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RESEARCH ARTICLE

Enhanced propagation of adult human renal epithelial progenitor cells to improve cell sourcing for tissue-engineered therapeutic devices for renal diseases

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Abstract

Renal cell therapy employing cells derived from adult renal epithelial cell (REC) progenitors promises to reduce the morbidity of patients with renal insufficiency due to acute renal failure and end stage renal disease. To this end, tissue engineered devices addressing the neglected biologic component of renal replacement therapy are being developed. Because human donor tissue is limited, novel enhanced progenitor cell propagation (EP) techniques have been developed and applied to adult human kidney transplant discards from six donors. Changes include more efficient digestion and the amplification of progenitors prior to terminal epithelial differentiation promoted by contact inhibition and the addition of retinoic acid. Differentiated morphology in EP populations was demonstrated by the ability to form polarized epithelium with tight junctions, apical central cilia and expression of brush border membrane enzymes. Evaluation of lipopolysaccharide stimulated interleukin-8 secretion and γ–glutamyl transpeptisade activity in EP derived cells was used to confirm therapeutic equivalence to REC obtained using published techniques, which have previously shown efficacy in large animal models and clinical trials. Yield exceeded 10^{16} cells/gram cortex from the only kidney obtained due to an anatomical defect, while the average yield from diseased kidneys ranged from 1.1×10^{9} to 8.8×10^{11} cells/gram cortex, representing an increase of more than 10 doublings over standard methods. Application of the EP protocol to REC expansion has solved the problem of cell sourcing as the limiting factor to the manufacture of cell based therapies targeting renal diseases and may provide a method for autologous device fabrication from core kidney biopsies. **Copyright © 2012 John Wiley & Sons, Ltd.**

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Supporting information may be found in the online version of this article.

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1. Introduction

Acute renal failure (ARF) and chronic renal disease (CRD) are distinctly different disease processes and current therapy for both disorders is suboptimal. ARF arises from toxic or ischaemic (usually simultaneous) tubule damage from antibiotics, chemotherapeutic agents or shock during infectious or major operative procedures. The development of ARF in a hospitalized patient results in a five- to eight-fold higher risk of death (Humes, 1995; Chertow *et al*., 1998; Bates *et al*., 2001), with overall mortality rates exceeding 50%. However, if the patient survives the episode of ARF, the regenerative repair processes within the kidney can result in a return of kidney function in 90–95% of patients with this acute disorder. The cause of death in patients with acute renal failure is usually the development of Systemic Inflammatory Response Syndrome (SIRS), with resulting cardiovascular

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collapse, ischaemic damage to vital organs and multiple organ failure (MOF) (Humes, 2000; Humes *et al*., 2002). The propensity of patients with ARF to develop SIRS and sepsis suggests that renal metabolic function, specifically renal tubule cell function secondary to Acute Tubular Necrosis (ATN), plays a critical immunomodulatory role in individuals under stress states. This function is not replaced with standard dialysis therapy.

Unlike ARF, CRD is an irreversible process of progressive kidney damage, commonly from diabetes and hypertension, which leads to end-stage renal disease (ESRD). Patients with ESRD on dialysis continue to have major medical, social and economic problems with life expectancies of only 4–5 years (Iglehart, 1993; Xue *et al*., 2001; Cukor *et al*., 2007). While the ideal standard for kidney replacement therapy for ESRD remains organ transplant, with less than ten thousand kidney transplants completed each year and the current number of patients awaiting transplantation approaching sixty thousand (Collins *et al*., 2005), the need far exceeds availability. For all disease processes, available renal replacement therapies consisting of haemofiltration, haemodialysis or chronic ambulatory peritoneal dialysis are non-physiological and fail to address the homeostatic, regulatory, metabolic and endocrine functions of the kidney. Patients with ESRD are at high risk for cardiovascular and infectious diseases, despite conventional renal replacement therapy. A recent clinical trial failed to show survival benefit from increased doses of dialysis above the current standard of care (Humes, 1995), suggesting that there are important metabolic derangements not adequately treated with conventional dialytic treatment. Survival of renal transplant recipients far exceeds that of age-, sex- and risk-matched controls awaiting transplant, clearly demonstrative of the metabolic function provided by the kidney beyond filtration. ESRD currently affects over 430 000 US patients and has an annual cost of more than $25 billion, and patient numbers are predicted to increase to 2.24 million by 2030 (Schrier and Wang, 2004).

The potential success of renal cell therapy lies in the growing appreciation that most disease processes are not due to the lack of a single protein, but develop due to alterations in complex interactions of a variety of cell products. The renal tubule cell's roles in glutathione reclamation, glutathione peroxidase synthesis and activation of vitamin D3, with its important immunoregulatory functions, are well-recognized pathways to maintain important tissue integrity and host defence under stress conditions (Humes, 2000; Humes *et al*., 2002). A less recognized role of the kidney, chiefly the renal tubule, is its potential immunoregulatory function. The kidney is derived embryologically from dorsal mesoderm, a collection of cells also important in the development of bone marrow stem cells (Turpen and Knudson, 1982; Saxen, 1987). The maturation of cells responsible for erythropoietin synthesis and activation of 1,25-$(OH)_2$ vitamin D3 in the kidney is reflective of this embryonic origin. Phylogenetically, in bony fish and amphibians without lymph systems, the kidney is the major antibody-producing organ (Smith *et al*., 1967; Zapata, 1979; Turpen and Knudson, 1982). Not surprisingly, mammalian renal proximal tubule cells are immunologically active. They are antigen-presenting cells (Bishop *et al*., 1988; Wuthrich *et al*., 1990) that possess co-stimulatory molecules (Wahl *et al*., 2002) and that synthesize and process a variety of inflammatory cytokines (Prodjosudjadi *et al*., 1995; Leonard *et al*., 1999; van Kooten *et al*., 1999).

