Engineering Kidney Organoids Derived From Human Induced Pluripotent Stem Cells Under 3-Dimensional Conditions

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Abstract:

Human induced pluripotent stem cells (iPSCs) are a powerful tool for conducting research, as they have the potential to differentiate into all three germ layers, which can serve as the basis for generating organoids. In the last decade, progress has been made in developing protocols to generate kidney organoids from iPSCs, as in vitro kidney models can enable disease modelling, drug screening, and potentially organ regeneration. This study aims to investigate the initial phase of renal differentiation and the influence of culture medium, spatial configuration, and the effect of various small molecules in the final cell population. To achieve this, the Morizane, Takasato, and Uchimura protocols have been fully adapted to 3D conditions. Furthermore, this project proposes the development of a novel hybrid protocol “Morisato”, inspired by the concept of separately inducing the kidney progenitor populations and then co-culturing them to form complex structures. Finally, the utility of the resulting renal organoids as an option to model drug-injury responses was evaluated. Obtained kidney organoids showed heterogeneity in shape and size but demonstrated capably of self-organize into impressive tubular structures, found to be distal tubes, early proximal tubules, and showed existence of glomeruli cells. However, organoids revealed to be immature, with an inconclusive drug-injury response and no collecting duct formation. As main conclusions, kidney organoids can be generated from human iPSCs under fully 3D conditions and demonstrate that development of different populations within the kidney is a highly dynamic process that can be controlled by external factors.

Keywords: human induced pluripotent stem cells; kidney organoids; renal differentiation; 3D culture; 2D culture

Introduction

Pluripotency can be defined as capacity to differentiate into tissues of all three germ layers: ectoderm, mesoderm, and endoderm, as well as the germ lineage (Bradley et al., 1984). The knowledge of genetic hallmarks in pluripotent stem cell populations let to an important breakthrough in stem cell technology: the method of nuclear reprogramming adult cells into pluripotent stem cells (PSCs). This discovery shifted the stem cell research paradigm by demonstrating that differentiation is not a unidirectional process. iPSCs were obtained for the first time in 2006 through the introduction of the expression of transcription factors, found to be increased in embryonic stem cells (ESCs), specifically, OCT4, SOX2, c-Myc, and Klf4, delivered by retroviral vector constructs. iPSC-derived organoids have taken the spotlight of stem cell research as they are not limited to accessibility to tissues, in comparison to adult tissue-derived organoids. The current rational of *in vitro* organoid generation from iPSC is based on closely mimicking the complete process of the organ development during embryogenesis, in which the first step is the germ layer specification into ectoderm, mesoderm or endoderm. What triggers the iPSC differentiation is the introduction of specific combinations of exogenous agents like small molecules and growth factors, even though it is impossible the exact imitation of all biochemical factors that drive cell differentiation at the right timing, concentration, and localization at which they occur throughout embryogenesis. Luckily, this process is facilitated by the nearly autonomous differentiation trajectory that the stem cells follow, displayed both *in vivo* and *in vitro*. Kidney organoids were observed to differentiate and self-assemble spontaneously in response to environmental stimuli similar to those seen in the developing kidney (Grobstein & Dalton, 1956; Takasato & Little, 2016). By mimicking nephrogenesis, the first step on the differentiation protocol is late primitive steak induction, that can be achieved with the presence of WNT signaling. CHIR99021, a GSK-3 inhibitor, is used to induce late primitive streak via canonical WNT signaling pathway

activation (Nusse, 2008). The anterior intermediate mesoderm (AIM) is known to differentiate into ureteric bud (UB), whilst posterior intermediate mesoderm (PIM) gives rise to the metanephric mesenchyme (MM), the nephron progenitor and to posterior mesonephric mesenchyme. Reciprocally inductive interactions between these two populations (UB and MM) are key to nephrogenesis. Therefore, to mimic kidney development *in vitro*, it is crucial to recapitulate the interactions between AIM and PIM populations. This precise concept is used in the Uchimura protocol, which is examined and applied in this study under fully 3D conditions. In addition, special attention is given to the type of populations present in intermediate mesoderm (IM) phase and structures that they give rise in future kidney organoids. Additionally, this project aims to develop a framework for a novel hybrid protocol "Morisato". Different conditions will be tested to determine which are most favorable for producing each of the two main populations: AIM and PIM. Then, the concept of the Uchimura protocol will be applied to develop a protocol that uses fewer small molecules and a more cost-effective culture medium.

Materials & Methods

Cell Line and Maintenance of human iPSC

All the experiments employed the human iPSC line DF6-9-9T.B (DF6) obtained from WiCell Research Institute (Wisconsin, United States of America). DF6 cell line was generated from a healthy donor's foreskin fibroblasts with a normal karyotype (44+XY) through retroviral transduction using seven reprogramming factors (OCT4, SOX2, Nanog, LIN28, L-Myc, Klf4 and SV40 T). The cell culture in the 6-well tissue culture plates coated with Matrigel using mTeSR Plus was kept in a humified incubator at 37°C, 5% CO_2, 20% O_2 and culture medium was changed daily. Human iPSCs were passaged at 1:3-1:5 split ratio when 60%-70% cell confluence was achieved by using 0.5 mM EDTA solution (Invitrogen) diluted in phosphate-buffered saline (PBS, Sigma-Aldrich). Cell passages between 48 and 64 were used for all experiments.

