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Controlling cellular plasticity to improve *in vitro* models for kidney regeneration

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Abstract

Given the increasing prevalence of end-stage kidney disease, the high morbidity and mortality of dialysis treatment, and the shortage of donor kidneys, the field of nephrology is progressively shifting its focus to regenerative medicine. In particular, both the development of a bioartificial kidney and the improvement of kidney-mimicking systems developed *in vitro* (*e.g.* organoids or tubuloids) for implantation purposes are attractive therapeutic strategies. However, a major hurdle to overcome with the current kidney cell models available is the limited control over cellular plasticity to augment cell-type-specific functionality. In this review, we summarize the main knowledge on important factors known to drive or affect maturation of kidney epithelial cells. This might aid in the advancement of *in vitro* kidney models to enable their use in regenerative medicine.

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Introduction

For decades, there is a persistent tension between the high demand for kidney transplantations and the scarce availability of donors. According to the United States Organ Procurement and Transplantation Network, as of April 2021, 90,937 candidates are on the waiting list for a new kidney in the United States [1]. Most patients suffer from chronic kidney disease or end-stage kidney disease and are fully dependent on long and frequent dialysis sessions. Dialysis poses not only an economic burden to the healthcare system but also an extreme psychological burden to the patient. Moreover, dialysis remains ineffective for the removal of protein-bound toxins in the blood, which leads to higher morbidity and mortality.

To circumvent or limit the use of dialysis and ultimately aid in the shortage of kidney donations, the field of nephrology is increasingly interested in regenerative medicine and the development of bioartificial kidneys. In the last decade, several *in vitro* kidney systems have emerged, giving rise to new opportunities to progress in replacing defective kidneys *in vivo*. The most well-known kidney model that could be suitable for regenerative applications is induced pluripotent stem cell (iPSC)-derived kidney organoids [2,3]. These are complex three-dimensional cellular structures that resemble the morphology of the nephron. One of the major advantages of kidney organoids is that they contain, in an organized fashion, a variety of epithelial and mesenchymal cell types, including tubular epithelial cells, nephron progenitor cells, podocytes, and endothelial cells [4]. However, because of their incomplete level of maturity and off-target differentiation [5], iPSC-derived kidney organoids are, to date, mostly suitable for studies concerning embryonic nephron development. Another model that has recently been developed is adult kidney tubuloids [6,7]. Unlike organoids, which are usually cultured on transwells or low-attachment plates [8], kidney tubuloids are embedded in hydrogels. Furthermore, they differ from kidney organoids in the fact that they exclusively contain epithelial kidney cell types, and their structure is spheric. Although being unsuitable to study nephrogenesis, tubuloids have a better capacity to mimic kidney regeneration as they are directly derived from adult kidney tissue, and they show high expression levels of several maturity markers together with the absence of precursor and immature cell types that are found in iPSC-derived kidney organoids. Additional advantages for the regenerative application of tubuloids are easy cell sourcing from urine, which allows autologous tissue growth, as well as the lack of genetic modification and off-target differentiation [6,7].

