

# Directed Differentiation of Pluripotent Stem Cells into Kidney



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**ABSTRACT:** Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), represent an ideal substrate for regenerating kidney cells and tissue lost through injury and disease. Recent studies have demonstrated the ability to differentiate PSCs into populations of nephron progenitor cells that can organize into kidney epithelial structures in three-dimensional contexts. While these findings are highly encouraging, further studies need to be performed to improve the efficiency and specificity of kidney differentiation. The identification of specific markers of the differentiation process is critical to the development of protocols that effectively recapitulate nephrogenesis *in vitro*. In this review, we summarize the current studies describing the differentiation of ESCs and iPSCs into cells of the kidney lineage. We also present an analysis of the markers relevant to the stages of kidney development and differentiation and propose a new roadmap for the directed differentiation of PSCs into nephron progenitor cells of the metanephric mesenchyme.

**KEYWORDS:** kidney, stem cells, iPSC cells, ES cells, directed differentiation

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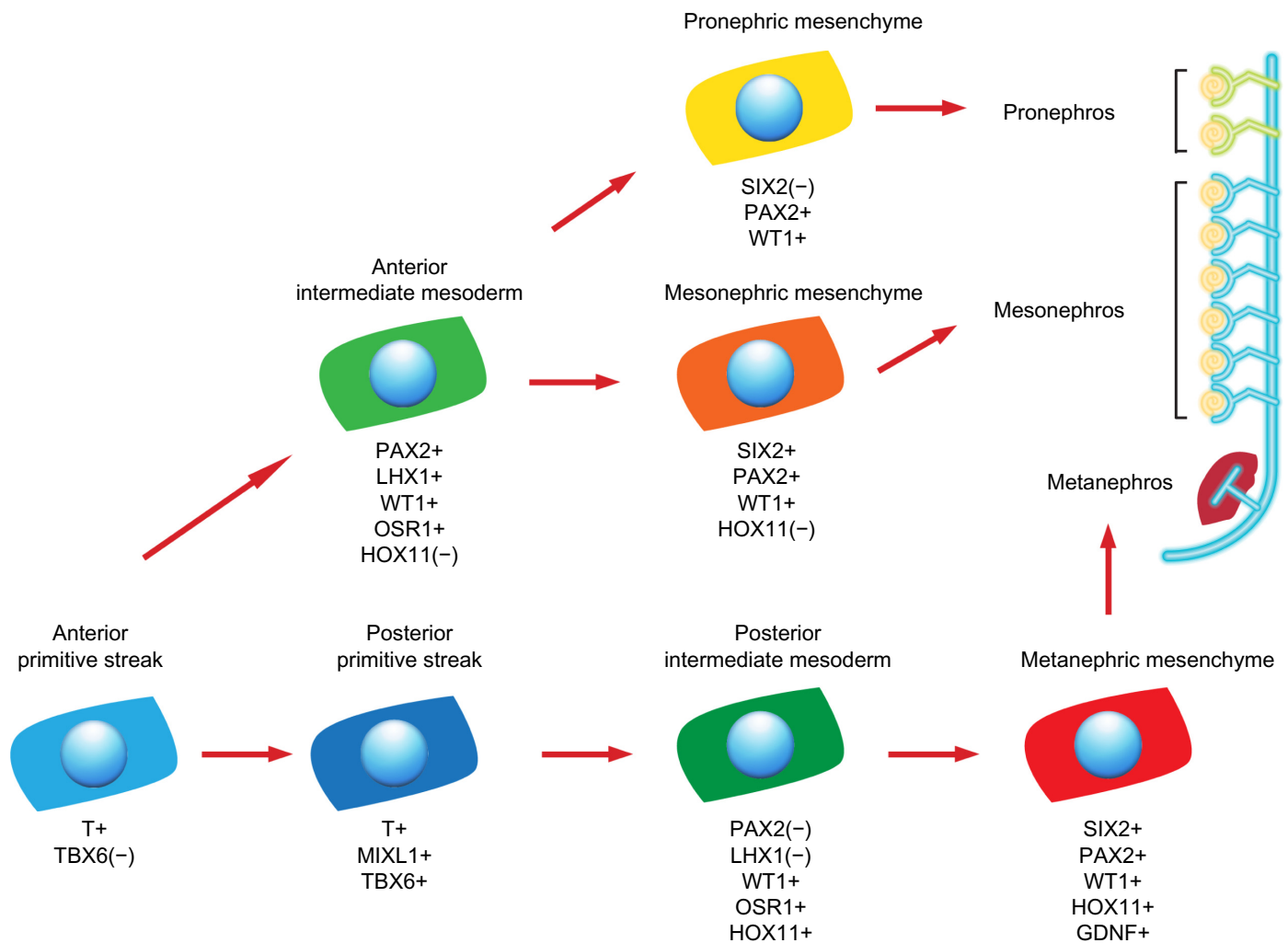
## Introduction