Differentiation of human iPSCs into renal lineage

Culture media

In all experiments of differentiation of human iPSCs into renal linage, one of the following culture media was used: Advanced Roswell Park Memorial Institute medium 1640 (Advanced RPMI 1640, Gibco) or STEMdiff APEL 2 (STEMCELL Technologies). Both media were supplemented with 0.5% (v/v) penicillin/streptomycin, stored at 4ºC and pre-warmed at room temperature before use. Advanced RPMI 1640 medium was supplemented with 1:100 (v/v) GlutaMAX-I (100X) (Gibco) before use. Advanced RPMI 1640 medium was used during Morizane and "Morisato" protocols. STEMdiff APEL 2 medium is a fully defined, serum-free and animal component-free medium. This medium was used during Takasato and Uchimura protocols.

Aggregate 3D culture setting

For the 3D differentiation of DF6 human iPSCs and their promotion to assemble into cell aggregates, AggreWell 800 plates (STEMCELL Technologies) were used (Ungrin et al., 2008). For the process of cell seeding, iPSCs were collected, dissociated into single cells using Accutase. Then, resuspended in mTeSR Plus supplemented with 10μM ROCKi and the adequate number of cells was transferred into AggreWell 800 and spun down at 1,000 rpm for 3 minutes, to force cells to settle into the microwells. Upon successful seeding, cells were kept in a humified incubator at 37°C, 5% CO_2, 20% O_2. After a 24-hour period, the differentiation protocol was initiated.

Air-liquid interface 3D culture setting

Air-liquid interface 3D culture or Transwell culture was possible with the use of 6-well Costar Transwell 3450 clear plates (Corning) that in which each well contains a 24 mm polyester membrane with 0.4 μm pore, treated for optimal cell attachment. Transwell culture setting was only applied during the Morizane modified protocol (Morisato protocol) and Uchimura protocol after day 7 of differentiation in aggregate culture. Each Transwell filter was seeded with 5 different aggregates with $5x10^5$ cells each.

Differentiation protocols of human iPSCs into renal lineage

Takasato protocol

At day 0 of differentiation (24h after seeding)

culture media was replaced with STEMdiff APEL-2 medium supplemented with CHIR99021 (8 or 11 µM) for 4 days, followed by APEL-2 supplemented with 200 ng ml^{-1} FGF9 (R&D Systems) and 1 µg ml^{-1} heparin (Sigma-Aldrich) for another 3 days, replacing the medium every other day. Both in the monolayer differentiation and in 3D culture differentiation aggregates, the protocol was halted at day 7 timepoint prior to fixation in 4% (v/v) PFA in PBS at 4ºC, for 20 min and stored in PBS for posterior analysis. Additionally, approximately $1x10^6$ whole cells were recovered and stored at -80ºC for RNA extraction.

"Morisato": Novel hybrid protocol

"Morisato" is a portmanteau of the words "Morizane" and "Takasato", which reflects on its hybrid nature as it was created based on both protocols referenced. The medium used during this differentiation protocol is the Advanced RPMI1640, which was supplemented with 11 µM CHIR99021 for 2-4 days, followed by the same medium supplemented with 200 ng ml^{-1} FGF9 and 1 µg ml^{-1} heparin for another 3-5 days, replacing the medium every other day. The protocol was halted at day 7 timepoint prior to aggregate fixation in 4% (v/v) PFA in PBS at 4ºC, for 20 min and stored in PBS for posterior analysis. Additionally, approximately $1x10^6$ whole cells were recovered and stored at -80ºC for RNA extraction.

Uchimura protocol

For Uchimura protocol two independent but simultaneous differentiations were undertaken which have the objective of induction the two renal linages from iPSCs: AIM and PIM. Firstly, both AIM and PIM cultures followed in the Aggregate 3D culture setting until day 7. For the PIM linage differentiation, Takasato protocol was followed until day 7. Briefly, cells were treated with 11 µM CHIR99021 in basal medium STEMdiff APEL 2 for 4 days, followed by FGF9 (200 ng/mL) and heparin (1 mg/mL) for another 3 days. The AIM linage differentiation started with 11 µM CHIR99021 treatment for one day, followed by treatment with CHIR99021 (11 µM), Activin A (10 ng/mL), and BMP4 (1 ng/mL, R&D Systems) for two days, followed by FGF9 (200 ng/mL), heparin (1 mg/mL), Activin A (1 ng/mL), Retinoic Acid (100 nM, Sigma-Aldrich), and LDN193189 (100 nM, StemGent) for 4 days with the exception that LDN193189 was reduced to 30 nM after two days. In order to generate kidney organoids, the AIM and PIM lineage cells were dissociated into single cell suspension using Accutase, at day 7. A total of $5x10^5$ cells of each lineage and also a combination of the two lineages at 3:1 ratio was mixed and spun down at 1,000 rpm for 3 min to form a pellet. The pellets were transferred onto a Transwell membrane and incubated with CHIR99021 (5 µM) for 1 hour, then cultured with FGF9 (200 ng/mL), heparin (1mg/mL), Retinoic Acid (100 nM), GDNF (10 ng/mL, PeproTech), and EGF (10 ng/mL, R&D Systems) for 5 days. For the next 17 days, the organoids were cultured in only basal medium that was changed three times a week.