The development of renal epithelial cell (REC) therapy to replace the endocrine and immunological functions of the kidney, in addition to haemofiltration, promises to add significant value to the current suboptimal treatments of renal failure. Candidate biological markers that are known to be dysregulated in ESRD correlate with poor outcome and are linked to known mechanisms of disease, include markers of inflammation and oxidative stress.

A methodology to isolate and grow REC from adult human kidneys has already been developed (Smith *et al*., 2006) and the described methods used to provide the renal cell component of tissue-engineered devices evaluated in large animal and clinical studies (Tumlin *et al*., 2008). However, using standard cell-processing techniques, RECs isolated from human transplant discards do not have the expansion capability required to provide cell therapy for the large patient population suffering from ARF and ESRD. The ability to differentiate a totipotent embryonic stem cell down an intricately orchestrated kidney-specific path is still in its infancy and does not provide a timely solution to cell sourcing for renal replacement therapy. Clinical and experimental observations suggest that REC progenitor cells have immense proliferative potential, as evidenced by complete

Table 1. Comparison of standard vs. enhanced propagation protocols

Step	Standard method	Enhanced propagation
Enzymatic digestion	Collagenase $< 600\ \mu M$ particle size	Liberase/Blendzyme. Single cell $\rightarrow 212\ \mu M$ particle size
Centrifugation	50 g (exclusive)	300 g (inclusive)
Plating density	500 μl pellet/100 mm plate	4–32 μl pellet/100 mm plate
Media change	24 h post-plating, non-adherent cells removed by aspiration	7 days post-plating, non-adherent cells retained
Matrix	Collagen IV/adsorbed FCS	Endogenous matrix
Subculture	$\sim 1 \times 10^7$ cells seeded and 2×10^7 recovered per T75 flask. Pass ratio 1:2	1.25×10^6 cells plated and 2×10^7 cells recovered per T75 flask. Pass ratio ~1:16
Differentiation factor retinoic acid	Added prior to primary passage. Cells remain differentiated through passages	Signals the end of precursor amplification. Added on device or test well after confluence (contact inhibition)

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tubule regeneration to reform a fully functional and differentiated epithelium after severe nephrotoxic or ischaemic injury (Duffield and Bonventre, 2005). This regeneration potential has been attributed to haematopoetic or mesenchymal stem cells, rather than resident renal progenitor cells, with controversial results (Poulsom *et al*., 2001; Gupta *et al*., 2002; Kale *et al*., 2003). The most pertinent observation from these studies was that injection of mesenchymal stem cells following ischaemic injury enhances the regenerative potential and recovery of tubules, occurring without actual incorporation of these cells into the regenerated tubules, possibly due to a paracrine effect (Bi *et al*., 2007; Imai and Iwatani, 2007).

Current opinion is that precursors responsible for the repopulation of tubules reside within the kidney, possibly maintained within a specialized niche (Al-Awqati and Oliver, 2006; Sagrinati *et al*., 2006), although the defining characteristics and mechanism are still under investigation (Duffield and Bonventre, 2005; Al-Awqati and Oliver, 2006; Challen *et al*., 2006). Regardless of source, these resident kidney progenitor cells have stem cell-like characteristics, with a high capacity for self-renewal and the ability to differentiate under defined conditions into mature somatic cells, as evidenced by tubule regeneration (Hall and Watt, 1989; Potten and Loeffler, 1990). An approach to more closely mimic the *in vivo* environment promises to greatly enhance REC propagation potential and is the basis of these studies.

2. Materials and methods

2.1. Tissue procurement

Human cadaver kidneys, rejected for organ transplantation due to anatomical or fibrotic defects, were procured from the National Disease Research Interchange (NDRI). Additional donor acceptance criteria included: adults $<$ 80 years of age, blood tests negative for adventitious viruses, non-septic, normal creatinine and blood urea nitrogen levels, no other indices of kidney disease, and time between cross-clamp and the beginning of tissue processing $\leq$ 36 h. Tissue from six donors was simultaneously processed using enhanced propagation (EP) and the standard protocol. The method modifications are highlighted in Table 1.

