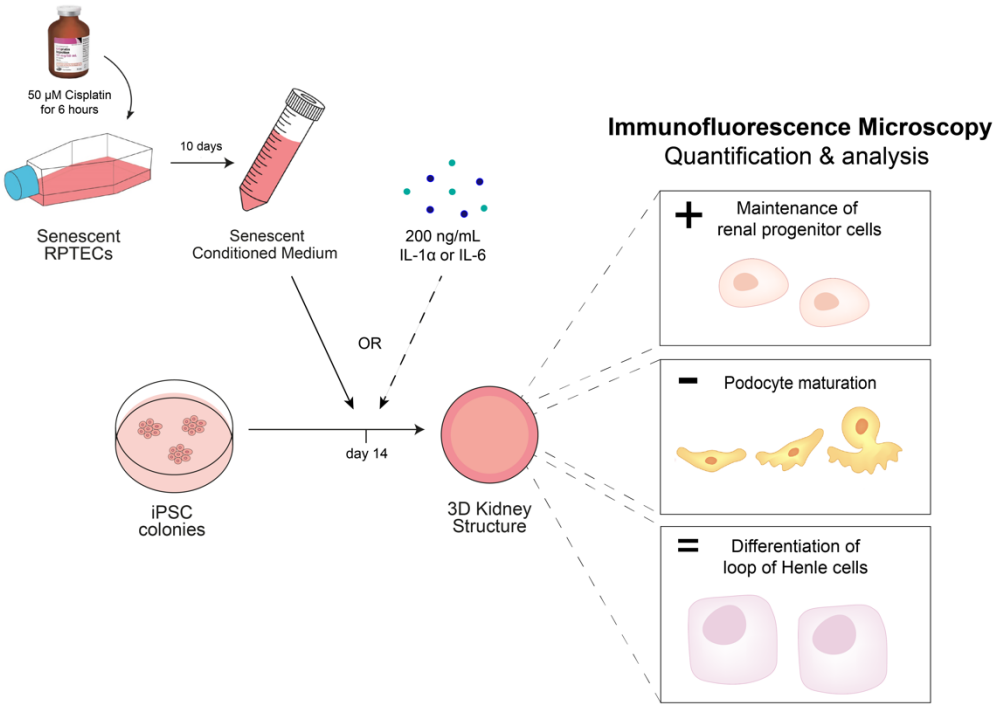


Figure 1: Graphical abstract

3. Abbrevations

AKI	Acute Kidney Injury
CD44	Cluster of Differentiation 44
CKD	Chronic Kidney Disease
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	Erythropoietin
IL	Interleukin
iPSC	induced Pluripotent Stem Cell
IRI	Ischemic Reperfusion Injury
IKKß2i	Inhibitor of IκB Kinase Beta 2
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NormCM	Normal Conditioned Medium
PEC	Parietal Epithelial Cell
RPTEC	Renal Proximal Tubular Epithelial Cell
SASP	Senescence-Associated Secretory Phenotype
SA-ß-gal	Senescence-Associated Beta-Galactosidase
SCM	Senescent Conditioned Medium
UMOD	Uromodulin

4. Introduction

4.1 Aging, kidney health and the burden of Chronic Kidney Disease

Over the years, advances in healthcare have led to an ongoing increase in life expectancy worldwide. By 2050, the global population aged 65 and above is expected to surpass 1.5 billion (1). As longevity continues to rise, this increase would worsen the already heavy burden of age-related diseases, which impact quality of life and put pressure on healthcare systems. Aging itself is characterized by a gradual decline in physiological functions, and the reduced ability to maintain homeostasis in case of stress (2).

The kidneys, with their high metabolic activity and constant exposure to oxidative stress, are particularly susceptible to the detrimental impact of aging. Gradually, they become less resilient to injury and disease, while losing their capability of repair. Studies have indicated that after the age of 50, renal mass goes down 10% per decade and after the age of 80, there is a drop in function of 30-40% as measured by creatinine clearance (3).

A rapid decrease in kidney function due to damage, regardless of age, is referred to as Acute Kidney Injury (AKI). It is often caused by the destruction of renal tissue cells, resulting in the accumulation of waste products in the blood, mainly urea (4). In most cases, repair mechanisms are triggered and induce renal tissue regeneration. The widely accepted concept for repair after AKI is that tubular or glomerular epithelial cells dedifferentiate, proliferate and re-differentiate to repair damage of functional kidney cells. During dedifferentiation, the epithelial cells lose transporters, as well as their brush border, and upregulate markers indicative of renal progenitor cells such as CD44 (5, 6).

Due the route of renal excretion, the kidneys are considered major targets of chemical toxic agents that are not first metabolized by the liver. In most cases, AKI is induced by nephrotoxic agents, such as antibiotics and chemotherapeutic drugs. Moreover, drug-induced AKI is a frequent phenomenon in hospital admissions (7). In particular, the chemotherapeutic drug cisplatin has been found to be highly nephrotoxic, though it is still commonly used to treat patients with solid malignant tumors. Within days of initiating cisplatin chemotherapy, 30% of patients develop AKI (9). In ongoing research, cisplatin has been used to study AKI in mice, but also aging itself, as it is known to accelerate ageing related biological processes (10).

In case the damage of AKI is not properly resolved due to maladaptive repair, it can progressively develop into Chronic Kidney Disease (CKD) (11). CKD forms a major burden among the elderly, as its prevalence in the population of people aged over 65 has reached about 44% in the US (12). Though there are remedies to slow the progression of CKD, there are no curative treatment options available. Patients with late-stage CKD rely heavily on kidney dialysis or renal transplantation (7). Although dialysis is life-sustaining, quality of life of patients that receive dialysis is very poor. This is because current dialysis methods only serve to compensate for diminished glomerular filtration, without effectively replacing tubular function, leading to inadequate removal of waste products (8).

To gain a better understanding of how CKD can be prevented in case of AKI, it is crucial to conduct further studies that explore the fundamental aspects of maladaptive repair in the aging kidney. This research could then also provide valuable insights into new therapeutic targets to improve the well-being and long-term survival of patients that suffer from CKD.

4.2 Cellular senescence and the Senescence Associated Secretory Phenotype

In tissues such as the kidney, the liver, the lungs and the skin, aging comes with an increase in cells that have entered a state of senescence. Cellular senescence can be induced by various stressors, resulting in a state of permanent cell cycle arrest. DNA damage, oncogene activation, oxidative stress and the loss of the protective telomeres are all potential inducers of senescence (2).

Senescent cells exhibit morphological and metabolic changes, and notably, they develop distinct secretory phenotypes. The Senescence-Associated Secretory Phenotype (SASP) comprises a heterogenous pool of molecules, that varies depending on the type of stressor and the type of cell that has become senescent. SASP molecules mainly include pro-inflammatory cytokines, chemokines, matrix metalloproteases, growth factors and angiogenic factors (Figure 2) (2).

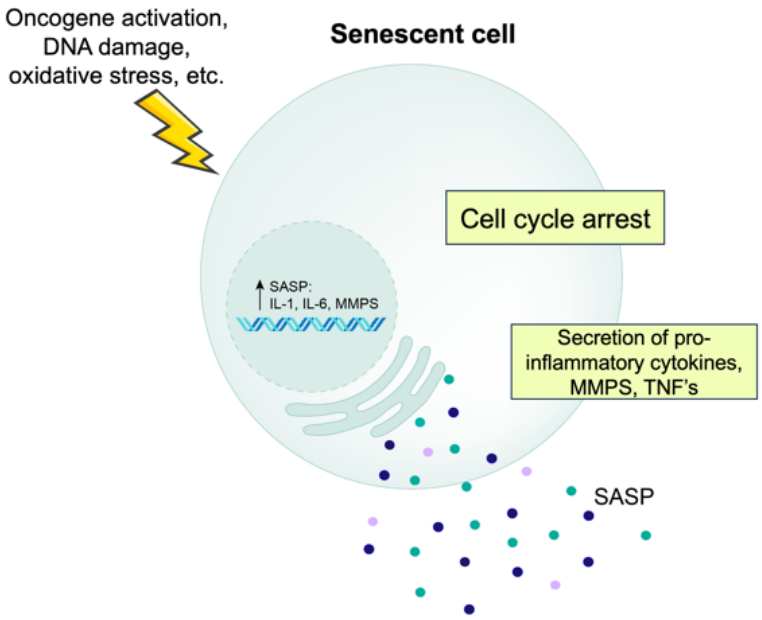


Figure 2: Schematic drawing of a senescent cell and potential inducers of senescence.

To some extent, the SASP is thought to be beneficial in case of tissue damage, as certain SASP components have been found the be chemoattractant of immune cells that can eliminate damaged cells (13). Moreover, the SASP is known to induce senescence in surrounding cells (14). Many studies have raised the concern that, if senescent cells do not get cleared up and remain present, chronic secretion of SASP molecules may have detrimental effects, as it can contribute to fibrosis, adverse tissue remodeling and chronic inflammation in the surrounding tissue (15, 2).

4.3 Senescence induces renal dysfunction

Senescent cells have been found to play a causative role in the development of renal dysfunction in aging, as evidenced by several mouse studies (16, 17, 11). In a study from our group, kidney tissue slides were stained for SA-ß-Gal, a marker for senescence. This experiment showed that senescent cells accumulated mainly in the tubular regions of the kidney, when mice reached 130 weeks of age (Figure 3A). This is equivalent to 80-90 human years. These mice also exhibited higher levels of urea in the plasma, which is indicative of a decline in filtration capacity of the kidneys (15). Increased urea levels were also observed in progeric mice that exhibit early senescence ($Xpd^{TTD/TTD}$), indicating that senescent cells are at the cause of this form of renal dysfunction (Figure 3B). Subsequently, when senescent cells were targeted using a FOXO4/p53-interfering peptide (FOXO4-DRI), an improvement in renal function was observed. In more detail, the FOXO4-DRI selectively induces apoptosis in senescent cells by blocking the interaction between FOXO4 and the p53 apoptotic signaling pathway.