Pluripotent stem cells (PSCs), which include embryonic stem cells (ESCs)<sup>1,2</sup> and induced pluripotent stem cells (iPSCs),<sup>3,4</sup> are attractive sources of cells for regenerative medicine, *in vitro* disease modeling, and drug screening. Successful application of PSCs to achieve these goals is dependent on the ability to differentiate PSCs into specific cell types. PSCs, by definition, are capable of stochastically differentiating into cells of all three germ layers, but the efficient derivation of a particular cell or tissue type requires directed differentiation, a process by which PSCs are treated with growth factors and chemicals in a stepwise manner that frequently mimics the stages of organ development. In this review, we will summarize the current approaches to induce kidney cells from PSCs and discuss the future direction of kidney differentiation from PSCs.

## Kidney Development and the Identification of Stage-Specific Markers of Differentiation

Mammalian kidney development involves the sequential induction of three distinct kidneys: the pronephros, mesonephros, and metanephros, all of which are derivatives of the intermediate mesoderm (IM) (Fig. 1). First to arise is the pronephros, which has no known excretory function in human beings and

disappears almost as quickly as it is formed. The mesonephros, which forms as the pronephros degenerates, functions transiently as an excretory organ, but it ultimately regresses and contributes in part to the formation of the male genital system. The metanephros begins to form in the fifth week of gestation in human beings and persists as the permanent adult kidney. The formation of nephrons, the individual functional units of the adult kidney, is initiated through reciprocally inductive signals between two embryonic tissues in the IM, the ureteric bud and metanephric mesenchyme. While the development of the pronephros, mesonephros, and metanephros is separated in both time and space, certain features appear to be shared between them. For instance, the expression of particular developmental transcription factors such as PAX2, PAX8, and LHX1 is common to all three structures.<sup>5–7</sup> SIX2, the transcription factor expressed in the nephron progenitor cell population of the developing metanephros, is also expressed in the mesonephros, though its role there is uncertain.<sup>7</sup> To make matters more complicated, certain markers such as PAX8 and WT1 are expressed at the early stages of kidney development and downregulated, only to be activated again at later stages of nephron formation.<sup>8</sup> These overlapping and temporally dependent patterns of marker expression during kidney development



**Figure 1.** Marker analysis of the stages of kidney development. Schematic diagram showing the major stages of kidney development beginning with the primitive streak and markers known to be expressed or absent at each stage.

pose a challenge to efforts to derive kidney cell types from PSCs. Since expression of these markers is frequently used to monitor the progress of directed differentiation, it is critical that these markers be specific for the respective stage of differentiation. Importantly, markers known to be expressed at a particular stage of differentiation should be present and colocalized in the same cell population. Markers not representative of a particular stage should be absent, and demonstration of this lack of expression is arguably as important as demonstration of markers that should be present. Careful characterization of marker expression in differentiating cell populations is the first critical step to prove the developmental identity and physiologic relevance of cell populations derived from PSCs.

### Differentiation of Mouse PSCs into Kidney Cells

Initial attempts to differentiate PSCs into cells of the kidney lineage were performed using mouse ESCs and relied more on stochastic rather than directed differentiation. Yamamoto and colleagues differentiated mouse ESCs in the presence of serum

into embryoid bodies, which, when transplanted into mice, formed teratomas containing structures with features characteristic of mesonephric ducts and ureteric buds.<sup>9</sup> Subsequent efforts focused on identifying growth factors that could more specifically promote kidney differentiation from mouse ESCs. Though the precise signals involved in the earliest stages of kidney differentiation are not completely understood, certain growth factors such as retinoic acid (RA) and activin have been shown to have kidney inductive roles in *Xenopus*.<sup>10</sup> Kim and Dressler demonstrated that the combination of activin, RA, and bone morphogenetic protein 7 (BMP7) could induce the expression of the IM markers Pax2 and WT1. Vigneau and colleagues reported that treatment of mouse ESCs with activin induced Brachyury<sup>+</sup> mesodermal cells with nearly 50% efficiency.<sup>11</sup> In both these studies, the authors transplanted the differentiated cells into mouse embryonic kidneys and demonstrated evidence of incorporation into the forming tubular structures. Since the publication of these two studies, other groups have supported their findings that activin, BMPs, and RA are putative nephrogenic factors.<sup>12-15</sup>