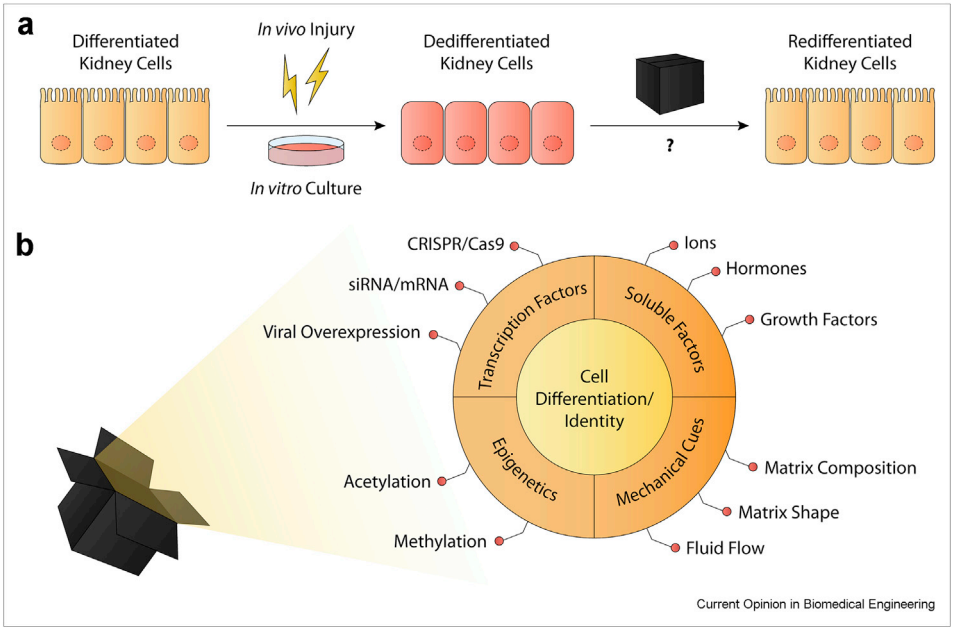
The major limitation for the clinical applicability of organoids/tubuloids is the lack of adequate differentiation owing to limited control over cellular plasticity. Cellular plasticity refers to the phenomenon in which terminally differentiated cells are able to transform into another cell type, either by dedifferentiation (*i.e.* transformation into a cell with a lower maturity state) followed by redifferentiation or by transdifferentiation (*i.e.* transformation into a different mature cell type). In kidney cells, plasticity occurs frequently, both *in vivo* and *in vitro*, as adaptive response to changes in the environment. Regarding plasticity related to transdifferentiation, an example can be taken from recent findings by Howden et al. (2021), who showed that distal tubule (DT) cells in iPSC-derived kidney organoids are able to transdifferentiate into ureteric epithelium under certain culture conditions [9]. With respect to plasticity related to dedifferentiation, a clear example occurs during acute kidney injury *in vivo*, when cells in the affected area enter a more immature and proliferative state to repopulate the nephron with their subsequent redifferentiation [10]. Similarly, when primary kidney cells are cultured *in vitro*, they rapidly dedifferentiate. However, although most cells *in vivo* quickly redifferentiate upon dedifferentiation, when cultured *in vitro*, they do not regain a fully differentiated, mature phenotype [11,7]. In tubuloids, addition of specific growth factors can augment the proximal tubule (PT) phenotype, whereas withdrawal of growth factors promotes preferential differentiation toward distal kidney segments. Yet, in both cases, the expression of many segment-specific differentiation markers remains below the levels found in mature kidney tissue *in vivo* [12,13,7]. To this day, the exact drivers for full redifferentiation remain elusive.

Control over both the dedifferentiated and proliferative state, as well as the functional and fully matured cell state, is crucial, on the one hand, to obtain sufficient cell numbers for a bioartificial device, and on the other hand, to differentiate the cells toward the required functional phenotype. Understanding and modulating cellular plasticity can help upgrade the physiological mimicking capacity of *in vitro* models. In this short nonexhaustive review, we provide an overview of key stimuli — both physical and chemical — that have been described to direct adult kidney epithelial cell (re)differentiation, and factors that have been shown to transcriptionally modulate the cell state of adult kidney cells *in vitro* (Figure 1).

Regulators of adult kidney epithelial cell maturity

Recently, Morris [14] proposed a framework to conceptualize cell identity, distinguishing between (1) phenotype and function, (2) lineage, and (3) cell state. For regenerative purposes, cell identity is best defined by function, which depends on the adequate expression

Figure 1



Cell plasticity during kidney regeneration and *in vitro* culture. (**a**) Upon injury *in vivo* or after introduction into an *in vitro* culture, kidney epithelial cells become dedifferentiated. By unknown mechanisms ('black box'), these cells are able to redifferentiate *in vivo*. (**b**) Opening this black box could unravel how kidney cell redifferentiation can be promoted *in vitro*.

of specific proteins; along the nephron, each cell type is primarily distinguished by the expression and function of segment-specific transporters. Because dedifferentiation *in vitro* is driven by drastic transcriptional changes, we examined per nephron segment which signaling pathways and transcription factors are crucial regulators for cell maturity and function (Section 2.1, Figure 2). The second pillar of cell identity, lineage, is an interesting concept to study at developmental stages to identify cells based on their origin, but in this review, we focus on cells at the other end of the maturity scale and how their functionality can be influenced *in vitro*. Here, the third pillar is more interesting: cell state is described by Morris [14] as 'the range of cellular phenotypes arising from the interaction of a defined cell type with its environment'. This review provides an overview of the main soluble factors (Section 2.2) and matrix cues (Section 2.3) that have shown to influence the (functional) state of kidney cells (Figure 2).

