Tubular epithelial progenitors are excreted in urine during recovery from severe acute kidney injury and are able to expand and differentiate *in vitro*

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Background. Acute kidney injury (AKI) is a serious condition associated with chronic kidney disease, dialysis requirement and a high risk of death. However, there are specialized repair mechanisms for the nephron, and migrated committed progenitor cells are the key players. Previous work has described a positive association between renal recovery and the excretion of tubular progenitor cells in the urine of renal transplant recipients. The aim of this work was to describe such structures in non-transplanted AKI patients and to focus on their differentiation. **Methods.** Urine sediment gene expression was performed to assess which part of the tubular or glomerular segment was affected by injury, along with measurement of neprilysin. Urine output and sediment morphology were monitored, viable hyperplastic tubular epithelial clusters were isolated and characterized by antibody or cultured *in vitro*. These cells were monitored by phase contrast microscopy, gene, and protein expression over 9 days by qPCR and confocal immunofluorescence. Furthermore, UMOD secretion into the supernatant was quantitatively measured.**Results.** Urinary neprilysin decreased rapidly with increasing urinary volume in ischemic, toxic, nephritic, and infection-associated AKI, whereas the decrease in sCr required at least two weeks. While urine output increased, dead cells were present in the sediment along with debris followed by hyperplastic agglomerates. Monitoring of urine sediment for tubular cell-specific gene transcript levels AQP1 and AQP6 (proximal tubule), NSPH2 (podocyte), and SLC12A1 (distal tubule) by qPCR revealed different components depending on the cause of AKI. Confocal immunofluorescence staining confirmed the presence of intact nephron-specific epithelial cells, some of which appeared in clusters expressing AQP1 and PAX8 and were 90% positive for the stem cell marker PROM1. Isolated tubule epithelial progenitor cells were grown *in vitro*, expanded, and reached confluence within 5-7 days,

while the expression of AQP1 and UMOD increased, whereas PROM1 and Ki67 decreased. This was accompanied by a change in cell morphology from a disproportionately high nuclear/cytoplasmic ratio at day 2-7 with mitotic figures. In contrast, an apoptotic morphology of approximately 30% was found at day 9 with the appearance of multinucleated cells that were associable with different regions of the nephron tubule by marker proteins. At the same time, UMOD was detected in the culture supernatant.
Conclusion. During renal recovery, a high replicatory potential of tubular epithelial progenitor cells is found in urine. *In vitro* expansion and gene expression show differentiation into tubular cells with marker proteins specific for different nephron regions.

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Abstract

Background. Acute kidney injury (AKI) is a serious condition associated with chronic kidney disease, dialysis requirement and a high risk of death. However, there are specialized repair mechanisms for the nephron, and migrated committed progenitor cells are the key players. Previous work has described a positive association between renal recovery and the excretion of tubular progenitor cells in the urine of renal transplant recipients. The aim of this work was to describe such structures in non-transplanted AKI patients and to focus on their differentiation.
Methods. Urine sediment gene expression was performed to assess which part of the tubular or glomerular segment was affected by injury, along with measurement of neprilysin. Urine output and sediment morphology were monitored, viable hyperplastic tubular epithelial clusters were isolated and characterized by antibody or cultured *in vitro*. These cells were monitored by phase contrast microscopy, gene, and protein expression over 9 days by qPCR and confocal immunofluorescence. Furthermore, UMOD secretion into the supernatant was quantitatively measured.
Results. Urinary neprilysin decreased rapidly with increasing urinary volume in ischemic, toxic, nephritic, and infection-associated AKI, whereas the decrease in sCr required at least two weeks. While urine output increased, dead cells were present in the sediment along with debris followed by hyperplastic agglomerates. Monitoring of urine sediment for tubular cell-specific gene transcript levels NSPH2 (podocyte), AQP1 and AQP6 (proximal tubule), and SLC12A1 (distal tubule) by qPCR revealed different components depending on the cause of AKI. Confocal immunofluorescence staining confirmed the presence of intact nephron-specific epithelial cells, some of which appeared in clusters expressing AQP1 and PAX8 and were 90% positive for the stem cell marker PROM1. Isolated tubule epithelial progenitor cells were grown *in vitro*, expanded, and reached confluence within 5-7 days, while the expression of AQP1 and UMOD increased, whereas PROM1 and Ki67 decreased. This was accompanied by a change in cell morphology from a disproportionately high nuclear/cytoplasmic ratio at day 2-7 with mitotic figures. In contrast, an apoptotic morphology of approximately 30% was found at day 9 with the appearance of multinucleated cells that were associable with different regions of the nephron tubule by marker proteins. At the same time, UMOD was detected in the culture supernatant.
Conclusion. During renal recovery, a high replicatory potential of tubular epithelial progenitor cells is found in urine. *In vitro* expansion and gene expression show differentiation into tubular cells with marker proteins specific for different nephron regions.

Introduction

Acute kidney injury (AKI) occurs due to various conditions such as sepsis, hypoxia, trauma, and exposure to toxins. A classification into three stages according to KDIGO (KDIGO, 2012) has become established. The most severe AKI-form stage 3 is often associated with the need for renal replacement therapy (RRT) and can lead to persistent chronic kidney disease. However, during an episode of AKI, it is unpredictable whether a patient will recover or develop persistent kidney failure. Chronic kidney disease not only leads to an increased need for dialysis in a number of patients, but also to a dramatic increase in mortality due to associated cardiovascular consequences, and only kidney transplantation (KTX) can provide improvement in this respect. Renal recovery relies upon a local renal epithelial progenitor pool and former studies have shown that appearance of renal epithelial progenitor clusters, called nephrospheres, in urine of kidney transplanted AKI (KTX-AKI) patients indicate recovery (Knafl et al. 2019). Therefore, there seems to be an urgent need to investigate whether nephrospheres can also be found in non-KTX-AKI patients and if so, whether their occurrence is also an indication of recovery in this patient population.

