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Research Article

Explanting Is an *Ex Vivo* Model of Renal Epithelial-Mesenchymal Transition

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Recognised by their *de novo* expression of alpha-smooth muscle actin (SMA), recruitment of myofibroblasts is key to the pathogenesis of fibrosis in chronic kidney disease. Increasingly, we realise that epithelial-mesenchymal transition (EMT) may be an important source of these cells. In this study we describe a novel model of renal EMT. Rat kidney explants were finely diced on gelatin-coated Petri dishes and cultured in serum-supplemented media. Morphology and immunocytochemistry were used to identify mesenchymal (vimentin+, α-smooth muscle actin (SMA)+, desmin+), epithelial (cytokeratin+), and endothelial (RECA+) cells at various time points. Cell outgrowths were all epithelial in origin (cytokeratin+) at day 3. By day 10, $50 \pm 12\%$ (mean $\pm$ SE) of cytokeratin+ cells double-labelled for SMA, indicating EMT. Lectin staining established a proximal tubule origin. By day 17, cultures consisted only of myofibroblasts (SMA+/cytokeratin−). Explanting is a reproducible *ex vivo* model of EMT. The ability to modify this change in phenotype provides a useful tool to study the regulation and mechanisms of renal tubulointerstitial fibrosis.

1. Introduction

Over recent years, considerable clinical and laboratory work has focused on the role of tubulointerstitial pathology in progressive renal disease and the cellular basis of its pathogenesis [1, 2].

Many of these studies have now indicated that interstitial fibroblasts are a major determinant of progression of all human and experimental models of end-stage renal disease. Fibroblasts can be stimulated by a wide variety of agents derived from stimulated tubular cells, leukocytes, or from the fibroblasts themselves. Activated fibroblasts, the so-called myofibroblasts, are usually recognized by their *de novo* expression of alpha-smooth muscle actin (SMA), a protein usually only found in vascular smooth muscle cells [3, 4]. Fibroblasts are fundamentally important to the pathogenesis of tubulointerstitial fibrosis, with animal studies showing that incorporation of a suicide transgene can conditionally minimise fibrogenesis through depletion of fibroblasts [5].

It is in turn increasingly recognised that myofibroblasts may be derived from a number of sources, including resident fibroblasts, migrating perivascular (adventitial cells), recruitment of circulating progenitor cells, and injured tubular cells through a process of epithelial mesenchymal transition (EMT). EMT can be defined as the acquisition of phenotypic as well as functional properties of mesenchymal cells by epithelial cells [6]. In this process, epithelial cells lose their phenotypic markers and characteristics and migrate into the surrounding matrix where they acquire phenotypic characteristics typical of mesenchymal cells. *In vivo* models have shown that EMT is an orchestrated sequence of events which relies not only on an interplay of different cytokine and noncytokine mediators but also on the integrity of the tubular epithelial cell, its intact basement membrane and cell adhesive proteins, and the nearby interstitium [7]. The repeated observation of EMT in both human renal biopsies [8] and animal models [9] has suggested that tubular epithelial

Table 1: Cytochemical markers of renal cell phenotype.

Antigen	Supplier	Clone	Source	Specificity
Vimentin	Dako	V9	Mouse	Mesenchymal cells
αSMA	Dako	1A4	Mouse	Smooth muscle cells, myofibroblasts, mesangial cells
αSMA (Cy3 conjugate)	Sigma	IA4	Mouse	Smooth muscle cells, myofibroblasts, mesangial cells
Desmin	Dako	D33	Mouse	Myofibroblasts, smooth muscle cells, glomerular podocytes
Cytokeratin	Dako	LP34	Mouse	Epithelial cells
RECA	Serotec	HIS 52	Mouse	Endothelial cells
E-cadherin	BD Biosciences	36	Mouse	Epithelial cells

cells are a major source of interstitial myofibroblasts in the kidney. Indeed, elegant experiments from Iwano et al. [7] have shown that after unilateral ureteric obstruction (UUO) approximately 40% of fibroblasts are derived from EMT. This suggests that a process that was once thought to be confined to embryogenesis may in hindsight be a fundamentally important process in the pathogenesis of renal scarring. However, the role of EMT in renal fibrosis is not without controversy [10], which has recently culminated in back-to-back editorial debate [11]. Recent lineage tracing studies from Humphreys and colleagues [12] have shown that pericytes, not tubule cells, are the predominant source of fibroblasts in UUO.