2.2. Cell isolation

Kidneys were dissected clean of all external tissue, the capsule removed, the cortex cut from the medulla and the cortex weight documented. Finely minced cortex was digested at 1 g tissue/ml prewarmed enzyme (collagenase for the standard protocol or Liberase for EP)/DNase (0.239 Wunchst units and 250 Kunitz units/ml) at 37 °C for 20 min cycles. Using the standard protocol, the digestion was quenched with cold Dulbecco's modified Eagle's medium (DMEM) and all tissue aggregates $>$ 850 μm were returned to the digestion flask and the cycles repeated. The quenched slurry was subsequently sieved without further enzymatic digestion and remaining tissue aggregates $>$ 600 μm were discarded. For EP, the enzymatic digestion proceeded uninterrupted with only the $<$ 212 μm sieved filtrate being quenched as it passed into the collection beaker containing cold DMEM. To evaluate the relationship of aggregate and cell yield, EP slurry was further fractionated by differential sieving into 150–212, 90–150, 38–90 and $<$ 38 μm fractions. For the standard method, the tissue was washed by centrifugation at 50 $\times$ *g* to preferentially retain intact tubules, while EP tissue was centrifuged at 300 $\times$ *g*, which was inclusive of single cells and aggregates that may have contained damaged and therefore more buoyant tubules.

2.3. Primary plating

RECs were cultured in UltraMDCK medium (UM, Lonza) supplemented with half the manufacturer's recommended dilution for insulin, transferrin, ethanolamine and selenium supplement (ITES; Lonza), 60 ng/l epidermal growth factor (hrEGF; R&D Systems), 10^{-9} M triiodothyronine and 1$\times$ penicillin–streptomycin. Standard plating density was 1 ml 50 $\times$ *g* pellet/24 ml medium (20 ml/T75 flask), and the non-adherent fraction was aspirated as a result of the first medium change after 24 h in culture. For EP plating, the cells were further diluted 1:16 by suspending 1 ml of the 300 $\times$ *g* pellet/384 ml medium (20 ml/T75 flask). For three donors, initial plating density was evaluated over a wide range by continued 1:2 dilution until 1:516 dilution was achieved. Partial medium changes (50%) were performed on an as-needed basis, with need assessed by monitoring glucose (Stanbio), lactate (Van Den Hamer and Elias, 1958; Krieg *et al*., 1967) and pH of the culture medium. Non-adherent cells were retained from primary EP culture through the first passage.

For both methods, subculture was accomplished as follows: cells were rinsed free of divalent cations, enzymatically released from plates using 0.05% trypsin–versene (Lonza) by incubation for 20 min at room temperature, and the reaction inhibited by 0.1% w/v soybean trypsin inhibitor (Gibco). Upon reaching tight confluence, retinoic acid (RA) was added to standard cultures 24 h before the first passage and cultures passed at 1:2, thus maintaining confluency in the range 50–100%. Enhanced propagation cultures did not receive RA at confluence and were subcultured at 10^5 cells/ml, 12 ml/100 mm plate, and passaged when 50–75% confluent, maintaining confluency in the range 6.25–75%. For both standard and EP cultures, RA was added to the medium just prior to use, at a concentration of 10^{-7} M.

2.4. Assessment of identity and function

Tests were made on increasingly passaged EP cultures, while those generated using standard protocols were tested at the fifth passage, the point at which standard populations were integrated into devices for clinical studies. For all tests, EP cells were seeded at confluent density, 10^6 cells/ml/

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5 cm^2. RA was added after 2 days and the cells cultured for an additional 5 days prior to testing. For cytokine evaluation, the cell medium was supplemented with 0.1% bovine serum albumin, both with and without 10 μg/ml lipopolysaccharide (LPS); the supernatants were removed at 24 h, clarified by centrifugation and the concentration of IL-8 determined by ELISA (R&D Systems). A quantitative γ-glutamyl transpepsidase (γGT) enzyme assay was developed using γ-glutamy-*p*-nitoanalide, a commercially available, γGT-specific colorometrc substrate (Stanbio) (Szasz, 1974). Activity was assayed in whole cell lysates prepared using a non-ionic detergent buffer and normalized with protein concentration (BioRad). One unit of activity is defined as the conversion of 1 μM reagent/min/mg protein at 37 °C. For immunohistochemistry (IHC), cells were fixed in 4% paraformaldehyde, permeated with 0.1% Triton X-100 and subsequently labelled with antibodies to REC-specific markers, including acetylated tubulin (AT1; Zymed), zona occludens-1 (ZO1; Zymed), γGT (Lab Vision) and aminopeptidase-N (APN; Becton Dickinson), and the nuclei labelled with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

2.5. Cryopreservation

Trypsinized cells were mixed at 10^7 cells/ml in cryoprotectant medium (10% DMSO in UM), placed in a controlled-rate freezing apparatus (Nalgene, Mr. Frosty) at −80 °C overnight and then transferred to the vapour phase of liquid nitrogen. The cells remained frozen for at least 2 weeks prior to use in cryopreservation studies.