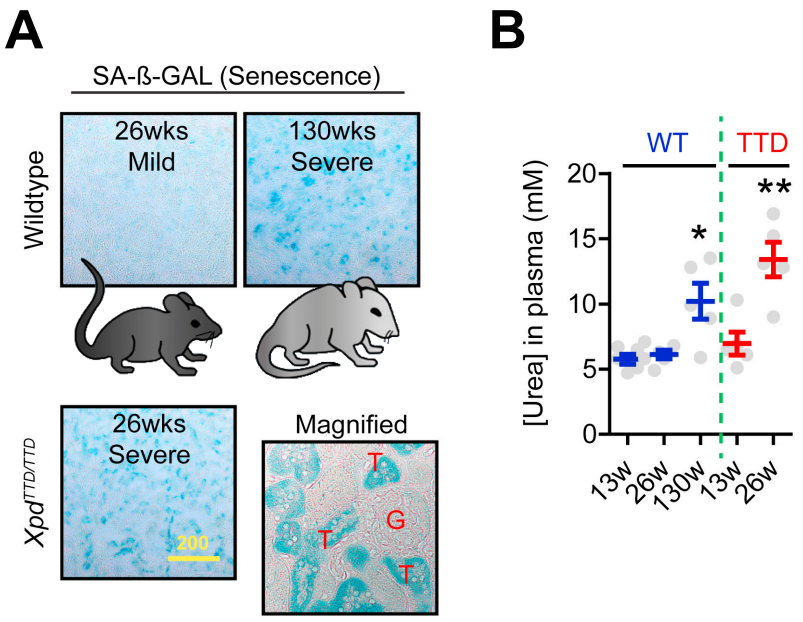


Figure 3: Senescent staining of renal tissue slides and plasma urea levels from young, aged and progeric mice. (A) *"SA-ß-Gal staining of renal tissue slides from mice aged 26 and 130 weeks, as well as progeric ($Xpd^{TTD/TTD}$) mice. T=tubuli, G=glomeruli.* ***(B)*** *Urea plasma levels in WT and progeric mice. * p<0.05, ** p<0.01. Modified from (16).*

Although these experiments do demonstrate that senescence induces renal dysfunction in aging, it remains unclear whether this is a direct effect or not, and which signaling mechanisms are at play within this link.

4.4 Senescent cells are involved in maladaptive repair in the kidney

Several studies have suggested that senescence in the kidney affects regenerative processes, as their accumulation is associated with hindered intrinsic repair mechanisms (12, 16, 17, 18). In a mouse model, in which AKI was induced by ischemic reperfusion injury, it was found that targeting senescent cells could ameliorate age-related maladaptive repair. The kidneys exhibited reduced fibrosis, and notably, a restored regenerative phenotype. This was based on an increase in cell proliferation and kidney weight in the mice treated with the compound (18). Similar results were observed in mice in which AKI was induced using cisplatin (12). Interestingly, another mouse study found that this rescue of the regenerative phenotype was dependent on the time at which senescent cells were targeted. Here, renal ischemia was induced by stenosis of the renal artery, and it seemed that targeting senescent cells in the first week appeared to impair full recovery. When the senescent cells were targeted after two weeks, it did seem to improve renal function (17). This finding is in line with the thought that the SASP does have a beneficial role in regeneration in the short term, but has a detrimental effect in case of prolonged secretion.

Altogether, these experiments suggest that when senescent cells persist in the tissue, they hinder intrinsic repair mechanisms and thus contribute to maladaptive repair in aged mice, ultimately leading to renal dysfunction.

4.5 The SASP can keep surrounding cells in a progenitor-like state

In addition to the previously known effects of the SASP, such as its ability to induce senescence in other cells and to attract immune cells, a new role has been described in literature that it also seems to be able to induce, or maintain, stemness-like properties in surrounding cells (15). Preliminary evidence from our group has indicated that the SASP is capable of keeping differentiating cells locked in a progenitor-like state. This effect was observed when differentiating liver organoids were treated with conditioned medium from senescent stellate cells, resembling the hepatic SASP. When this senescent conditioned medium (SCM) was added to liver organoids that were still in the process of differentiation, it maintained high levels of expression of a stem cell marker called CD44 (Thomas Brand, data not shown). CD44 is a surface glycoprotein, known to be expressed by hepatic progenitor cells (19). Normally, once liver organoids are fully differentiated, CD44 expression decreases. Therefore, this observation suggests that SASP factors secreted by senescent cells can keep surrounding cells in a progenitor-like state.

In line with this are the findings of another study, which showed that induction of senescence resulted in the expression of stem cell markers in the liver *in vivo*. Interestingly here, prolonged exposure to the SASP, seemed to counter the regenerative stimuli, limiting full repair (15). Together these findings indicate that the accumulation of senescent cells can impair regeneration processes of the surrounding cells by keeping them locked in a progenitor like state, through to chronic secretion of SASP molecules.

4.6 iPSC-induced 3D kidney structures to study renal differentiation

The model used for this study is based on a hybrid protocol derived from studies by Takasato et al. and Morizane et al., which focus on the development of 3D kidney-like structures from human induced pluripotent stem cells (iPSCs). Several studies have used these protocols for human disease modelling, for instance for podocytopathies (20), as well as for the study of the fundamental aspects of human renal regenerative processes (21). Moreover, glomerular differentiation in disease has been well studied in mice but remains poorly understood in humans.

In this study, iPSC-derived 3D kidney structures are used to investigate the effect of the SASP on renal differentiation processes. During development, the 3D kidney structures facilitate communication between the different renal cell types and more accurately represent *in vivo* processes in comparison to traditional 2D models (22, 23). To assess the expression levels of certain markers, immunofluorescence microscopy is used for analysis, as it offers visualization at high resolution and sensitivity.

4.7 The effect of the renal SASP on differentiation and regeneration

Considering senescent cells primarily accumulate in the proximal tubules in the kidney, and that it has been proven they can impair filtration capacity in aging, one might question whether this is due to the SASP impairing regeneration processes, similar to what has been observed already in the liver. Thus, the main question of this study is: How does secretion of the renal SASP affect the differentiation of surrounding kidney cells? (Figure 4).

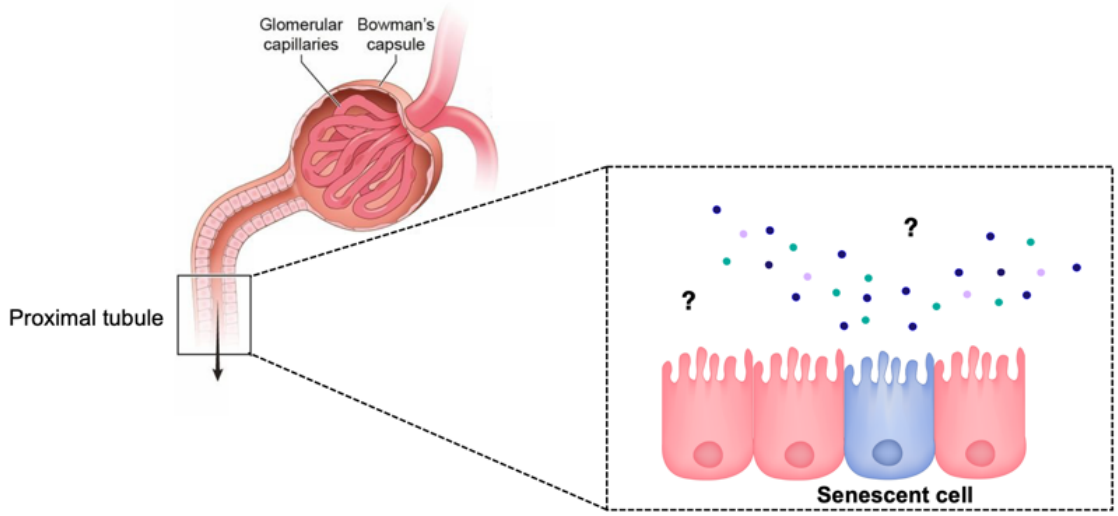


***Figure 4: SASP release to the surrounding cells by senescent proximal tubular epithelial cells**. Adapted from (17).*

CD44 is expressed by progenitor cells in the kidney (24). To determine whether the renal SASP is capable of maintaining kidney cells in a progenitor-like state, in this study, CD44 expression serves as an indicator of progenitorness. Moreover, CD44 is expressed by glomerular parietal epithelial cells (PECs) during the regeneration of podocytes after damage in the adult kidney (25). Fully differentiated podocytes express NPHS1. Therefore, studying the effect of the SASP on these markers in relation to each other could provide valuable insights. Double positives for NPHS1 and CD44 have been described in literature to be in a

transitional phase from PECs to podocytes (Figure 5). Differences in the amount of CD44 and NPHS1 double positive cells could indicate to which extent progenitorness is maintained.

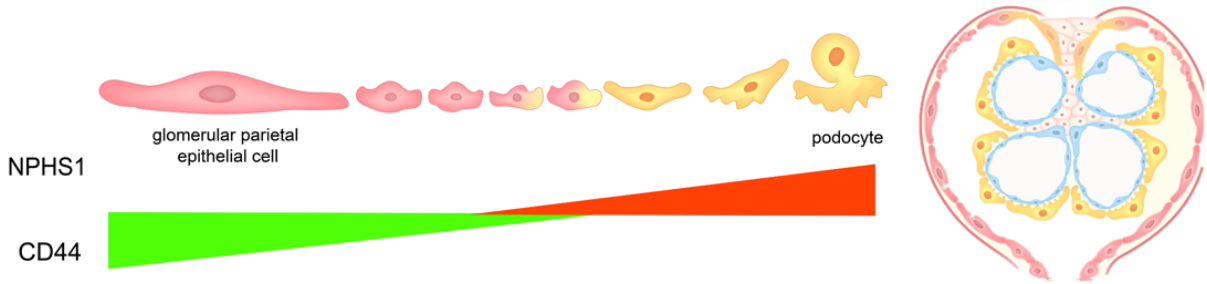


Figure 5: Expression of CD44 and NPHS1 during the regeneration of podocytes. *Adapted from (26).*

Furthermore, fully differentiated cells of the loop of Henle express UMOD. Comparing changes in the expression of different differentiation markers gives an idea of which functional kidney cell types might be affected because of the renal SASP.

4.8 Studying the effect of the renal SASP using conditioned media

To study the effects of the renal SASP, conditioned media were obtained from senescent renal proximal tubular epithelial cells (RPTECs). Proximal tubular epithelial cells are the most prone in the kidney to becoming senescent with aging, as they become exposed to nephrotic compounds. Mirroring cisplatin-induced acute kidney injury (AKI), RPTECs can be made senescent by administration of cisplatin (Figure 6) (27).

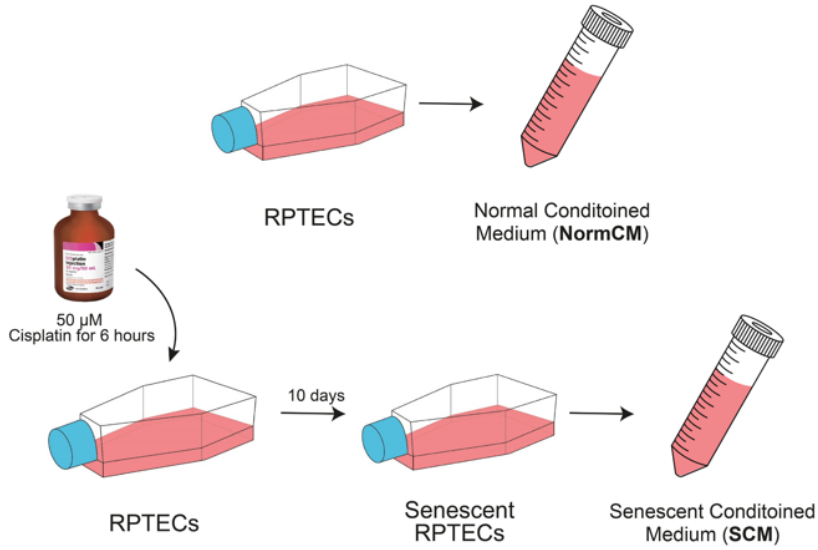


Figure 6: Conditioned media are obtained from Renal Proximal Tubular Epithelial Cells in 2D culture.