Consequently, the study of renal tubulointerstitial fibrosis requires robust experimental models that accurately reproduce EMT in experimental conditions. While most *in vivo* models have proven invaluable to delineating mechanisms of EMT, the kinetics of EMT has limited their usefulness. In reality, few cells undergo this process at any one time *in vivo*. Likewise, although EMT can be readily induced *in vitro*, this is at least in one case quite different from what happens to the same cells *in vivo* [12]. Furthermore, the complexity of EMT and its dependence on other cellular programmes [13] and the microenvironment [14] raises important questions about the usefulness of isolated cell lines.

In this paper we describe the characterisation and validation of a new *ex vivo* model of EMT. It is our contention that this provides an accurate and reproducible model for the study of this important process.

2. Materials and Methods

2.1. Ex Vivo Culture of Renal Explants. EMT was studied using cell explant outgrowths from normal rat kidneys using explanting methods described previously [15]. Renal cortex for explants was excised from Sprague-Dawley rats asphyxiated with an 80% : 20% mixture of $CO_2 : O_2$. Tissue was collected in ice-cold Hanks' salt solution with gentamycin (MP Biomedicals, Solon, Ohio, USA). Cultures were established by dicing cortical tissue onto gelatin- (Sigma, St. Louis, Mo, USA) coated Petri dishes and covering with DMEM (CSL, Parkville, Vic, Australia) supplemented with 20% foetal calf serum (FCS; CSL) and Penicillin/Streptomycin antibiotics (ICN). Tissue was maintained at 37°C overnight with further medium supplementation the following day. Explant outgrowths were then cultured for 3–17 days, with medium changed every third day. In each case cells were fixed by flooding Petri dishes with ice-cold methanol for 10 min.

2.2. Cell Phenotype. Cell outgrowths were phenotyped by immunocytochemistry, using standard techniques [16]. Fixed cells were consecutively incubated with primary antisera against cell-specific proteins using vimentin, SMA, pan-cytokeratin (all Dako, Glostrup, Denmark), rat endothelial cell antigen (RECA; Serotec, Oxford, UK), or E-cadherin (BD Biosciences Pharmingen, San Jose, Calif, USA) (Table 1). Explants were then rinsed in PBS, incubated with appropriate species-specific biotinylated secondary antisera (Vector Laboratories, Burlingame, Calif, USA), washed in PBS, and incubated with avidin-biotin complex (ABC; Vector) and diaminobenzidine (DAB; Dako). DAB enhancing solution (Vector) was used to enhance the reaction product, and cells were then counterstained with Harris haematoxylin and mounted with Gurr Aquamount (BDH, Poole, UK). Cells with positive staining were enumerated and expressed as a percentage of total cells counted.

2.3. Double Labelling of Epithelial and Mesenchymal Cells. In the case of double labelling, cells were prepared, treated, and fixed as above. Cells were then washed in PBS, blocked with normal serum, and incubated with a murine antibody against anticytokeratin. An anti-mouse FITC (Dako) was then applied followed by an anti-SMA Cy3 conjugate (Sigma). Cells were mounted in aqueous mounting media (Dako) and viewed with a fluorescent microscope (Leica Microsystems, Wetzlar, Germany) using appropriate filters for 520 nm (FITC) and 570 nm (Cy3) emissions. Representative images at the two different wavelengths were captured with a digital microscope camera (DP10, Olympus, Tokyo, Japan) and merged using the combine function in PaintShop Pro (Jasc Software, Minnetonka, Mich, USA).