Repair mechanisms are extremely complicated and orchestrated as the biological function of each nephron segment is complex, relying upon the expression of highly specialized ion channels, ion sensing proteins, enzymes, and ciliary motor proteins. These proteins define into which segment of the nephron the cells have differentiated (Abedini et al. 2021; Harari-Steinberg et al. 2020). Therefore, bioengineering of nephrons seems almost impossible due to the detailed and sophisticated architecture of the organ (Harari-Steinberg et al. 2020; Rahman et al. 2020). In the human fetus, nephron development relies on multipotent stem cells and nephron formation completes by the gestational week 34 (Pleniceanu et al. 2018). However, committed progenitors remain interspersed within the nephron epithelia in Baumann's capsule and tubular segments until adulthood (Romagnani & Remuzzi 2013). Renal recovery does not depend on bone marrow-derived stem cells (Duffield et al. 2005), as was assumed in the past, but rather on the replicative potential of these epithelial progenitor cells (Humphreys et al. 2008; Romagnani & Remuzzi 2013). Thereby, after AKI, only renal progenitor cells proliferate and are responsible for cellular regeneration while other tubular cells endoreplicate and become polyploid leading to tubular cell hypertrophy (Lazzeri et al. 2018) in mouse models. It is estimated that both processes are required to ensure renal recovery. In this process, balance is key as exuberant hypertrophy leads to fibrosis and CKD, while enhancement of renal progenitor proliferation has been shown to induce clonal papillary adenoma and renal cell carcinoma (RCC) (Peired et al. 2020). This observation, that AKI may lead to papillary RCC, has also been made in two different human cohorts (Peired et al. 2020; Peired et al. 2021). In accordance therewith, nephrogenic adenomas have been found in KTX patients and it has been shown that they are derived from tubular cells of the renal transplant.

Nevertheless, cellular repair is key after AKI to prevent from the development of CKD. To achieve this, renal progenitors can proliferate and differentiate into different functional segments of the nephron. In this context, hyperplastic tubular epithelial cell clusters together with

apoptotic cells derived from over-proliferation have been shown in a rat model of AKI *in vivo* (Shimizu & Yamanaka 1993) and have also been found in human urine (Nguyen & Smith 2004). As mentioned above, they can cause engraftments in urinary bladder and initiate nephrogenic adenomas (Mazal et al. 2002). In an earlier study, our group was able to isolate hyperplastic clusters of renal progenitor cells from urine of KTX patients with AKI stage 3 and demonstrate that their appearance in urine showed a positive correlation with recovery from AKI, as 100% of all patients with nephrospheres in urine recovered, while only 39.3% without nephrosphere-excretion recovered from AKI (Knafl et al. 2019; Shimizu & Yamanaka 1993). We therefore addressed the question if nephrospheres could also be delineated in non-KTX-AKI patients and whether their presence is also indicative of recovery in this patient population.

In this study, renal progenitors were isolated from human morning urine of patients with recent AKI stage 3 and need for RRT. Kidney-specific gene expression using qPCR, morphological cell monitoring and measurement of urine and serum biomarkers were performed over the period of recovery. In parallel, a tissue culture of isolated tubular cells was established, and the *in vitro* proliferation and differentiation were monitored by protein immunostaining and gene expression.

Materials & Methods

Patient enrollment and sample collection

Patients admitted to the Department of Nephrology presenting with stage III AKI were enrolled on a continual basis after giving oral and written informed consent. The study was approved by the Ethics committee of the Medical University of Vienna under the number 1043/2016. Catheter urine was obtained every morning from the designated port and was immediately processed.

Cell isolation and *in vitro* culture

Morning urine was centrifuged in 50ml conical tubes at 1500 RPM in order to pellet cells. The resultant cell pellet was suspended in tissue culture medium (RPMI 1640 supplemented with 10% fetal calf serum) which was layered onto Ficoll-Paque Plus (GE Healthcare) and centrifuged at 2000 RPM for 20 minutes. The interface was taken off and washed using tissue culture medium. The cell pellet was then resuspended and plated into 12-well plates. The second day the culture medium was replaced with tubular cell culture medium. This type of medium was prepared immediately before cell feeding by combining the tissue culture medium with (30%) ProxUp RPTEC Growth Medium (Evercyte, Vienna Austria). Cell feeding was carried out every second day, thereby dead cells were washed off and taken from the well together with old tissue culture medium.

Preparation of cytoslides

Epithelial cells monolayer was washed twice with PBS and then incubated with trypsin/EDTA over 3 minutes. Trypsin was then inactivated by tissue culture medium and suspended cells were washed with tissue culture medium. One hundred µl of cell suspension was applied into the cytocentrifuge funnel which was spun at 1200 RPM for three minutes. The resultant cytopreparation was air-dried and either immediately stained using a routine H/E staining for visualization of cell density or frozen at -20°C wrapped in aluminum foil.

Immunofluorescence staining

Cytopreparations of *ex vivo* urinary cells and of cultured epithelial cells were fixed in acetone for 5 minutes. A water repellent barrier was drawn around the area where cells had been placed by the cytocentrifuge using a PAP Pen (SCI, science service, München Germany). Following wetting of the cell containing area with 20µl PBS the antibodies were applied. CD133/1 (Prominin 1), AQP1 (aquaporin 1), PAX8 a kidney specific transcription factor (Kaminski et al. 2016), CD10 (neprilysin). The dilution is described below. Incubation was carried out at room temperature for 2-3 hours under constant shaking in a moist chamber. Before application of the secondary antibodies, slides were washed in PBS for 10 minutes. As secondary antibodies, goat anti-rabbit Alexa Fluor 488 (diluted 1:400) and Rhodamine (TRIC)-conjugated AffiniPur F(ab)2 goat anti-mouse (diluted 1:200) were applied and incubated for one hour under constant shaking at room temperature. As nuclear staining, DAPI/PBS was incubated for 5 minutes before final

washing for 10 minutes twice. Slides were mounted in Vectashield mounting medium for immunofluorescence (Vecotor Laboratories, Burlingham CA) and covered with a coverslip. A Zeiss Axiovert confocal microscope was used for picture recording. Pictures were further processed using Photoshop Version 6.

RNA isolation

The pellet of 7-10ml morning urine was lysed in 1000 µl of Trizol and kept for 10 minutes at room temperature and frozen at -20°C until RNA isolation. Before addition of 200µl chloroform the Trizol lysate was thawed. The content was then vigorously shaken and spun at 12000g for 10 minutes for phase separation. The aqueous phase was pipetted into a separate tube and mixed with 500µl isopropanol. Following an incubation period of 10 minutes at RT tubes were spun at 12000 g for 10 minutes at 4°C. The RNA pellet was washed with 500µl 75% ethanol, and the pellet was shortly dried and finally dissolved in about 20µl RNAse free water. The RNA concentration was evaluated at a Nano drop device.