"Morisato" in Transwell protocol

Morizane "long" protocol follows a similar principle of independent but simultaneous induction of the two renal linages AIM and PIM in 3D conditions. For the first 7 days of differentiation for the PIM aggregates, the "Morisato" protocol was applied. Firstly, Advanced RPMI1640 supplemented with 11 µM CHIR99021 for 4 days, followed by the same medium supplemented with 200 ng ml^{-1} FGF9 and 1 µg ml^{-1} heparin for another 3 days. In terms of AIM linage differentiation, the protocol Morizane was followed in the first 7 days. Aggregates were cultured in Advanced RPMI1640 supplemented with 11 µM CHIR99021 for 2 days, following supplementation with Activin A (10 ng/ml) for the next 5 days. At day 7 of differentiation, both AIM and PIM aggregates were collected and dissociated into single cell suspension using Accutase. A total of $5x10^5$ cells of each lineage and also a combination of the two lineages at 3:1 ratio was mixed and spun down at 1,000 rpm for 3 min to form a pellet. The pellets were transferred onto a Transwell membrane and incubated with CHIR99021 (5 µM) for 1 hour, then cultured with FGF9 (200 ng/mL for 5 days. For the next 17 days, the organoids were cultured in only basal medium that was changed three times a week.

Cell characterization

Immunocytochemistry

Cell cultures in 6-well plates were previously fixed in 4% PFA at 4ºC for 20 minutes.

Sectioned cell aggregates in glass sides were washed with 0.1 M Glycine (Sigma-Aldrich) in PBS then, permeabilized with 0.1% (v/v) Triton X-100 in PBS and blocked with blocking solution composed with 10% (v/v) fetal bovine serum in TBST, constituted by 20 mM Tris-HCl pH 8.0 (Sigma), 150 mM NaCl (Sigma) and 0.05% (v/v) Tween-20 (Sigma-Aldrich) in PBS. Cells were incubated with the primary antibody diluted in blocking solution at 4ºC overnight. Secondary antibody was diluted in blocking solution and incubated with the cells, in the dark, at room temperature for 30 minutes. After this, cells were incubated with 15:10000 dilution of 4',6-diamidino-2-phenylindole (DAPI) dye (Sigma-Aldrich) at room temperature for 5 min. Mowiol (Sigma-Aldrich) was added using a glass-cover and was left overnight to dry. Cells were examined using a fluorescence microscope Leica DMI3000B and a digital camera Leica DFC7000T, or with Zeiss LSM 710 Confocal Laser Point-Scanning Microscope using 20x objective and a digital zoom of 80%.

Quantitative RT-PCR

Total RNA from cell samples of both in monolayer and in 3D cultures were extracted using High Pure RNA Isolation Kit (Roche Diagnostics) following manufacturer's instructions. After RNA quantification with nanodrop, 1 µg of RNA was converted into cDNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) also following manufacturer's instructions. The RNA-cDNA conversion was done resorting to a T100™ Thermal Cycler (Bio-Rad) with the following program: 10 minutes at 25ºC, 120 minutes at 37ºC, 5 minutes at 85ºC and then, 4ºC until storage (at -20ºC). All PCR reactions were run in triplicate on Step One Plus Real-time PCR System (Applied Biosystems) or on ViiA™ 7 RT-PCR Systems (Applied Biosystems) using NZY Supreme qPCR Green Master Mix Rox Plus (NZYTech), with 12.5 ng of cDNA and 250 µM of each primer of each gene. Data obtained was first normalized to housekeeping gene GAPDH and then normalized to control samples (day 0) by applying the ΔΔCt method. The final results of gene expression are represented as $2^{-\Delta\Delta CT}$. Graphs were made using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, USA).

Results & Discussion

Uchimura protocol in fully 3D conditions

The original Uchimura protocol starts with separate induction of AIM population, the progenitor of MM and PIM, progenitor to ureteric bud-like cells. PIM induction, based on the Takasato protocol, and AIM protocol induction, a novel procedure, were followed till day 7. To access off-target populations at day 7 of differentiation, pluripotency marker OCT4, several mesoderm markers (KDR, PDGFRA-α, OSR1, PAX2 and WT1) and endoderm marker SOX17 were analyzed (Figure 1A). Results shown that in both AIM and PIM day 7 aggregates there is predominance of gene expression of IM makers, namely the early IM induction marker OSR1, also PAX2 and WT1 genes, which are vital for IM formation and later play a major role in kidney nephrogenesis (Figure 1A).

