Synthetic biomaterials as coatings for kidney tissue engineering applications

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SYNTHETIC BIOMATERIALS AS COATINGS FOR KIDNEY TISSUE ENGINEERING APPLICATIONS

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Mak and Abah, this is for you.

A handwritten signature in black ink, appearing to read 'Mohammed Shahrudin Bin Ibrahim', with a stylized flourish at the end.

Mohammed Shahrudin Bin Ibrahim, 2018

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Abstract

Poorly-defined animal-derived extracellular matrices (ECMs) used as substrates for pluripotent stem cell expansion and differentiation are expensive, suffer from batch to batch variations, and have low relevance with respect to clinical applications. Recently, our lab has synthesized two types of synthetic chemically-defined nanoparticles, NP1 and NP2 respectively, that were able to support the expansion of pluripotent stem cells for up to 23 passages [Erathodiyil *et al.*, unpublished results]. Herein, we show that these synthetic substrates can effectively support the differentiation of induced pluripotent stem cells (iPSCs) into human primary renal proximal tubule cell (HPTC)-like cells with a similar efficiency as Matrigel. The resultant HPTC-like cells differentiated on NP2 expressed similar or higher levels of proximal tubule cell (PTC) markers SLC34A1 (1.65-fold), PEPT1 (8.43-fold), AQP1 (6.23-fold), CD13 (10.02-fold), GGT (3.93-fold), VITD3 (2.27-fold), NBC1 (4.16-fold), SGLT2 (5.60-fold) and GLUT5 (7.35-fold) in comparison to cells differentiated on Matrigel. However, elevated levels of mesenchymal markers and markers expressed by other renal cell types were also observed. Fluorescence-activated cell sorting (FACS) analysis revealed that HPTC-like cells differentiated on NP2 substrates have a high percentage of cells positive for PTC markers PEPT1, OAT3, AQP1, URO10 and GLUT1. Results obtained by compound screening suggested that HPTC-like cells obtained on NP2 would be a suitable *in vitro* cell model for screening of PTC-specific nephrotoxics. *Keywords: Extracellular matrix, induced pluripotent stem cell, proximal tubule cells, toxicology, in vitro cell model*

List of Acronyms

This list excludes acronyms already listed as primers for qPCR analysis (Table 1).

μL	Microlitre
μm	Micrometre
AUC	Area under curve
BMP	Bone morphogenetic protein
cDNA	Complementary deoxyribonucleic acid
cm	Centimetres
DAPI	4',6-diamidino-2-phenylindole
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-to-mesenchymal transition
FACS	Fluorescence-activated cell sorting
FESEM	Field emission scanning electron microscope
GLUT1	Glucose transporter 1
HCl	Hydrochloric acid
hESCs	Human embryonic stem cells
HPTC	Human primary renal proximal tubule cell
IF	Immunofluorescence
iPSC	Induced pluripotent stem cells
iPS-F4	iPS (Foreskin)-4

kV	Kilovolts
mg	Miligram
mL	Mililitre
mV	Milivolts
ND	Could not be determined
nm	Nanometres
NPV	Negative predictive value
PBS	Phosphate-buffered saline
PPV	Positive predictive value
PSC	Pluripotent stem cell
PTC	Proximal tubule cell
qPCR	Quantitative polymerase chain reaction
REGM	Renal Epithelial Growth medium
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
ROCK	Rho Kinase
ROS	Reactive oxidation species
sd	Standard deviation
SILAC	Stable isotope labeling by amino acids in cell culture
TN	True negative
TP	True positive
URO10	Urothelium 10
UV	Ultraviolet

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Thesis Outline

The thesis is structured as a series of chapters modified from journal articles that have or will result from this thesis and summarized accordingly,

Chapter 1: Literature review

Chapter 2. NP1 and NP2 nanoparticulate coating as a synthetic ECM to efficiently support the differentiation of human iPSC to human primary renal proximal tubule cell (HPTC)-like cells

Chapter 3. Application of differentiated HPTC-like cells as an *in vitro* cell model for identification of proximal tubule cell (PTC)-specific nephrotoxics

Chapter 4. The main findings and achievements of this project are summarized, and suggestions for future work are presented

Chapter 1 Cell-Matrix Interactions

1.1 Importance of cell-matrix interactions

Extracellular matrices (ECMs) are biological scaffolds onto which a cell in its physiological environment adheres to in all tissues and organs [1]. The ECM is a synergy of various structural proteins, examples of which include glycoproteins, fibronectin, collagens, laminins, proteoglycans, as well as non-structural proteins such as growth factors [2-6]. Various cell surface receptors in the forms of integrins mediate the cell-matrix interaction via interactions with specific corresponding ligands found on the ECM substrate itself (Figure 1) [5]. These interactions induce specific intracellular activation of discrete signalling pathways, which in turn elicits changes in cellular behavior ranging from physical responses such as cell spreading and migration due to changes in their cytoskeletal structure, as well as biochemical responses such as activation of pathways that induces genetic expression and the resultant cellular protein expressions [6]. Cell-matrix interactions thus play a critical role in cellular survival, migration, tissue organization and differentiation and hence, in development, homeostasis and tissue remodeling [6-9].

Figure 1. Regulation of cell behaviour by ECM. Various extracellular cues can influence the fate of cells, and they are mainly acted upon the cells via means of surface receptors and co-receptors (depicted in red, orange and black). Activation of these receptors may mediate anchorage of the cells, remodelling of intracellular cytoskeletal structures such as actin (purple), or activation of downstream cascade pathways which may lead to activation or silencing of genes, thus leading to changes in cell metabolism or cell fate [5]. Soluble ligands from the ECM (blue) may also activate non-canonical signalling pathways. Remodelling of cell-ECM interaction via enzymes (yellow) may also alter cell behavior [5].

1.2 Polymer coatings as synthetic ECMs

Surface coating strategies that enhance protein and cell interactions are often inspired by the chemical and topographical features of the biological ECM. The components that make up a rationally-designed biomaterial coating should be able to achieve similar interactions and response with cells for adhesion, propagation or differentiation [10]. These can be achieved by chemical means such as functionalization with specific biochemical ligands, or by physical means such as regulation of the elasticity of the microenvironment [10, 11].

Recently, tremendous amounts of research have been done to formulate cell-compatible biomaterials using synthetically-derived polymers as ECMs. Although natural polymers such as chondroitin sulfate, chitosan, collagen and hyaluronic acid have been previously formulated as ECMs in the form of hydrogels [12-15], ECMs derived from synthetic biomaterials have many advantages such as ease in designing tailorable biodegradation rate, predictability of physical properties [16], presentation of specific moieties and ease of fabrication, which would allow scaling up with high reproducibility [10, 17]. The absence of biologically-derived components would also decrease immunogenicity and cross-contaminations issues and hence facilitate potential clinical applications.

1.3 Synthetic ECM for manipulation of pluripotent stem cell fate

The extensive technological developments in work on human pluripotent stem cells (PSCs) have created enormous potential for applications in regenerative medicine [18-21], with much research being devoted to culturing and significantly expanding human PSCs to clinically relevant amounts. A major consideration for the cultivation of stem cell are specialized substrates that will enable the former to propagate while retaining their pluripotency. Classically, mouse fibroblast feeder layers have been utilized as base substrates to serve this function [18], but have since been replaced in most stem cell labs by Matrigel, a complex mixture of proteins derived from murine sarcoma [20, 22, 23]. Matrigel is commonly used as a feeder-free substrate for the maintenance and propagation of human PSCs, as well as the basal substrate for the differentiation of PSCs to committed cell lineages.

However, recombinant ECM proteins or animal-derived matrices are expensive. ECM coatings obtained from poorly defined animal-derived proteins such as Matrigel, vitronectin, fibronectin or laminin also commonly suffer from batch-to-batch variations, making reproducibility of findings difficult [24]. These ECMs may also be incompatible with humans and may cause debilitating immunogenic responses [25]. Thus, resultant products obtained from use of these ECMs would require costly and time-consuming pre-transplantation screening [25]. Although co-culture with human feeder cells represent a xeno-free option for the *in vitro* expansion of PSCs [26], such feeder cell environments are still undefined, may contain latent pathogens, and will also require costly and labor-intensive screening [27]. Therefore, tissue engineering applications for PSC cultures on these

substrates remain limited due to high cost, immunogenicity, variability and reproducibility issues [28-30]. Hence, there is a real need for PSCs to be cultured in xeno-free conditions to realize their applications in clinical practice.

In order to overcome some of the limitations of these biologically-derived extracellular matrices, synthetic ECM for the culture of hPSCs have recently been developed. Li and colleagues were the first to report a fully synthetic ECM that was able support self-renewal of human embryonic stem cells (hESC) [31]. Since then, various groups have formulated other synthetic substrates, ranging from chemical polymers [32-34] to peptide-based surfaces [25, 35, 36], with the vitronectin-derived peptide acrylate substrate Synthemax®-R and Synthemax® II-SC being available commercially [25, 37].

Several of these synthetic ECMs have been applied as amenable substrates for the differentiation of stem cells to specific specialized cell lineages such as definitive endoderm [38], insulin-producing cells [39], retinal epithelial-like cells [37] and mesenchymal stem cells [27]. Some of these substrates are based on peptides, and their applications are thus limited by well-known problems of polypeptide matrices, such as difficulties in sterilization, difficulties in storage due to their tendency to quickly degrade [40], and the high costs of scaling up [41]. As such, ECM coatings based on chemical polymers are a cost-effective alternative to expand PSCs and potentially provide a permissive environment for their targeted differentiation as they are much less expensive as compared to peptide-based approaches.

