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A 3-D organoid kidney culture model engineered for high-throughput nephrotoxicity assays

Anna I. Astashkina [a], Brenda K. Mann [b,1], Glenn D. Prestwich [b,c,1], David W. Grainger [a,b,*]

[a] Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT 84112-5820, USA
[b] Department of Bioengineering, University of Utah, Salt Lake City, UT 84112-5820, USA
[c] Department of Medicinal Chemistry, University of Utah, Salt Lake City, UT 84112-5820, USA

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ABSTRACT

Cell–cell and cell-matrix interactions control cell phenotypes and functions *in vivo*. Maintaining these interactions *in vitro* is essential to both produce and retain cultured cell fidelity to normal phenotype and function in the context of drug efficacy and toxicity screening. Two-dimensional (2-D) cultures on culture plastics rarely recapitulate any of these desired conditions. Three dimensional (3-D) culture systems provide a critical junction between traditional, yet often irrelevant, *in vitro* cell cultures and more accurate, yet costly, *in vivo* models. This study describes development of an organoid-derived 3-D culture of kidney proximal tubules (PTs) that maintains native cellular interactions in tissue context, regulating phenotypic stability of primary cells *in vitro* for up to 6 weeks. Furthermore, unlike immortalized cells on plastic, these 3-D organoid kidney cultures provide a more physiologically-relevant response to nephrotoxic agent exposure, with production of toxicity biomarkers found *in vivo*. This biomimetic primary kidney model has broad applicability to high-throughput drug and biomarker nephrotoxicity screening, as well as more mechanistic drug toxicology, pharmacology, and metabolism studies.

1. Introduction

In vivo cell–cell interactions, and more broadly, the contextual communication characteristics of the tissue microenvironment (i.e., matrix-mediated, haptotactic, chemotactic, biomechanic, paracrine, autocrine signals), dictate cell phenotype and are required to produce and maintain tissue-like responses *in vitro* [1–8]. Many physiological variables including cell–cell interactions, cell-matrix communication, matrix chemical composition, matrix mechanics, matrix porosity, chemotactic gradients of soluble cell signals, cell oxygenation, and 3-D matrix architectures are critical for producing *in vivo*-like cell behavior [1–4,6]. Cell proliferation, polarization, migration, signaling, and gene expression *in vitro* depend on both presence and proper presentation of these signals. However, no single variable alone achieves tissue-like organization to elicit tissue-like functions; many (if not all) of these chemical, mechanical and structural parameters must be satisfied in any *in vitro* culture to recapitulate *in vivo* cell responses. Available *in vitro* bioreactors using highly sophisticated control systems and dynamic inputs to control cell growth in 3-D scaffold systems [9] is not informed at the appropriate cell level with requisite design criteria to recapitulate *in vivo* complexity in several tissue types, particularly at levels of organ heterogeneity. Hence, a compelling need remains for improved cellular models that capture the complexity of tissue organization *in vitro* in a reproducible manner to reproduce key traits (e.g., responses to drugs, regenerative tissue function) and study processes only accessible currently in mammalian animal models.

Development of a physiologically accurate and functionally high-fidelity kidney tissue surrogate is of particular interest for kidney transplant clinical demands [10–13]. Another impact lies in pharmaceutical drug development needs, as many drugs and new drug candidates inadvertently cause kidney toxicity [14]. Currently, commercially marketed drugs account for 25% of acute kidney injury in critical care patients [15]. Next to liver toxicity, kidney toxicity is the second leading indicator for drug candidate attrition [15]. Furthermore, since current clinical methods such as blood urea nitrogen (BUN) and creatinine measurements suffer from an intrinsic inability to recognize early signs of kidney damage [16,17], a great need exists for cell-based systems capable of reliable and sensitive assessment of kidney toxicity. Such systems would benefit from numerous advances in high-throughput (HTS) assessment as

* Corresponding author. Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT 84112-5820 USA. Tel.: +1 801 581 3715; fax: +1 801 581 3674.
E-mail address: david.grainger@utah.edu (D.W. Grainger).

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well as recent developments in biomarker validation using toxicogenomics, proteomics, and metabolomics [14]. The few extensive biomarker studies conducted – the Innovative Medicines for Europe (InnoMed) PredTox project, ILSI Health and Environmental Sciences Institute initiative, Predictive Safety Testing Consortium – were limited by their reliance on costly *in vivo* models due to limitations inherent in *in vitro* cell culture screens. Additionally, these efforts required consortia of pharmaceutical industry, academia, and regulatory agencies due to the excessive expenses and workload associated with the studies [14]. Hence, development of improved *in vitro* culture approaches better reflective of *in vivo* cell behaviors and reactions to drugs would significantly impact both the cost and accessibility of future HTS work.

Reliable kidney toxicity assessment requires retention of cellular structural and biochemical pathways; nephrotoxicity is a complex process involving drug metabolism using cytochrome (CYP) P450, biotransformation using intra- and extracellular enzymes, dynamic changes in cellular accumulation as a function of uptake and drug removal via specific transporters and inflammatory process regulation [18–20]. Currently, the gold standard for kidney-like culture models involves use of primary or immortalized cell lines grown on conventional 2-D tissue culture plastic surfaces. Although convenient and relatively simple to employ, the 'petri dish' approach leads to quick de-differentiation of primary cells *in vitro*, most probably through loss of tissue-like interactions on 2-D plastic [21,22]. Furthermore, transformed kidney cell lines have limited cellular machinery, including transporters, ligands associated with endocytosis, enzymes involved in biotransformation of molecules, and CYP P450 metabolism, making them intrinsically incapable of reacting to complex external pathophysiological stimuli with *in vivo*-relevant biomarkers [3,7,22–26]. Due to the limiting functional cell equivalence on 2-D surfaces, cell assay data obtained in response to toxic drug and environmental agents is typically limited to simple observations of changes in cell viability and proliferation, both of which have little predictive value *in vivo*. Furthermore and perhaps most importantly, no tangible improvements of 2-D cell culture are feasible as studies will always be constrained by the fundamental limitations of rudimentary cell monolayer and (frequently) monoculture interactions on tissue culture plastic. More complex *in vitro* cell cultures that preserve tissue-like 3-D cell–cell interactions, tissue architecture and communicative biochemistry are needed to better assess complex cellular mechanisms in a clinically significant fashion.