2.4. Lectin Staining. Explant outgrowths were stained with lectins to determine the origin of the epithelial cell outgrowths. This methodology has been used previously in a number of studies to identify epithelial tubular segments [17–19].

Specificity of lectin binding was first confirmed by staining normal paraffin-embedded kidney tissue sections. Tissue sections were incubated for 2 hr with biotin-conjugated *phaseolus vulgaris* leukoagglutinin (Pha-L; Vector) (proximal

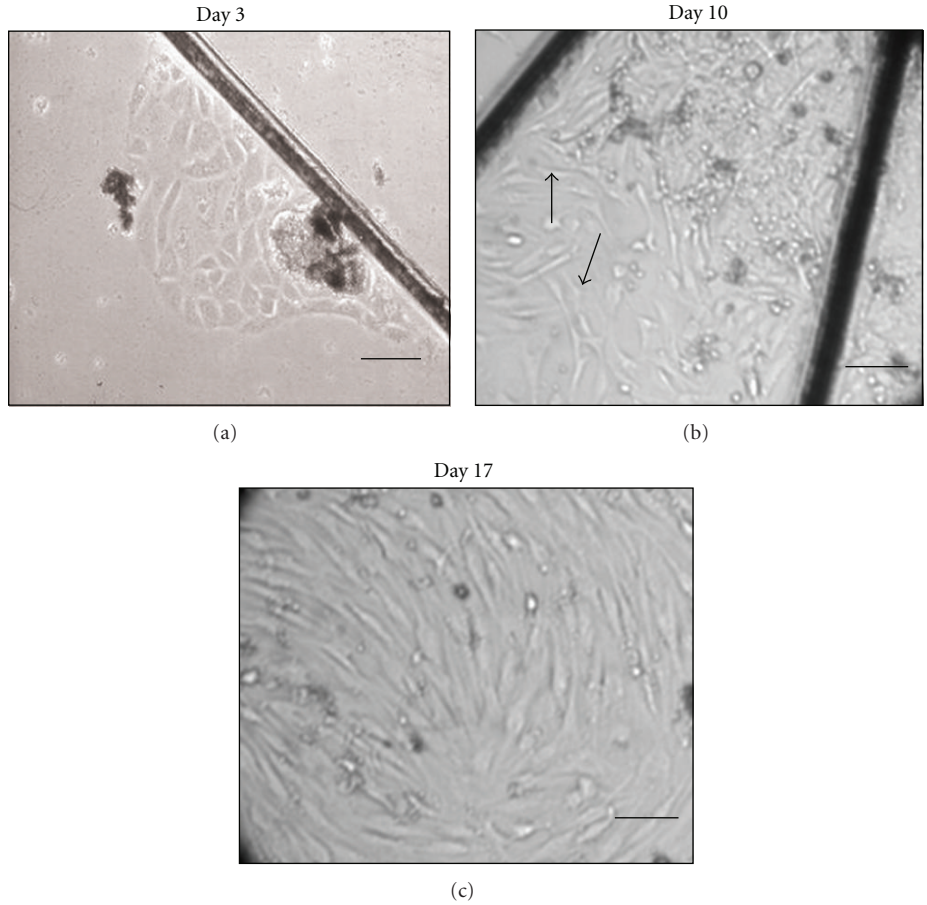

Figure 1: Phase contrast light microscopy of tissue at 3, 10, and 17 days after explanting. (a) Cuboidal-shaped cells are clearly seen propagating from explanted tissue at day 3. (b) By day 10 cells at the periphery have a more elongated morphology (arrows). (c) At day 17, confluent areas of cells form fingerprint patterns, characteristic of fibroblast culture. Diagonal line shows scratch in Petri dish used to mince tissue and anchor tissue to culture surface. Scale bar = 25 μm.

tubules and thick loop of Henle), *phaseolus vulgaris* erythroagglutinin (Pha-E; Vector) (proximal tubules), *Bandeiraea simplicifolia* I (BSL-I; Sigma) (collecting ducts, *vasa recta*), or *Arachis hypogaea* (Sigma) (distal convoluted tubules and collecting ducts). This was followed by incubation with ABC and DAB. Finally, tissue sections were dehydrated, counterstained with Harris haematoxylin, and mounted with Gurr Aquamount.