1.4 Research objectives and proposal

The objective of this thesis was to investigate the suitability of synthetic polymers as potentially clinically-relevant substrates for the differentiation of human induced pluripotent stem cells (iPSCs) to human primary renal proximal tubule cell (HPTC)-like cells. Another objective was to address the applicability of such cells for *in vitro* nephrotoxicology. For the applications of interest, the synthetic polymers should have the following properties:

1. Synthesis via simple free-radical polymerization using synthetic monomers.
This would allow inexpensive synthesis and potential scale-up.
2. Physical immobilization via solvent evaporation to allow coating to be conveniently performed at normal ambient conditions.
3. Capability to support differentiation of stem cells to differentiated cell lineages for tissue engineering and *in vitro* toxicology and to provide a xeno-free substrate for translational applications.

Recently, novel chemically-defined synthetic nanoparticles were synthesized in our lab. These NP1 and NP2 nanoparticles have been shown to support the attachment and propagation of human PSCs while maintaining their pluripotency for up to 23 passages [Erathodiyil *et al.*, unpublished results]. These nanoparticles were synthesized via Michael addition polymerization of Chemicals “A”, “B” and “C” in a water-in-oil reverse emulsion, and were thus free from products of animal origin or costly materials such as proteins and peptides. However, their applications for use as a substrate for human PSC differentiation had not been validated.

These fully synthetic nanoparticles NP1 and NP2 were chosen to coat surfaces as they can be homogeneously dispersed in water or ethanol and immobilized onto glass substrates simply via immersion into the nanoparticle suspension followed by solvent evaporation [Erathodiyil *et al.*, unpublished results]. This coating process can be safely and effectively performed at room temperature conditions without use of any special equipment. The evaporation process can be facilitated by subjecting the immersed substrates to slightly elevated temperature (such as in an oven at 50°C).

Dynamic light scattering (DLS) analysis revealed NP1 and NP2 particles to be of approximately 100 nm in size and positively-charged with a zeta potential reading of +88 mV and +96 mV, respectively (Table 1) [Erathodiyil *et al.*, unpublished results]. Glass coverslips were coated with the prepared nanoparticles according to Figure 3. The nanoparticle-coated coverslips exhibited a highly hydrophilic surface, with a resultant contact angle of 38° and 24° for NP1 and NP2-coated coverslips, respectively (Table 1) [Erathodiyil *et al.*, unpublished results]. Scanning electron microscopy imaging shows that the thoroughly washed coverslips were evenly coated with the NP1 and NP2 nanoparticles (Figure 2).

The ease of synthesis, scale-up and preparation of these NP1 and NP2 nanoparticles make them an ideal candidate for a permissive substrate for the differentiation of human iPSCs to HPTC-like cells. Success of this project would not only open up a new door to the applications of these xeno-free substrates, but could potentially be a step towards a sustainable clinically-relevant source of HPTC-like cells.

	Monomer	Cross-Linker	Functional Group (10%)	Size (nm)	Zeta Potential (mV)	Contact Angle (°)
NP1	"A"	"B"	"C"	88 ± 10	+88 ± 4	38 ± 6
NP2	"A"	"B"	"C" (post-treatment with 1N HCl)	106 ± 12	+96 ± 5	24 ± 4

Table 1. Composition and properties of NP1 and NP2 [Erathodiyil *et al.*, unpublished results]. "A" was used as the base monomer with "B" utilized as the polymeric crosslinker and 10% molar ratio of "C" to provide the functional group. For NP2 preparation, the nanoparticles were treated with 1N HCL after synthesis of the nanoparticles. NP1 and NP2 were approximately 100 nm in size and stable with a zeta potential of more than +85 eV. Glass coverslips coated with NP1 or NP2 were highly hydrophilic, with a contact angle of 38° and 24°, respectively. The detailed chemical composition cannot be revealed due to confidentiality issues.

Figure 2. FESEM images of coated glass coverslips taken at a) 1000X magnification show ridges formation on the surface of the glass coverslips due to the slow-drying process of the nanoparticle solution at room temperature [42-44]. Panel b) shows a 100,000X magnification of the region marked by the white box in a). NP1 and NP2 nanoparticles (white arrows) were seen deposited on the surfaces.

1.5 Goals

The goals of the thesis were as follows

- To evaluate the suitability of NP1 and NP2 substrates in supporting the differentiation of human iPSCs into HPTC-like cells through the following,
 - Physical characterizations of NP1 and NP2 materials
 - Optimization of parameters for differentiation of iPSCs by addressing
 - Pre-conditioning of base substrates to improve efficiency of cell attachment,
 - iPSC cell seeding density.
 - Characterization of stem cell-derived HPTC-like cells by
 - Morphological characterization of HPTC-like cells via microscopy,
 - Genetic expression profiling of derived HPTC-like cells via quantitative polymerase chain reaction (qPCR),
 - Qualitative analysis of expression of PTC-specific marker proteins on the derived HPTC-like cells via immunofluorescence (IF) staining, and
 - Analysis of efficiency of the differentiation process by quantifying the derived HPTC-like cells via fluorescence-activated cell sorting (FACS).
- To evaluate the suitability of the differentiated HPTC-like cells as a cell model for compound nephrotoxicity screening through the following,

- Exposure of HPTC-like cells to compounds with known nephrotoxicity in humans
- Evaluate the response of HPTC-like cells to such compound treatment by
 - Morphological characterization via microscopy,
 - Evaluating compound-induced expression of Interleukin (IL)-6 and IL-8 / C-X-C motif chemokine ligand (CXCL) 8 pro-inflammatory markers by qPCR [45-47]
 - Comparing the performance of HPTC-like cells differentiated on synthetic substrates versus HPTC-like cells differentiated on Matrigel

1.6 Relevance of current project to original proposed research

The project was focused on application of synthetic materials for the differentiation of iPSCs into HPTC-like cells. The work in this thesis addressed surface coatings that could potentially provide a defined, xeno-free environment for the differentiation and maturation of human iPSCs into HPTC-like cells. A synthetic surface coating that could successfully support the differentiation and propagation of human HPTC-like cells would be beneficial for various relevant applications such as *in vitro* diagnostics, bioartificial kidneys, kidney organoids, and *in vitro* cell models for screening of compound safety and efficacy [48].

This thesis also evaluated potential applications of HPTC-like cells differentiated on synthetic substrates in *in vitro* screening of compounds. Cell-based *in vitro* platforms are of increasing importance in toxicology [49, 50]. Success of this project would also shed light on the suitability of the synthetic materials for differentiation of HPTC-like cells that retain the functionalities of PTCs *in vivo*. Such cells would be critical for the other biomedical applications mentioned above, such as applications in bioartificial kidney [48].

1.7 Materials and Methods

1.7.1 Preparation of NP1 and NP2-coated substrates

Nanoparticles NP1 and NP2 were synthesized by water-in-oil reverse microemulsion method. Typically, a 250-mL round-bottom flask was charged with a well-stirred solution of surfactants dioctyl sulfosuccinate and Brij 30 in hexanes under argon atmosphere. In a 20 mL scintillation vial, monomer “A”, crosslinker “B” (10 mol% cross-linking) and the “C” monomer (10 mol%) were dissolved in phosphate-buffered saline (PBS, pH 7.2) by sonication to obtain a clear solution. This ratio of reactants was previously reported to yield the best substrate for hESC attachment and propagation [Erathodiyil *et al.*, unpublished results]. The resulting monomer solution was added to the surfactant solution and the mixture was stirred under an argon atmosphere at room temperature for 10 minutes to form a clear homogeneous reverse microemulsion. The reaction mixture was treated with freshly prepared aqueous ammonium persulfate and N,N,N',N'-tetramethylethylenediamine to initiate polymerization, and stirred further at room temperature overnight under argon to ensure complete polymerization. The hexane solvent was evaporated under reduced pressure to obtain a thick residue, which was suspended in absolute ethanol by sonication. The resultant NP1 precipitated particles were filtered and thoroughly washed with ethanol in a Millipore ultrafiltration system equipped with a Millipore polyethersulfone filter membrane (100 kDa). The solid material was gently crushed into a fine powder, air dried, suspended in water and sonicated to obtain a homogeneous solution. The solution

was purified by ultrafiltration using Millipore cellulose filter and water. The free functional groups on NP1 nanoparticles were conferred a positive charge via treatment of NP1 nanoparticles with 1N hydrochloric acid (HCl) to obtain NP2. The respective concentrated samples were then reconstituted into an aqueous solution with a concentration of 20 mg/mL. 100 μ L of the respective prepared NP1 and NP2 suspension was carefully pipetted onto the surface of a glass coverslip and air-dried at room temperature over 48 hours. The NP1 and NP2-coated coverslips were sterilized by ultraviolet (UV) irradiation overnight, rinsed with phosphate-buffered saline (PBS, pH 7.4) and conditioned with mTeSR1 medium (StemCell Technologies, Singapore) for 48 hours prior to cell seeding (Figure 3). Coverslips were coated with platinum using a JFC-1600 Auto Fine Coater (JEOL, Japan) (20 mA, 60 s). A field emission scanning electron microscope (FESEM, JEOL JSM-7400F, Japan) was used to obtain high magnification images of the treated surfaces at an acceleration voltage of 10.0 kV.