## Differentiation of Human PSCs into Kidney Cells

Early efforts to direct the differentiation of human PSCs (hPSCs) into kidney cells were guided by the findings with mouse PSCs, employing similar approaches, and applying combinations of previously tested growth factors with variable success. Batchelder and Lin were the first to demonstrate the differentiation of human ESCs into cells expressing developmental kidney markers.<sup>16,17</sup> Other groups attempted to differentiate hPSCs directly into mature kidney epithelial cell types without the generation of intermediate kidney precursor cells. Song and colleagues used a combination of embryoid body and monolayer differentiation methods and treated human iPSCs with RA, activin, and BMP7, producing podocyte-like cells expressing podocin and synaptopodin.<sup>18</sup> Narayanan and colleagues showed the differentiation of human ESCs into renal proximal tubular-like cells expressing aquaporin-1 (AQP1) using Renal Epithelial Cell Growth Media (REGM, Lonza) supplemented with BMP2 and BMP7.<sup>19</sup>

Several recent reports have demonstrated the differentiation of hPSCs into populations of nephron progenitor cells, specifically cells of the IM and the metanephric mesenchyme. Employing an *OSR1*-GFP reporter human iPSC line to monitor differentiation into *OSR1*<sup>+</sup> cells of the IM, Mae and colleagues were able to induce *OSR1*<sup>+</sup> cells with 90% efficiency in 11 days using a two-step protocol involving the addition of the GSK-3 $\beta$  inhibitor CHIR99021 (CHIR) and activin to induce mesendoderm, followed by CHIR and BMP7 to induce IM.<sup>20</sup> The authors showed that *OSR1*<sup>+</sup> cells could give rise to expressing markers of kidney, gonad, and adrenal cortex, all of which are IM derivatives. However, the efficiency of kidney-specific differentiation was not quantified. Furthermore, the use of *OSR1* as a kidney-specific marker may be confounded by the fact that *OSR1* is also expressed during heart, limb, and branchial arch development.<sup>21,22</sup> Therefore, expression of *OSR1* by itself may not be the ideal marker to enrich kidney progenitor populations from hPSCs.

Three different groups have used the co-expression of the transcription factors PAX2 and LHX1 as a more specific marker for IM differentiation from hPSCs.<sup>23–25</sup> Although PAX2 and LHX1 are individually expressed in multiple embryonic tissues during development, co-expression of these markers is limited to the developing kidney and spinal cord. Our group developed a monolayer culture protocol to generate PAX2+LHX1+ cells with 70%–80% efficiency within three days by sequentially treating hPSCs with CHIR to induce mesendoderm followed by the combination of FGF2 and RA.<sup>25</sup> With extended culture, PAX2+LHX1+ cells spontaneously formed polarized tubular structures expressing the proximal tubular markers kidney-specific protein (Ksp), N-cadherin, and *Lotus tetragonolobus* lectin. Further treatment of PAX2+LHX1+ cells with FGF9 and activin induced the expression of SIX2, SALL1, and WT1, markers of the metanephric cap mesenchyme. Similarly, Takasato and colleagues showed that the addition of BMP4+activin (or

CHIR) and FGF9 could induce PAX2+LHX1+ IM cells within six days.<sup>24,26</sup> Sustained treatment of these cells with FGF9 resulted in the generation of SIX2+ cells within 14 days with 10%–20% efficiency. The authors showed that PAX2+E-cadherin+ cell clusters were also present in the same cultures with the SIX2+ cells, suggesting that cells of both metanephric mesenchyme and ureteric bud lineages existed in the same culture. Mixing these heterogeneous cultures with dissociated and reaggregated mouse embryonic kidneys resulted in a small proportion of human cells contributing to mouse tubular structures. Three-dimensional pelleting of the SIX2+ cells produced aggregates that contained tubular structures expressing markers such as AQP1, AQP2, JAG1, E-cadherin, WT1, and PAX2.

Xia and colleagues induced PAX2+LHX1+ cells from hPSCs within four days using a combination of BMP4 and FGF2 followed by RA, activin, and BMP2.<sup>23,27</sup> Though the authors initially labeled these as IM cells, they concluded that they were ureteric bud progenitor cells based on their spontaneous upregulation of transcripts of the markers *GDNF*, *HOXB7*, and *RET* within two days. Furthermore, incorporation of these putative ureteric bud progenitor cells with dissociated-reaggregated mouse embryonic kidneys resulted in partial integration of the human cells with mouse ureteric bud structures. While these findings are encouraging, a protocol to specifically generate ureteric bud cells from hPSCs alone has yet to be established.