However, in AIM and PIM aggregates we can see also the expression of paraxial mesoderm marker PDGFRA-α (Sakurai et al., 2006). This indicates that there are some populations that in were not enough exposed to CHIR99021, the regulator mesendodermal patterning of iPSCs. This population is certainly difficult to avoid since paraxial mesoderm is derived from anterior primitive streak, just like IM. Interestingly, in AIM aggregates there is also expression of SOX17, a marker of definitive endoderm. This probably because some populations of cells were exposed high levels of Activin A, which leads to endoderm differentiation (Wang et al., 2015). Overall, there is efficient IM induction in both AIM and PIM aggregates, the off-target populations are expected to be residual because of the low gene expression that they display. Additionally, relative gene expression of GATA3 and HOXD11 was examined on day 7 of differentiation to confirm the anteroposterior fate of each population (Figure 1B). As expected, in the AIM aggregates had a strong expression of GATA3, but not HOXD11 (Uchimura et al., 2020). However, in PIM aggregates there was similar expression of the two markers. Suggesting that there is population of both progenitors of MM and UB. This is not totally unexpected as the original protocol from which PIM differentiation is based - Takasato protocol - reports the simultaneous induction of all progenitors of kidney lineage (Takasato et al.,

2016). Additionally, relative gene expression can change in the course of the differentiation as RNA can still be degraded before being translated to a protein, and this can be resolved by analyzing resultant population in the kidney organoid.

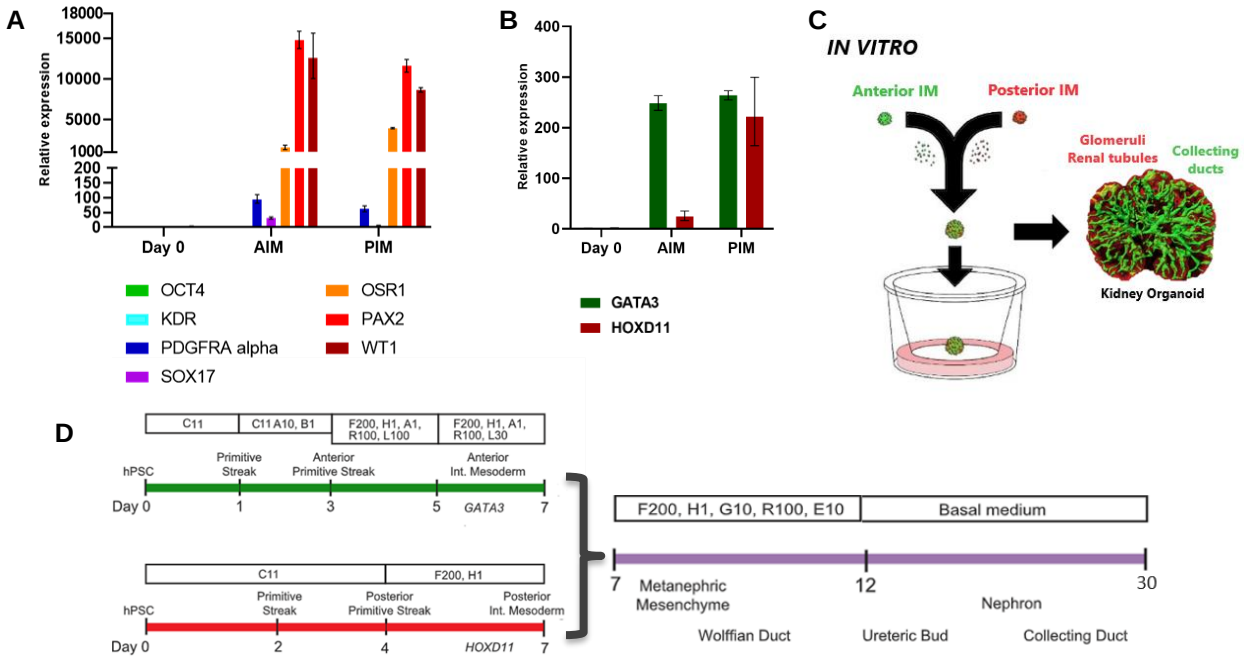

Figure 1 – (A) Relative expression profiles of pluripotency, renal lineage, and different mesoderm markers during renal differentiation under monolayer conditions at day 7. Values are normalized to GAPDH expression and plotted relative to gene expression levels in iPSCs (D0). Data are represented as means ± SD from technical triplicates. (B) Relative expression profiles of IM markers during renal differentiation under monolayer conditions at day 7. Values are normalized to GAPDH expression and plotted relative to gene expression levels in iPSCs (D0). Data are represented as means ± SD from technical triplicates. (C) Schematic representation of the overall procedure of mixing of the two-progenitor population of renal tissue to achieve complex kidney organoids. (D) Outline of Uchimura protocol from day 0 to day 30 of differentiation. C, CHIR99021 (mM); E, EGF (ng/mL); F, FGF9 (ng/mL); G, GDNF (ng/mL); H, heparin (mg/mL); R, nM retinoic acid. Adapted from (Uchimura et al., 2020).

At day 7, the two populations (AIM and PIM) were mixed and seeded in transwell plates (Figure 1C) at a ratio of 3:1, with a total of $5x10^5$ cells per aggregate. Additionally, aggregates with only one of the cell lineages were also seeded. A 1-hour CHIR99021 pulse was applied to all cell aggregates (Figure 1D) to optimize nephron formation, as the ureteric epithelium secretes WNT and stimulates nephron formation during kidney development (Takasato et al., 2015). Aggregates in the transwell filter stayed in air-liquid interface to maintain a 3D conformation. Outline of the protocol can be seen in Figure 1D.