Reverse transcription and quantitative PCR

Eight hundred ng of RNA was combined with dNTPs and random primers and incubated for 5 minutes at 65°C followed be rapid chilling on ice-water. First stand buffer, DTT, and RNAse OUT and Superscript III was added before increasing incubation temperature to 42°C for cDNA synthesis for 50 minutes. Enzyme activity was stopped by heating the sample at 70°C for 10 minutes. The resultant cDNA concentration was diluted up top 80µl and out of this 2µl were added to the qPCR setup mix. This consisted of TaqMan mastermix and gene specific probe sets. Each sample was set up in duplicate in a final volume of 10µl.

UMOD ELISA from tissue culture

The human uromodulin Elisa was purchased from BioVendor (RD191163200R) and performed as described in the supplied test manual. In brief: tissue culture fluid was applied to the test well accompanied with a standard series as supplied in the test kit. Following 60 minutes of incubation at room temperature under constant shaking the plate was washed three times with wash buffer. Following this, the diluted biotinylated detection antibody was applied to each well and again incubated for 60 mins at room temperature. Following the same washing procedure as above, the streptavidine/HRP was applied to each well and reacted for 30 minutes. After a final washing step, the ELISA was developed with TMB substrate incubating for 10 minutes under light protection. The reaction was stopped using the stop solution and read with an ELISA reader. Concentrations were calculated according to the standard curve.

Neprilysin ELISA using urine

The human neprilysin ELISA was purchased from RayBiotech and performed according to the description in the test manual. In brief: frozen urine was thawed mixed and centrifuged at 3000 RPM before loading. Twenty µl of assay diluent were dispensed in each well before loading 80µl

of urine. Samples from time points with disease maximum had to be diluted up to 1:5 in provided assay diluent. Following 2 hours incubation at room temperature under constant shaking the plate was washed three times with the provided wash buffer. This was followed by incubation with the biotinylated antibody using a dilution of 1:100 in assay diluent. Following an incubation period of 60 minutes and washing the plate three times on the plate washer, the streptavidine/HRP conjugate was dispensed into each well at the recommended dilution of 1:300. Following an incubation period of 45 minutes and washing of the plate (3 times) on the plate washer the antibody reaction was developed with TMB at room temperature under light protection. Following the application of 50µl stop solution the reaction intensity was measured on the ELISA reader and neprilysin concentrations in samples were calculated according to the included standard curve.

Results

The proximal tubular epithelial cell marker protein neprilysin and nephron derived epithelial cell clusters are found in urine of AKI stage 3 patients during recovery

On the first day that patients resumed urine production after anuria, urine collection was performed with morphological examination of urine sediment and measurement of gene expression. Morphological follow-up pictures of the urinary sediment are shown from a patient with toxic AKI in **Fig. S1** and one with infection-associated AKI in **Fig. S2**. During the initial phase of renal recovery, patients with AKI of different etiologies (ischemic, toxic, nephritic, and infectious AKI) experienced a steady increase in urine output accompanied by decreasing urinary excretion of the cell injury marker neprilysin (**Fig. 1A, 1C, 1E, 1G**). The decrease in serum creatinine (sCr) showed a delay of about five days compared with urinary neprilysin as marker of tubular cell damage (Bernardi et al. 2021; Knafl et al. 2017; Pajenda et al. 2017). Peak urinary neprilysin levels were significantly higher in infection-associated AKI (**Fig. 1G**). Renal recovery in terms of sCr levels and time was comparable among all four types of AKI.

In addition, RNA was extracted from 10ml of urine sediment, reverse transcribed and analyzed for expression of renal epithelial genes. The transcriptome of the four tubular epithelial genes, AQP1, AQP6, NPHS2, SCL12A1, was examined over a period of 11 days for toxic and ischemic and of up to 24 days for nephritic and infection-associated AKI. Expression of the podocyte marker gene podocin (NPHS2) varied but was highest on the first day of observation in patients with ischemic AKI (**Fig. 1B**) and increased by the fifth day in those with toxic AKI (**Fig. 1D**). Of note, no podocin was detected in the sediment of patients with nephritic AKI (**Fig. 1F**) and infection associated AKI (**Fig. 1H**). Expression of the proximal tubule marker AQP1 increased in all four variants of AKI, toxic, ischemic, nephritic, and infection-associated, reflecting increasing numbers of hyperplastic epithelial cells in the urine (**Fig. 1B, 1D, 1F, 1H**). A similar pattern was found for the distal tubule cell-specific marker gene SLC12A1. Peak detection was found in patients with nephritic AKI and infection-associated AKI (**Fig. 1F and 1H**) at day 24, whereas in toxic AKI, peak levels were found during the early phase of recovery.

Naive epithelial cell clusters express the kidney-specific transcription factor PAX8 and the stem cell marker CD133 (PROM1) in confocal immunofluorescence staining

In a first attempt of investigating the origin of urinary sediment cells, naive isolated cells from urine were examined. Immunofluorescence and confocal microscopy were performed to confirm the gene expression pattern and to evaluate the morphology of the excreted cells contributing to the expression landscape shown by qPCR (**Fig. 1B, 1D, 1F, 1H**). Most of the cell clusters examined expressed AQP1, and a subpopulation was positive for the stem cell marker CD133 (PROM1) (**Fig. 2**). All of cells visualized in cluster formation showed positive staining for PAX8, a kidney-specific transcription factor (**Fig. 2**). In the patient with ischemic AKI, no podocin-expressing cells could be detected.

Isolated nephron derived epithelial cells from urine of patients recovering from stage 3 AKI proliferate in *in vitro* cell culture for a minimum of 7 days

Viable epithelial cells consisting of hyperplastic cell clusters (**Fig. 3A**) and individual progenitor cells were isolated by density gradient sedimentation of urine sediment cells and incubated in culture medium as described in the methods section. A rapid expansion from proliferating foci and migration over the entire free tissue culture plate was observed over the initial 5 days (**Fig. 3B**). As demonstrated in **Fig. 3C** a minor number of cells already started to show apoptotic morphology with condensed chromatin and apoptotic bodies on day seven. Fifty percent of the cell population continued to exhibit disproportionate nuclear/cytoplasmic ratios, indicating mitotic and DNA synthesis stages of the cell cycle. This scenario changed with day 8-9 when 70% of adherent growing cells started to round up and detach from the culture plate.