Figure 3. Coating and pre-conditioning of NP1- and NP2 coated glass coverslips.

a) Glass coverslips were immersed in respective NP1 or NP2 suspension in deionized water (20 mg/ml) and allowed to dry at room temperature over 72 hours. b) The dried coated coverslip surface yielded a visible white opaque layer. The coated glass coverslips were kept dry until use. Prior to use, the coated glass coverslips were sterilized via exposure to ultraviolet light overnight. c) The coated glass coverslips were then immersed in PBS wash solution for one hour leading to d) delamination of excess coating material, which was then removed upon aspiration of the PBS wash solution. The now lightly-coated glass coverslips were then e) pre-conditioned in complete mTeSR1 medium for 48 hours in a cell incubator at 37°C, 5% CO₂. The medium was aspirated out prior to subsequent characterizations and experiments of the prepared coverslips.

1.7.2 Propagation and differentiation of iPS (Foreskin)-4 (iPS-F4) cells

iPS-F4 cells were obtained from the WiCell Research Institute (Madison, WI, USA). Undifferentiated cells were continuously cultured as colonies in mTeSR1 medium (StemCell Technologies, Singapore) in tissue culture plates coated with hESC-qualified Matrigel (BD, Franklin Lakes, NJ, USA) with daily changes of medium. Undifferentiated iPS-F4 colonies are defined by a flat colony with distinct and defined borders. These tight colonies are populated by cells with large nuclei to cytoplasm ratio [51, 52].

iPS-F4 colonies were passaged periodically by first observing the culture under a microscope to identify and mark regions of differentiated cells. These differentiated cells were scraped off using a micropipette tip in a sterile manner and the medium was discarded. Dispase (StemCell Technologies, Singapore) was added to the culture and incubated in a humidified incubator at 5% CO₂, 37°C for maximum of 5 minutes. The dispase solution was then removed and the culture was rinsed once with KnockOut™ Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-Invitrogen, Carlsbad, CA). The required volume of mTeSR1 medium was then added into the culture well and pipetted several times to resuspend and gently dissociate the iPS-F4 colonies into aggregates of approximately 100 µm in size. The prepared suspension was then gently aliquoted into freshly prepared Matrigel-coated tissue culture plates. Seeding of iPS-F4 colonies followed the same protocol except that the prepared suspension was aliquoted into wells containing prepared NP1- and NP2-coated glass coverslips.

Differentiation of iPSC was performed as previously described [47]. iPS-F4 cells were resuspended at day 0 in commercial Renal Epithelial Growth medium (REGM BulletKit; Lonza Bioscience Singapore) supplemented with 10 μ M Rho Kinase (ROCK) inhibitor (Y-27632) (Calbiochem, Merck, Darmstadt, Germany) as single-cell suspension. The prepared cells were seeded at a density of 8,000 cells/cm² or 32,000 cells/cm² onto 24-well tissue culture plates coated with growth-factor reduced Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) or onto the prepared glass coverslip substrates coated with NP1 or NP2. The medium was exchanged on days 1, 3, 5 and 7 with REGM supplemented with 10 ng/mL of bone morphogenetic protein (BMP) 2 (Sigma- Aldrich, St. Louis, MO, USA) and 2.5 ng/mL of BMP7 (Life Technologies, Carlsbad, CA, USA) without ROCK inhibitor (Figure 4).

Figure 4. Timeline of differentiation of iPS-F4 to HPTC-like cells

1.7.3 qPCR

qPCR was performed to evaluate expression levels of specific genes in differentiated HPTC-like cells. On day 8 of differentiation, cells were rinsed gently with PBS before RNA was extracted using Qiagen RNeasy ribonucleic acid (RNA) Extraction Kit (Qiagen, Hilden, Germany) as per manufacturer's protocol. To generate complementary deoxyribonucleic acid (cDNA) strands, reverse transcription of the extracted RNA was performed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA) on ProFlex PCR System Thermal Cycler (Applied Biosystems, Foster City, CA) as per manufacturer's protocol. qPCR was performed using iTaq Supermix (Bio-rad, Hercules, CA, USA) on iCycler iQ5 (Bio-Rad, Hercules, CA, USA) with the respective forward and reverse primers. The sequences of these primers are given (Table 2). Data were normalized to gene expression level of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene.

Acronym / Gene ID	Primer Pairs	Amplicon (bp)
AQP1 / AQP1 Aquaporin 1	F 5'-AAGCTCTTCTGGAGGGCAGT-3' R 5'-CACCTTCACGTTGTCCTGGACCG-3'	137
ATPase / ATP1B1 ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	F 5'-GCTGACCCGCCATCGCCAT-3' R 5'-ACCAACTGCCACCGGTCCTG-3'	114
CD13 / ANPEP Alanyl (membrane) aminopeptidase	F 5'-CACACACCGTTCCTGGATCTCCTCT-3' R 5' -GCTCCAACAGGCGAAGGTCACT-3'	76
E-CAD / CDH1 Cadherin 1	F 5'-GAGGACCAGGACTTTGACTT-3' R 5'-AGATACCGGGGGACACTCAT -3	107
GAPDH / GAPDH Glyceraldehyde-3-phosphate dehydrogenase	F 5'-ACCCCTTCATTGACCTCAACTACA-3' R 5'-CTTGACGGTGCCATGGAATT-3'	80
GGT / GGT Gamma-glutamyl transferase 1	F 5'-TGAGCCCAGAAGTGAGAGCAGTTG-3' R 5' -ATGTCCACCAGCTCAGAGAGGGT-3'	85

GLUT5 / SLC2A5 Solute carrier family 2 (facilitated glucose / fructose transporter), member 5	F 5'-CCCCAGCTCTTCATCACTGTTGGC-3' R 5'-TTTGGAACACAAGGAGGGGGCC-3'	148
IL-6 / IL-6 Interleukin 6	F 5'-TGGCTGCAGGACATGACAAC-3' R 5'-TGAGGTGCCCATGCTACATTT-3'	100
IL-8 / CXCL8 C-X-C motif chemokine ligand 8	F 5'-TTGGCAGCCTTCCTGATTTCT-3' R 5'-GGGTGGAAAGGTTTGGAGTATG-3'	110
KIM-1 / HAVCR1 Hepatitis A virus cellular receptor	F 5'-CAGGCTGATCCCATAATGCA-3' R 5' -CTGCCTCTCCACCAACCTTTAC-3'	100
MEG / LRP2 Low density lipoprotein receptor-related protein 2 (Megalin)	F 5'-AGACTGGTTCTAACGCCTGTAATC-3' R 5'-GCTCTGTGGGTGGTTCATTGG-3'	171
NCAD / CDH2 Cadherin 2	F 5'-CCCATACACCAGCCTGGAACGC-3' R 5'-TGGGTCGGTCTGGATGGCGA-3'	80
NBC1 / SLC4A4 Solute carrier family 4 (sodium bicarbonate co-transporter), member 4	F 5'-CCAAACTGGAGGAGCGACGGAAG-3' R 5'-CACACACATGCTTGAGGAAGGA -3'	90

NCCT / SLC12A3 Thiazide-sensitive sodium chloride co-transporter	F 5'-CACCAAGAGGTTTGAGGACATG-3' R 5'-GACAGTGGCCTCATCCTTGAA-3'	70
OAT3 / SLC22A8 Solute carrier family 22 (organic anion transporter), member 8	F 5'-GCCCTTGGACTTGCAGACCG-3' R 5'-ACCTGTTTGCCTGATGACTG-3'	127
OCTN2 / SLC22A5 Solute carrier family 22 (organic cation/carnitine transporter), member 5	F 5'-GGTTTGGCCGGAAGAATGT-3' R 5'-CCATGCCTACAAGGACAAACAG-3'	120
PEPT1 / SLC15A1 Solute carrier family 15 (oligopeptide transporter), member 1	F 5'-CAGTGGGCCGAGTACATTCTATT-3' R 5'-TCTCCGCTGGGTTGATGTAAG-3'	100
PODXL / PODXL Podocalyxin-like	F 5'-ACCTACCCTGCCAGAGACCAT-3' R 5'-AGATCCTCACACTTTGCCCAGTT-3'	120
SGLT2 / SLC5A2 Solute carrier family 5 (sodium/glucose co-transporter), member 2	F 5'-ACGCCTGATTCCCGAGTTCT-3' R 5'-AGAACAGCACAATGGCGAAGT-3'	110
SLC34A1 / SLC34A1 Solute Carrier Family 34 (Type II Sodium/Phosphate Co-transporter), Member 1	F 5'-CTGGGTCACAGGCTACTTTGC-3' R 5'-GCCCTCTCAATGCTGATCACA-3'	126

SMA / ACTA2 Actin, alpha 2, smooth muscle, aorta	F 5'-TCATCACCAACTGGGACGAC-3' R 5'-ATGCTCTTCAGGGGCAACAC-3'	80
UMOD / UMOD Uromodulin	F 5'-TGGCTTCAGGACACCAGACATCAG-3' R 5'-AGCACCTGCCCAAAGGAAAGACG-3'	77
VIM / VIM Vimentin	F 5'-ACCTGAGGGAACTAATCTG-3' R 5'-CGTTGATAACCTGTCCATCT-3'	105
VIT D3 / CYP27B1 Cytochrome P450, family 27, subfamily B, polypeptide 1 (synthesizes 1-alpha, 25-dihydroxyvitamin D3)	F 5'-GGAAATTCTCGTGTCCCAGA-3' R 5'-TGACACAGAGTGACCAGCGTA-3'	80
WT1 / WT1 Wilms tumor 1	F 5'-AACAGCAACAGCAAGAAATAAATCA-3' R 5'-GACCTCGGGAATGTTAGACAAGAT-3'	71
ZO1 / TJP1 Tight junction protein 1	F 5'-GAGAGGATTTGTCCGCTCAG-3' R 5'-AGGCCTCAGAAATCCAGCTT-3'	86

Table 2. Sequence of forward and reverse primers of the genes used in qPCR analysis. The table was derived with slight modifications from Kandasamy *et al.*, (2015) [47]. The table provides widely used acronyms that are used throughout this thesis as well as the HUGO nomenclature and acronyms.