Although the exact differences in composition between the various conditioned media are not known, the SCM should be enriched in SASP factors, compared to the NormCM. It should be noted that proliferating RPTECs might consume more nutrients compared to senescent RPTECs, making the SCM more deficient in base medium nutrients. On the other hand, the expression of the SASP also requires the uptake of nutrients.
Despite that the expression of the SASP requires nutrient uptake, proliferating RPTECs likely demand greater nutrients in order to divide, than senescent ones. This potentially results in a higher depletion of base medium nutrients in the SCM. Overall, the exact difference in the levels of base medium nutrients between the SCM and NormCM remains to be clarified.

4.9 Identification of specific SASP components which affect renal differentiation

The secondary aim of this research is to identify the specific individual SASP components responsible for any effects exerted by the SCM on renal differentiation. In the 130 week old mice, that exhibited renal dysfunction due to senescence in the kidney, an increase was observed in interleukin-6 (IL-6) expression (16). This elevation in IL-6 levels was localized to the tubular regions where senescent cells had accumulated, locally extending to the glomerular regions (Figure 7). IL-6 has been recognized as a major component of the SASP (16).

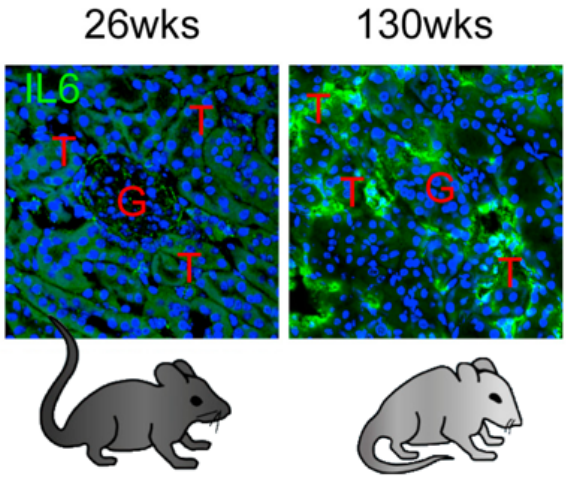


Figure 7: IL-6 expression levels in 26 week and 130 week old mice. *T=tubuli and G=glomeruli. Modified from (16).*

From a small preliminary screen conducted in liver organoids, six interleukins have been identified to exert similar effects as the SCM in maintaining surrounding cells in a progenitor-like state. These interleukins include IL-1α, IL-1ß, IL-4, IL-6, IL-13 and IL-17 (Thomas Brand, data not shown and unpublished). From literature, it is known that some of these interleukins, mainly IL-1α, IL-1ß, and IL-6, are also secreted by senescent cells in the kidney. Therefore, they are proper candidates as renal SASP molecules to study their effect on the differentiation on the kidney (27, 28, 29, 30, 31, 51). Interleukin-induced CD44 expression has already been documented for IL-4 and IL-13, but this has thus far only observed in colonic epithelial cell lines (35).

Altogether, determining whether these individual interleukins have a similar effect on their own as the SCM, would contribute to existing knowledge on the effects of the renal SASP in aging. Additionally, it may reveal new potential therapeutic targets in the case of AKI. Since these interleukins are known to participate in various signaling mechanisms, answering these questions would provide valuable insight into the fundamentals of kidney regeneration, in the context of aging.

5. Results

5.1 Podocyte maturation occurs during development of 3D kidney structures derived from iPSCs

To establish 3D kidney structures, human iPSCs were induced to differentiate towards the kidney lineage using a hybrid differentiation protocol based on studies from Takasato et al. and Morizane et al. (Figure 7A). Within 21 days, these studies have shown that the kidney structures develop the majority of cell types as found in the adult human kidney (22, 23). These include podocytes, which are characterized by increased expression of NPHS1 (Nephrin). During unperturbed differentiation, NPHS1 expression levels increase from day 17 and onwards and reach a peak at day 21 (Figure 7B, D). Furthermore, renal progenitor cells express CD44, which decreases from day 14 to day 21 (Figure 7B, C). Given that the relevant changes for CD44 and NPHS1 occur starting from day 14, this seemed an appropriate time point at which to test the effect of senescent factors on podocyte differentiation.

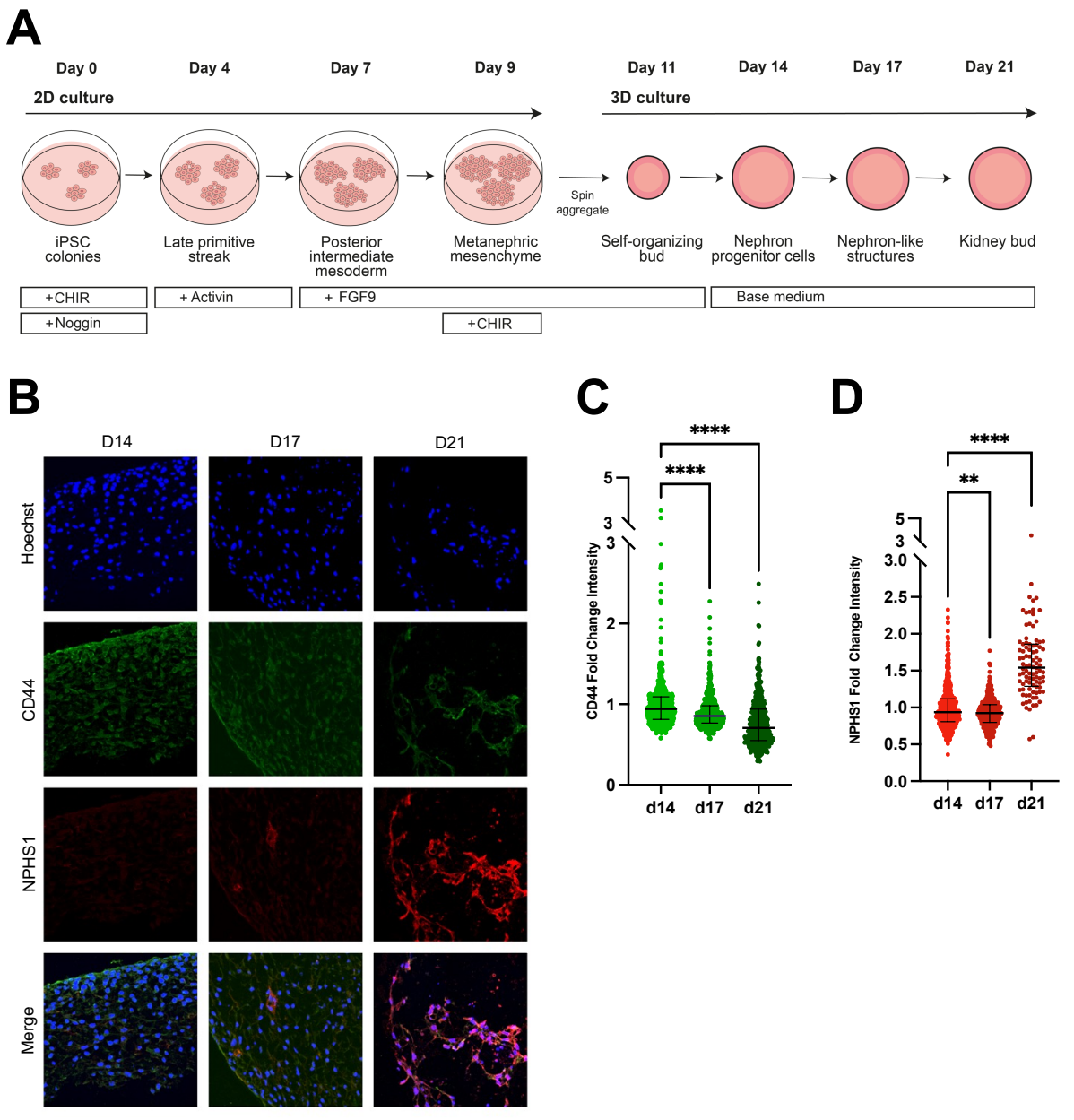
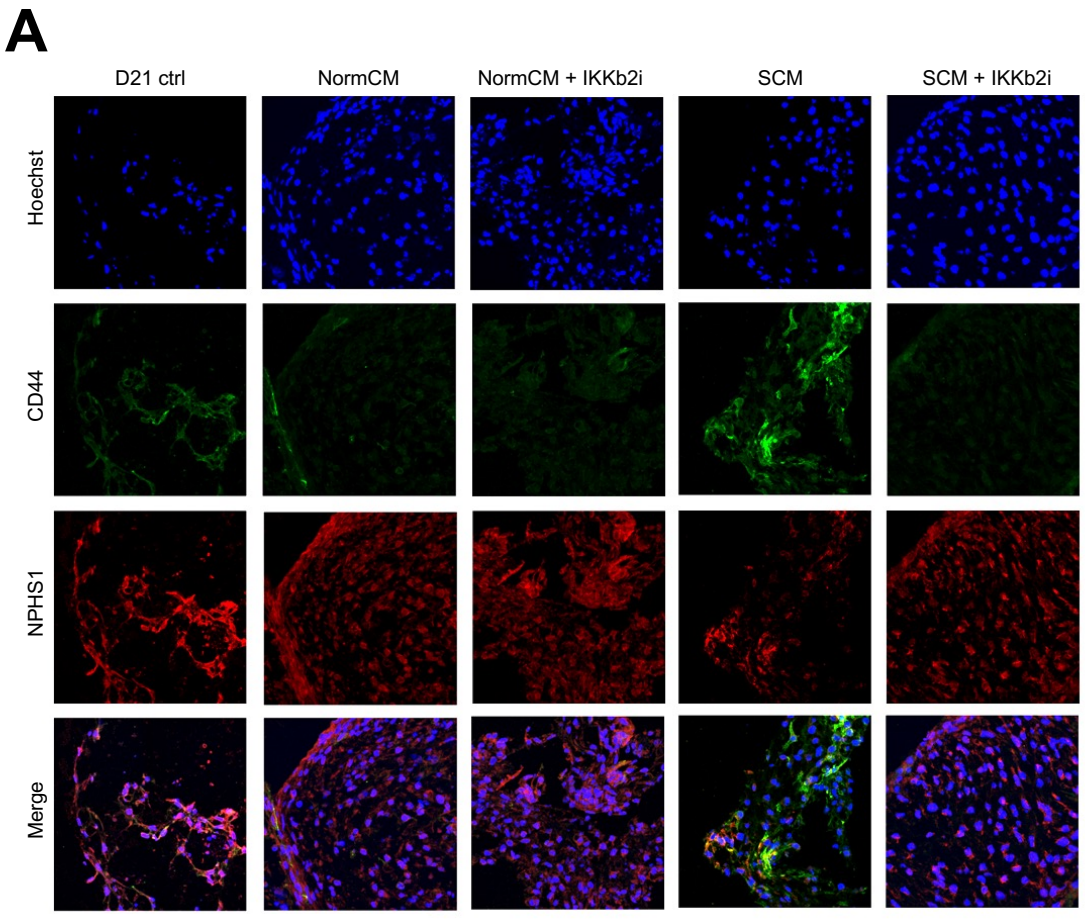