Likewise, to characterise explant outgrowths, explants were fixed in methanol at day 10, washed in PBS, and incubated with Pha-L, BSL-I, or AH conjugates before being treated as above.

2.5. Statistical Analysis. Data is represented as mean ± SE.

3. Results

The basis for presenting this *ex vivo* model of EMT lies in the typical growth patterns that have been observed during explanting of the renal cortex. Once cortical renal tissue is minced into gelatin-coated Petri dishes, it takes approximately 3 days before cell growth can be identified. Of those cells that grow out initially, most are cuboidal in shape (Figure 1(a)). Typical of epithelial cell culture, they grew in a uniform manner consistent with being tightly bound by cell-cell junctions and adhesions [20]. In contrast with this epithelial cell-like phenotype, from day 10, cells at the periphery of this outgrowth had a spindle shape appearance and were less organized in the surrounding matrix (Figure 1(b)). This was more consistent with a mesenchymal phenotype, their non-uniformity and weak cellular adhesion sites being indicative of a migratory capacity [20]. Cells proliferated rapidly over the next 1-2 weeks until most regions became confluent (Figure 1(c)).

3.1. Phenotype of Cells Grown from Tissue Explants. To characterise the cells that grow out of explant tissue, cells were stained with a panel of phenotype markers (Table 1). These included cytoskeletal proteins (vimentin, SMA, desmin), RECA, pan-cytokeratin, and the epithelial cell-cell junction protein E-cadherin. In accordance with the staining characteristics, cells were defined as mesenchymal (vimentin+, SMA+, desmin+), epithelial (cytokeratin+), or endothelial (RECA+) cells. At day 7, 66 ± 9% (mean ± SE) of cells