1.7.4 Immunofluorescence (IF) Staining

IF staining was performed to visualize and localize the presence of specific marker proteins in the differentiated HPTC-like cells. Differentiated cells were washed in PBS and fixed in 4% formalin (Sigma- Aldrich, St. Louis, MO, USA) for 15 minutes at room temperature. Fixed cells were washed and stored in PBS at 4°C prior to staining. Where necessary, fixed cells were permeabilized with PBS supplemented with 0.1% (v/v) Triton-X (Sigma- Aldrich, St. Louis, MO, USA) for 5 minutes. Blocking was carried out by incubating the cells with 5% (w/v) bovine serum albumin (BSA, Sigma- Aldrich, St. Louis, MO, USA) and 0.25% Tween 20 (Sigma- Aldrich, St. Louis, MO, USA) in PBS. Primary antibodies against AQP1, SGLT2 (Abcam, Cambridge, MA, USA), OAT3, urothelial glycoprotein 10 (URO10) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or ZO1 (Invitrogen, Carlsbad, CA, USA) were used for IF staining (for full gene / protein names, see Table 1). The primary antibodies were diluted in blocking buffer and incubated with cells overnight at 4°C. Secondary antibodies used were Alexa Fluor 594- or Alexa Fluor 488-labelled goat anti-rabbit or anti-mouse antibodies (Invitrogen, Carlsbad, CA, USA). 4',6-diamidino-2-phenylindole (DAPI) was used to stain the cell nucleus.

1.7.5 FACS

FACS was performed to quantify the percentage of successfully differentiated HPTC-like cells within the total cell population at the end of day 8. Differentiated cells were dissociated by treatment of the cells with trypsin/ ethylenediaminetetraacetic acid (EDTA) (0.025%/ 0.01%) for 5 minutes. The

trypsin was neutralized with equivalent volume of REGM and transferred into 15 mL falcon tubes. The cell suspension was centrifuged at 500 g for 5 minutes and the supernatant was decanted. The cells were then resuspended in PBS. Primary antibodies against AQP1, GLUT1 (Abcam, Cambridge, MA, USA), PEPT1, OAT3 and URO10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Secondary antibodies used were Alexa Fluor 594- or Alexa Fluor 488-labelled goat anti-rabbit or anti-mouse antibodies (Invitrogen, Carlsbad, CA, USA).

1.7.6 Compound treatment and determination of interleukins IL-6 and IL-8 expression levels

To further evaluate the suitability of HPTC-like cells differentiated on the synthetic substrates as a cell model for nephrotoxic compound screening, compound treatment was performed directly on the differentiated cells as outlined by Kandasamy *et al.* [47]. Briefly, cell culture medium was exchanged with REGM containing respective test compounds with a final concentration of 10, 50 and 100 µg/mL on day 8 of differentiation and followed by incubation for 16 hours. Subsequently, the cells were rinsed gently with PBS and RNA extraction, cDNA synthesis and qPCR were performed as outlined earlier, with IL-6- and IL-8-specific primers (forward and reverse), respectively. Data were normalized to gene expression levels of GAPDH housekeeping gene. Delta delta Ct method was utilized for the quantification of IL-6 and IL-8 levels.

1.7.7 Statistics

Statistical analyses were performed using Microsoft® Excel (Microsoft Corp., Redmond, WA). Shapiro-Wilk normality test was chosen to determine normal distribution of the data due to small sample size. The two-tailed Student's *t*-test was used for all statistical analyses, with $P < 0.05$ being considered as statistically significant.

Chapter 2 Synthetic materials as substrates for the differentiation of human induced PSCs (iPSCs) to HPTC-like cells

2.1 PSCs as a sustainable source of HPTC-like cells

HPTCs are cells derived from the renal tubules, which is the portion of the tubule system of the kidney's nephron leading from the Bowman's capsule to the loop of Henle. HPTCs are relevant in many applications including disease modelling [53, 54], *in vitro* toxicology [55-57] and bioartificial kidney development [58-61]. However, cell source limitations compromise translational applications of HPTCs [62]. Although PTC-derived cell lines and animal-derived PTCs could potentially alleviate this, a variety of problems related to the use of PTC-derived cell lines such as functional changes associated with cell immortalization procedures [60], including dedifferentiation of the cells under *in vitro* conditions are major obstacles [63, 64]. Animal-derived PTCs are associated with interspecies variability and safety issues are major concerns. Therefore, to solve the problems of availability, compatibility and translatability, it would be desirable to obtain HPTC-like cells from the directed differentiation of human PSCs [65].

Yielding relatively mature renal cells *in vitro* from PSCs would be necessary. Vigneau *et al.* (2007) and Kim *et al.* (2005) had previously shown the differentiation of renal progenitor-like cells from mouse embryonic stem cells [66, 67]. Other protocols developed by Bruce *et al.* (2007) gave rise to cells that expressed markers of mature PTCs as well as markers of other mature renal cell types [68]. Batchelder and co-workers had worked with human-derived

embryonic stem cells and reported on the differentiation of these cells into cells expressing renal-precursor markers [69]. Kang and Han had also previously demonstrated the differentiation of human PSCs into nephron progenitor cells that further matured into populations of renal tubular-like cells and glomerular podocyte-like cells [70].

Narayanan *et al.* (2013) was the first to report a 20-day protocol that allowed the differentiation of human ESCs into HPTC-like cells [65]. However, ethical issues governing the use of human ESCs limits their translational use and hence, their clinical relevance. More recently, Kandasamy *et al.* (2015) developed a one-step protocol that allowed the differentiation of human iPSCs into HPTC-like cells in 8 days and with more than 90% purity [47]. However, due to the utilization of Matrigel as substrate, the applications of such cells are limited to *in vitro* applications.

2.2 Optimizing the attachment of iPS-F4 cells to NP1- and NP2-coated coverslips

To evaluate the performance of NP1 and NP2 with respect to the attachment and expansion of iPS-F4 cells, iPS-F4 colonies were seeded onto prepared NP1- and NP2-coated glass coverslips, respectively. Both NP1- and NP2-coated coverslips allowed adherence of these colonies at day 1. These substrates also allowed the cells to proliferate, as observed by the expansion of the colonies 7 days post-seeding (Figure 5a), while maintaining the large nuclei-to-cytoplasm ratio morphology characteristic of PSC colonies (data not shown) [51, 52].

Before initiating the differentiation process, the attachment of single-celled iPS-F4 on NP1 and NP2 needed to be assessed, as outlined by Kandasamy *et al.* [47] on Matrigel. iPS-F4 single-cell suspension were seeded onto the NP1- and NP2-coated coverslips and allowed to attach overnight. Matrigel-coated polystyrene tissue culture plates were used as a positive control. Attachment of iPS-F4 cells on the respective NP1- and NP2-coated coverslips was observed on day 1, comparable to that seeded on Matrigel (Figure 5b), thus making NP1 and NP2 permissible base substrates to commence differentiation of iPS-F4 to HPTC-like cells.

Figure 5. Phase contrast images of attachment and expansion of iPS-F4 cells on synthetic substrates. (a) Colonies of iPS-F4 cells seeded on NP1 (top panel) and NP2 (bottom panel) on day 1 (left column) and day 7 (right column). (b) iPS-F4 single cells attached NP1, NP2 and Matrigel substrates respectively on Day 1 post-seeding. Adherence of iPS-F4 single cells on NP1 and NP2 were comparable to those on Matrigel substrate. Scale bars represent 500 μm .

2.3 Morphology of iPS-F4 cells differentiated into HPTC-like cells

Recently, Kandasamy *et al.* (2015) had shown that single-cell seeded hiPSC could be efficiently differentiated into HPTC-like cells in a span of 8 days on Matrigel-coated tissue culture plates [47]. This protocol was adopted and Matrigel was substituted with NP1- and NP2-coated coverslips as a xeno-free base substrate. iPS-F4 single-cells were seeded on Matrigel-coated multi-well plates, NP1- or NP2-coated coverslips at a density of 8,000 cells/cm² with REGM supplemented with BMP2 and BMP7 (Figure 6).

The expansion of iPS-F4 on NP1 and NP2-coated coverslips was observed to be significantly less on day 3 as compared to those being differentiated on Matrigel. On day 5, the iPS-F4 cells on NP1 and NP2-coated coverslips remained sparse, while cells on Matrigel were almost confluent. However, it was interesting to note that the day 5 iPS-F4 cells on NP1 and NP2-coated coverslips looked similar to day 3 iPS-F4 cells on Matrigel (Figure 6). At the end of the differentiation process on day 8, iPS-F4 differentiated on NP2-coated coverslips were observed to form a tight epithelial-like layer, almost comparable to the cells on Matrigel, whereas such a confluent layer of epithelial-like cells was not observed on NP1-coated coverslips.

Figure 6. Morphology of iPS-F4 cells throughout the differentiation process to HPTC-like cells on NP1, NP2 and Matrigel. Cells on NP1 and NP2 substrates took 5 days to attain morphology of Day 3 cells on Matrigel. At Day 8, there was visibly a higher number of cells on NP2 than on NP1, although not as confluent as cells on Matrigel. Scale bars represent 500 μm .