A longer, 24-day protocol to generate nephron progenitor cells from hPSCs was introduced by Kang and Han, who also proposed a stepwise system transitioning through states of primitive streak and IM differentiation before generating SIX2+ cells with 38% efficiency.<sup>28</sup> In addition to SIX2, these putative nephron progenitor cells expressed other metanephric mesenchyme markers, including PAX2, SALL1, and WT1. The authors then demonstrated that these SIX2+ cells could differentiate into renal tubular epithelial-like cells or podocyte-like cells when treated with REGM or VRAD medium, respectively, and cultured on fibronectin-coated surfaces for 21 days. Culturing the SIX2+ cells in 3D collagen I gels induced the formation of KRT18+ tubular structures.

An alternative approach to generating nephron progenitor cells was proposed by Taguchi and colleagues, who postulated that the embryonic origins of the metanephric mesenchyme were distinct from those of other IM structures such as the pronephros, mesonephros, and ureteric bud.<sup>29</sup> Using mouse lineage tracing techniques, these authors demonstrated that the origin of the cells of the metanephric mesenchyme could be traced back to T (Brachyury)+ cells of the posterior primitive streak, which subsequently give rise to *OSR1*<sup>+</sup> cells of the posterior IM. The origin of the cells that form the ureteric bud, however, is the anterior IM, which is not capable of contributing to the metanephric mesenchyme. The authors devised a multistep protocol using embryoid body culture methods to differentiate mouse and human iPSCs sequentially into



posterior nascent mesoderm (T+CDX2+TBX6+HOX11+), posterior IM (OSR1+WT1+HOX11+), and ultimately metanephric mesenchyme cells expressing SIX2 (62.0%), PAX2 (19.9%), WT1 (72.4%), and SALL1 (44.0%). Differentiation into metanephric mesenchyme could be achieved within 8.5 and 14 days using mouse and human iPSCs, respectively. Co-culture of these putative nephron progenitor cells with mouse embryonic spinal cord induced differentiation into tubular and glomerular structures.

As summarized above, significant progress has been made in the directed differentiation of hPSCs into cells of the kidney lineage. A comparison of these different differentiation methods reveals obvious and significant heterogeneity in terms of the culture methods used (eg, embryoid body versus monolayer conditions), growth factor and/or small molecule combinations, number of steps involved, duration of the protocol, and the markers used to identify the kidney lineage. Overall, the efficiency of nephron progenitor cell generation remains relatively low. As there is no existing gold standard to test the functional capacity of kidney progenitors derived from hPSCs, it remains unclear as to which of these protocols generates cells that are closest in identity to nephron progenitor populations *in vivo*. Definitive evidence of differentiation into metanephros rather than mesonephros is also lacking, since the process of nephrogenesis up to the S-shaped body stage is also observed in the mesonephros. In addition, while most of these protocols demonstrate the ability of PSC-derived nephron progenitor cells to give rise to structures resembling nephron rudiments, none have been able to generate mature nephron segments or a complete, multicompartiment nephron. Establishing a differentiation roadmap that recapitulates *in vivo* metanephric development as closely as possible is most likely to efficiently generate nephron progenitor cells and mature kidney cells that appear and function most closely to their *in vivo* counterparts.

### A New Developmental Roadmap for Generating Metanephric Mesenchyme

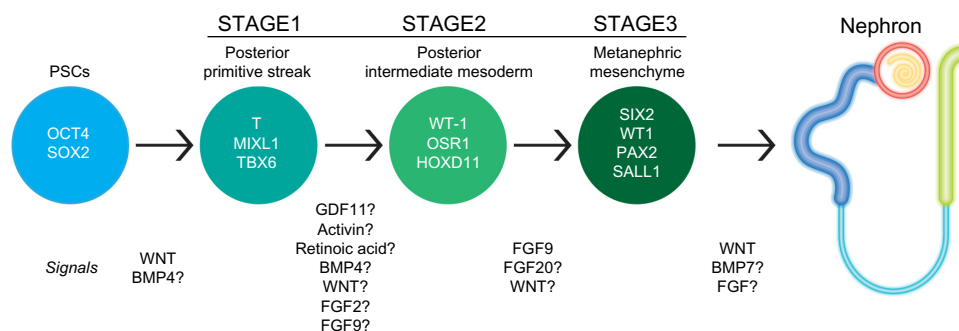
We hereby propose a differentiation roadmap based on what is known about the *in vivo* development of metanephric mesenchyme (Fig. 1). In the first step, PSCs are differentiated into cells of the posterior primitive streak, the earliest tissue fated to give rise to the metanephric mesenchyme, and monitored for the expression of T, a marker expressed along the length of the primitive streak, and TBX6, a marker more specific for the posterior region of the primitive streak (Fig. 2). The combination of these two markers should be used as a marker to distinguish posterior primitive streak. Wnt/ $\beta$ -catenin signaling is most likely to induce this step of differentiation,<sup>30</sup> though consideration should also be given to the time dependence of cell fate determination during gastrulation, since posterior IM is derived from late-stage primitive streak.<sup>29</sup>