In vitro grown epithelial cells express genes affiliated with podocytes, the proximal and distal tubule, and the loop of Henle

In order to further define the *in vitro* growth and differentiation of tubular epithelial cells, total RNA of *in vitro* cultured cells was reverse transcribed, and the resultant cDNA was investigated by qPCR using gene specific probe sets indicative for defined nephron segments such as for proximal tubular epithelial cells (AQP1), the loop of Henle (SLC12A1) and distal tubular epithelia (SLC12A3) and podocytes (NSHP2). This revealed remarkable AQP1 expression in addition to SCL12A1 and NSHP2 (**Fig. 4**). NPHS2 was detected from day 8 of culture.

In vitro grown cells exhibit PAX8, PROM1 and increasing levels of UMOD

As a next step, we thought it would be of interest to establish whether subpopulations of cells would differentiate into region-specific epithelia by translating specific marker genes into proteins. Therefore, immunofluorescence staining of *in vitro* cultured cells was performed.

In vitro grown cells also showed positive staining for PAX8, and the protein distribution was spread throughout the cells, with more than 50% of cells also positive for PROM1 (**Fig. 5**). This changed rapidly over the time of *in vitro* culture. While the nuclear size decreased, the proportion of PROM1 positive cells minorized, the expression levels of AQP1 and of UMOD increased, reaching a maximum on day 9 (**Fig. 5**). When testing for the proliferation marker Ki67 on day five, 15% of cells stained positive (**Fig. S3**), the percentage of nuclear staining decreased and was absent on day 9, while tubular protein expression markers AQP1/UMOD increased (**Fig. 5**, bottom panel). Testing into different regions of the nephron the SLC12A1 expression was found in about 3-5% of the outgrowing cells at day 8 of culture. Interestingly, these cells were also positive for PROM1 in about 10%. Among these cells were 8% undergoing endocycling (**Fig. 6**). Measurement of UMOD in the culture supernatant increased starting from day 5 and reaching a maximum on days 8-9 (**Fig. S3**).

Discussion

Acute kidney injury represents a major risk for the development of chronic kidney disease, need for dialysis and death. In the past, renal epithelial progenitor cells in urine have shown to be an excellent prognostic sign for recovery from AKI in kidney transplanted patients, however up to now, none of these cell agglomerates have been described in non-transplanted AKI patients (Knafl et al. 2019). Therefore, the presence of renal epithelial progenitor cells from urine sediment at baseline and during the period of renal recovery in AKI stage 3 was investigated and their differentiation into different nephronic segments was studied. The renal injury marker neprilysin in urine and the course of serum creatinine were monitored during this period. The proliferation potential of the isolated progenitor cells and their initial protein and gene expression were investigated and followed by *in vitro* propagation for 9 days until apoptosis. The *in vitro* production of UMOD was also detected in the tissue culture supernatant in this study.

There is no specific therapy for AKI in patient care to heal tubular injury and cure the microvascular damage. However, the tubule's capacity to self-repair is remarkable, especially in AKI stage 1 and 2 or at a young age (Humphreys et al. 2008). The mechanisms have been studied in mouse and rat models (Romagnani et al. 2015). As mentioned above, there are two types of cells found within the kidney tubules: renal progenitor cells, which proliferate and are responsible for cellular regeneration and cells that endoreplicate and generate hypertrophy (Lazzeri et al. 2018). Exuberant hypertrophy appears to lead to fibrosis and CKD, while an overshooting progenitor proliferation leads to induce clonal papillary adenomas and RCC (Peired et al. 2020; Peired et al. 2021; Venkatachalam et al. 2015). It is therefore highly likely that the proliferative rate of epithelial progenitors could also be coupled with a certain rate of mutations coinciding with papillary RCC. When these tubular progenitors are incorporated into organoids, the overall growth and cell survival is much prolonged and even can give rise to nephrogenic structures when injected into SCID mouse kidney capsules (Harari-Steinberg et al. 2020). However, it seems inconceivable that this could lead to the formation of entire nephrons, although it has been a long-standing vision to create a self-organ from autologous mesenchymal stem cells (Lang et al. 2013; Rahman et al. 2020). Given the very long transplant waiting list of dialysis patients, various experiments have been performed with the injection of human mesenchymal stem cells into the nephrogenic site of embryonic rats and with the cultivation of ureteric buds in the omentum of uni-nephrectomized rats (Yokoo et al. 2006). Although there was progress, a clinically relevant step does not appear to be in the near future.

In this study, we were able to isolate viable progenitor cells from urine in the phase of recovery from AKI. The number of excreted committed stem cells was variable from day to day but persisted for up to 24 days. In addition, the excretion rate of regenerative stem cells was highly variable among different individuals. Whether this depended on the renal age or related to the individual genetic background has to be worked out in future studies. It would be of great interest, whether this proliferative rate is of relevance for renal function recovery or determining the time span of a patients CKD degenerating to CKD5. Kidney organoids might support this type of research (Bonventre 2018).

Urinary granulocyte and monocyte excretion has been demonstrated in renal recovery from ischemic injury and toxic tubular necrosis (**Fig. S2**). It is noteworthy that CSF-1 signaling was associated with tubular epithelial cell repair in mice (Menke et al. 2009). This suggests that this seemingly inflammatory process involving granulocyte diapedesis from juxtatubular capillaries is directly linked to the pathway of epithelial repair in renal tubules (Goligorsky 2008). In addition, specific proteins - such as stanniocalcin-1 - can inhibit exuberant reactive oxygen species production and thereby protect from extensive ischemic injury (Huang et al. 2012). Moreover, the renal cell injury marker neprilysin in urine (Bernardi et al. 2021; Pajenda et al. 2017) decreased rapidly after elimination of nephrotoxic substances or restoration of oxygenation. The course of urinary neprilysin showed that excretion of hyperplastic epithelial cells began with a short delay of approximately two days when urinary neprilysin had flattened. Based on immunostaining and qPCR, it must be assumed that there is heterogeneity among the outgrowing tubular epithelial cells. Most likely, they do not reflect the *in vivo* conditions.