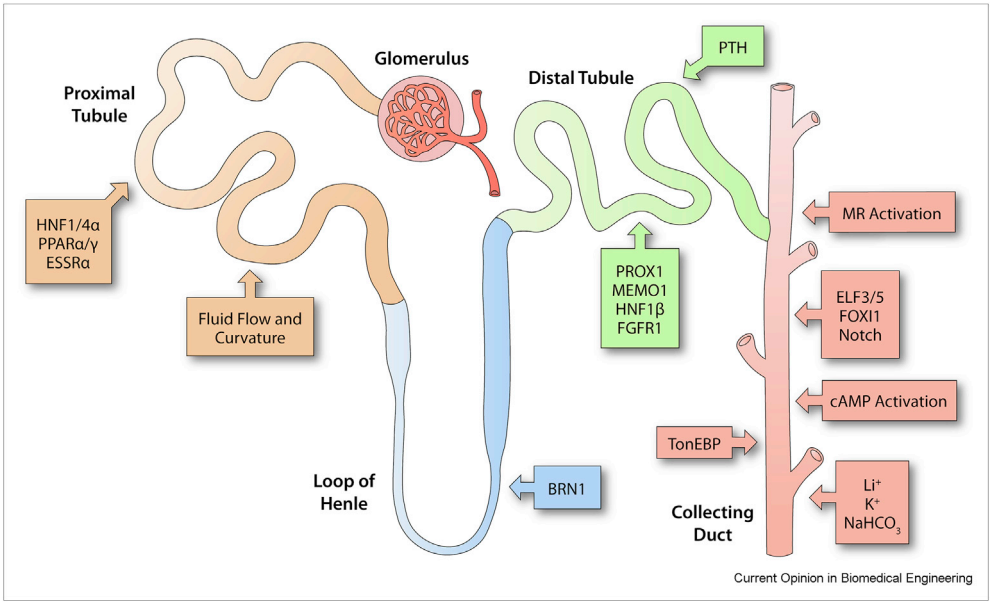
Signaling pathways and transcription factors

Transcription factors are the primary regulators of gene expression, and there is a plethora of transcription factors and pathways that either promote differentiation or preserve a mature state of kidney epithelial cell types in a cell-type specific manner. Current knowledge is mainly based on knockout studies in adult mice, and on *in vitro* experiments using human and murine cell lines, summarized in Table 1.

In PT cells, major drivers of differentiation include the hepatocyte nuclear factors 1α/4α (HNF1α/4α), the peroxisome proliferator-activated receptors α and γ (PPARα/γ), and the estrogen-related receptor α (ESRRα). In mice, *Hnf1a* regulates the expression levels of the chloride channel chloride voltage-gated channel 5 (*Clcn5*), the sodium and glucose cotransporter 2 (SGLT2, *Slc5a2*), and the sodium-phosphate cotransporters 1 and 3 (Npt1/3, *Slc17a1/3*) [15–17]. In addition, *in vitro* studies have demonstrated that *Hnf1a*, together with *Hnf4a*, is able to promote expression of a major PT transporter, the organic anion transporter 1 (Oat1, *Slc22a6*) [18,19]. *HNF4A* alone can also induce the expression of other PT-specific carriers, such as the transporters *SLC7A7* and *SLC16A4*, and the endocytic receptor megalin (*LRP2*) [20].

The roles of PPAR and ESRRα on PT maturation have been recently investigated by Dhillon et al. (2021): activation of Pparα in mice resulted in increased expression levels of PT markers such as the solute carriers *Slc22a30*, *Slc27a2*, *and Slc16a11*, whereas knockout of *Esrra* led to reduced expression of a variety of genes, including *Slc22a6*, *Slc7a13*, and *Slc6a13* [21]. Confirming the positive effects of PPAR activation on PT cell maturation, treatment of LLC-PK1 cells with either a PPARα or PPARγ agonist increased *LRP2* expression levels, whereas the antagonists showed an opposite effect [22]. Of note, the effects of PPAR and ESRRα on

Figure 2



Key factors involved in regulating maturation of the different nephron segments in the kidney. PTH, parathyroid hormone; MR, mineralocorticoid receptor.

Table 1

Main transcription factors and signaling pathways involved in the differentiation of the different kidney epithelial segments.