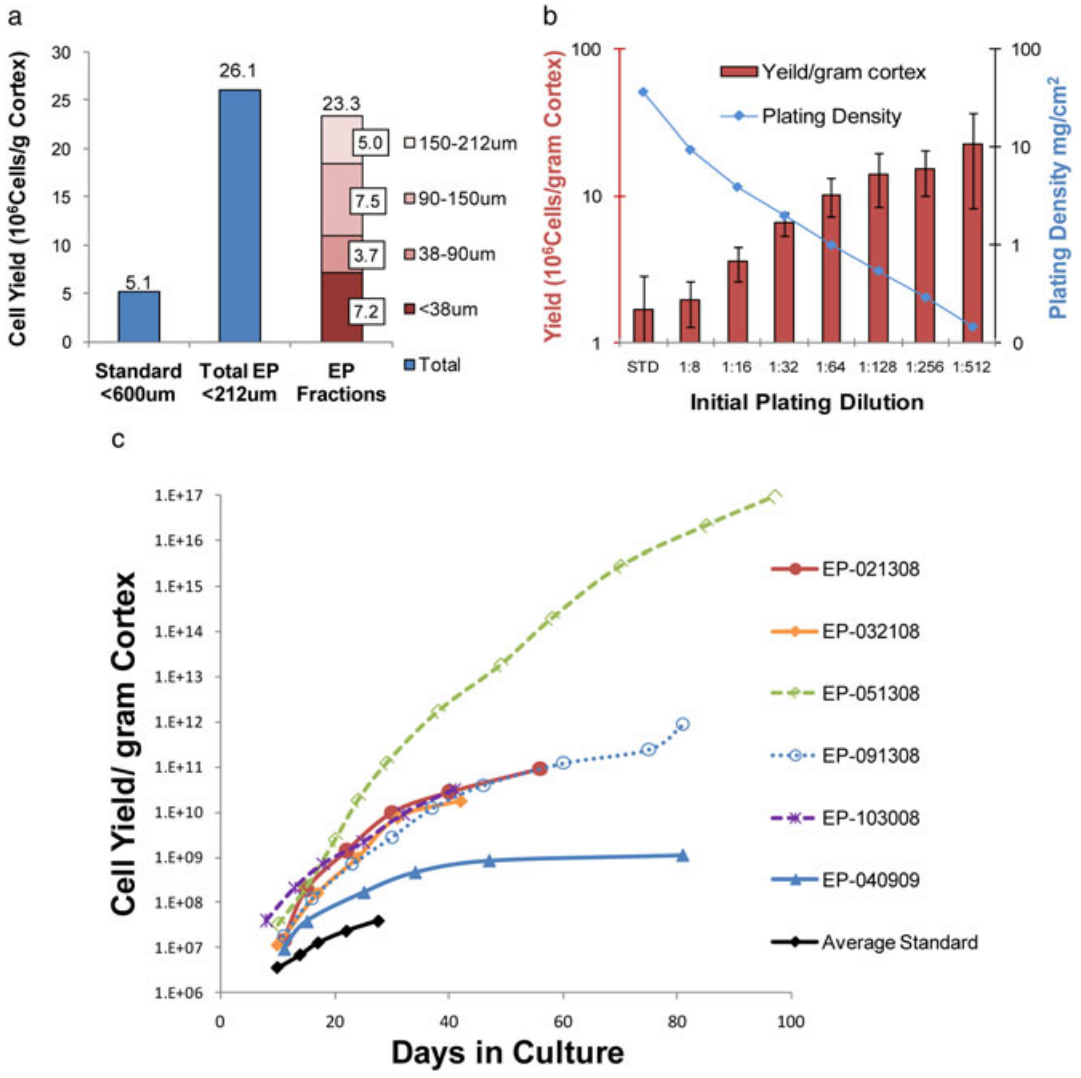

Figure 1. Cell yield calculations. Application of the enhanced propagation (EP) protocol for the isolation and propagation of renal epithelial progenitor cells to human cadaver kidneys greatly increased yield. (a) Yield increase is observed at the first passage. Progenitor cells are present in all sieved cell fractions, shown by fraction (red) far right, including the < 38 μm portion, in which any 3D stem cell niche would be destroyed. Representative results from the 13 September 2008 isolate are shown. (B) Cell yield/g cortex was proportional to dilution factor at primary plating. The results shown are average ± SE for $n = 3$ isolates. (C) The growth curves of six individual EP isolates and the average standard are plotted as total cell number through time. Although growth potential varies greatly between isolates, the plots clearly demonstrate that a far greater cell mass was obtainable using EP techniques; note the logarithmic scale displayed on the vertical axis (cell yield/g cortex). In (A, B) primary yield is defined as the number of cells resulting from the initial plating, counted post-trypsinization prior to subculture

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The cells were allowed to recover by plating and passaging using the EP propagation protocol before seeding at confluent density for characterization.

3. Results and discussion

Independent culture of sieved fractions confirmed that all aggregate pools contributed significantly to the EP precursor pool. A representative plot of the yield from the primary plating of each sieved portion is shown in Figure 1A. The proliferation potential of the $< 38\,\mu m$ portion, in which any three-dimensional (3D) stem cell niche would be destroyed, was essentially equivalent to the 38–90 μm portion and persisted through continued passaging. Plating density was investigated in four of six isolates and representative results are shown in Figure 1B. Yield/g cortex increased inversely to plating density at primary passage. However, cultures plated at dilutions > 1:16 of the standard protocol were subject to an island effect, in which cells become contact inhibited at the centres of islands. For continued expansion, these dilute cultures required passage before the tissue culture medium became depleted. Cells plated at 1.25×10^6 cells/20 ml/75 cm^2 flask (a concentration calculated from 1:16 of a confluent 75 cm^2 flask $= 2 \times 10^7$ cells) required weekly passage, essentially eliminating the need for medium changes between passage days and dramatically decreasing the reagents, supplies, effort and therefore cost of maintenance over more dilute cultures. For the remainder of this report, all assays for identification and efficacy focus on EP cells plated at 1:16 and subcultured at 1.25×10^6 cells/20 ml/75 cm^2 flask.