Fig. 7. Expression of CD44 and NPHS1 during unperturbed differentiation of iPSC-derived 3D kidney structures (A) A schematic illustrating each stage of differentiation during development of iPSC-induced 3D kidney structures, along with the growth factors and small molecules necessary for each stage. **(B)** Representative immunofluorescence images showing CD44 and NPHS1 stainings on iPSC-derived 3D kidney structures, at the indicated differentiation timepoints **(C)** Quantification of CD44 expression levels. ****P<0.0001 (one-way ANOVA analysis followed by Bonferroni post-test). **(D)** Quantification of NPHS1 expression levels. ***P<0.001, ****P<0.0001 (one-way ANOVA analysis followed by Bonferroni post-test). Data are presented as mean+ sd. from one experiment using at least two biological replicates per condition.

5.2 Podocyte maturation from renal progenitor cells is hindered by senescent conditioned medium

To investigate the effects of the renal SASP on differentiating kidney cells, conditioned media from either healthy or senescent renal proximal tubular epithelial cells (RPTECs) were added to the kidney structures, starting from day 14 of the differentiation process. First, cisplatin was used to induce senescence in renal proximal tubular epithelial cells (RPTECs) in 2D culture. Previous studies have shown that time-dependent cisplatin treatment of RPTECs induces senescence within 3 days (27). 7 days after cisplatin treatment, the medium conditioned by these senescent RPTECs (SCM) was collected, containing the renal SASP. For control conditions, NormCM was obtained from proliferating RPTECs that were not treated with cisplatin. The conditioned media were added in a 1:1 ratio with normal differentiation media, with or without an IKKß2 inhibitor, which is known to inhibit the NF-κB pathway involved in the appearance of CD44.



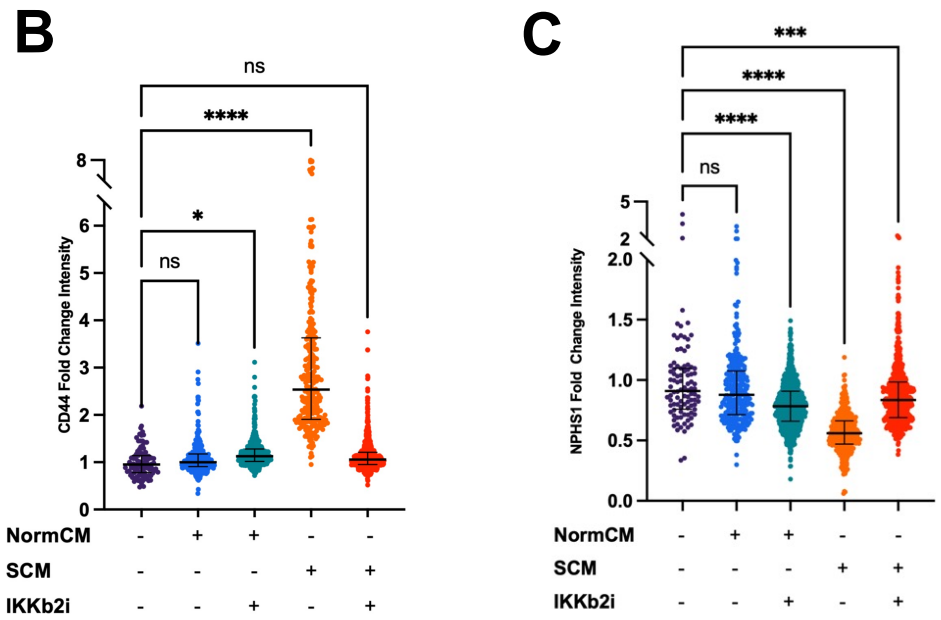


Fig. 8. Expression of CD44 and NPHS1 at day 21 after treatment with conditioned media from day 14. **(A)** Representative immunofluorescence images showing CD44 and NPHS1 stainings on iPSC-derived 3D kidney structures, after treatment with different conditioned media and/or an IKKß2 inhibitor (IKKß2i) from day 14. **(B)** Quantification of CD44 expression levels. ns: not significant, *P<0.05, ****P<0.0001 (one-way ANOVA analysis followed by Bonferroni post-test). **(C)** Quantification of NPHS1 expression levels. ns: not significant, ***P<0.001, ****P<0.0001 (one-way ANOVA analysis followed by Bonferroni post-test). Data are presented as mean+ sd. from one experiment using at least two biological replicates per condition.

There was a significant increase in the expression of CD44, indicative of renal progenitor cells (Figure 8B). This increase was not observed when the SCM was added from day 17, instead of from day 14 (Suppl. Fig 1A). The expression of NPHS1, indicative of podocytes, was reduced to half the expression levels of the control conditions (Figure 8C). When the SCM was added in combination with the IKKß2i, there was no alteration in CD44 expression and a slighter decrease in NPHS1 expression compared to SCM only, indicating a significant rescue effect. When the SCM and IKKß2i were added from day 17, instead of from day 14, this rescue effect was less significant (Suppl. Fig 1B). Cells that were high in the expression of CD44 were, either in proximity to, or overlapping with the cells that expressed lower NPHS1, which suggests podocytes might still be in a transitional phase from renal progenitor cells.

5.3 Renal progenitor cells can still differentiate to cells op the Loop of Henle in presence of senescent conditioned medium

UMOD (Uromodulin) is expressed by renal tubular cells which line the ascending limb of the loop of Henle. In iPSC-induced 3D kidney structures, UMOD expression is known to increase in these cells around day 21 of differentiation (Androniki Orfanidou, data not shown) (22, 23). Immunofluorescence images showed that the kidney structures treated with SCM from day 14 did not differ in UMOD expression levels at day 21, though again renal progenitor cells expressing CD44 were kept high (Figure 9A). CD44 expression in these kidney structures increased twofold, except in the presence again of the IKKß2i (Figure 9B). UMOD expression levels were similar in the different treatment conditions, indicating differentiation of tubular loop of Henle cells was not affected by the SCM (Figure 9C).

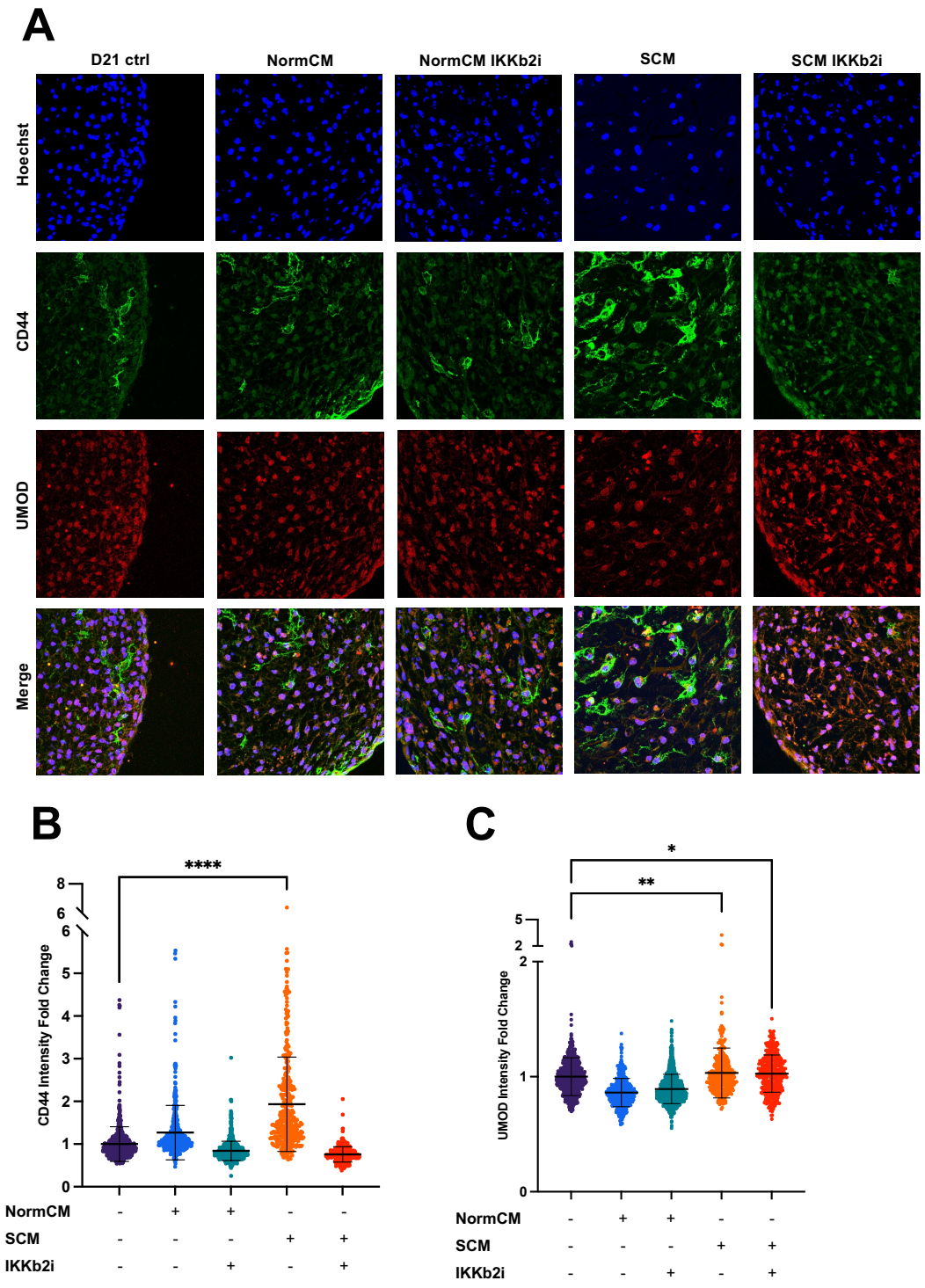
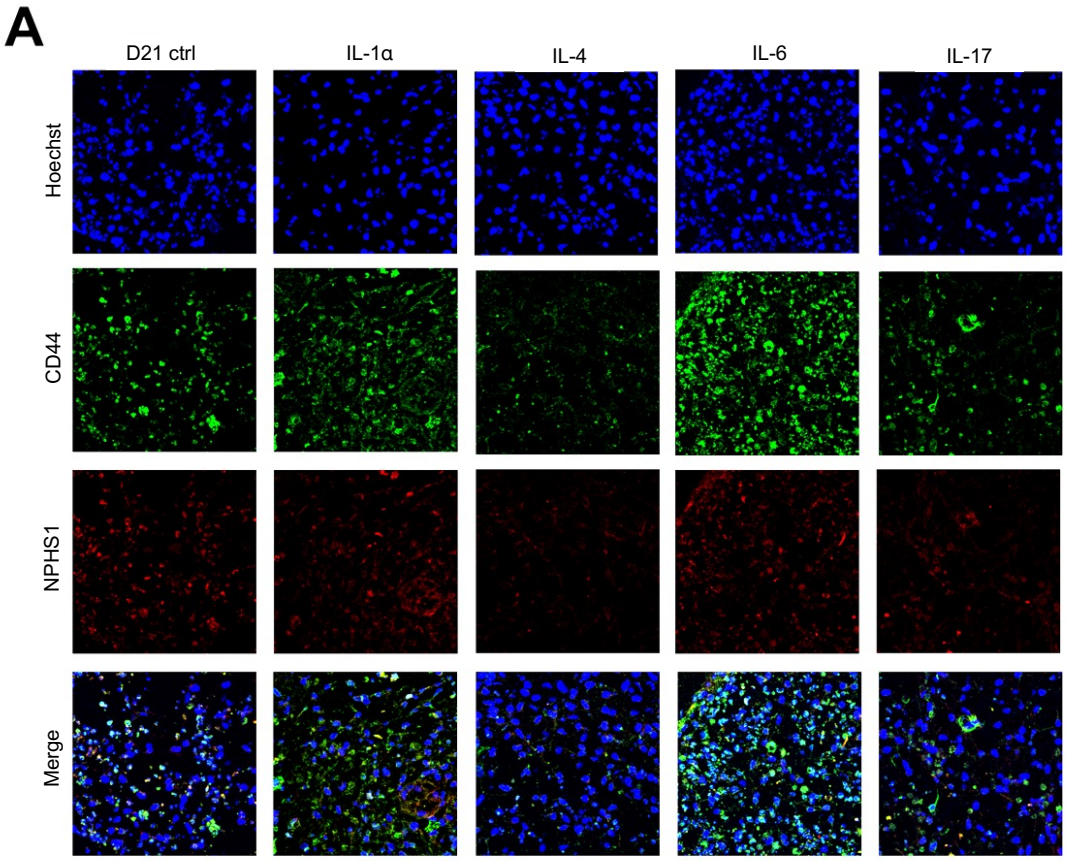