Differentiation was halted at day 30 and immunofluorescence staining was performed in the sections of resulting kidney organoids to access which population were present. In PIM aggregates, immunofluorescence shows PAX8 expression but not LHX1 (Figure 2). There is no confirmation of the presents of renal vesicles (PAX8+ LHX1+) but PAX8 is abundantly expressed by renal blastema cells during nephrogenesis (Bouchard et al., 2002). Additionally, PAX8 staining was previously reported in renal epithelial cells in all segments of renal tubules from the proximal tubules to the renal papillae in the adult kidney (Tong et al., 2009).

There is the possibility of subsequent GATA3 gene downregulation, as in PIM differentiation is not expected to give rise to AIM derivates. The expression of LTL in tubular structures and the lack of expression of ECAD marker confirms the rise of early proximal tube (LTL+ ECAD-). In contrast, tubular structures with only ECAD marker expression that lack both LTL and GATA3 staining point to early distal tube (LTL- GATA- ECAD+) formation (Figure 2) Interestingly, expression of both markers in the

same tubular structure was noted but in distinct segments as there was never the simultaneous staining of LTL and ECAD. Nevertheless, ECAD marker is expressed both in immature proximal tube as well in the distal tube. Thus, it is difficult distinguish between these two structures. WT1 and NPHS1 markers both stain podocytes but only WT1 expression can be seen in the cells around tubular-shaped cells (Figure 2), which is enough to suggest the presence of glomeruli cells.

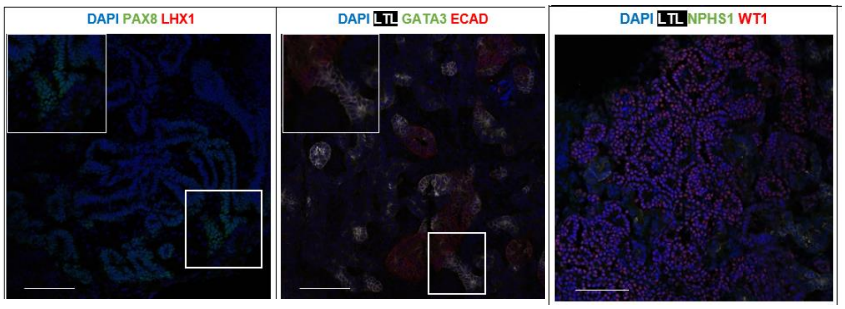

Figure 2 - Immunofluorescence staining in cryosections of PIM aggregate-derived kidney organoids at day 30 of Uchimura differentiation. Scale bars represent 100 µm.

Drug-injury model: cisplatin assay

On day 30 of differentiation, kidney organoids generated were treated with cisplatin (5 µM) for 24 hours to test their applicability as a model for research of drug-induced kidney injury. Cisplatin is an anti-cancer drug that was observed to cause proximal and distal tubular damage. It induces caspase-mediated acute apoptosis of proximal tubule cells in the kidney (Cummings & Schnellmann, 2002; Mese et al., 2000). Cisplatin nephrotoxicity assay is considered a maturation analysis on kidney organoids as nephrotoxicity is expected in mature kidney tubules. PIM-derived kidney organoids were immunostained for kidney injury molecule-1 (KIM-1), a biomarker that is substantially upregulated in the proximal tubules following acute kidney injury (Vaidya et al., 2010), as well as LTL and ECAD to distinguish between the proximal and distal tubules. In both control and exposed to cisplatin aggregates, there is LTL staining, which indicates the presence of early proximal tubes. However, there is no KIM-1 expression at the luminal surface of LTL+ tubules in organoids exposed to cisplatin that would indicate cisplatin-induced injury in proximal tubules (Figure 3A). It was previously reported that only in mature proximal tubules (LTL+ ECAD+) was possible to detected cisplatin-induced apoptosis, whereas in the immature early proximal tubules (LTL+ ECAD-) do not undergo apoptosis (Takasato et al., 2015). From this it is concluded that the proximal tubules of the PIM-kidney organoids are immature. Curiously, in organoids treated with cisplatin, there is almost no ECAD+ cells. Only a small cluster of ECAD+ cells can be seen in Figure 3A and is enough to confirm that ECAD staining was successful. The observed absence of ECAD+ cells could indicate injury in distal tubules as it was previously reported that cisplatin causes ECAD suppression in kidney organoids generated (Morizane et al., 2015). However, the absence of ECAD+ cells might be due to organoid heterogeneity that cause differences in the structures that formed inside the kidney organoids.