Single-cell RNA sequencing would have allowed determination of the stretch of the tubular part of the nephron from which single excreted urinary cells could have originated (Abedini et al. 2021; Menon et al. 2020). This could not be performed, which is a limitation of our study. Beyond that, the causes of AKI varied among patients, so did the time of recovery. In addition, there was a small sample size.

In our previous work, these hyperplastic epithelial progenitor clusters were only observed in KTX patients with ischemic reperfusion injury and were positive predictors of renal recovery (Knafl et al. 2019). This needs to be extended to severe ischemic and toxic AKI in the endogenous kidney. To our knowledge, this is the first study describing the occurrence of hyperplastic epithelial cell clusters in non-KTX patients recovering from AKI stage 3.

Conclusions

The aim of this study was to investigate the presence of urinary tubular epithelial progenitor cells in patients during recovery from AKI stage 3 and to explore their growth behavior and differentiation *in vitro*, as these structures have been shown to be strongly associated with recovery from AKI in KTX patients in the past (Knafl et al. 2019). Thereby, we detected hyperplastic epithelial cell agglomerates in urine which expressed tubular marker proteins associated with podocytes (NSPH2), the proximal tubule (AQP1, AQP6), and the distal tubule (SLC12A1) by qPCR. Confocal immunofluorescence staining confirmed that these epithelial cells were intact and nephron-specific, staining positive for AQP1 and PAX8. In addition, 90% of these cells expressed the stem cell marker PROM1, indicating their replicatory potential. Isolated in cell culture, these tubular epithelial progenitors grew, expanded, and reached confluence within five to seven days, continuing to express AQP1 and UMOD, while the proliferation markers PROM1 and Ki67 decreased over time. Accordingly, the cells showed signs of senescence after nine days in culture.

Single-cell RNA sequencing would have been beneficial, as it would have allowed determination of the section of the tubular part of the nephron from which single excreted urinary cells could be derived, but this was not possible in our laboratory. This represents a limitation of the study.

In conclusion, this is the first study describing hyperplastic tubular epithelial progenitor cell clusters in non-transplanted humans after AKI stage 3. Renal recovery appears to be reflected in high numbers of tubular cells with high replicative potential in the urine. These appear to be a positive indicator of renal recovery, as all patients in this study recovered from AKI despite the severity of the disease, which is consistent with previously published data from a kidney transplant cohort.

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Figure 1

Correlation of urinary neprilysin- and sCr-levels, and urinary expression of AQP1, AQP6, NPHS2 and SLC12A1 during recovery from AKI of different etiologies.

Urinary neprilysin, sCr and urinary expression of AQP1, AQP6, NPHS2, and SLC12A1 were measured during recovery from ischemic (**A**,**B**), toxic (**C**,**D**), nephritic (**E**,**F**) and infection-associated (**G**,**H**) AKI stage 3. The sCr levels were monitored, and fluctuations within the first 13 days were attributable to the use of RRT. Urinary sediment cell and cell fragment gene expression was measured by qPCR. The first day of urine excretion was arbitrarily taken as the reference value (1st day in **B** and **D**, 6th day in **F** and **H**). All other days were compared with this value.

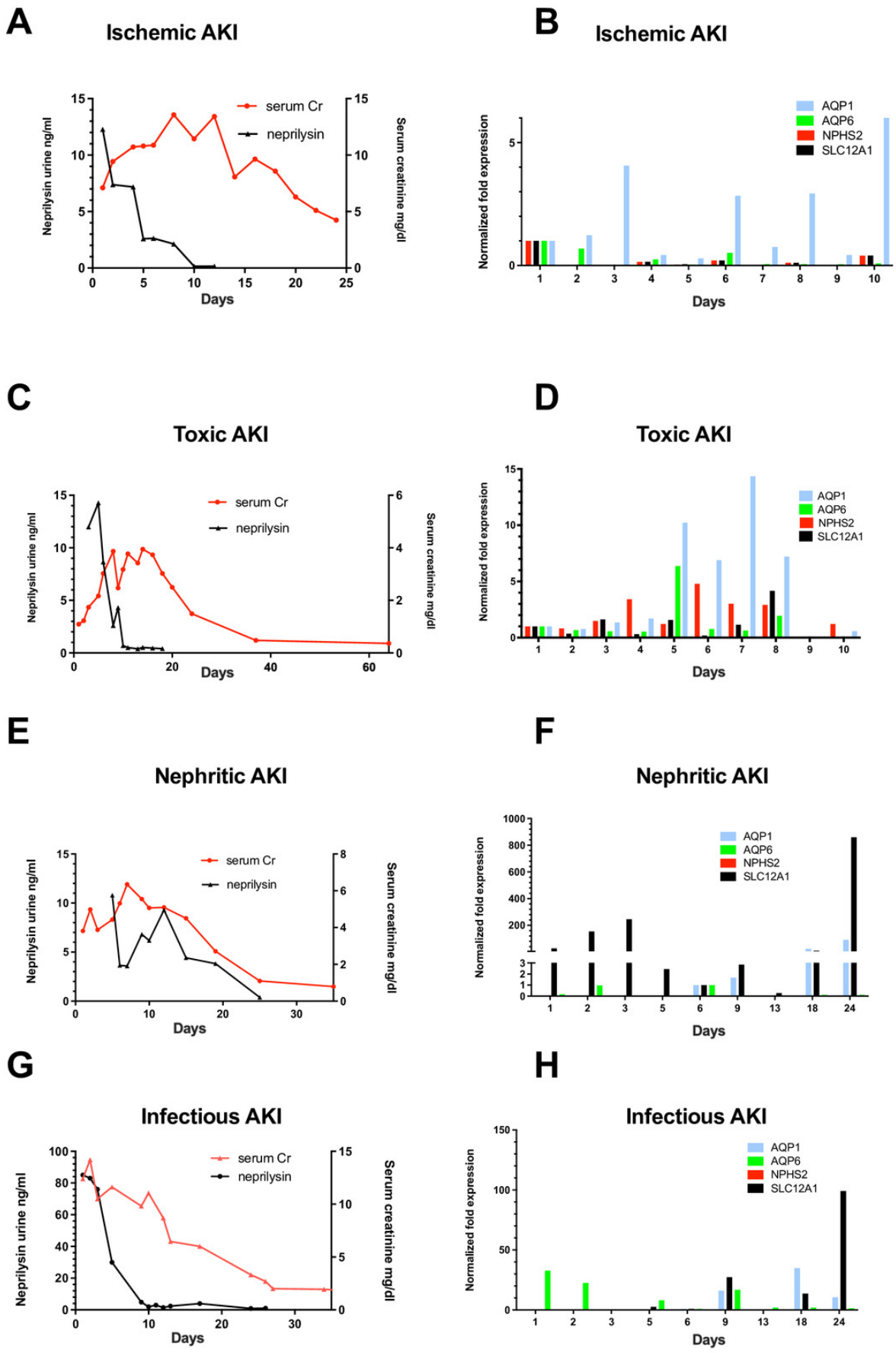
A
Ischemic AKI
serum Cr
neprilysin
Neprilysin urine ng/ml
Serum creatinine mg/dl
Days
B
Ischemic AKI
AQP1
AQP6
NPHS2
SLC12A1
Normalized fold expression
Days
C
Toxic AKI
serum Cr
neprilysin
Neprilysin urine ng/ml
Serum creatinine mg/dl
Days
D
Toxic AKI
AQP1
AQP6
NPHS2
SLC12A1
Normalized fold expression
Days
E
Nephritic AKI
serum Cr
neprilysin
Neprilysin urine ng/ml
Serum creatinine mg/dl
Days
F
Nephritic AKI
AQP1
AQP6
NPHS2
SLC12A1
Normalized fold expression
Days
G
Infectious AKI
serum Cr
neprilysin
Neprilysin urine ng/ml
Serum creatinine mg/dl
Days
H
Infectious AKI
AQP1
AQP6
NPHS2
SLC12A1
Normalized fold expression
Days