2.4 Effect of cell seeding density on efficiency of differentiation

To investigate the effect of cell seeding density on the differentiation process, an increased iPS-F4 seeding density of 16,000 cells/cm² and 32,000 cells/cm² were studied. As expected, the density of the cells at the end of the 8-day protocol was significantly higher. The cell monolayer was more closely packed when seeding density was increased to 16,000 cells/cm². However when seeding density was further increased at 32,000 cells/cm², no multilayer of epithelia was seen, probably due to contact inhibition typical of epithelial proximal tubule monolayers [71].

Figure 7. Phase contrast images of cell confluency and morphology at end of 8-day differentiation to HPTC-like cells. iPS-F4 at a density of 8,000 cells/cm² (first row), 16,000 cells/cm² (second row) and 32,000 cells/cm² (third row) were seeded onto NP1 (first column) and NP2 (second column). A seeding density of 8,000 cells/cm² on Matrigel was used as a positive control (third column). Cells differentiated on NP1 with a seeding density of 8,000 cells/cm² were still subconfluent on day 8, while those on NP2 substrate at a seeding density of 8,000 cells/cm² showed similar confluency to those differentiated on Matrigel. Scale bars represent 500 μ m.

The gene expression profiles of cells from seeding densities of 8,000 cells/cm² and 32,000 cells/cm² on NP1- and NP2-coated coverslips were analyzed against those seeded at a density of 8,000 cells/cm² on Matrigel (Figure 8). A panel of markers typically expressed by PTCs, SLC34A1, PEPT1, AQP1, CD13, GGT, VITD3, ATP1B1 [47, 72-77], were used to characterize and compare these cells. SLC34A1 codes for a type II sodium/phosphate co-transporter, which is expressed only in fully differentiated PTC [78]. PEPT1 codes for renal peptide transporters located mainly at the apical brush border of PTCs [79]. AQP1 codes for water channels that are normally enriched in the cell membrane [80] while GGT codes for γ -glutamyltransferase [81]. In the kidney AQP1 and GGT expression is characteristic for PTCs [80, 81]. The brush border enzyme aminopeptidase N or CD13 is also a marker specific for PTCs, and has been utilized for isolation of PTCs [75]. The expression of PTC-specific transporter proteins (NBC1, SGLT2, and GLUT5) and epithelial proteins involved in transport (OAT3, OCTN2 and MEG) were also studied. The sodium bicarbonate cotransporter, NBC1 and glucose transporters SGLT2 and GLUT5 are commonly associated with PTCs [82-84]. OAT3 is localized at the basolateral membrane of the renal proximal tubule [85] and involved in drug disposition and renal clearance of endogenous compounds. OCTN2 is the main organic cation transporters in PTCs and is a transporter for organic cations as well as carnitine [86, 87]. MEG is part of the megalin/cubilin endocytotic receptor complex [88], important for the uptake of nephrotoxic aminoglycoside antibiotics [89]. Therefore, a similar or higher expression levels of

these genes in the cells processed on the synthetic substrates compared to that of cells processed on Matrigel would indicate the similarity of these cells.

A higher expression of PEPT1, AQP1, CD13, GGT and comparable expression of SLC34A1, VITD3 and all the other markers were observed when NP1 was seeded with a density of 8,000 cells/cm² (Figure 8). When the seeding density was increased to 32,000 cells/cm², a higher expression of PEPT1, CD13, GGT, GLUT5 and OAT3 were observed, while AQP1, VITD3, NBC1, SGLT2 and OCTN2 had comparable expression to the cells processed on Matrigel.

Remarkably, NP2 seeded with a density of 8,000 cells/cm² showed higher expression levels of SLC34A1 (1.65-fold), PEPT1 (8.43-fold), AQP1 (6.23-fold), CD13 (10.02-fold), GGT (3.93-fold), VITD3 (2.27-fold) and all the other markers (Figure 8, Table 3). However, it was observed that only PEPT1, AQP1, CD13, GGT, OAT3 and MEG show higher expression levels when 32,000 cells/cm² were seeded onto NP2, while VITD3, NBC1, SGLT2, GLUT5 and OCTN2 showed comparable expression to that of cells differentiated on Matrigel. In all the cells processed on the synthetic substrates NP1 and NP2, the expression of ATP1B1, encodes the β 1 essential subunit of the Na⁺/K⁺-ATPase pump [90], was lower with respect to cells differentiated on Matrigel, even at a higher seeding density. Moreover, these results show that an increase in cell seeding density from 8,000 cells/cm² to 32,000 cells/cm² consistently led to a low expression level of SLC34A1.

Figure 8. qPCR of PTC markers of cells processed on NP1, NP2 and Matrigel. 8K and 32K denotes the cell seeding densities of 8,000 cells/cm² and 32,000 cells/cm² on the respective substrates. (n=3)

	Gene	Substrate		
		Matrigel	NP2	NP1
PTC-specific Markers and Transporters	SLC34A1	1	1.65	0.73
	PEPT1	1	8.43	3.44
	AQP1	1	6.23	3.41
	CD13	1	10.02	4.23
	GGT	1	3.93	2.28
	VITD3	1	2.27	1.94
	ATP1B1	1	0.30	0.23
	NBC1	1	4.16	1.13
	SGLT2	1	5.60	1.44
	GLUT5	1	7.35	1.35
	OAT3	1	4.44	1.66
	OCTN2	1	1.23	0.58
	MEG	1	3.03	0.76

	Gene	Substrate		
		Matrigel	NP2	NP1
Epithelial Markers	ZO1	1	3.48	1.29
	NCAD	1	19.19	11.66
	ECAD	1	0.03	0.01
Nephron Progenitor Marker	WT1	1	0.44	0.10
Kidney Injury Markers	KIM-1	1	0.91	0.17
	VIM	1	14.22	0.64
	SMA	1	56.84	19.98
Other Renal Markers	PODXL	1	0.08	0.09
	NCCT	1	2.56	1.49
	UMOD	1	4.44	2.68

Table 3. Normalized gene expression levels of cells differentiated on NP1 and NP2 relative to gene expression levels of cells differentiated on Matrigel.

Given that there was a higher number of PTC markers with a higher expression level when a cell density of 8,000 cells/cm² was seeded onto NP2 as compared to 32,000 cells/cm², a seeding density of 8,000 cells/cm² was therefore the most efficient condition for the differentiation of iPS-F4 cells to HPTC-like cells as the resultant cells show PTC marker expression that were higher than those expressed by HPTC-like cells differentiated on Matrigel substrate.

Taken together with the morphological analysis, iPS-F4 seeding density of 8,000 cells/cm² was thus the optimized cell seeding density for differentiation of iPS-F4 to HPTC-like cells on the synthetic substrates.

2.5 Comparative analysis of HPTC-like cells differentiated on NP1, NP2 and Matrigel

To further characterize these cells, expressions of other genes associated with PTCs were analyzed. Of particular interest were the tight junction epithelial marker ZO1 and NCAD, which is the predominant cadherin in human proximal tubules [91]. In addition, ECAD, which is mostly found in distal tubules and collecting ducts [91, 92] was also analyzed. Elevated levels of expression of ECAD had also been reported for PTCs cultured *in vitro* [93]. A ~3.5-fold upregulation of expression of ZO1 in HPTC-like cells differentiated on NP2 substrate was observed as compared to those differentiated on Matrigel, while expression of ZO1 was comparable for those derived on Matrigel and NP1 (Figure 9a, Table 3). An increase in expression of NCAD (19.19 and 11.60-fold) and a lower expression of ECAD (0.03 and 0.01-fold) in HPTC-like cells differentiated on NP2 and NP1 as compared to those differentiated on Matrigel was observed. This closely resembles the expression patterns of human PTCs *in vivo* [94]. A lower expression of WT1, which is a tumour suppressor gene and a nephron progenitor marker [95], was observed on HPTC-like cells differentiated on NP1 and NP2 in comparison to those on Matrigel. This suggests a more mature PTC-like phenotype.

In addition, expression of kidney injury markers KIM-1 and VIM [96-98] and SMA was studied. While KIM-1 expression of HPTC-like cells on NP2 was comparable to that on Matrigel, the expression level was lower in NP1. This was also observed with respect to VIM, whereas this marker expression was elevated on NP2. SMA is a marker for epithelial-to-mesenchymal transition (EMT) [99, 100].

This marker was highly expressed on cells processed on NP1 and NP2. Also, VIM is expressed in mesenchymal cells [100, 101]. (Figure 9, Table 3). Together, these results suggest a more mesenchymal phenotype of cells processed on NP1 and NP2.

HPTC-like cells differentiated on the synthetic substrates also expressed markers for other renal epithelial cell types that are normally present under *in vivo* conditions such as PODXL (glomerulus) [102], NCCT (distal tubule) [103], and UMOD (Loop of Henle) [104]. Expressions of these markers have been observed in HPTCs and iPSC-derived PTC-like cells cultured *in vitro* [47]. In addition, PODXL is also expressed in undifferentiated iPSCs [105, 106]. PODXL expression was ~10-fold lower as compared to HPTC-like cells differentiated on Matrigel (Table 3). NCCT expression was higher for cells on NP2 while UMOD expression was higher in cells on both synthetic substrates. These results suggests a mixed phenotype of cells with a strong suppression of stem cell markers, in comparison to cells differentiated on Matrigel.

Figure 9. qPCR analysis of levels of marker expression. ZO1, NCAD, SMA and UMOD were highly expressed in cells differentiated on NP2 and NP1, while lower levels of expression ECAD, WT1 and PODXL was observed. Expression levels for VIM and NCCT were elevated only for cells on NP2. Expression level for KIM-1 was comparable between both HPTC-like cells differentiated on NP2 and those on Matrigel, and lower in cells on NP1. (n=3)

Figure 10. PTC-specific markers indicated on the top of the respective panels were detected by IF staining. DAPI (blue) was used to stain nuclei. Scale bars represent 100 μm .