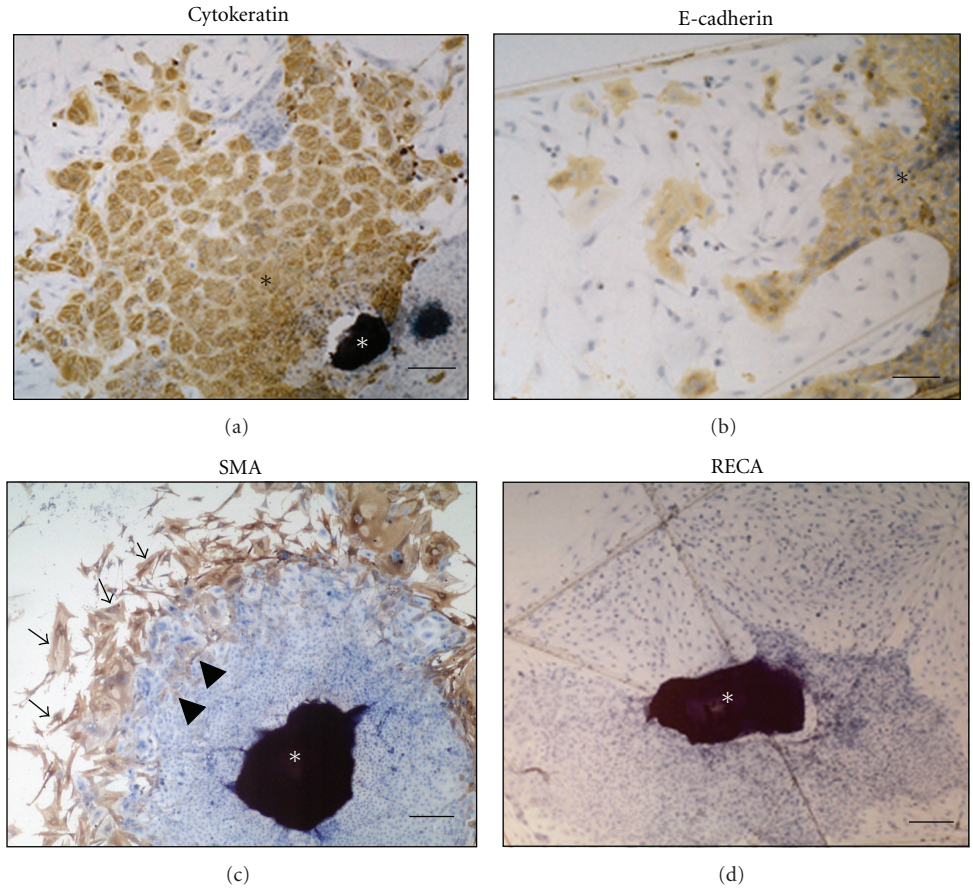

Figure 2: Immunohistochemical staining characteristics of cells 10 days after explanting. Two distinct areas are seen—an inner most area of cuboidal-shaped cells staining for (a) cytokeratin and (b) E-cadherin (black asterisk) and stellate-shaped cells at the periphery staining for (c) the myofibroblast marker SMA. Occasional cells at the junction of the two areas stain for SMA (arrow heads). (d) All cells were negative for the endothelial marker RECA. Remnants of the tissue explant from which cells have propagated can be seen (white asterisk). Scale bar = 50 μm.

were vimentin+, 21 ± 8% SMA+, 26 ± 8% desmin+, and 79 ± 5% cytokeratin+. Cells did not express RECA. By day 10, explants typically contained clusters of E-cadherin and cytokeratin-positive cells, surrounded by a peripheral region of cells staining for the mesenchymal marker SMA (Figure 2). Accordingly, it was apparent that explants consisted of epithelial cell outgrowths, with myofibroblasts present at the periphery. The acquisition of SMA, a myofibroblast marker, at the periphery of the outgrowths suggests that cells that previously expressed epithelial markers may be undergoing transition to a mesenchymal cell type.

3.2. Double Labeling Indicates That Cell Outgrowths from Explants Undergo Progressive EMT. To determine if the mesenchymal and epithelial cells shown in Figure 2 were autonomous cell populations or cells undergoing EMT, immunofluorescent double labelling was performed at 3, 10, and 17 days after explanting using red (SMA) and green (cytokeratin) fluorochromes (Figure 3). Merged fluorescent micrographs confirmed that at day 3 outgrowths from tissue were almost exclusively epithelial cells (cytokeratin+/SMA−) with myofibroblasts (cytokeratin−/SMA+ cells) recognised from day 10. This was most likely due to EMT as 50 ± 12% (n = 5 explants) of cytokeratin-positive cells costained for SMA at day 10. By day 17 cultures were all uniformly myofibroblasts, each with a well-organised SMA cytoskeleton.

3.3. Lectin Staining Indicates That Cell Outgrowths from Explants Have a Predominantly Proximal Tubular Derivation. Little is known about the specific derivation of tubular epithelial cells during EMT. In attempt to determine the nephron origin of the cells undergoing EMT, cell explant outgrowths were labelled for sugar moieties expressed by various nephron segments. AH is specific for distal tubule epithelium, whilst Pha-E and Pha-L are specific for proximal tubule epithelium (data not shown). Staining of explants with biotinylated lectins illustrates that, of those cells that grow out of explant tissue, the majority are derived from proximal tubular epithelium (Figure 4) with no cells staining positive for distal tubular epithelium lectin markers.