The current nephrotoxicity assay design focused on toxicity processes associated with the PT, an essential central component of the functional nephron. The PT is a hollow tube comprising a basement membrane lined by epithelial cells, and is the primary site of clinical kidney toxicity instigated by external stimuli, such as heavy metals, dyes and drugs [27]. Susceptibility of PTs to environmental assaults is most likely due to the significant epithelial surface area provided in this part of the nephron, as well as the large number of active transporters possibly hijacked by small molecules entering the nephron. Encapsulation of harvested viable intact PTs, and not simply the epithelial cells that line their lumens, directly into a well-characterized biologically derived matrix avoids unpredictable, confounding cell-polymer matrix interactions, while preserving native 3-D cell-matrix environments and cell–cell physiological interfaces. Together this cellular context ensures cell maintenance, signaling and more accurate, extended *in vivo*-like epithelial cell responses.

Hyaluronic acid (HA)-based hydrogels used to encapsulate PTs are already well-established in preserving tissue-like architectures and cell interactions in several tissue regeneration and 3-D bioreactor culture models [28–31]. Furthermore, HA is a natural polymer present is almost all bodily tissues, including the kidney. Primary proximal tubule epithelial cell viability, differentiation potential and functionality were all used to assess and validate this newly developed 3-D organoid culture for up to 2 months. This culture response to known nephrotoxic agents compared cytochrome P450 enzyme induction, metabolite production, and kidney injury-1 (Kim-1) protein up-regulation – accepted markers for typical drug toxicity screens.

2. Methods

Male C57BL/6 mice (6–8 weeks) were purchased from Jackson Laboratory (Bar Harbor, USA). All animals euthanized using carbon dioxide according to approved University of Utah IACUC protocols and PTs were isolated immediately following literature-established procedures using standard aseptic conditions in a BSL2-certified laminar flow hood [32]. Briefly, murine kidneys were removed surgically and cleaned of the kidney capsule, blood vessels and ureter. Between all steps tissue was stored in ice-cold KREBS solution (145 mM NaCl, 10 mM HEPES, 5 mM KCl, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.8 mM $MgSO_4$, 5 mM glucose, pH 7.3) [33]. The tissue was then mechanically disrupted using sharp razor blades and enzymatically digested for 30 min in enzyme solution (10 ml KREBS, 2 mg/ml hyaluronidase (Worthington Biochemical Corporation, USA), 3 mg/ml collagenase IV (Worthington Biochemical Corporation, USA), 0.1 mg/ml DNase I (Sigma–Aldrich Chemical, USA)) at 37°C [33]. PTs were enriched by sequential 250 μM and 80 μM sieving of the resulting digested nephron sections. PTs were then pelleted from the resulting suspension in KREBS by centrifugation for 15 min at 12,000 rpm. Proximal tubule yield was estimated using a hemocytometer.

The 3-D organoid proximal tubule cultures were fabricated by combining purified PTs with 1.5% commercial thiol-modified carboxymethylated hyaluronic acid (HA) (carboxymethyl, thiol-modified: CMHA-S) and 7.5% commercial poly(ethylene glycol) diacrylate (PEGDA) bifunctional polymer electrophile [29,31,34]. Both gel-forming GMP biomedical-grade polymers were generously provided by Sentrx Animal Care (Salt Lake City, USA). CMHA-S and PEGDA were resuspended in PBS^{++}, sterile filtered using 0.22 μm cell culture syringe filters (ISC Bioexpress, USA), and mixed in 4:1 ratio with PTs. Constructs were made by casting 50 μL of PT/gel mixture into TeflonAF® -coated 96-well plates (see below). Then the 3-D organoid gel precursor matrix was crosslinked for 35 min in a cell incubator at 37°C (5% CO_2 and 95% air). Proximal tubule media consisting of 1% fetal calf serum (Invitrogen, USA), 5% sodium pyruvate (Invitrogen, USA), 10% non-essential amino acids (Invitrogen, USA), 10% insulin/transferrin/selenium (Invitrogen, USA), 1% antibiobic-antimycotic (Invitrogen, USA), 0.9 μg of hydrocortisone (Invitrogen, USA), and Dulbecco's modified Eagle medium (DMEM)-Ham's F-12 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and L-glutamine (Invitrogen, USA) [32] was then added to final HA hydrogel constructs containing viable tubules (100 μL/well) and exchanged every 2 days for maximum cell viability.

The Teflon AF®-coated 96-well culture plates (150 μL/well) were prepared according to previously published protocols [35] using non-tissue culture polystyrene plates (ISC Bioexpress, USA) and TeflonAF® (Dupont, USA) dissolved in FC-40 (3 M, USA) organic solvent. Solvent was removed using vacuum drying in elevated temperature (56 °C), and coated plates were sterilized using UV for 30 min before use [35].

2.1. Analysis of cell viability

Proximal tubule epithelial cell viability in the 3-D organoid constructs was assessed using CyQuant NF® (Invitrogen, USA) and