	Gene	Modification	Species/cell Line	Effect	References
Proximal tubule	*Hnf1a*	Knockout	Mice	↓ *Clcn5* expression ↓ *Slc5a2* expression ↓ *Slc17a1/3* expression	[17] [16] [15]
	Hnf4a+Hnf1a	Overexpression	Mouse embryonic fibroblasts	↑ *Slc22a6* expression	[18,19]
	HNF4A	Overexpression	HK-2/HEK-293T cells	↑ *SLC4A1* expression ↑ *SLC7A7* expression ↑ *SLC16A4* expression ↑ *LRP2* expression	[20]
	PPARA	Activation	Mice	↑ *Slc22a30* expression ↑ *Slc27a2* expression ↑ *Slc16a11* expression	[21]
	PPARA/G	Activation Inhibition	LLC-PK1 cells	↑ *LRP2* expression ↓ *LRP2* expression	[22]
	ESRRA	Knockout	Mice	↓ *Slc22a6* expression ↓ *Slc7a13* expression ↓ *Slc6a13* expression	[21]
Loop of Henle	*Brn1*	Heterozygous knockout	Mice	↓ *Umod* expression ↓ *Ptger3* expression ↓ *Slc12a1* expression ↓ *Kcnj1* expression ↓ *BSND* expression	[23]
Distal tubule	*Prox1*	Knockout	Mice	↓ *Slc12a3* expression ↓ *Trpm6* expression	[27]
	Memo1	Knockout	Mice	↑ *Trpv5* expression ↑ *Slc8a1* expression ↑ *Calb1* expression	[26]
	Hnf1b	siRNA Knockout	mpkDCT cells Mice	↓ *Kcnj16* expression ↓ *Kcnj10* expression ↓ *Fxyd2a* expression ↓ *Kcnj16* expression ↓ *Kcnj10* expression ↓ *Slc12a3* expression ↓ *Pkhd1* expression	[25]
	Fgfr1	Knockout	Mice	↑ *Trpv5* expression ↑ *Trpv6* expression ↑ *Calb1* expression	[24]
Collecting duct	*Elf3*	Knockdown Overexpression	mpkCCD cells	↓ *Aqp2* expression ↑ *Aqp2* expression	[29]
	Elf5	Overexpression Knockout	mpkCCDC14 cells Mice	↑ *Aqp2* expression ↑ *Avpr2* expression ↓ *Aqp2* expression ↓ *Avpr2* expression	[28]
	Notch1+Notch2 Hes1	Knockout	Mice	↓ *Aqp2* expression ↓ *Elf5* expression ↓ *Avpr2* expression ↓ *Aqp4* expression ↑ *Foxi1* expression ↑ *Atp6v1b1* expression ↑ *Slc26a4* expression	[31,32]
	Foxi1	Knockout	Mice	↓ *Slc4a1* expression ↓ *Slc26a4* expression	[30]

PT cell differentiation are very tightly linked to their effects on cellular metabolism, which highlights the importance of cellular metabolism as a major driver of PT cell differentiation [21].

In the loop of Henle (LoH), transcriptional regulation of maturation is still not well understood; to our knowledge, the only factor linked to higher LoH differentiation to date is POU class 3 homeobox 3 (*POU3F3*, or *BRN1*). A heterozygous knockout of *Brn1* in adult mice decreased the expression levels of important markers in that segment, including uromodulin (*Umod*), the prostaglandin E receptor 3 (*Ptger3*), the sodium-potassium-chloride cotransporter 2 (Nkcc2, *Slc12a1*), the renal outer medullary potassium channel (Romk, *Kcnj1*), and barttin (*Bsnd*) [23].

The maturation of the DT in mice is affected by the expression of the transcription factors prospero homeobox 1 (*Prox1*), mediator of cell motility 1 (*Memo1*), hepatocyte nuclear factor 1β (Hnf1b), and the fibroblast growth factor receptor 1 (Fgfr1). The individual deletion of these genes affects the expression of several DT-specific markers, such as the calcium-handling genes *Trpv5* and calbindin 1 (*Calb1*), or the potassium channels *Kcnj16* and *Kcnj10* [24–27].