In Figure 1C, growth curves of REC progenitors from each donor are plotted as cell yield/g cortex through culture time, along with the average yield from the standard method for comparison. All EP cultures maintained exponential growth for at least 6 weeks, with the exception of the 9 May 2009 isolate, the only one that was terminated due to senescence. The others were terminated because they did not form epithelial monolayers when plated at higher density on tissue culture surfaces. Instead, they had a greater affinity for each other and aggregated into 3D structures in suspension. Yield exceeded 10^{16} cells/g cortex from the only kidney obtained due to an anatomical defect, while the average yield from diseased kidneys was in the range 1.1×10^9–8.8×10^{11} cells/g cortex, representing an increase of 10.3 ± 1.4 doublings over standard methods. Because the cell numbers obtained using the EP methods were sufficient by the time the effect was observed, further propagation and analysis of these cells was not pursued. The plots clearly demonstrate the far greater cell mass obtainable with EP techniques; note the logarithmic scale displayed on the vertical axis (cell yield/g cortex). The cell yields for both methods and relevant donor information are provided in Table 2.

During primary propagation at low density, REC progenitor cells are highly motile, forming adherent islands, floating rafts and non-adherent spheroids (Figure 2A–C). Using standard methods, part of the progenitor pool is discarded as a result of the initial medium change and the non-adherent structures do not have time to form. As cell density increases, the cells coalesce into islands with cuboidal centres and spindle-shaped cells at the outer edges (see Supporting information, Video S1). When plated at higher density with RA for functional testing, the cells quickly develop the familiar cobblestone appearance associated with REC, with occasional doming (Figure 2D, E; see also Supporting information, Video S2). As shown in Figure 3A, advanced EP cultures retained the ability to form polarized epithelium, as evidenced by the formation of apical central cilia, revealed by punctuate AT1 labelling, and the distinctive spider-web pattern of tight junctions was demonstrated with ZO1. The brush border enzymes APN and γGT persisted in advanced EP cultures (Figure 3B, C). The results from the γGT enzyme assay complemented IHC results, with 160 ± 43 and 242 ± 37 U/mg protein for the standard and EP cells, respectively. No trend was observed as the cultures aged; however, for both populations there was a significant decrease in γGT enzyme activity detected in cells post-cryopreservation, dropping to 78 ± 14 and 99 ± 23 U/mg protein for the standard and EP cells, respectively (Figure 4A).

Table 2. Relevant donor history and yield

Isolation date	Yield cell number/g cortex			Additional doublings Fresh/CRYO	Cause of death	Age/sex	Relevant donor history[a]	Glomerular sclerosis
	Standard	EP	EP–CRYO					
13 February 2008	6.3×10^7	9.2×10^{10}	3.0×10^8	10.4/5.6	Anoxia	57 F	D, HT, T, EtOH, A	6%
21 March 2008	1.3×10^7	3.7×10^{10}	2.7×10^{10}	11.5/11.0	Trauma	50 M	HT	4%
13 May 2008	2.6×10^7	9.2×10^{16}	6.3×10^{12}	31.7/17.9	CVA[b]	65 M		0%
13 September 2008	4.3×10^7	8.8×10^{11}	2.2×10^{10}	14.3/9.0	Anoxia	51 F	HT, T, EtOH	Biopsy not performed
30 October 2008	5.3×10^7	3.2×10^{10}	6.6×10^{10}	9.2/10.3	CVA	52 F	D, HT, T, EtOH, A	14%
9 May 2009	2.0×10^7	1.1×10^9	Not determined	5.8/ND	CVA	78 M	D, HT, T, RS	20%

Cell mass generated from enhanced propagation (EP) methods is diminished by cryopreservation (CRYO), but still far exceeds that of the standard protocol. Yield is highly dependent on prior disease state of donor and ranges from $> 10^{16}$ cells/g cortex derived from a relatively healthy donor to $< 10^{10}$ from a kidney known to be diseased. At a projected cell therapy dose of 10^8 cells, the 13 May 2008 donor would yield over 10^8 devices from a single g of cortex.

[a]D, diabetes; HT, hypertension; T, tobacco use; EtOH, alcohol use; A, asthma; RS, renal stent.

[b]CVA, cardiovascular accident.