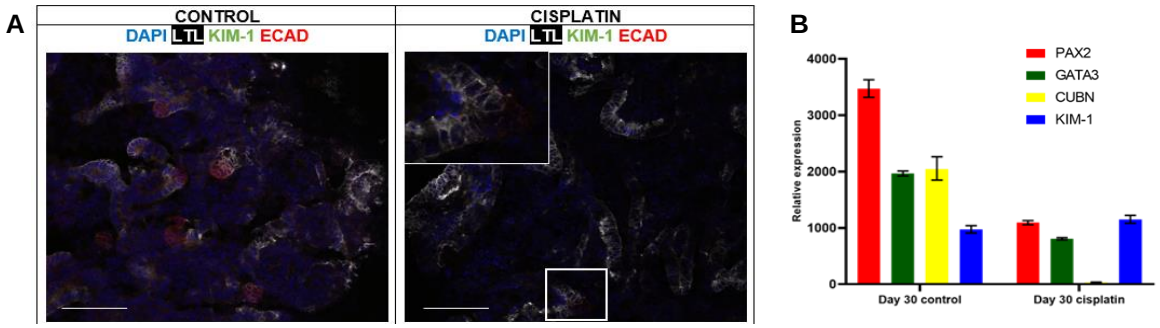

Figure 3 - Immunofluorescence staining in cryosections of control PIM aggregates derived kidney organoids at day 30 of Uchimura differentiation (left) and PIM aggregates derived kidney organoids at day 30 treated 24h with cisplatin (right). Scale bars represent 100 µm. Relative expression profiles of renal markers at day 30 of

(Continuation) differentiation in AIM+PIM aggregates treated with cisplatin and in control aggregates. Values are normalized to GAPDH expression and plotted relative to gene expression levels in iPSCs (D0). Data are represented as means ± SD from technical triplicates.

Additionally, in control and cisplatin-treated AIM+PIM kidney organoids, relative mRNA expression was assessed in different genes related to kidney structures and development PAX2, GATA3, CUBN and also the injury-related KIM-1 (Figure 3B). The levels of gene expression of PAX2, GATA3 and CUBN decreased significantly which in cisplatin-treated organoids, compared to the control organoids. This might be due to the cisplatin effect in the renal cells that might increase the renal cell stress response to a toxicant and thus, diminish the levels of genes related to synthesis of new structures inside the kidney. However, KIM-1 mRNA levels only increased slightly in cisplatin-treated organoids. In case proximal tubule injury, this molecule would be increased but this fact did not occur. A similar conclusion was reached after immunocytochemistry, in which KIM-1 staining was not detected. As discussed previously, results reveal that the proximal tubule injury is not detected by KIM-1 biomarker potentially because the proximal tubules developed in the organoids are still immature and do not sustain injury by cisplatin.

Development of "Morisato" protocol in fully 3D conditions

"Morisato" protocol uses Advanced RPMI medium as basis, the same as used in Morizane protocol. Furthermore, the novel "Morisato" protocol uses a limited set of small molecules (CHIR99021, Activin A, FGF9 and heparin) throughout differentiation to induce AIM and PIM populations, inspired by the protocols of Morizane and Takasato (Morizane & Bonventre, 2017; Takasato et al., 2016). In order to assess the optimal conditions to differentiate AIM and PIM populations, three main adaptations were subjected to modification: the initial size of the aggregate, the duration of CHIR99021 exposure and the following supplementation of either Activin A (similarly to Morizane protocol) or supplementation of FGF9 and heparin (similarly to Takasato protocol). Briefly, initial duration of WNT signaling was varied by manipulation of CHIR99021 exposure length in the culture medium (2 or 4 days). After primitive streak induction with CHIR99021 exposure, the next step of IM induction was the addition of either Activin A (during Morizane protocol) or FGF9 and heparin (during "Morisato" protocol) until day 7 of differentiation. The initial size of the aggregate as 1,000 cells per aggregate ("1K") or 2,000 cells per aggregate ("2K") was also taken into consideration. After the selection of the most suitable protocols and respective modifications: Morizane 1K2CHIR for the differentiation of AIM population and "Morisato" 1K4CHIR for the differentiation of PIM population (Figure 4), differentiation was started by two separate but simultaneous differentiations in 3D environment.

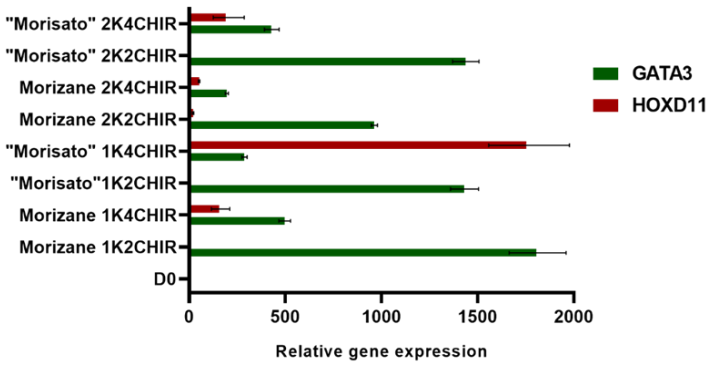

Figure 4 - Relative expression profiles of intermediate mesoderm markers during renal differentiation under distinct conditions at day 7 with Morizane or "Morisato" protocols. Values are normalized to GAPDH expression and plotted relative to gene expression levels in iPSCs (D0). Data are represented as means ± SD from technical triplicates.

At day 7, the resulting aggregates were mixed and exposed to 5µM of CHIR99021 pulse for 1h. Additionally, only AIM and PIM populations (without mixing) were also seeded in the transwell. FGF9 and heparin was supplemented until day 12 of differentiation and after that, only basal medium was supplied until day 30 (Figure 5C).