In the second step, cells of the late-stage posterior primitive streak are specified into cells of the posterior IM, which

are characterized by the co-expression of the markers WT1 and OSR1. PAX2 and LHX1, markers of anterior IM, should not be expressed in posterior IM. This is a key feature in distinguishing metanephric from mesonephric mesenchyme, since the mesonephric mesenchyme arises from anterior IM that expresses all the four of these markers. In addition, the expression of HOX11 genes is also important in distinguishing between metanephric and mesonephric mesenchyme, since HOX11 gene expression is restricted to the region of the metanephric mesenchyme. HOXD11 has been shown to be an important transcription factor for metanephric mesenchyme induction.<sup>7</sup> TBX6 expression at this stage is localized to paraxial mesoderm and should be absent from posterior IM. In this second step, the signals to induce HOX11 genes are not well defined. Gaunt and colleagues reported that Gdf11/Smad2/3 signaling activated axial expression of Hoxd11<sup>31</sup>; therefore, activin, which also signals via Smad2/3, is a potential inducer of Hox11 genes. In addition to activin, BMP4, CHIR, FGF2, FGF9, and RA have been used in different combinations in published protocols to induce IM differentiation,<sup>24,25,29</sup> suggesting that these signals could also be involved.

The third step is the specification of posterior IM into the nephron progenitor cells of the metanephric mesenchyme. Nephron progenitor cells should be characterized by the co-expression of SIX2, SALL1, WT1, and PAX2. In addition, the production of GDNF, which promotes ureteric bud invasion into metanephric mesenchyme, might also help to identify nephron progenitor cells.<sup>32</sup> During reciprocal interaction between metanephric mesenchyme and ureteric bud tips, metanephric mesenchyme forms dense clusters called cap mesenchyme in which Cited1 expression is activated.<sup>33</sup> In this step, FGF9 and FGF20 are likely candidate factors to induce SIX2 expression, given that they have been shown to play an important role in the *in vivo* maintenance of Six2+ nephron progenitors in mice.<sup>34</sup> Following the induction of the nephron progenitor cell population, the final step is the mesenchymal-to-epithelial transition (MET) of metanephric mesenchyme into nephrons consisting of podocytes, proximal tubules, loops of Henle, and distal tubules along with loss of mesenchymal markers such as Vimentin. This process is mediated by Wnt9b/Wnt4 signaling that originates from the ureteric bud.<sup>35</sup> Embryonic spinal cord has also been shown to induce tubulogenesis in *ex vivo* metanephric mesenchyme explants, presumably also via Wnt signals.<sup>36</sup> However, studies to date have not been able to demonstrate that Wnt signaling alone is sufficient to induce MET in PSC-derived nephron progenitor cells. Further studies will be required to identify the necessary and sufficient signals to induce SIX2+ nephron progenitors to form nephrons.

The establishment of this roadmap defining the stages of differentiation as well as specific combinations of markers that should be present and absent at each stage should facilitate the development of protocols that can more efficiently produce metanephric nephron progenitor cells.



**Figure 2.** Diagram for kidney differentiation from hPSCs. Markers and signals for each stage of differentiation.

## Conclusions

The recent progress in kidney differentiation from PSCs advances us closer to the ultimate goal of generating a human nephron entirely from human cells. Protocols must be further refined and optimized to enhance the efficiency of nephron progenitor cell generation, and the signals to induce nephron progenitor cells to form renal epithelia *in vitro* need to be defined. Construction of a complete human nephron will also require more defined methods of generating cells of the ureteric bud lineage in order to reproduce the collecting duct system. More investigation is also needed in how to integrate a vascular supply and stromal compartment into nephron structures derived from PSCs. The ability to regenerate human nephrons will hopefully translate into novel therapies for patients with kidney disease.

## Author Contributions

Conceived and designed the experiments: RM, AQL. Analyzed the data: RM, AQL. Wrote the first draft of the manuscript: RM, AQL. Contributed to the writing of the manuscript: RM, AQL. Agree with manuscript results and conclusions: RM, AQL. Jointly developed the structure and arguments for the paper: RM, AQL. Made critical revisions and approved final version: RM, AQL. Both authors reviewed and approved of the final manuscript.

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