Figure 2

Confocal immunofluorescence of clusters of renal epithelial cells excreted in urine during the phase of renal recovery.

AQP1 staining (**green, upper panel**) indicated derivation from proximal tubular origin. Positive PROM1 (**red**) directed towards progenitors. PAX8 (**green, lower panel**), a kidney-specific transcription factor, demonstrated the nephronic origin of the cells.

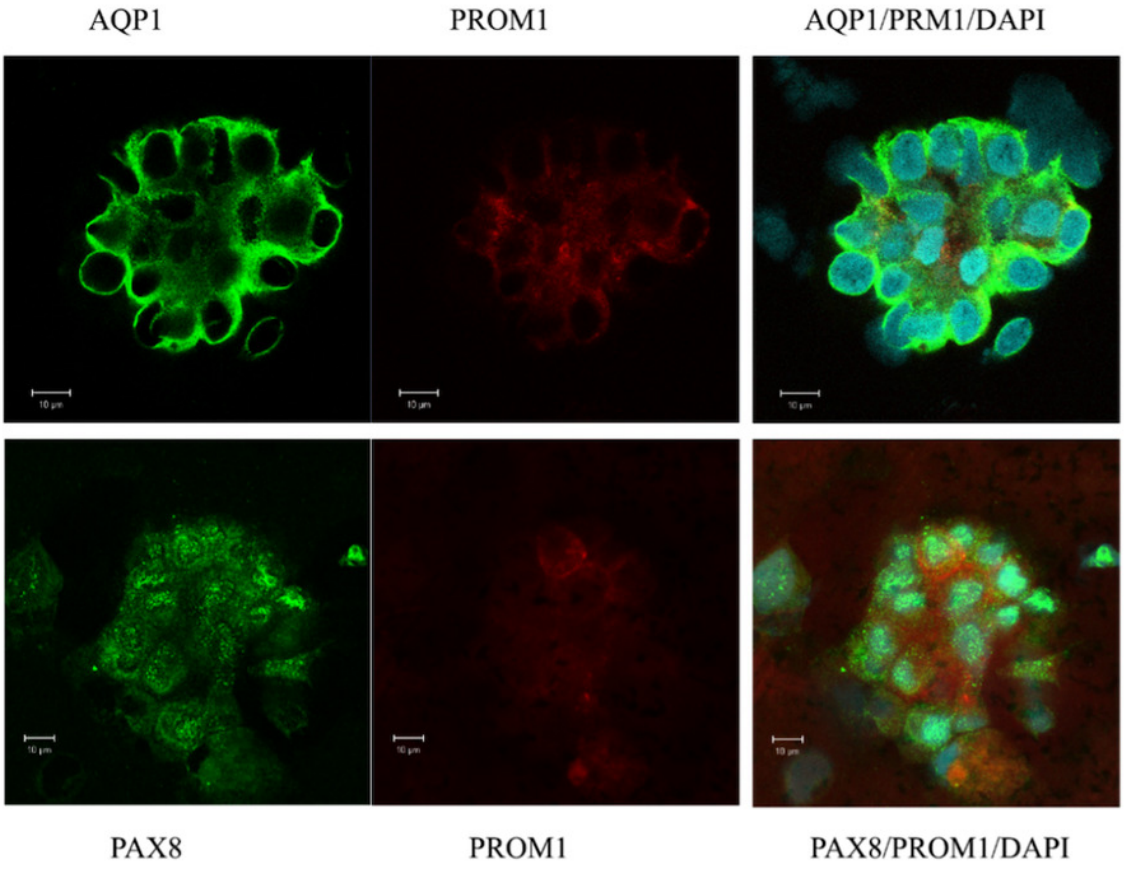

Figure 3

Hyperplastic tubular epithelial cell cluster isolated from urine.

(A) Hyperplastic tubular cell agglomerates from a patient with ischemic AKI stage 3 including a cast. **(B)** Hyperplastic tubular epithelial cell cluster from a patient with infection-associated tubular necrosis. **(C)** Tubular epithelial cell clusters and progenitor cells isolated from urine by density gradient centrifugation grown in culture dishes in adherent mode. During mitosis in the phase of cytokinesis epithelial cells loosened from the dish and formed mitotic doublets (**arrow**). Mitotic cells were seen starting day 3 through day 8. This was followed by predominant apoptotic structure starting with day 9. Cells containing two or three nuclei (endocycling arrow) were observed. **(D)** H/E staining of cytopreparations of 8 day cultured human renal epithelial progenitor cells. Some of them appeared with disproportionate cytoplasmic nuclear size in mitosis, others possessed condensed nuclear chromatin and apoptotic bodies as typical indicators of apoptosis (**arrow**). Original magnification 400x.