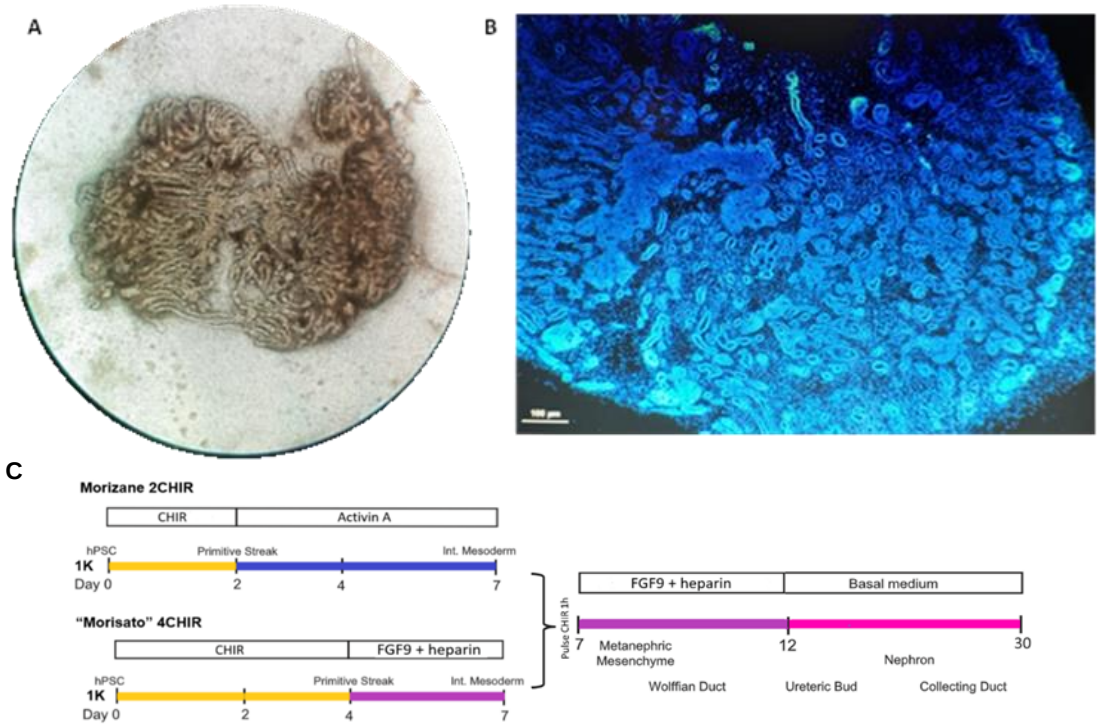

Figure 5 – (A) Brightfield image of a kidney organoid derived from Morizane 1K2CHIR aggregates at day 29 of differentiation. Image at 10x magnification. (B) Immunofluorescence staining with DAPI in a cryosection of the same 1K2CHIR Morizane-derived kidney organoid as in (A) at day 30 of differentiation. Scale bars represent 100 µm. (C) Outline of modification to Morizane and "Morisato" protocols that rise from modifications of conditions, from day 0 to day 7 of differentiation.

After the seeding the transwell, only separate populations developed into microscopically visible structures associated with the starting of the nephrogenesis. AIM+PIM populations assumed a monolayer layer of cells with no complexity in the transwell and did not develop further. This translated to only organoids derived from only one of the populations: Morizane 1K2CHIR and "Morisato" 1K4CHIR.

The resulting kidney organoids were heterogenous in size and shape. Nevertheless, the resulting 1K2CHIR Morizane derived organoids revealed unexpected results, as the structures formed were suspected to not be originated from AIM population only (Figure 5A). Only AIM-derived kidney organoids were possible to cryosection and immunostain (Figure 5B). Immunostaining revealed the presence of renal vesicles (PAX8+ LHX1+), early proximal tubules (LTL+ GATA3+) and distal tubules (LTL- ECAD+). However, where was no WT1 and NPHS1 staining, suggesting no glomeruli generation. Similarly, the lack GATA3 expression indicates that collecting duct (GATA3+ ECAD+) formation is absent (Figure 6).

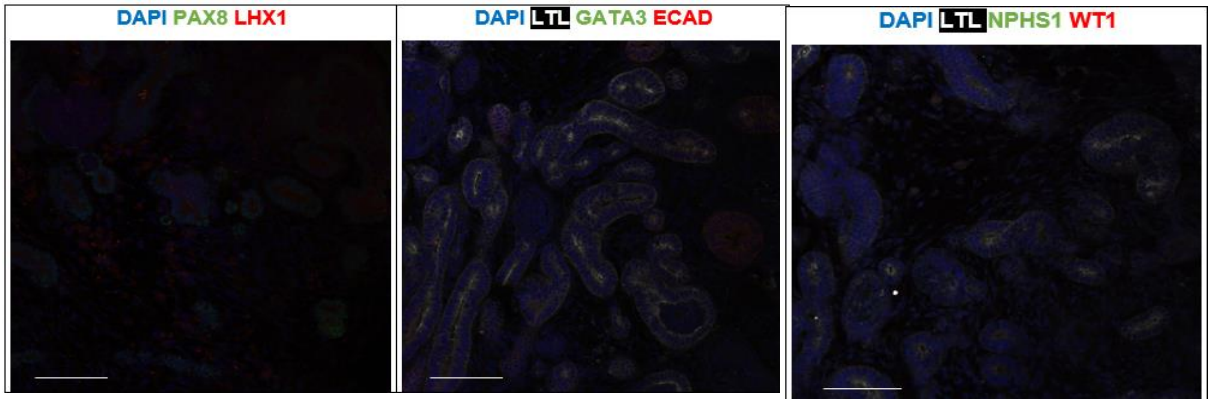

Figure 6 - Immunofluorescence staining in cryosections of AIM aggregate-derived kidney organoids at day 30 of "Morisato" differentiation. Scale bars represent 100 µm.