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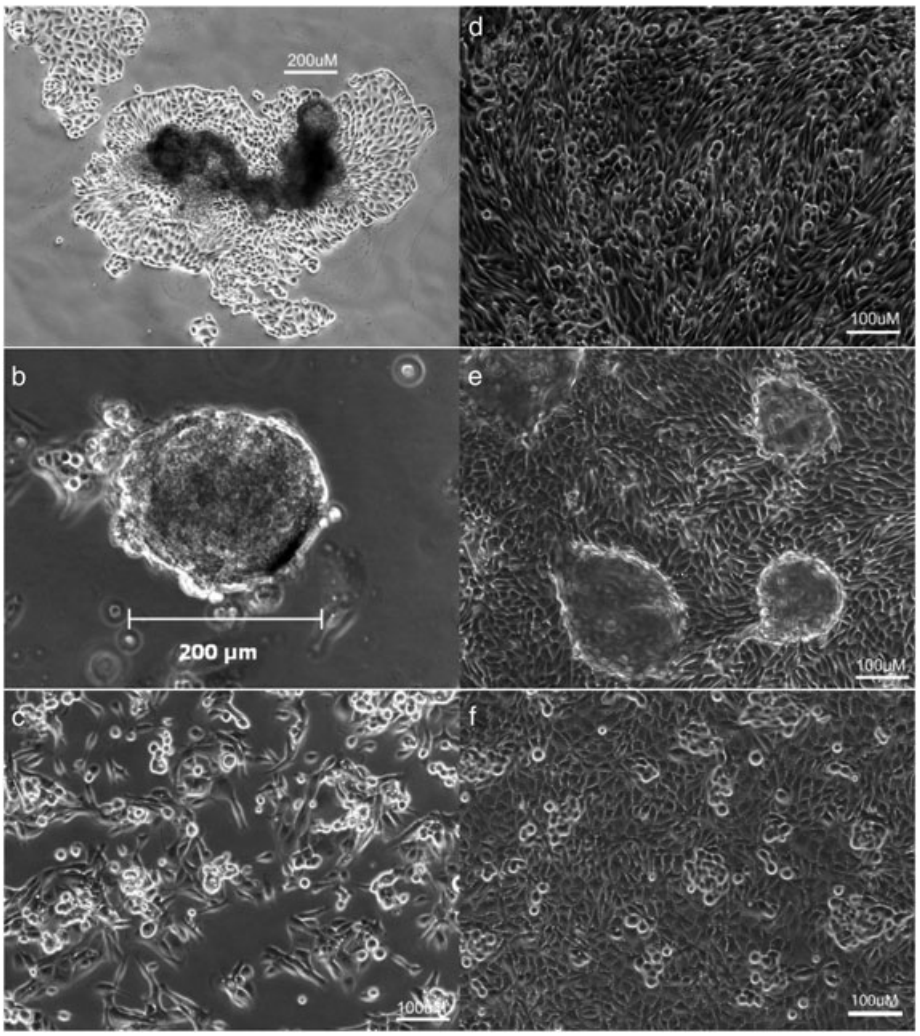

Figure 2. Morphology of REC propagated using the EP protocol. In primary culture REC progenitors form non-adherent floating rafts (A) and hollow spheroids (B), as well as adherent spindle-shaped cells. Post-cryopreservation, progenitor cells kept at low density remain in the exponential growth phase, retaining the spindle-shaped morphology (C). When plated at density with RA, previously cryopreserved cells acquire the cobblestone morphology of differentiated RECs (D), with occasional doming (E). Standard culture at passage 5 is shown for comparison (F)

EP-generated REC retained the ability to respond to endotoxin challenge through advanced culture. LPS stimulated IL8 secretion for all populations tested; however, the amount of IL8 released was highly variable across donors for the standard populations, averaging 6.3 ± 3.4 and 17.6 ± 8.6 ng/10^6 cells/24 h for basal and stimulated, respectively. EP rates were more consistent between donors, with the average being 3.4 ± 1.1 and 19.7 ± 3.5 ng/10^6 cells/24 h for basal and stimulated, and remained within the secretion ranges detected for the standard cultures. The ability to respond to endotoxin challenge persisted through cryopreservation, with basal and stimulated rates being 6.3 ± 3.4 and 19.1 ± 9.6 ng/10^6 cells/24 h for standard and 5.0 ± 2.1 and 18.7 ± 4.2 ng/10^6 cells/24 h for basal and stimulated EP populations (Figure 4B).

Cryopreservation had a detrimental effect on proliferation potential. In addition to a low initial recovery rate of 80% (8×10^6 viable cells from each vial containing 10^7 cells upon cryopreservation), the overall growth potential was greatly diminished. For the two isolates with the lowest potential, growth rates were unaffected. However, for the isolates having the greatest proliferation potential prior to cryopreservation (13 February 2008, 13 May 2008 and 13 September 2008), the growth potential was reduced between two and four orders of magnitude (Table 2).

Application of EP protocols to human kidneys has significantly increased cell yields compared to the standard method. In aggressively digested kidney cortex, the < 38 µm fraction, which consists of mostly single cells, still retained a high potential for proliferation, indicating that the retention of a 3D stem cell niche is not a requirement for transient amplification of renal progenitors. Yield was inversely proportional to plating density, while partial medium changes increased yields, indications that more than one factor may be influencing REC progenitor propagation.

Unfortunately, the available kidney pool for REC therapy consists of donors having pre-existing serious health