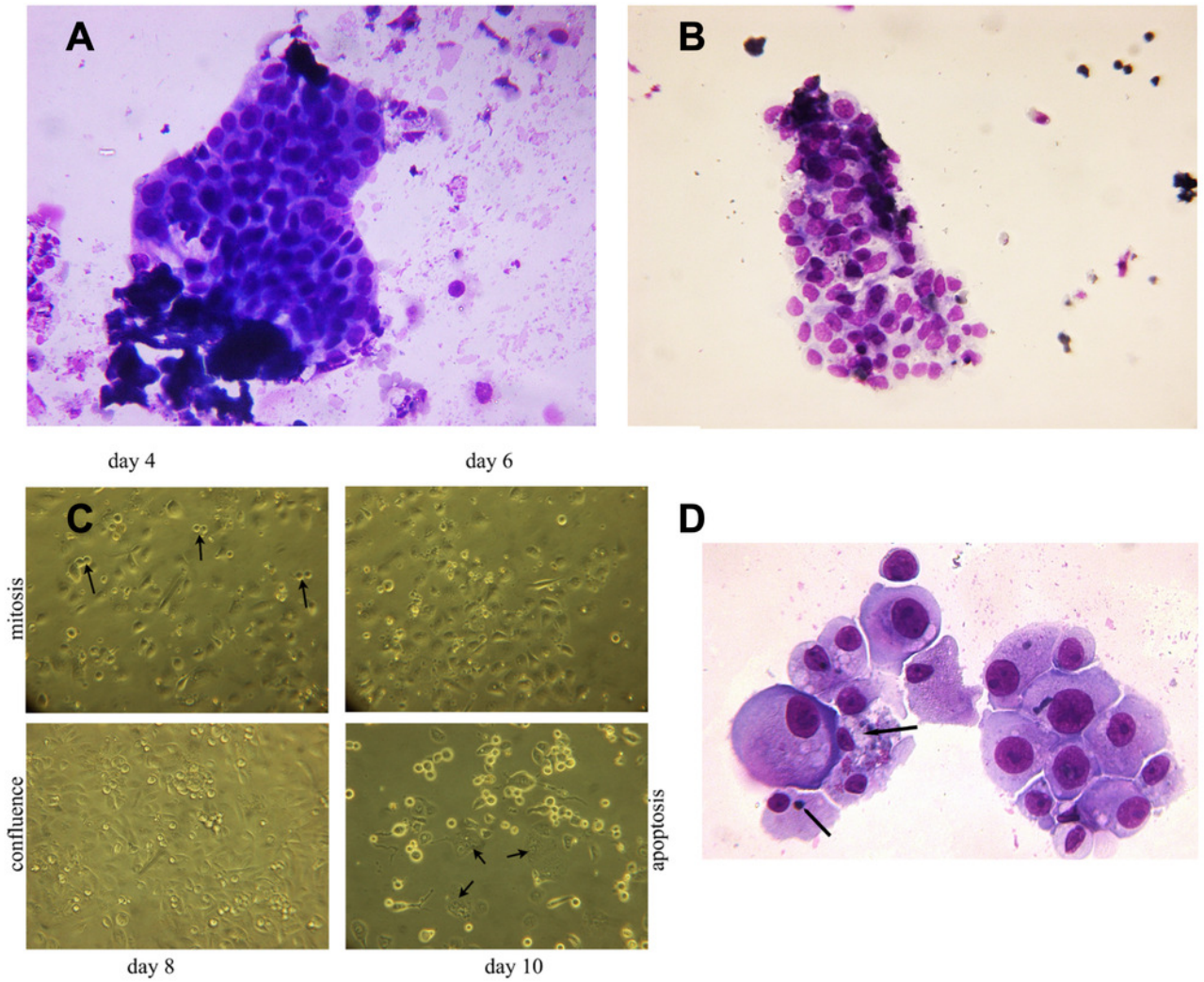
A
B
day 4
day 6
C
D
mitosis
confluence
apoptosis
day 8
day 10

Figure 4

Gene expression of epithelial cells *in vitro* grown for 7, 8, 9 and 10 days in tissue culture media.

Total RNA was isolated from cultured cells and qPCR was performed. Expression data of day 10 were arbitrarily chosen as reference and data from other days were compared to this. *In vitro* grown cells showed affiliation to podocytes (NPHS2) with a maximum on day 8 and to the proximal tubule (AQP1), the loop of Henle (SCL12A1) as well as the distal tubule (SLC12A3).