Finally, collecting duct (CD) maturity and identity are mainly regulated by the transcription factors E74 like ETS factors 3 and 5 (*Elf3/5*), forkhead box I1 (*Foxi1*), and the Notch signaling pathway. Elf3/5 and Notch are important for the maturation of principal cells (PCs) [28,29], whereas Foxi1 is essential for the maturation of intercalated cells (ICs) [30,31]. Furthermore, these factors have shown to orchestrate the trans-differentiation between both cell types in the CD [31]. One of the most important markers in PCs demonstrating maturity is the channel aquaporin-2 (*Aqp2*). Both *Elf3* and *Elf5* can bind to the *Aqp2* promoter, thereby regulating its basal expression levels [28,29]. *Aqp2* expression can also be greatly modulated by Notch signaling: dual knockout of Notch1 and Notch2 in mice has shown to significantly downregulate the expression of this channel [32]. Similar results have been obtained with the deletion of the Notch target *Hes1*, which not only leads to a reduction in *Aqp2* expression but also of *Elf5*, *Avpr2*, and *Aqp4*. Simultaneously, inhibition of Notch increases the expression of IC-related markers, such as *Foxi1*, the ATPase H+ transporting V1 subunit B1 (*Atp6v1b1*), and pendrin (*Slc26a4*) [31,32], indicating that Notch inhibition promotes transdifferentiation of PCs into ICs. Finally, Foxi1, the major IC transcription factor, is able to regulate the mRNA expression of the IC transporters anion exchanger 1 (AE1, *Slc4a1*) and *Slc26a4* [30].

Soluble factors

Ions and osmolality

Apart from the direct action of transcription factors, cells react to systemic signals with transcriptional changes to maintain homeostasis. For example, kidney cells in the medulla are exposed to very high levels of osmolality, and thus possess mechanisms to reduce ionic strength, for example, by accumulating intracellular osmolytes [33]. This adaptive process is partly regulated at the transcriptional level. *In vitro*, induction of hyperosmolality has shown to increase expression of a variety of genes in different kidney cells, including the chloride voltage-gated channel 5 (*Clcn5*), *Aqp2*, *Aqp3*, and *Slc6a12* [34–36]. Furthermore, hyperosmolality upregulates the transcription factor tonicity-responsive enhancer-binding protein (TonEBP), which has been linked to the hyperosmolality-induced upregulation of *Aqp2* [33]. Interestingly, impairment in Notch signaling was reported to cause a significant decrease in urine osmolality [32]. Therefore, the effects of Notch on CD (trans)differentiation might be partly dependent on osmolality changes.

The differential abundance of specific ions has shown to transcriptionally affect the levels of transporters in, especially, CD cells. For instance, lithium (Li^+) treatment is known for its effects on transdifferentiation of PCs into ICs, reflected by a decrease in cellular *Aqp2* expression and an increase in IC marker expression, such as *Slc4a1* [37,32,38]. Similarly, the dietary depletion of potassium (K^+) leads to the downregulation of *Aqp2* expression and the promotion of expression of IC-specific markers. Importantly, K^+ depletion also suppresses Notch signaling [39]. Finally, treatment of mice with bicarbonate ($NaHCO_3$), together with an aldosterone analog, has shown to upregulate mRNA levels of both *Slc4a1* and *Slc26a4* [40].

Hormones

The major function of hormones in the distal segments of the kidney is the regulation of transporters and channels in regulatory systems for homeostasis and osmolality control. Therefore, hormones in these segments (especially the CD) have a crucial effect on the cell state, even though this might not be *per se* related to differentiation.

Especially hormones binding to the mineralocorticoid receptor (MR) display a wide array of effects in these segments. For instance, several studies have demonstrated that aldosterone, an MR ligand, is able to not

only upregulate the protein levels of the sodium-chloride cotransporter (Ncc) and Aqp2, but also the mRNA expression of the epithelial sodium channel α (ENaCα, *Scnn1a*) [41–43]. Fludrocortisone and dexamethasone, which can also bind to MR, were similarly able to increase protein levels of Ncc and expression of *Aqp2* in rodent models, respectively [42,44].

The antidiuretic hormone vasopressin, which acts through cAMP, is also a well-known modulator of CD cell maturation. Vasopressin stimulates mRNA expression of *Aqp2* in a dose- and time-dependent manner. This effect can also be mimicked by using other cAMP activators, such as forskolin or 8-bromo-cAMP [45,46]. A recent article by Uchimura et al. (2020) [4] demonstrated an improved differentiation of iPSC-derived kidney organoids toward kidney cells of the CD — based on the significant increase in markers specific for the CD — by treating them with both vasopressin and aldosterone.

Another hormone with a putative but conflicting role in kidney maturation is the parathyroid hormone (PTH). In rat kidneys, PTH positively regulates expression of *Trpv5*, *Calb1*, and *Ncx1* [47]. In contrast, specific knockout of *Pth1r* in the LoH, DT, and CD of mice caused an increase in the expression of *Trpv5* and *Calb1* [48]. These differential effects could be owing to the fact that PTH is also able to activate cAMP [49] and might thus exert its positive effects on differentiation independently of its receptor.