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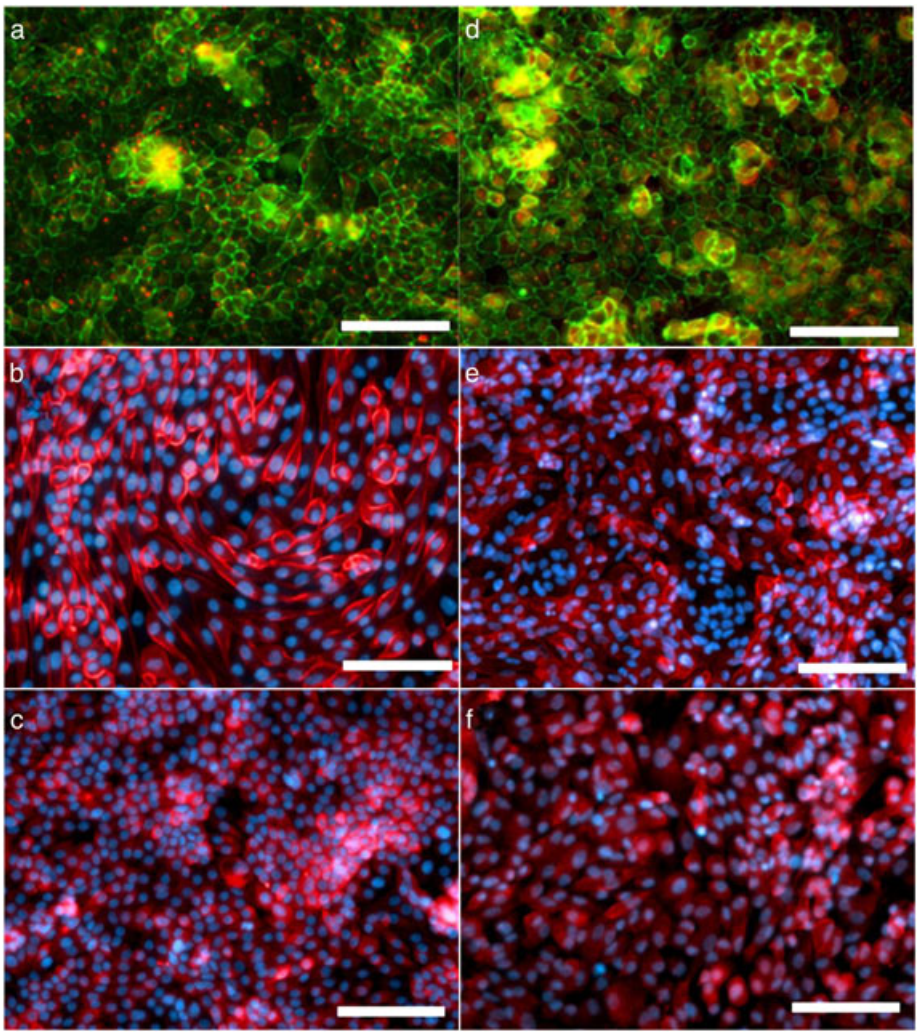

Figure 3. Immunohistochemical comparison of REC propagated using the EP protocol (A–C) compared to the standard protocol (D–F). EP-generated RECs form polarized epthelial monolayers, as demonstrated by acetylated tubulin-positive apical central cilia (red), while zona occludens (green) is concentrated in tight junctions having a distinct spider-web pattern (A, D). Expression of the renal brush border enzymes aminopepsidase N (B, E; red) and γGT (C, F; red) persists. DAPI counterstaining is shown in (B, E, C; blue).The photographs are representative of advanced EP cultures at yields > 100 times that possible with the standard protocol, compared to passage 5 standard cultures (= passage historically used for therapy). Scale bar = 100 μm

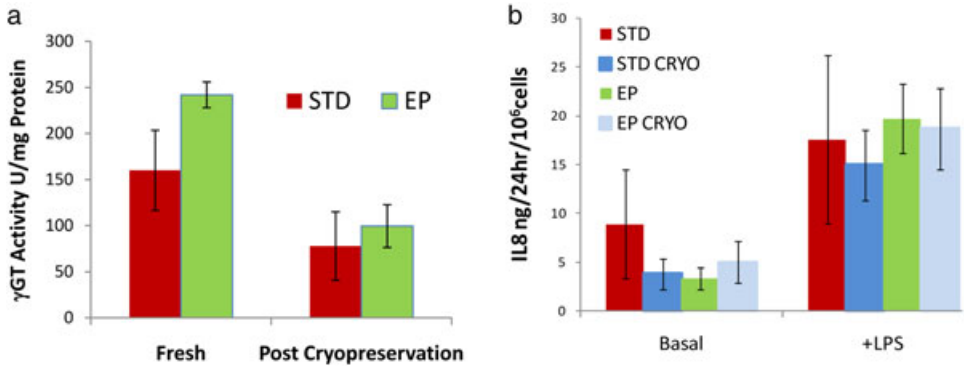

Figure 4. Retention of therapeutic potential through cryopreservation. (A) γ-Glutamyl transpepsidase (γGT) enzyme activity was detected in renal epithelial cells generated from both standard (STD) and EP cultures but was greatly diminished post-cryopreservation (CRYO). (B) Addition of lipopolysaccharide (LPS) stimulated IL8 secretion over basal secretion level in all cultures tested and was not affected by cryopreservation. Average results ± SE for $n = 4$ donors are shown

issues, including diabetes, hypertension and sclerosis of the kidney. Although donor health had a large impact on progenitor cell amplification, the post-cryopreservation cell yields from suboptimal donors exceeded 10^{10} cells/g cortex while retaining the appropriate morphology and ability to express surrogate efficacy markers. With a

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cell load/device expected to be 10^8 cells, and cortex weight of about 50 g/kidney, this correlates to more than 5000 devices from a single donor kidney, a vast improvement to the 1–10 devices demonstrated using standard methods. The only kidney donor with no known disease (13 May 2008) yielded over 10^{16} cells/g cortex.