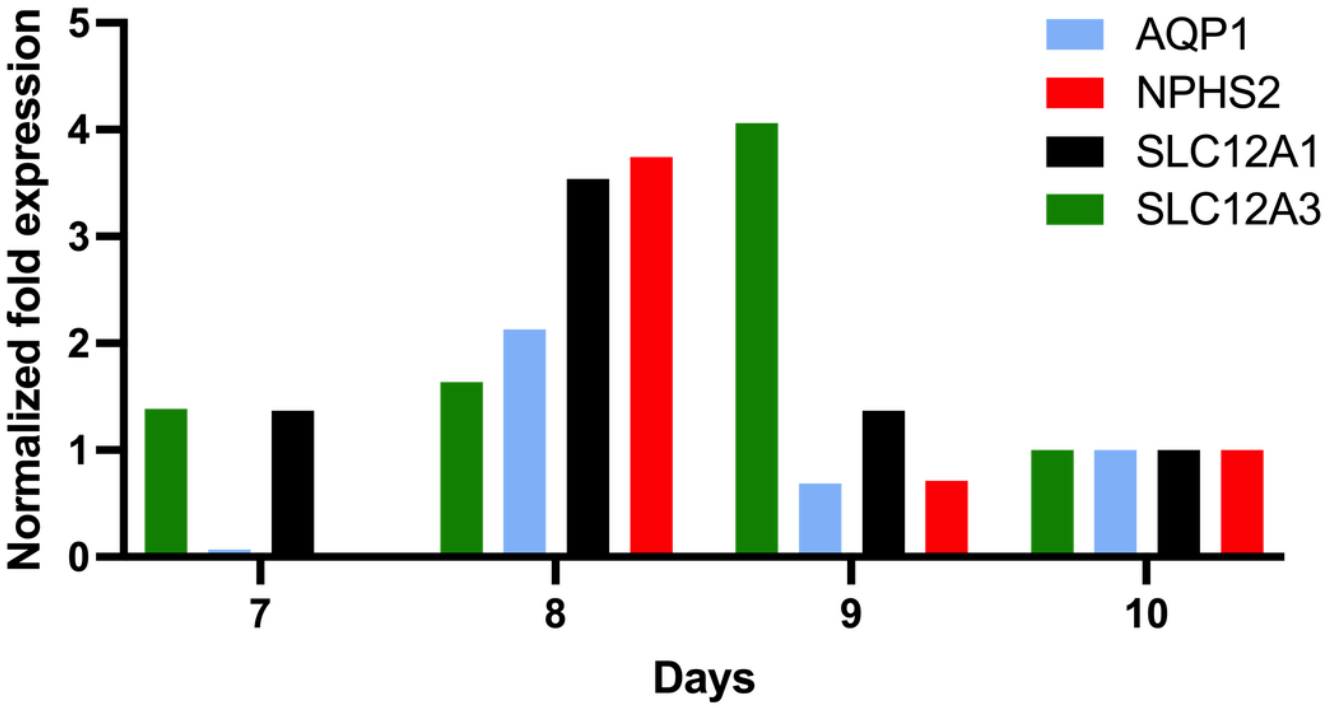

Figure 5

Confocal immunofluorescence of renal epithelial cells 5, 7 and 9 days in culture.

Renal epithelial cells 5 days in culture stained positive for AQP1 (**green**) and PROM1 (**red**). Cells cultured for 7 days showed increased AQP1 staining (**green**) and reduced staining for PROM1 (**red**). Cells cultured for 9 days exhibited strong staining for UMOD (**green**), while Ki67 was absent (**red**).

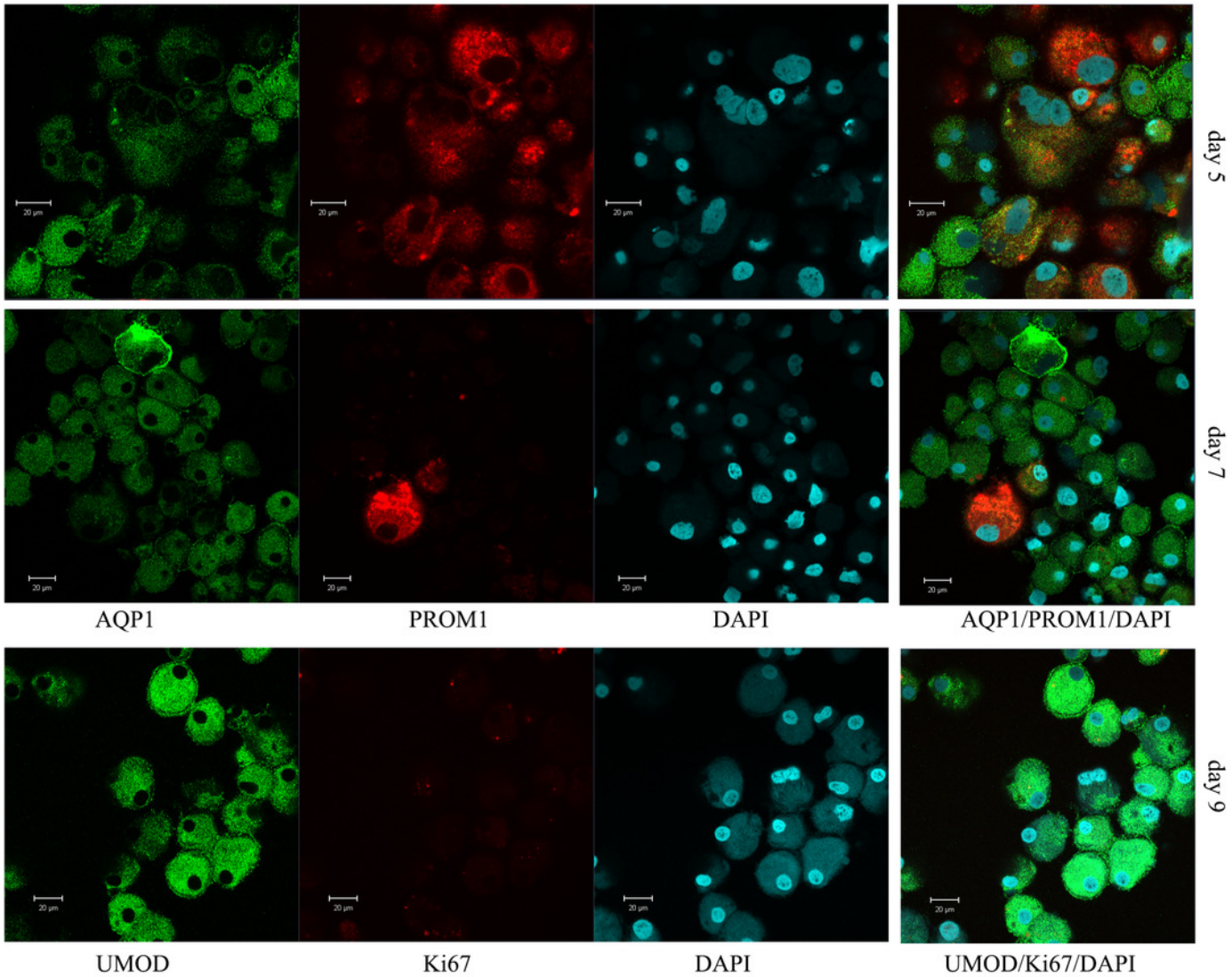

Figure 6

Confocal immunofluorescence of renal epithelial cells 8 days in culture.

Ten percent of SCL12A1 (**green**) positive cells also expressed PROM1 (**red**). The * represents an apoptotic SCL12A1-expressing cell containing apoptotic corpuscles.