Structures like renal vesicles, proximal tubules, distal tubules and also the presence of nephron progenitor cells indicates that the gene expression AIM population at day 7 in 1K2CHIR Morizane-derived aggregates is not truly revealing in terms of future cell population inside the aggregate. The process of antero-posterior patterning in IM is more dynamic than expected. Additionally, batch-to-batch heterogeneity between aggregates may play a role in the derivation of populations that ultimately bring distinct outcomes in terms of final cell populations inside the kidney organoid.

Conclusion & Future Remarks

In the adaptation of Uchimura protocol in fully 3D conditions revealed that in day 7, there was no significant gene expression of off-target genes in the differentiated populations AIM and PIM. However, unexpectedly the AIM population revealed both gene expression of markers characteristic of both AIM and PIM population. Indeed, it was observed the rise of some rudimentary tubular structures as early as in day 15 of differentiation in the AIM-derived kidney organoids. The identification of this structures requires more study since organoids did not survive cryosectioning method. It is proposed that whole-mount immunohistochemistry is probably a better alternative to cryosectioning in this type of kidney organoids. Kidney organoids derived from AIM+PIM population and PIM populations revealed impressive tubular structures that could be easily seen under the microscope. Immunostaining revealed distal tube (LTL- GATA- ECAD+) and early proximal tube (LTL+ ECAD-) formation, surrounded by WT1+ glomeruli cells. With no GATA3 expression, it was concluded that kidney organoids obtained were still immature.

Furthermore, the cisplatin assay used to confirm organoid viability as proximal tubule injury model revealed no KIM-1 expression, hypothesized that this is due to the fact that the proximal tubules present in the kidney organoid are still immature. However, it is suspected that cisplatin halted the growth of distal tubules, observed in immunostaing and down-regulated genes related with tubular genesis as revealed in the RT-PCR analysis. In future experiments, I propose to access the gene expression of neutrophil gelatinase-associated lipocalin (NGAL). This could shed light into distal tubule injury by cisplatin, since NGAL is an early biomarker for nephrotoxic injury specifically in distal tubules of kidneys. NGAL protein expression was induced specifically in ECAD-positive distal tubules while subjected to cisplatin, as it was reported in Uchimura protocol article (Uchimura et al., 2020). Alternatively, apoptosis in cells treated by cisplatin can be detected by cleaved caspase 3 antibody-staining (CASP3) and give a more general answer about the overall damage in the organoids (Takasato et al., 2015). There is also the question if there is sufficient exposure of cisplatin in kidneys organoids, as a 24-hour exposure to 5 μM concentration of cisplatin may not be enough to assess significant injury in the tubules, but only the downregulation of genes related to tubule growth. Future research should certainly test further whether cisplatin injury is dose-dependent and at what threshold there is simultaneous distal tube and proximal tubes injury. Additionally, whole-mount immunostaining can potentially expand the understanding if there is specific zones or gradients that are damaged by cisplatin in the kidney organoids that are grown in transwell. Since air-liquid interface of transwells imply limited contact with the culture medium, where the cisplatin is supplemented. Development of “Morisato” protocol is set out to be more economically viable option as Uchimura protocol, with less small molecules supplemented and at the same time using the same rationale of separate induction followed by co-culture. “Morisato” rises as a promising protocol but requires more optimization, namely in the number of cells of each population (AIM and PIM) seeded in the transwell and their respective ratio as well, as a response to the problem inviable populations seeded in transwell. Nevertheless, surviving AIM-derived kidney organoid at day 30 of differentiation shown structures canonically derived from PIM populations, such as renal vesicles (PAX8+ LHX1+), early proximal tubules (LTL+ GATA3-) and distal tubules (LTL- ECAD+). However, where was no WT1 and NPHS1 staining, suggesting no glomeruli generation.

Kidney organoids at day 30, both in Uchimura and “Morisato” protocols, revealed to be highly heterogenous in size and shape. This may be

related with the number of cells that survive seeding in transwell and quality of the populations. This problem can be resolved by bioprinting that could allow precise manipulation of biophysical properties, including organoid size, cell number and conformation in the transwells. One example is the application of extrusion-based 3D cellular bioprinting made possible high-throughput generation of kidney organoids with highly reproducible cell number and viability (Lawlor et al., 2021).

Overall, the limitations of the present study include low number or absence of replicates in the performed experiments, as a greater number of replicates for each experiment would provide a more accurate comparison. Additionally, working with 2 or 3 different pluripotent stem cell lines would be beneficial to demonstrate proof-of-concept of the experiments.

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