Fig. 9. Expression of CD44 and UMOD at day 21 after treatment with conditioned media from day 14. (A) Representative immunofluorescence images showing CD44 and UMOD stainings on iPSC-derived 3D kidney structures, after treatment with different conditioned media and/or an IKKß2 inhibitor (IKKß2i) from day 14. **(B)** Quantification of CD44 expression levels. ****P<0.0001 (one-way ANOVA analysis followed by Bonferroni post-test). **(C)** Quantification of UMOD expression levels. *P<0.05. **P<0.01 (one-way ANOVA analysis followed by Bonferroni post-test). Data are presented as mean+ sd. from one experiment using at least three biological replicates per condition.

5.4 Podocyte maturation is slightly hindered by SASP-associated interleukins

From a small screen, 6 interleukins associated with the SASP had already been shown to maintain high CD44 expression during the differentiation process of liver organoids. After addition of a concentration between 50-1000 ng/mL of IL-1α, Il-1ß, IL-4, IL-6, IL-13 and IL-17, the liver organoids showed hepatocyte de-differentiation (Thomas Brand, unpublished and data not shown). Some of these interleukins, including IL-1α, IL-1ß, IL-6, and IL-17, have previously been reported to be secreted by senescent cells in the kidney in other studies (16, 28, 29). Altogether, this raised the question whether these interleukins could singlehandedly induce a similar effect to the SCM in the kidney structures. The interleukins were added during the medium changes on day 14, and 17 and left for three days. Due to technical difficulties with the 6 wells plate containing the IL-1ß and IL-13 treated kidney structures, data on these treatment conditions could not be obtained and are therefore not visualized. Immunofluorescence images showed that the single interleukins did not appear to maintain high CD44 expression upon full differentiation of the kidney structures (Figure 10A). CD44 expression was slightly, but significantly, higher in IL-1α and IL-6 treatment conditions. CD44 expression was significantly decreased in IL-4 and IL-17 treatment conditions (Figure 10B). IL-1α and IL-6 treatments thus resulted in a slight maintenance of the progenitor-like state in renal progenitor cells. NPHS1 expression showed a significant decrease in all single interleukin treatment conditions. The strongest decrease was observed when the kidney structures were treated with IL-4, IL-6 and IL-17, suggesting a stronger inhibition of podocyte maturation (Figure 10C).



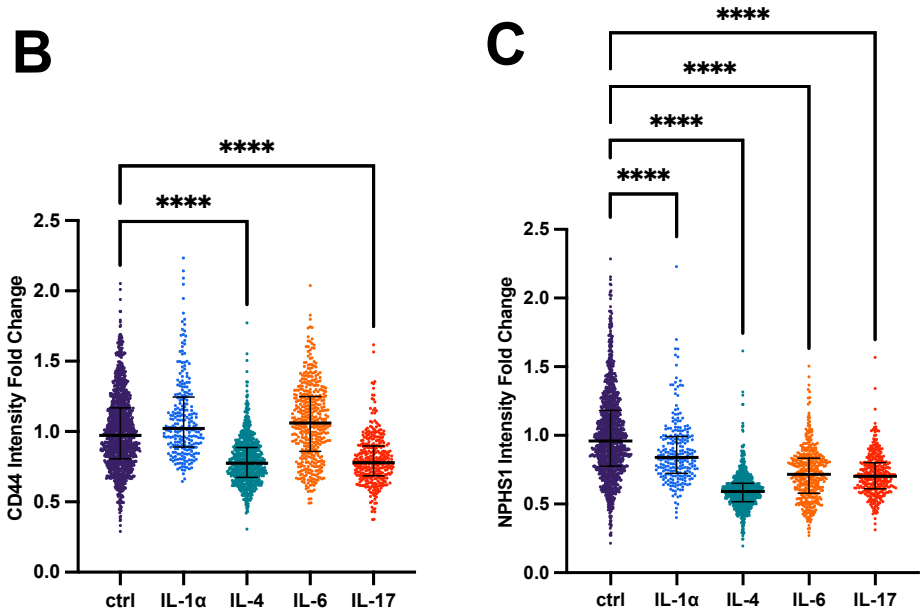


Fig. 10. Expression of CD44 and NPHS1 at day 21 after treatment with single interleukins, associated with the renal SASP. (A) Representative immunofluorescence images showing CD44 and NPHS1 stainings on iPSC-derived 3D kidney structures, after treatment with different single interleukins from day 14**. (B)** Quantification of CD44 expression levels. ns: not significant, ****P<0.0001 (one-way ANOVA analysis followed by Bonferroni post-test). **(C)** Quantification of NPHS1 expression levels. ns: not significant, ****P<0.0001 (one-way ANOVA analysis followed by Bonferroni post-test). Data are presented as mean+ sd. from one experiment using at least three biological replicates per condition.

5.5 Erythropoietin secretion levels by kidney structures remain minimal in the presence of senescent conditioned medium

Human Erythropoietin (EPO) levels were quantified in the culture media of 3D kidney structures between different medium changes: days 11-14, days 14-17, and days 17-21 (Figure 11). Along, either the SCM or NormCM was added during these timepoints, to assess whether the renal SASP influences EPO production, which is an important function of mature kidney cells. The analysis revealed minimal EPO concentrations, with no significant variation observed across the different conditions. This suggests that there was no production of EPO during these stages of differentiation, neither in the control nor in the experimental conditions.

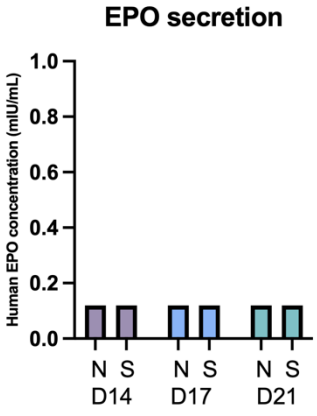


Fig. 11. Human EPO concentrations in culture media of 3D kidney structures. Measured EPO concentrations in the medium from day 11 to day 14 (D14), from day 14 to day 17 (D17), and from day 17 to day 21 (D21). Comparison between Normal Conditioned Medium (N) and Senescent Conditioned Medium (S). Data are presented from one experiment with two samples per condition.

6. Discussion

Life expectancy is increasing worldwide, and along with it rises the prevalence of CKD. There are no effective treatment options available for CKD, nor biomarkers for its early detection. This issue underscores the importance of fundamental research focusing on the mechanisms of decreased repair capability in the kidney after damage, particularly in the context of aging (32). At the cellular level, a knowledge gap remains regarding how senescent cells contribute to age-related maladaptive repair and why this is rescued when these cells are targeted using senescence-targeting.
iPSC-derived kidney structures are a promising model for studying human renal differentiation processes in health and disease, as they allow real-time communication to take place between the differentiating cells in a 3D environment (22, 23). The primary aim of this study was to elucidate how the renal SASP affects the differentiation of kidney cells in a 3D model and which specific SASP molecules might be able to affect renal differentiation on their own.

6.1 The SASP is able to maintain renal progenitor cells in their undifferentiated state

Our data suggest that the secretion of SASP molecules from senescent RPTECs impairs full differentiation of functional kidney cells, by keeping them in a progenitor-like state. Earlier studies had suggested that when senescent cells are only transiently present, the SASP can promote processes of regeneration (15). This is considered beneficial because the regenerated cells can then replace the senescent cells, after clearance by immune cells. However, in case of chronic secretion of the SASP, it has been theorized that a regenerative block can occur, as the dedifferentiated cells do not differentiate back into functional cells (15). The thought that long-term exposure to the SASP impairs regeneration could potentially explain why tissues lose regenerative capacity over time in aging. The findings of this research align with this overarching theory, as the SCM was able to maintain progenitor-like cells in what should have been fully differentiated kidney structures, while it hindered podocyte development. Although the renal SASP seems to be able to maintain cells in a progenitor-like state, it's important to note that if progenitor-like cells aren't initially present, the renal SASP can likely not induce the initial dedifferentiation process towards renal progenitor cells. This is evident from the supplementary evidence, which shows CD44 expression did not increase when the SCM was added starting from day 17.