With amplification of renal progenitor cells to this magnitude, the EP protocol could be successfully applied to tissue obtained from a kidney biopsy for autologous device manufacture for chronic applications. Kidney biopsies are routinely taken for diagnostic purposes and range from a few milligrams for a needle biopsy to around to 50 mg for a wedge biopsy. If the method was applied successfully to the non-diseased 13 May 2008 isolate, 6.3×10^{12} cells, or approximately sixty three thousand autologous devices, could theoretically be manufactured from a 50 mg wedge biopsy. For proof of principle, the EP method was applied to an 11 mg simulated needle biopsy taken from the whole donor kidney prior to dissection on 9 April 2009. Even though the simulated biopsy was from the kidney of the oldest donor with the most pre-existing health issues, and had the lowest overall yield, application of the EP method still yielded enough cells ($> 10^8$) for one autologous renal cell therapy device (data not shown).

To compare cells from standard methods and those generated using the EP protocol, surrogate markers were evaluated in addition to establishing equivalent morphology. LPS-stimulated IL8 secretion and γGT enzyme activity were chosen because they correlate with the ability to respond to inflammation and oxidative stress, mechanisms that are known to be dysregulated in ESRD and correlate with poor outcomes. Additionally, basal IL8 secretion was used as a release criterion for previous clinical trials involving REC (Tumlin *et al*., 2008). Because the immunoregulatory role of the kidney is very complex and response is highly individualized to the animal or patient (Humes *et al*., 2003), the usefulness of a surrogate marker panel in relation to actual therapeutic benefit remains unclear. Nevertheless, correlation of *in vitro* measurements to efficacy will be required for the transition of REC therapy to the clinical setting, and therefore the pursuit of surrogate markers remains a topic of emphasis. Because γGT enzyme activity and the ability to respond to endotoxin challenge in expanded EP cultures are retained through cryopreservation, these functions provide very promising efficacy markers. EP-derived cells in a bioartificial renal cell system are currently being evaluated using a porcine model of sepsis, where cells are in contact with the ultrafiltate in a haemodialysis circuit. In this model, renal cell therapy with standard cells increased survival time from 6.7 to 11.3 h (Buffington *et al*., 2009). Additionally, cells are being evaluated in an ovine model of ESRD, where cell therapy is delivered via peritoneal dialysis (Song *et al*., 2009). Results from these lines of experimentation will be used to further validate the use of IL8 secretion and γGT activity as efficacy markers.

The EP method was developed to provide an adequate and reliable supply of functional REC for integration into a cell therapy device. While cryopreservation affected the potential cell yield, only the very basic cryopreservation techniques were employed. Cell function and yield are expected to improve with optimized cryopreservation protocols. Cells derived from EP populations reconstituted post-cryopreservation were successfully integrated into a renal cell therapy device and retained the correct morphology through long-term *in vitro* maintenance in a perfusion circuit and *ex vivo* extracorporeal haemodialysis and peritoneal dialysis circuits. Application of the EP protocol to human cadaver kidneys has greatly enhanced cell proliferation, clearly demonstrating a robust cell source for renal cell therapy in two prospective delivery systems.

Emphasis was not placed on understanding the mechanisms responsible for the observed increase in yield. More investigation into the factors influencing REC amplification is merited and may provide further advancement in renal cell progenitor technology, as well as insight into progenitor amplification in other organ systems.

4. Summary

As envisioned, application of the EP protocol to human transplant discard kidneys increased cell yield dramatically over the standard method for all six donors tested. The generated cell number from each kidney was highly variable and based on available information, correlated with overall donor health. Yield exceeded 10^{16} cells/g cortex from the only kidney obtained due to an anatomical defect, while the average yield from diseased kidneys was in the range 1.1×10^9–8.8×10^{11} cells/g cortex, representing an increase of 10.3 ± 1.4 doublings over standard methods. Surrogate markers of efficacy, LPS-stimulated IL8 secretion and γGT enzyme activity, were chosen because they represent the ability to respond to endotoxin challenge and oxidative stress. IL8 secretion and γGT activity persisted through advanced EP cultures. Stimulated IL8 secretion was unaffected by cryopreservation, but γGT activity and overall cell yield were significantly reduced. However, the negative effects of cryopreservation may be resolved with the application of more advanced cryopreservation methods. Cells derived from EP populations reconstituted post-cryopreservation were successfully integrated into a renal cell therapy device and retained the correct morphology through long-term *in vitro* maintenance in a perfusion circuit and *ex vivo* extracorporeal haemodialysis and peritoneal dialysis circuits. Application of the EP protocol to REC expansion has solved the problem of cell sourcing as the rate-limiting factor to the manufacture of cell-based therapies targeting renal diseases, and may provide a method for autologous device fabrication from core kidney biopsies.

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Disclosure statement

H. D. Humes is a shareholder of Innovative BioTherapies and CytoPherix Inc.

Supporting information on the internet

The following supporting information may be found in the online version of this article:

Video S1. Adherence and outgrowth of renal epithelial progenitor cells, obtained using Zeiss AxioVision software and 10× objective (1sec=40minutes).
Video S2. Doming behavior exhibited by differentiated renal epithelial cells, obtained using Zeiss AxioVision software and 10× objective (1sec=40minutes).

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