IF staining of the iPS-F4 cells differentiated on NP1 and NP2-coated coverslips and Matrigel was performed for PTC markers OAT3 [85, 107], ZO1 [108], SGLT2 [83], URO10 [47] and AQP1 [80]. OAT3 staining was seen to be distributed over the cell area and was comparable between HPTC-like cells differentiated on NP1, NP2 and Matrigel. Chicken-wire staining of ZO1 was also present in HPTC-like cells differentiated on the three substrates, indicating a tight epithelia formation for the differentiated cells, which is essential for its barrier properties [109] (Figure 10a). Staining of SGLT2 was seen throughout the cell area. SGLT2 is localized on the apical membrane of PTCs in humans [83]. URO10 staining showed the expected sub-cellular localization with cell surface enrichment [65], and this was comparable for the cells differentiated on all three substrates (Figure 10b). AQP1, present on both the apical brush border as well as the basolateral membrane of the proximal tubules [110], are typically enriched on the cell membranes. Cells processed on all three substrates stained positive for AQP1 (Figure 10c), with similar nuclear enrichment, which is an artifact. These results demonstrate that protein localization of PTC-specific markers in cells differentiated on the synthetic substrates were comparable to those differentiated on Matrigel.

In summary, the results suggest that HPTC-like cells differentiated on NP1 and NP2 have a mixed phenotype. The resultant cells have higher gene expression of PTC-specific markers, but also increased expression of EMT and mesenchymal markers (VIM and SMA) and markers for other renal cell types (NCCT and UMOD). However, protein localization of the PTC-specific markers was similar to HPTC-like cells differentiated on Matrigel. It was concluded that NP2 to be a permissible

substrate than NP1 for iPS-F4 differentiation to HPTC-like cells and a seeding density of 8,000 cells/cm² was optimal.

FACS was performed to quantify the percentages of cells expressing PTC-specific markers (Figure 11). Results revealed that URO10 and GLUT1 were expressed in more than 90% of the cells differentiated on NP2 and Matrigel. However, percentage of PEPT1⁺, OAT3⁺ and AQP1⁺ cells on Matrigel were about 10% to about 45% lower than on NP2 (Figure 11). These FACS results were in agreement with the earlier gene expression analysis (Figure 8 and 9a). The findings show that NP2 effectively supports the differentiation of iPS-F4 cells to HPTC-like cells.

Figure 11. FACS analysis of PTC markers on HPTC-like cells differentiated on NP2 versus Matrigel. The percentages of cells expressing the respective markers are indicated.

Chapter 3 Application of differentiated HPTC-like cells for *in vitro* nephrotoxicology screening

3.1 Nephrotoxicants screenings are lacking

The kidney plays an important role in the maintaining the balance of water and electrolytes, and the regulation of metabolic wastes and compounds, including drugs, from the blood plasma [111]. As a result, the kidney is a main target for compound-induced nephrotoxicity, and PTCs are frequently affected due to their roles in concentration of the glomerular filtrate and transport of compounds and molecules [112, 113].

Acute kidney injury or chronic kidney disease frequently result from compound-induced nephrotoxicity, which may ultimately progress to end-stage kidney disease [114, 115]. However, detection of nephrotoxicity usually occurs during the late stages of drug development, leading to 19% of drug being rejected in phase 3 of clinical trials [116]. Effective pre-clinical prediction and identification of PTC-specific nephrotoxicants would help in the development of drugs with reduced nephrotoxicity [117], greatly increase the success rates of clinical trials, and accelerate drug development [118].

Recently, a cell-based *in vitro* model that predicts PTC-specific toxicity in humans was developed. By analysis of compound-induced expression of IL-6 and IL-8, an end-point assay with high predictivity was achieved [45, 46]. Upregulation of IL-6 and IL-8 had been previously shown to be closely associated to kidney injury and disease *in vivo* [119, 120]. Kandasamy *et al.* (2015) had

applied these methods for evaluating the predictive performance of iPS-F4-derived HPTC-like cells differentiated on Matrigel. These cells were shown to predict PTC-specific nephrotoxicity with high accuracy [47]. It would thus be interesting to compare the predictive performance of HPTC-like cells differentiated on NP2 and on Matrigel respectively.

3.2 HPTC-like cells differentiated on NP2 as a cell model for nephrotoxicant screening

18 compounds were selected for exposure to the obtained HPTC-like cells. Detailed information on the nephrotoxicity of the compounds in humans had previously been summarized by Li and colleagues [46]. These compounds were classified as follows: PTC-specific nephrotoxicants, which are compounds known to be directly toxic to proximal tubules in humans; non-PTC-specific nephrotoxicants, which are compounds known to cause kidney damage via injury to other tissues within the kidney; and non-nephrotoxic compounds, which are compounds that do not induce kidney injury in humans. Compound concentrations of 10 µg/mL, 50 µg/mL and 100 µg/mL were chosen to reduce the probability of cell death at high concentrations. Similarly, as the purity of HPTC-like cells differentiated on NP2 were high, no harvesting of the cells would be required and compounds could be tested on the HPTC-like cells directly at the end of the 8-day differentiation protocol [47]. The respective HPTC-like cells were incubated with the individual compounds at the specified concentrations for 16 hours. The gene expressions of IL-6 and IL-8 in response to the compound exposure were analyzed via qPCR and compared between the HPTC-like cells differentiated on Matrigel and those differentiated on NP2. Results were summarized in Tables 4, 5 and 6.

The expression of IL-6 and IL-8 upon PTC-specific nephrotoxicant exposure was overall comparable between two sources of HPTC-like cells in cases where no cell death was observed. HPTC-like cells differentiated on NP2

showed a higher susceptibility to aristolochic acid and cephalosporin C at 100 µg/mL, gold (I) chloride and potassium dichromate at 50 µg/mL and above, and cadmium chloride, puromycin and tacrolimus at 10 µg/mL and above, evidenced by massive cell death at these compound concentrations (Figure 12). On the contrary, no massive cell deaths were observed when HPTC-like cells on Matrigel were subjected to these compound concentrations, as expected (Tables 4 and 5).

These results suggested that exposure of similar concentration of the respective nephrotoxics had led to extensive cell death of HPTC-like cells differentiated on NP2 in most cases (Figure 12), while only eliciting an injury response that stimulated the increased expression of IL-6 and IL-8 in the HPTC-like cells differentiated on Matrigel. Thus, the HPTC-like cells differentiated on NP2 substrate may be more sensitive and susceptible to PTC-specific nephrotoxics.

When the compound concentration was increased from 50 µg/mL to 100 µg/mL, HPTC-like cells differentiated on NP2 had a reduced IL-6 expression when subjected to 5-fluorouracil (0.9 ± 0.0 to 0.5 ± 0.1) or gentamicin (3.6 ± 0.1 to 0.4 ± 0.0), while HPTC-like cells differentiated on Matrigel showed a consistent baseline level of IL-6 expression throughout the concentration range. This phenomenon was also seen in the IL-8 expression of HPTC-like cells differentiated on NP2 exposed to 50 µg/mL to 100 µg/mL 5-fluorouracil (0.8 ± 0.1 to 0.2 ± 0.1). An increase in concentration of 10 µg/mL to 50 µg/mL of gentamicin and rifampicin also gave a similar trend (0.6 ± 0.2 to 0.1 ± 0.0 and 0.9 ± 0.3 to

0.2 ± 0.1 respectively), although the expression level increased at concentration of 100 µg/mL. Also, a similar trend was observed in IL-6 expression of HPTC-like cells differentiated on Matrigel subjected to 50 µg/mL to 100 µg/mL aristolochic acid (2.9 ± 0.1 to 0.8 ± 0.1) and in the IL-8 expression of these cells subjected to 50 µg/mL to 100 µg/mL gentamicin (1.3 ± 0.7 to 0.9 ± 0.1). The reduction in expression of these interleukins with an increase in concentration of compounds had also been previously observed [46, 47], and may point to the cells being compromised to the point that elevated levels of the interleukins could not be sustainably expressed.

HPTC-like cells differentiated on NP2 to non-PTC-specific nephrotoxics and non-nephrotoxic compounds at the highest concentration showed no massive cell death. These observations indicate that although HTPC-like cells differentiated on NP2 were more susceptible, this effect was exclusively to the PTC-specific nephrotoxics tested.

IL-6 Expression

Compound Concentration	10 µg/mL		50 µg/mL		100 µg/mL	
	Matrigel	NP2	Matrigel	NP2	Matrigel	NP2
Aristolochic acid	2.1 ± 0.0	1.2 ± 0.2	2.9 ± 0.1	2.2 ± 0.1	0.8 ± 0.1	ND
Bismuth (III) oxide	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	0.4 ± 0.1
Cadmium chloride	4.9 ± 0.2	ND	5.1 ± 0.2	ND	7.6 ± 0.6	ND
Gold (I) chloride	1.1 ± 0.1	0.5 ± 0.0	0.9 ± 0.0	ND	2.4 ± 0.1	ND
Potassium dichromate	0.4 ± 0.0	1.8 ± 0.1	1.0 ± 0.1	ND	1.3 ± 0.1	ND
Puromycin	13.2 ± 1.5	ND	11.8 ± 0.3	ND	14.2 ± 0.5	ND
Cephalosporin C	1.2 ± 0.1	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.1	2.3 ± 0.0	ND
5-Fluorouracil	1.1 ± 0.1	1.4 ± 0.0	1.2 ± 0.1	0.9 ± 0.0	1.4 ± 0.0	0.5 ± 0.1
Gentamicin	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.0	3.6 ± 0.1	1.2 ± 0.1	0.4 ± 0.0
Rifampicin	1.3 ± 0.1	1.0 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.1
Tacrolimus	1.3 ± 0.0	ND	1.2 ± 0.1	ND	12.5 ± 0.6	ND

Table 4. Nephrotoxicant-induced IL-6 gene expression in HPTC-like cells differentiated on Matrigel and NP2. The cells were treated with the PTC-specific nephrotoxicants (listed on the left) at the end of the 8-day differentiation protocol. Each compound was applied at concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL. IL-6 expression levels after 16 hours of compound exposure were determined by qPCR. In some cases, expression levels of IL-6 could not be determined (ND) due to extensive cell death. Values in bold denote the highest fold level of expression obtained throughout the series of compound concentration. The results show the mean +/- standard deviation (sd) (n = 3). All values were normalized to the respective vehicle controls, which were set to 1.0.