6.2 The SASP hinders full differentiation of podocytes

In this study we provide evidence that the renal SASP hinders full differentiation of renal progenitor cells, specifically towards podocytes. The reduced expression of NPHS1 at day 21 of differentiation indicates that podocyte number was likely to be reduced. Moreover, the double immunostaining with CD44 showed that the glomeruli-like structures contained cells that were double positive, likely to be in a transitional phase, as well as cells that were positive for either CD44 or NPHS1. This suggests that differentiation of podocytes is withheld to some extent, preventing completion of the process. As UMOD expression levels remained the same in all conditions, the renal SASP likely does not seem to affect the differentiation process of cells that differentiate towards cells of the Loop of Henle.

In the context of regeneration in the adult kidney, our findings suggest that podocyte loss may not be properly resolved in presence of chronic SASP secretion. Dedifferentiated epithelial cells could remain stuck in progenitor-like state, as indicated by increased CD44 expression in presence of SCM (Figure 12). A decrease in the number of podocytes would result in a decreased efficiency of the renal filtration barrier, implicating the renal SASP in the progression of CKD.

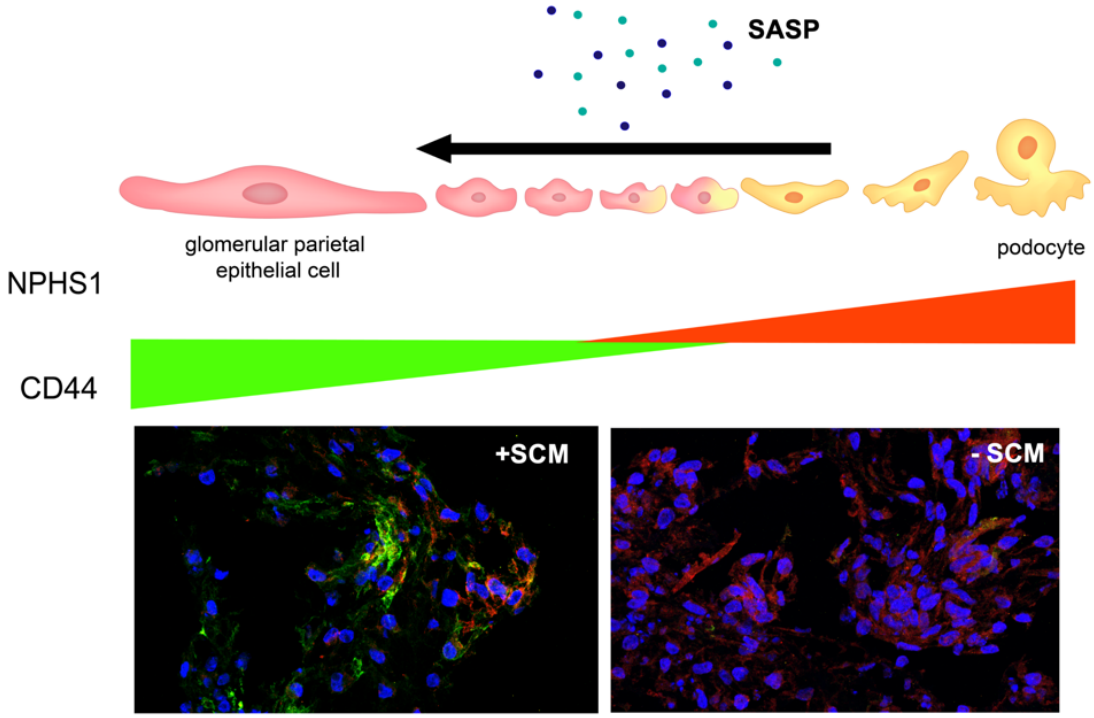


***Figure 12**: Hypothetical model on how the renal SASP might affect the regeneration process of podocytes.*

6.3 Activation of the NF-κB pathway is crucial for the effects exerted by the SASP

The effect of the SASP, hindering efficient differentiation, has only been described as of recently in literature (15). As we observe that this effect does not occur in the presence of an IKKß2 inhibitor, we suggest that it seems to be mediated by the activation of the NF-κB pathway in cells recipient of the SASP (figure 13). Previous studies have highlighted that NF-KB, as a transcription factor, is activated in cells exposed to pro-inflammatory cytokines such as IL-1ß (33). Additionally, it has been demonstrated that TNF-alpha, secreted by macrophages, can induce expression of CD44 in neighboring renal cancer cells through the activation of the NF-κB pathway (34). Our finding that the administration of an IKK inhibitor rescues differentiation of kidney cells, even when exposed to the SASP, links the observations of such studies on the NF-κB pathway. Altogether, these insights provide more clarity into the mechanisms on how senescent cells hinder differentiation and maintain renal progenitor cells in their undifferentiated state.

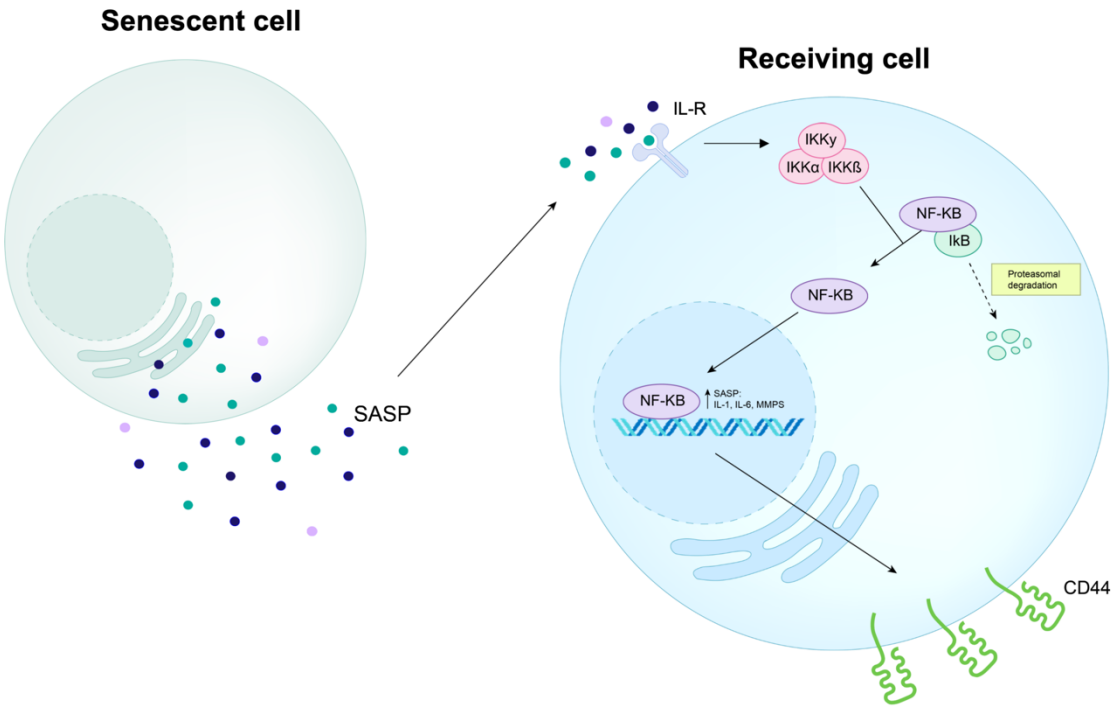


Figure 13: *Proposed mechanism on how the renal SASP induces progenitorness in surrounding cells through activation of the NF-κB pathway.*

6.4 Measuring CD44 expression in iPSC-derived kidney structures to study kidney regeneration

CD44, a typically minimally expressed surface protein in the healthy adult kidney, has been observed to be transiently upregulated during nephrogenesis in mice, as well in the adult kidney post-AKI and in various renal diseases in humans. Through lineage tracing techniques, it has been found that parietal epithelial cells in the kidney undergo dedifferentiation upon ischemic reperfusion injury, subsequently differentiating into new proximal tubular epithelial cells. During this regenerative process, the parietal epithelial cells express CD44 and adopt a proliferative progenitor-like phenotype (36). A similar mechanism involving CD44-positive parietal epithelial cells has been described for the regeneration of podocytes after injury (37). Regenerating glomerular parietal epithelial cells, that normally differentiate into new podocytes after injury in the adult kidney, might be hindered from full differentiation in case of chronic SASP. This could explain why damaged glomeruli are a common phenomenon in CKD patients which have undergone chemotherapy in the past.

Furthermore, a study on lupus nephritis revealed that cytokine release, specifically IL-1, TNF-alpha and IFN-y induced an increase in CD44 expression in tubular epithelial cells and glomerular cells. In contrast, only weak expression of CD44 was observed in the healthy kidney and this was mainly in the interstitial cells (38). These findings are consistent with our observations in the 3D kidney structures, indicating that the renal SASP, primarily composed of pro-inflammatory cytokines, impairs the differentiation of podocytes.

Another study proposed that CD44-positive renal progenitor cells are non-functional if they persist but have the potential to evolve into cancer stem cells upon dysregulation or mutation. This is because CD44+ positive cells have also been found in kidney tumor lesions (39). It could provide a reason why senescence, although tumor suppressing in the short term, can contribute to cancer formation in aging.

It is debatable whether the effect the SASP has on CD44 expression, as observed in the iPSC-derived kidney structures, would also occur during regeneration processes in the adult kidney. iPSC-derived kidney structures more closely recapitulate nephrogenesis rather than kidney regeneration. For the direct study of renal tissue regeneration in a 3D model, tubuloids have been preferred over iPSC-derived kidney structures (40). However, these structures lack podocytes, endothelial cells, and interstitial cells and therefore are less representative of the anatomical complexity of the adult kidney compared to iPSC-derived kidney structures. One might also wonder, why not directly differentiate iPSCs towards glomerular structures, to only study their process of differentiation. Although there are protocols for generation of iPSC-derived glomerular cells, most of these also lack internal vascular endothelial cells and other cell types found in the adult kidney (41).

Nonetheless, studying renal differentiation in nephrogenesis may still provide valuable insights into the mechanisms underlying regeneration in the adult kidney. The signaling cascades involved in the stages between progenitor cell recruitment, followed by proliferation, also play a role in nephrogenesis during embryonic development.
Several genes encoding components of the Wnt and BMP pathways are activated in the adult kidney after IRI-induced AKI in rats, as well as in the development of iPSC-derived kidney structures, in which they are induced by the Wnt-activator Chiron and BMP-activator Noggin (42, 43, 22, 23). The effects observed in the iPSC-induced kidney structures in our study may thus help decipher a mechanistic role of the SASP in hindering regenerative events in the adult kidney, by maintaining temporally intended signaling pathways activated in a chronic manner (44).