Mechanical cues

Matrix topography and stiffness

Especially for the development of bioartificial kidneys, it is crucial to choose the membrane chemistry and surface topography that better support cellular differentiation and functionality. Several studies have explored the effects of different substrate topographies and stiffnesses on the enhancement of kidney cell maturation. Microenvironmental curvature, as physiologically found in the nephron, has been shown to improve renal function, among others, through increased expression of PT transporters such as *SLC22A6* [50]. Besides curvature, anisotropic extracellular matrix architecture encourages structural arrangement of F-actin, which augments the expression of kidney transporters, including *SLC22A2* and *ABCB1* [51,52]. Stiffness has also shown to be a determinant of kidney cell maturity, with softer materials allowing for better differentiation of kidney organoids [53]. Finally, the findings of some studies suggest that decellularized tissues could be excellent candidates as natural kidney cell-supporting matrices, providing physiological topology, stiffness, and molecular cues. Both human and murine embryonic stem cells perfused into decellularized kidneys have shown to repopulate the scaffold and are able to differentiate [54,55]. However, the extent of such maturation has not yet been compared with that on standard culturing surfaces.

Fluid shear stress

The apical side of the kidney epithelium is constantly exposed to a pulsatile urine flow, generated by the heart's pumping action. Most kidney epithelial cells are equipped with microvilli and primary cilia, which act as mechanosensors that translate extracellular flow into intracellular signal transduction cascades. A recent study revealed that prolonged PT cell culture under fluid flow leads to selective activation of pathways involved in cell adhesion and polarization, leading to a phenotype that is transcriptionally more representative of the PT *in vivo* [56]. Moreover, flow has shown to positively affect the expression of various PT markers in human cell lines and organoids, including brush border enzyme γ-glutamyltransferase 1 (*GGT1*), the sodium/hydrogen exchanger 3 (NHE3, *SLC9A3*), the multidrug resistance protein 4 (MRP4, *ABCC4*), *LRP2*, and *AQP1* [57–59]. Transcriptional and metabolomics data showed upregulated aerobic metabolic pathways and decreased glycolytic flux under flow conditions, which might underlie a flow-induced increase in oxygen availability [60].

Conclusion and future outlook

Terminally differentiated kidney tubular cells are capable of de- or transdifferentiating owing to cellular plasticity. *In vivo*, this occurs primarily in response to injury, but a similar process is observed when cells are cultured *in vitro*. Cells in current kidney organoids and tubuloids remain mostly in an intermediate cell state with partial expression of cell-specific markers but functionality far below the capacity of fully differentiated cells. To generate highly functional tissue for kidney regeneration, a promising strategy is the exploitation of cellular plasticity by shifting cells to the most functional state possible. In this short review, we provide an overview of factors known to drive or affect the expression of cell-type-specific differentiation markers that determine cellular functionality. The induction of key transcription factors can promote function specific to the respective nephron segments, but also regulation through hormones and ions can induce cellular homeostatic responses. Moreover, mechanical cues from the extracellular matrix and fluid flow have been shown to potentiate cellular function. Therefore, defined culture media or microfluidic systems may be effective

approaches to enhance the differentiated phenotype of kidney cells. Interestingly, incorporation of supporting cells and/or vasculature in kidney organoids can further promote their maturation [61,62].

We limited this overview to the main information reported on kidney cell differentiation, but other stimuli, such as oxygenation, epigenetics, and the molecular composition of the extracellular matrix, should not be dismissed, as they have shown to affect plasticity of cells from other organs or even kidney cells during injury [63–67]. An exploration of how these factors could contribute to better kidney cell maturation is of great importance. In addition, kidney cell transdifferentiation remains to be an understudied subject. Future studies should investigate which specific factors are able to drive transdifferentiation of which kidney cell types. Last but not least, a large number of kidney single-cell RNA sequencing studies have recently been published that provide information on major transcription factors and other genes related to the maturation of the different epithelial kidney cell types [68,69]. The relevance of these candidates on kidney cell differentiation should now be confirmed experimentally.

In conclusion, a deeper mechanistic understanding of cellular plasticity in kidney organoids and tubuloids can ultimately be translated into advanced culture systems with functional and regenerative potential.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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