IL-8 Expression

Compound Concentration	10 µg/mL		50 µg/mL		100 µg/mL	
	Matrigel	NP2	Matrigel	NP2	Matrigel	NP2
Aristolochic acid	0.6 ± 0.0	0.4 ± 0.0	1.7 ± 0.1	3.2 ± 0.3	3.7 ± 0.6	ND
Bismuth (III) oxide	0.3 ± 0.2	0.4 ± 0.1	1.2 ± 0.1	0.4 ± 0.2	1.5 ± 0.6	0.9 ± 0.4
Cadmium chloride	1.8 ± 0.6	ND	0.0 ± 0.0	ND	0.9 ± 1.2	ND
Gold (I) chloride	2.4 ± 1.0	0.5 ± 0.1	5.2 ± 1.1	ND	12.4 ± 2.0	ND
Potassium dichromate	0.6 ± 0.2	0.0 ± 0.0	1.5 ± 0.1	ND	0.1 ± 0.1	ND
Puromycin	699.4 ± 24.0	ND	1832.4 ± 19.4	ND	654.7 ± 16.4	ND
Cephalosporin C	0.1 ± 0.0	0.4 ± 0.1	0.8 ± 0.1	0.0 ± 0.0	4.0 ± 1.2	ND
5-Fluorouracil	0.5 ± 0.3	0.1 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	2.5 ± 0.5	0.2 ± 0.1
Gentamicin	0.2 ± 0.1	0.6 ± 0.2	1.3 ± 0.7	0.1 ± 0.0	0.9 ± 0.1	0.5 ± 0.5
Rifampicin	0.5 ± 0.3	0.9 ± 0.3	0.7 ± 0.3	0.2 ± 0.1	21.0 ± 4.0	6.4 ± 1.1
Tacrolimus	0.2 ± 0.1	ND	228.8 ± 7.3	ND	9.1 ± 3.6	ND

Table 5. Nephrotoxicant-induced IL-8 expression in HPTC-like cells differentiated on Matrigel and NP2. IL-8 expression levels after 16 hours of compound exposure were determined by qPCR. In some cases, expression levels of IL-8 could not be determined (ND) due to extensive cell death. Values in bold denote the highest fold level of expression obtained throughout the series of compound concentration. The results show the mean +/- standard deviation (sd) (n = 3). All values were normalized to the respective vehicle controls, which were set to 1.0.

	IL-6 expression		IL-8 expression	
	Matrigel	NP2	Matrigel	NP2
Acetaminophen	0.6 ± 0.0	1.4 ± 0.2	2.3 ± 0.3	0.4 ± 0.2
Ethylene glycol	1.3 ± 0.1	0.7 ± 0.2	1.5 ± 0.5	1.3 ± 0.9
Valacyclovir	0.8 ± 0.0	1.2 ± 0.1	2.9 ± 0.6	0.6 ± 0.4
Vancomycin	1.1 ± 0.1	4.1 ± 0.2	1.0 ± 0.7	1.9 ± 0.7
Acarbose	1.0 ± 0.0	0.5 ± 0.0	0.8 ± 0.2	0.5 ± 0.2
Glycine	0.4 ± 0.0	0.5 ± 0.0	12.4 ± 1.0	1.0 ± 0.5
Ribavirin	0.3 ± 0.0	0.7 ± 0.1	1.8 ± 0.1	0.3 ± 0.1

Table 6. HPTC-like cells response to nephrotoxics that do not directly damage PTCs in humans and to non-nephrotoxic compounds. The cells were treated on the evening of day 8 with 100 µg/mL of the compounds listed on the left. Compounds highlighted in green are known nephrotoxics in humans that do not directly damage PTCs. Compounds highlighted in blue are not nephrotoxic in humans. IL-6 and IL-8 expression levels after 16 hours of exposure were determined by qPCR. The results show the mean +/- standard deviation (sd) (n = 3). All values were normalized to the respective vehicle controls, which were set to 1.0.

Figure 12. Phase contrast images of HPTC-like cells differentiated on NP2 after exposure to PTC-specific nephrotoxics for 16 hours. 10 µg/mL concentration of cadmium chloride, puromycin or tacrolimus (first row) and 50 µg/mL concentration of gold (I) chloride or potassium dichromate (second row) had extensively killed the HPTC-like cells, as compared to the vehicle control HPTC-like cells (Control). Scale bars represent 200 µm.

The expression levels of IL-6 and IL-8 of HPTC-like cells subjected to the highest tested concentration of the respective compounds from Tables 4, 5 and 6 were consolidated in Table 7. Cut-off values ranging from 1.0 to 4.0, were used for results classification. Fold changes of expression of either IL-6 or IL-8 or both that were equal to or above the indicated threshold level were classified as a positive result.

A result was classified as negative toxic if the expression levels of both IL-6 and IL-8 were below the cut-off value. Extensive cell death leading to unquantifiable IL-6 or IL-8 expression (ND) was always classified as a positive result. A true positive (TP) was defined as PTC-specific nephrotoxicant that gave a positive toxic response *in vitro* and a true negative (TN) was defined as a non-PTC-specific nephrotoxicant or a non-nephrotoxic compound that gave a negative response *in vitro*. Sensitivity and specificity were defined by the following formulae;

$$Sensitivity = \frac{TP}{Total\ 11\ (PTC - specific\ nephrotoxicants)} * 100\%$$

$$Specificity = \frac{TN}{Total\ 7\ (non - PTC - damaging\ compounds)} * 100\%$$

Sensitivity and specificity values obtained at the various cut-off values are shown in Table 7 and were graphically represented in Figure 13. An optimal ratio of sensitivity and specificity was obtained at a cut-off value of 2.5. At this cut-off

value, HPTC-like cells differentiated on Matrigel had a sensitivity and specificity of 72.7 % and 71.4% respectively, whereas HPTC-like cells differentiated on NP2 substrate gave sensitivity and specificity values of 72.7 % and 85.7% respectively.

Analysis of receiver operating characteristic (ROC) curves, where the rate of true positive is plotted against the rate of false positive (also defined as 100 – Specificity), have been widely used in evaluating the accuracy of diagnostic tests and in vitro toxicity screening platforms [121-123]. ROC curves were plotted to compare the performances of the HPTC-like cells differentiated on the respective substrates as an *in vitro* model in identifying PTC-specific nephrotoxics (Figure 14). A curve that is skewed closer to the upper left corner (the 0,100 coordinate) and giving a larger value of area under the curve (AUC) would indicate a model that has a better predictivity. The predictivity of HPTC-like cells differentiated on NP2 with an AUC value of 76.0% was similar to HPTC-like cells differentiated on Matrigel with an AUC value of 74.0%.

The positive predictive value and negative predictive value for the two HPTC-like cells as an *in vitro* cell model were also calculated according to the formulae defined below;

$$\text{Positive predictive value, PPV} = \frac{TP}{TP + FP} * 100\%$$

$$\text{Negative predictive value, NPV} = \frac{TN}{TN + FN} * 100\%$$

where *TP* denotes true positive, *FP* denotes false positive, *TN* denotes true negative and *FN* denotes false negative.

HPTC-like cells differentiated on NP2 achieved a PPV and NPV of 88.8% and 66.7% while those from Matrigel achieved a PPV and NPV of 72.7% and 62.5%, respectively. Collectively, these results obtained show that HPTC-like cells differentiated on NP2 substrate have a similar performance as those on Matrigel as a cell model for *in vitro* screening of PTC-specific nephrotoxics.

3.3 Identification of vancomycin as a PTC-specific nephrotoxicant

Given these findings, it was interesting to note that while no upregulation of either IL-6 or IL-8 were seen in HPTC-like cells differentiated on Matrigel subjected to vancomycin, those differentiated on NP2 had elevated expressions of both IL-6 and IL-8, which may point out to vancomycin having a toxic effect on PTCs, which was otherwise unobserved on HPTC-like cells differentiated on Matrigel.

Vancomycin had previously been reported to lead to detrimental effects on proximal tubules in rats [124, 125] and rabbits [126]. Lodise *et al.* and Nishino *et al.* had suggested that vancomycin acts as an oxidative stressor in these PTCs [127, 128]. More recently, Sakamoto *et al.*, had reported that vancomycin induced apoptosis in porcine renal tubule epithelial cells due to the production of intracellular reactive oxidation species (ROS) [129]. Using the stable isotope labeling by amino acids in cell culture (SILAC) approach, Li and Zhuo have shown that vancomycin may elicit nephrotoxic effect on the HK-2 HPTC line via the modulation of PI3K/AKT/mTOR cell cycle signalling pathway [130]. The work reported by Le Moyec and co-workers had also pointed out to vancomycin nephrotoxicity in humans being due to damage to both the medullary region as well as the proximal tubules [131], thus further indicating that vancomycin is indeed a PTC-specific nephrotoxicant.