6.5 Single SASP-associated interleukins impair podocyte differentiation but are insufficient to maintain renal progenitor cells in an undifferentiated state

Throughout the differentiation process of iPSC-derived kidney structures, the individual interleukins tested in this study, which have been associated with the renal SASP and shown to induce stemness in liver organoids, were not able to maintain renal progenitor cells in an undifferentiated state on their own. On the other hand, single administration of each interleukin tested slightly impaired differentiation of podocytes. Prior studies had already shown that IL-4 signaling can lead to podocyte damage (45). On the other hand, another study found that low-dose administration of IL-17 could prevent the loss of tubular epithelial cells and podocytes, suggesting that IL-17 secretion could be beneficial for regeneration. However, the dose administered in that study was much lower (10 ng/animal) compared to the doses used in this study, indicating that lower, more physiological secretion levels confer benefits in regeneration (46).

Moreover, administering different combinations of interleukins could offer insight into potential synergistic effects or other forms of signaling interplay that may influence differentiation. For instance the combination of IL-1α and IL-6, as they both are slightly able to maintain a progenitor-like state in renal progenitor cells and hinder podocyte maturation.

Lastly, while single interleukins were able to keep liver organoids in an undifferentiated state, it should be noted here that the regenerative potential of the kidney is much lower compared to that the liver. Therefore, kidney cells may require additional stimuli for regeneration (47).

6.6 Therapeutically targeting senescence to rescue renal regeneration

Currently, various forms of research are being conducted in the field of developing and testing senolytic compounds, which target senescent cells, as well as studies focused on preventing the onset of chronic kidney disease (CKD) from acute kidney injury (AKI) using different compounds. Our findings may shed light on the fundamental mechanisms to which extent some of these compounds may be beneficial to overcome aging-related maladaptive repair.

In a study in mice, it was found that the administration of N-acetylcysteine, an antioxidant, ameliorated cisplatin-induced renal senescence and fibrosis by activating sirtuin-1 (SIRT-1) and deacetylating p53 (27). This resulted in a decrease in senescent cells in the kidney and decreased levels of IL-1ß and IL-6. As our study has shown, these interleukins, mainly as part of the renal SASP, also hinder podocyte maturation in 3D kidney structures, which suggests that the described rescue of kidney function by N-acetylcysteine may in part also be explained by the prevention of podocyte loss after regeneration. Further studies are required to find out whether podocyte maturation can be rescued using N-acetylcysteine, in case of exposure to the renal SASP. The described amelioration of cisplatin-induced senescence by N-acetylcysteine appeared to be dependent on the activation of SIRT-1, particularly through the sirtuin-1/p53 axis. As SIRT-1 is also an upstream inhibitor of NF-κB, it implies that the rescue of kidney function by N-acetylcysteine in this study may also involve the inhibition of NF-κB in recipient cells of the SASP (27). In another study, involving mice which were subjected to IRI, it was found that N-acetylcysteine enhanced the progression of AKI to CKD, because it promoted metabolic dysfunction and dampening of necessary antioxidant responses. Taken together, these findings cast doubt on the suitability of N-acetylcysteine for further exploration as a therapeutic target, especially in the context of IRI-induced renal senescence (48).

Moreover, the use of senolytic compounds that directly target senescent cells holds the greatest promise in preventing the detrimental effects of senescent cells in CKD progression. The FOXO4-DRI peptide is able to selectively target senescent cells for apoptosis in the kidneys of mice with accumulated senescence. It has also been shown to decrease the secretion of IL-6, a prominent SASP molecule, while simultaneously maintaining renal tissue homeostasis. The kidneys of mice treated with this peptide maintained their weight after targeted apoptosis of senescent cells, suggesting that regenerative processes might have been able to take place (16). Another study in which the FOXO4-DRI was administered to mice, suggested that it is indeed able to reduce kidney fibrosis, though it did seem able to prevent tubular damage. Nonetheless, it would be interesting to determine whether the FOXO4-DRI peptide can still prevent podocyte damage, as apoptosis of senescent cells may prevent the podocyte differentiation-impairing effects exerted by the renal SASP (49).

6.7 Future aims

To characterize the composition of the renal SASP, a mass spectrometry experiment on the SCM could reveal which interleukins are elevated. Through ELISA experiments, no remarkable elevation of any interleukin was observed in the SCM, likely due to technical difficulties as all measured levels were nearly below detection limit. Interference by other components present in the SCM might potentially have affected antibody binding, resulting in false-negative results. Mass spectrometry could overcome this issue, as it does not rely on specific antibodies and can measure multiple interleukin concentrations simultaneously.

Although the SCM obtained from senescent RPTECs may share similarities with the renal SASP, there are limits to its use as its composition is not clear. To more accurately study what the effect is of SASP release in the kidney, it would be intriguing to induce endogenous senescence locally in the iPSC-derived kidney structures. This would also enable paracrine senescence to occur during differentiation. A potentially suitable approach for this could be through the use of iPSCs that contain the engineered H2B-DAAO construct. This construct enables intracellular H_2O_2 production localized to the nucleosomes. In the differentiated 3D kidney structures derived from iPSCs containing this construct, administration of a specific concentration of D-alanine could then induce senescence locally in the 3D kidney structures, resembling to some extent IRI-induced senescence (50). Furthermore, it would be interesting to investigate whether the SASP secreted from these intracellular H_2O_2-induced senescent cells elicits similar effects on the surrounding tissue as the SASP of the cisplatin-induced senescent RPTECS. If the effects differ depending on the inducer of renal senescence, it would add onto the knowledge of the fundamental characteristics that contribute to the heterogeneity of the renal SASP.

Altogether, the technique involving the H2B-DAAO construct requires more optimization before performing SASP-related experiments. Mainly on how to induce a specific percentage of senescence at the fully differentiated state of the 3D kidney structure, as well as on how to confirm that the cells positive for the DAAO-construct actually have become senescent. Ultimately, this model could be implemented to study the local effects of the SASP on kidney differentiation, with the potential of targeting senescent cells in the structures to assess the reversibility of the observed effects.

6.8 Conclusion

In conclusion, our study reveals new insights into the mechanisms of senescence-associated stemness in the kidney, as the SASP from senescent RPTECs hinders podocyte differentiation in iPSC-derived 3D kidney structures and keeps renal progenitor cells in an undifferentiated state. These effects are dependent on the activation of the NF-κB pathway in the cells recipient of the SASP. Additionally, single interleukins associated with the renal SASP are insufficient to induce a similar effect on their own, although they slightly impair the differentiation process of podocytes. This research illuminates a novel role of the SASP in regeneration, offering valuable insights for the identification of potential therapeutic targets in case of AKI. Senescence-targeting peptides like FOXO4-DRI could mitigate this effect of the SASP and hold promise in preventing early chronic kidney disease in elderly patients, post-cisplatin cancer treatment.

7. Materials and Methods

Passaging and maintaining iPSCs
Induced Pluripotent Stem Cells (iPSCs) were cultured in E8 complete (E8C) media, which consisted of 1X DMEM/F12 Medium (Gibco, #224260), sodium bicarbonate (543 µg/mL),1% penicillin-streptomycin, insulin-transferrin-selenium (ITS) (1X) (ThermoFisher, Ref #4140045), 2-Phospho-L-ascorbic acid (ASA) (64 µg/mL) (Sigma-Aldrich, Ref#49752-10G), Fibroblast Growth Factor 2 (FGF2) (100 µg/mL) (Immunotools, Ref #11343627), heparin (1 µg/mL) (Sigma-Aldrich, #H5515-25KU) and Transforming Growth Factor beta-1 (2 ng/mL) (Immunotools, Ref#11343161). iPSCs were passaged in 6-well tissue culture plates, pre-coated for 20 minutes with Vitronectin (500 µM) (ThermoFisher, Ref #A14700). E8C culture medium was refreshed daily. iPSCs were passaged upon 70% confluency, by dislodging them as cell clumps and discarding medium after adding EDTA-BPS for 5 minutes. This was done in a ratio of 1:6 or 1:8, twice a week.

iPSC-Derived 3D Kidney Structure Differentiation
iPSCs were differentiated to develop 3D kidney structures using a hybrid differentiation protocol, based on the ones developed by Takasato et al. and Morizane et al. Both of these studies have validated that the iPSCs undergo differentiation processes similar to nephrogenesis and that they ultimately contain among proximal and distal tubular epithelial cells, podocytes, endothelial cells and mesenchymal stromal cells. The differentiation process was initiated once the iPSCs showed 25% confluency, had well-defined borders and when there were no visible differentiated cells present. The entire differentiation process took 21 days and media changes took place at days 4, 7, 9, 11, 14 and 17. The Morizane-Takasato Base medium consisted of E8 Base (E8B) medium with additional factors depending on the day of medium change. From day 0 to day 4, Chiron (3 µM) and Noggin (5 ng/mL) were added to the medium. From day 4 to 7, Activin (5 ng/mL) was added. From day 7 to 9, Fibroblast Growth Factor 9 (FGF9) was added. At day 9, the cell aggregates were centrifuged at 250 g for 1 minute, counted using a Countess Autuomated Cell Counter and transferred to 3D NunclonSphera plates, at 100.000 cells per well. From day 9 to day 11, Chiron and FGF9 were added. From day 11 to day 14, only FGF9 was still added. From day 14, the medium did not contain any additional factors.

Treatment of 3D Kidney Structures with Conditioned Media
Renal Proximal Tubular Cells (RPTECs) (Sigma Aldrich, Ref#MMTOX1030 – 1VL) were cultured in 1X MEM Alpha medium (Gibco, Ref#2288910) with the addition of an RPTEC complete supplement (Sigma-Aldrich, Ref#MTOXRCSUP-30ML). RPTECs were made senescent by treatment with cisplatin (50µM) for 6 hours. Senescent conditioned medium (SCM) was obtained after 10 days of senescent RPTECs culture. Normal Conditioned Medium (NormCM) was obtained 24 hours after refreshing unperturbed RPTECs. During differentiation, 3D kidney structures were cultured with NormCM or SCM in a 1:1 ratio with the normal MTB medium, from day 14 and day 17 of the differentiation process.

Treatment of 3D Kidney Structures with single interleukins
3D kidney structures were treated separately with MTB media supplemented with single interleukins (200 ng/mL) from Immunotools. These included IL-1α (#11349013), Il-1ß (#11340013), IL-4 (11340043), IL-6 (#1130064), IL-13 (#11340133) and IL-17 (#11340174).

Use of IKKβ2 inhibitor
An IKKβ2 inhibitor (IKK inhibitor IV, CAS 507475-17-4, Santa Cruz Biotechnology) was added to 3D kidney structures at 1 µM, to inhibit IκBα phosphorylation as part of the NF-κB pathway.

Fixation of 3D Kidney Structures
On day 21 of differentiation, 3D Kidney Structures that were treated in the same conditions were transferred into 15 mL tubes with PBS. The kidney buds were then fixed with 3,7% formaldehyde diluted in PBS (pH 7.0), at 4 ºC. After fixation, the formaldehyde dilution was discarded and the structures were washed with TBS. After washing, the structures were quenched for 10 minutes with glycine (50 mM in TBS). The glycine was then discarded and the structures were kept in TBS. Finally, the tubes containing the structures were sealed with parafilm for storage at 4 ºC.

Agarose embedding, paraffin blocks and tissue sections acquisition
After fixation, the 3D kidney structures were washed twice in PBS and transferred to 70% ethanol. The samples were then dehydrated with the use of a tissue processor (Leica). The kidney buds were embedded in paraffin blocks. Paraffin sections were cut at 4 µm and deparaffinized.

Immunocytochemistry
To assess the expression of kidney progenitor and differentiation markers, immunocytochemistry (ICC) staining was performed on the kidney buds. Paraffin slides were rehydrated by dipping them two times for 3 minutes in 100% xylene, two times for 3 minutes in 96% ethanol, 3 minutes in 80% ethanol, 3 minutes in 70% ethanol and then in distilled water. Heat antigen retrieval was carried out at just below 100 ºC for 20 minutes, using a microwave. The antigen-retrieval buffer consisted of 1 mM EDTA in,50 mM TBS, at pH 9.4. The slides were allowed to cool in distilled water at RT. Then, the slides were blocked for 30 minutes in blocking buffer containing 2% horse serum, 0.1% fish gelatine and 2% BSA (in 50 mM TBS at pH 7.0). After blocking, the slides were incubated with the primary antibody (in blocking buffer) for 2 hours at RT. After discarding the primary antibody solution, the slides were incubated in TBS (50 mM) for 5 minutes at RT. Then, the secondary antibody (in blocking buffer) was added for incubation for 1 hour at RT. After incubation, ProLong Glass was used for permanent mounting.

Primary antibodies used were mouse anti-CD44 (1:100) (#156-3C11, Cell Signaling), sheep anti-NPHS1 (1:300) (#AF4269, R&D systems) and rabbit UMOD (1:1.000) (HPA043420, Sigma Aldrich). Secondary antibodies used were goat anti-Mouse Alexa FluorTM 488 (1:600) (#A11031, Invitrogen), donkey anti-Sheep Alexa FluorTM 568 (1:600) (A21099, Invitrogen) and goat anti-Rabbit Alexa FluorTM 568 (#A78955, Invitrogen). For nuclear staining, Hoechst 33342 (2.5 µg/mL) (Thermo Fisher) was used.

Erythropoietin ELISA
Erythropoietin levels were measured in the medium that the 3D kidney structures were incubated in from day 17 to day 21 of differentiation, following standard protocols of Human Erythropoietin/EPO DuoSet ELISA by R&D Systems, Inc. (Minneapolis, USA). EPO concentrations were quantified by measuring optical density at an absorbance wavelength of 450 nm. The concentration levels were calculated using a Four Parameter Logistic (4PL) regression curve, based on the standards. Each sample was analyzed in duplicate.

Interleukin ELISA
Single interleukin levels were measured in the NormCM and the SCM, following standard protocols of Human IL-1α DuoSet ELISA (#DY200), Human IL-1ß DuoSet ELISA (#DY201), Human IL-4 DuoSet ELISA (#DY204), Human IL-6 DuoSet ELISA (#DY206), Human IL-13 DuoSet ELISA by R&D Systems (#DY213), Inc. (Minneapolis, USA). Interleukin concentrations were quantified by measuring optical density at an absorbance wavelength of 450 nm. The concentration levels were calculated using a Four Parameter Logistic (4PL) regression curve, based on the standards. Each sample was analyzed in duplicate.

Confocal imaging and image processing
3D kidney structures were imaged on an LSM880 confocal microscope (Zeiss). For imaging Aexa488, excitation was set at 488 nm and emission measured between at 493 and 558 nm. For Alexa568, excitation was set at 568 nm and emission measured between 638 and 744 nm. All images were taken at a magnification of 40x. Z-Stacks of the images were made using FIJI. Mean signal intensity (Distance – N) was measured per cell using Cellprofiler-2. Fold changes in intensity were calculated based on control signal intensities.

Statistical Analysis
GraphPad Prism Pro 9.0 was used to perform one-way ANOVA and Bonferroni post-tests on the measured signal intensities.

8. Acknowledgements

I extend my sincere gratitude to everyone in the De Keizer group who have contributed to the completion of this research.

Foremost, to Dr. Peter de Keizer for granting me the opportunity to work on this project and for including me in the lab's weekly meetings. These sessions have been invaluable in shaping my understanding of how to actively participate in fundamental research endeavors, in this case focused on senescence.

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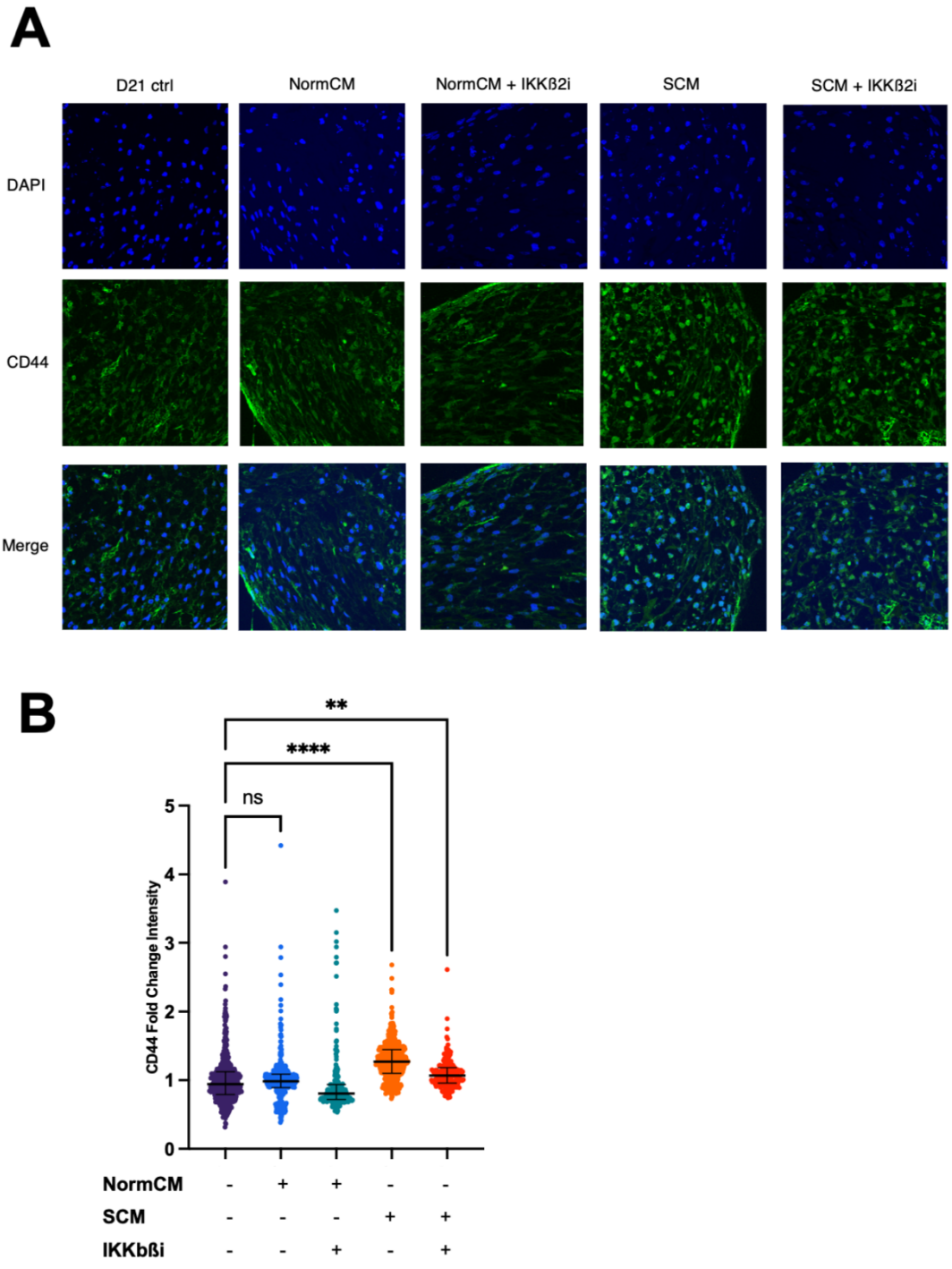
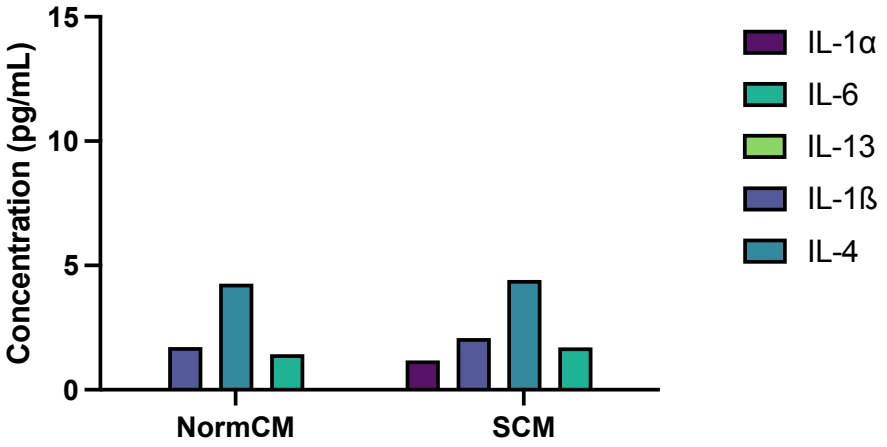
10. Supplementary data



Suppl. Fig 1. Expression of CD44 at day 21 after treatment with conditioned media from day 17.
(A) Representative immunofluorescence images showing CD44 staining on iPSC-derived 3D kidney structures, after treatment with different conditioned media and/or an IKKß2 inhibitor (IKKß2i) from day 17. **(B)** Quantification of CD44 expression levels. ns: not significant, **P<0.005 ****P<0.0001 (one-way ANOVA analysis followed by Bonferroni post-test). Data are presented as mean+ sd. from one experiment using at least two biological replicates per condition.



Suppl. Fig 2. ELISA of interleukins in the NormCM and the SCM. Measured concentrations of single interleukins in the NormCM and the SCM. Data are presented from one experiment with two samples per condition.