Herein, the results showed that HPTC-like cells differentiated on NP2 were able to elucidate vancomycin as a compound that induces damage to PTCs *in vitro*. Applying the same 2.5 cut-off value for a positive toxic response,

reclassification of vancomycin as a PTC-specific nephrotoxicant would hence give a sensitivity and specificity value of 75.0% and 100% respectively for HPTC-like cells differentiated on NP2 and a value of 66.7% and 83.3% respectively for HPTC-like cells differentiated on Matrigel.

		Matrigel									NP2								
		Expression Level		Cut-off Value							Expression Level		Cut-off Value						
		IL-6	IL-8	1.0	1.5	2.0	2.5	3.0	3.5	4.0	IL-6	IL-8	1.0	1.5	2.0	2.5	3.0	3.5	4.0
PTC-specific Nephrotoxicant (11 compounds)	Aristolochic acid	0.8 ± 0.1	3.7 ± 0.6	+	+	+	+	+	+	-	ND	ND	+	+	+	+	+	+	+
	Bismuth (III) oxide	1.2 ± 0.0	1.5 ± 0.6	+	+	-	-	-	-	-	0.4 ± 0.1	0.9 ± 0.4	-	-	-	-	-	-	-
	Cadmium chloride	7.6 ± 0.6	0.9 ± 1.2	+	+	+	+	+	+	+	ND	ND	+	+	+	+	+	+	+
	Gold (I) chloride	2.4 ± 0.1	12.4 ± 2.0	+	+	+	+	+	+	+	ND	ND	+	+	+	+	+	+	+
	Potassium dichromate	1.3 ± 0.1	0.1 ± 0.1	+	-	-	-	-	-	-	ND	ND	+	+	+	+	+	+	+
	Puromycin	14.2 ± 0.5	654 ± 16.4	+	+	+	+	+	+	+	ND	ND	+	+	+	+	+	+	+
	Cephalosporin C	2.3 ± 0.0	4.0 ± 1.2	+	+	+	+	+	+	+	ND	ND	+	+	+	+	+	+	+
	5-Fluorouracil	1.4 ± 0.0	2.5 ± 0.5	+	+	+	+	-	-	-	0.5 ± 0.1	0.2 ± 0.1	-	-	-	-	-	-	-
	Gentamicin	1.2 ± 0.1	0.9 ± 0.1	+	-	-	-	-	-	-	0.4 ± 0.0	0.5 ± 0.5	-	-	-	-	-	-	-
	Rifampicin	0.7 ± 0.1	21.0 ± 4.0	+	+	+	+	+	+	+	0.7 ± 0.1	6.4 ± 1.1	+	+	+	+	+	+	+
	Tacrolimus	12.5 ± 0.6	9.1 ± 3.6	+	+	+	+	+	+	+	ND	ND	+	+	+	+	+	+	+
Non-PTC Nephrotoxicant (7 compounds)	Nephrotoxics not damaging PTCs directly																		
	Acetaminophen	0.6 ± 0.0	2.3 ± 0.3	+	+	+	-	-	-	-	1.4 ± 0.2	0.40 ± 0.2	+	-	-	-	-	-	-
	Ethylene glycol	1.3 ± 0.1	1.5 ± 0.5	+	+	-	-	-	-	-	0.7 ± 0.2	1.3 ± 0.9	+	-	-	-	-	-	-
	Valacyclovir	0.8 ± 0.0	2.9 ± 0.6	+	+	+	+	-	-	-	1.2 ± 0.1	0.6 ± 0.4	+	-	-	-	-	-	-
	Vancomycin	1.1 ± 0.1	1.0 ± 0.7	+	-	-	-	-	-	-	4.1 ± 0.2	1.9 ± 0.7	+	+	+	+	+	+	+
	Non – Nephrotoxic compounds																		
	Acarbose	1.0 ± 0.0	0.8 ± 0.2	+	-	-	-	-	-	-	0.5 ± 0.0	0.5 ± 0.2	-	-	-	-	-	-	-
	Glycine	0.4 ± 0.0	12.4 ± 1.0	+	+	+	+	+	+	+	0.5 ± 0.0	1.0 ± 0.5	+	-	-	-	-	-	-
	Ribavirin	0.3 ± 0.0	1.8 ± 0.1	+	+	-	-	-	-	-	0.7 ± 0.1	0.3 ± 0.1	-	-	-	-	-	-	-
True positive				11	9	8	8	7	7	6	True positive		8	8	8	8	8	8	8
True negative				0	2	4	5	6	6	6	True negative		2	6	6	6	6	6	6
Sensitivity (%)				100.00	81.81	72.73	72.73	63.64	63.64	54.55	Sensitivity (%)		72.73	72.73	72.73	72.73	72.73	72.73	72.73
Specificity (%)				0.00	28.57	57.14	71.43	85.71	85.71	85.71	Specificity (%)		28.57	85.71	85.71	85.71	85.71	85.71	85.71

Table 7. Summary of IL-6 and IL-8 expression levels of HPTC-like cells differentiated on Matrigel or NP2 and subjected to compound treatment at 100 µg/mL. The corresponding classification of toxicity was based on cut-off value of IL expression, ranging from 1.0 to 4.0. A positive toxicity result (+) was determined by either one or both the expression level of IL-6 or IL-8 to be similar to or above the determined cut-off value. A negative toxicity result (-) was determined by expression of both IL-6 and IL-8 to be below the determined cut-off value. Extensive cell death (ND) was always classified as a positive result. The sensitivity and specificity percentages of the assay on both HPTC-like cells at the various cut-off values were calculated and tabulated under the respective columns.

Figure 13. Percentages of sensitivity and specificity obtained with HPTC-like cells differentiated on Matrigel or NP2 were plotted against a range of cut-off values ranging from 1.0 to 4.0. Optimal ratios of sensitivity and specificity were obtained at a cut-off value of 2.5.

Figure 14. ROC curves obtained with HPTC-like cells differentiated on Matrigel and NP2. Curves that are skewed further towards the upper left (in the direction of the blue arrow) indicate higher predictivity. Green dash depicts a curve for a test with no predictive value. AUC of HPTC-like cells differentiated on Matrigel and NP2 respectively were calculated to be 74.0% and 76.0% respectively.

Chapter 4 Summary and Future Work

The suitability of synthetic xeno-free nanoparticle substrate NP1 and NP2 as a suitable base substrate for the efficient differentiation of iPSCs into HPTC-like cells was investigated. iPS-F4-derived HPTC-like cells differentiated on these synthetic substrates formed a monolayer of cells comparable to those differentiated on traditionally-used Matrigel. Results from the analysis of PTC-specific protein markers on these HPTC-like cells showed similar staining patterns proximal tubule markers at protein levels. The resultant HPTC-like cells displayed tight epithelia, as seen by the chicken-wire ZO1 staining pattern at cell-cell junctions, which are typical features of proximal tubule epithelium essential for its barrier properties [109]. Gene expression analysis showed that HPTC-like cells differentiated on NP2 gave similar or higher expression of PTC-specific markers compared to those differentiated on Matrigel. Conversely, the elevated expression of other renal markers such as SMA, NCCT and UMOD also point out that the resultant HPTC-like cells to be of a mixed phenotype. FACS analysis of differentiated HPTC-like cells showed that NP2 substrate was equally efficient in generating URO10⁺, GLUT1⁺ and PEPT1⁺ cell population, while giving a higher yield of OAT3⁺ and AQP1⁺ HPTC-like cells as compared to those differentiated on Matrigel. It must however be noted that the yield of OAT3⁺ and AQP1⁺ HPTC-like cells obtained were lower than those obtained by Kandasamy *et al.* (2015) [47].

The predictive performance of HPTC-like cells obtained with Matrigel or NP2 was tested with a method that predicts PTC-specific toxicity of drugs and other compounds [45, 47]. The HPTC-like cells were exposed to compounds that are directly toxic or non-toxic to PTCs in human for 16 hours. Unexpectedly, compounds that are directly toxic to PTCs in humans frequently induced massive cell death of HPTC-like cells differentiated on NP2. This was not observed with cells differentiated on Matrigel. Cell death and compound-induced IL-6 and IL-8 expression were used as endpoints and the predictive performance of the two HPTC-like cells was determined using a cut-off value with respect to expression levels of compound-induced IL-6 and IL-8. Similar sensitivity, specificity and ROC AUC values for HPTC-like cells differentiated on NP2 versus Matrigel show the former to be an equally predictive cell model for *in vitro* nephrotoxicant screening.

Remarkably, the HPTC-like cells differentiated on NP2 substrates were able to identify vancomycin as a PTC-specific nephrotoxicant at the defined cut-off value. On the contrary, vancomycin was not positively identified by the HPTC-like cells differentiated on Matrigel, further pointing out the advantages of the NP2 substrates in deriving HPTC-like cells that were suitable for nephrotoxicant screening applications.

Recent published work by Takasato and colleagues have shown the generation of a ureteric bud and metanephric mesenchyme tissue and to the subsequent nephron organoid via differentiation of human embryonic stem cells [132]. Xia *et al.* have also previously demonstrated differentiation of PSCs to

ureteric bud kidney progenitor-like cells [133]. While greatly promising, the use of Matrigel as a base substrate in these studies may hamper its clinical application. It would therefore be feasible to investigate the compatibility of NP2 as a xeno-free, clinically-relevant substrate for the generation of nephron-like structures and organoids, toxicology studies, regenerative medicine and disease models.

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