4. Discussion

This study has shown that explanting of renal kidney tissue is a reproducible *ex vivo* model of EMT. Explanting methodologies consistently produced a population of cells

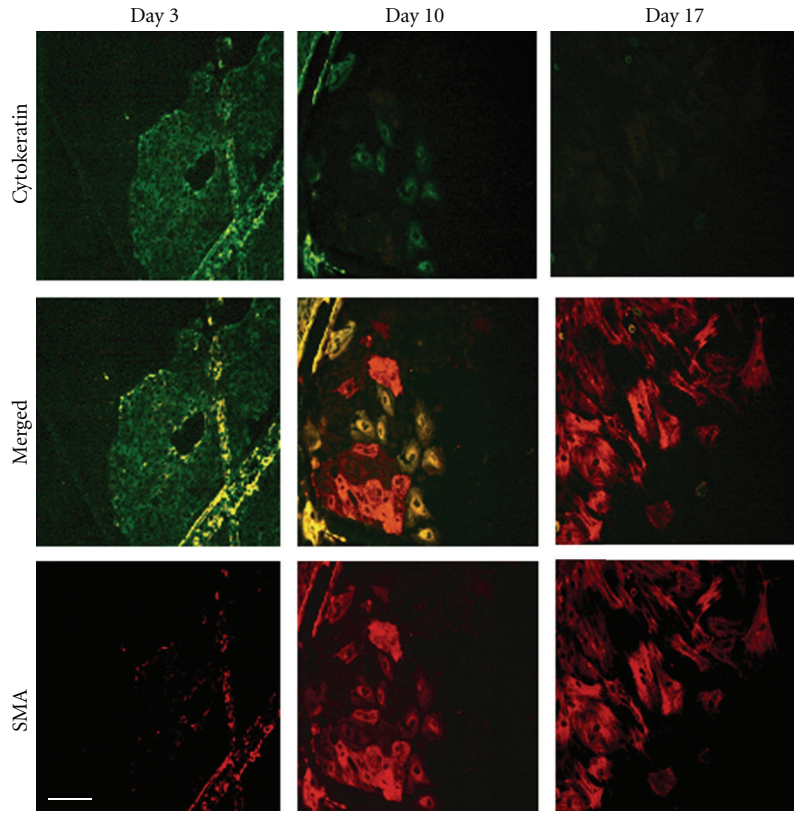

Figure 3: Double labeling for the epithelial marker cytokeratin and the myofibroblast marker SMA was used to determine if cell outgrowths were undergoing EMT. Two-colour immunofluorescence was performed at 3, 10, and 17 days after explanting. By labelling for both SMA (red) and cytokeratin (green), outgrowths were initially epithelial at day 3. By day 10 cell outgrowths expressed both SMA and cytokeratin (yellow) indicating that cells were coexpressing epithelial and mesenchymal markers. By day 17, cells had developed a mesenchymal phenotype, and all stained positive for SMA. Scale bar = 25 μm.

displaying the transitional features of EMT. Lectin studies suggest that the majority of these cells are derived from proximal tubule segments.

During embryogenesis, EMT gives rise to an array of fully differentiated adult cell types derived from pluripotential cells present in the developing embryo [21]. Accordingly, EMT in the kidney can be viewed as a reversal of renal embryogenesis where the metanephric mesoderm gives rise to the majority of nephron segments [22]. In the adult such transitions have generally been confined to those seen in wound healing and angiogenesis [21]. An increasing amount of evidence, however, suggests that cellular plasticity in the adult has been underestimated. Embryonic EMT can be recapitulated during certain adult disease states such as cancer and fibrosis [13] where dramatic morphological and functional changes are required to allow cells to migrate and invade.

Although the importance of EMT in the kidney has only been recognised relatively recently, the potential for EMT in adult cell types has been long known [23] and may be a relatively ubiquitous phenomenon in many labile cell types. EMT of tubular epithelia is the direct consequence of the release of a plethora of growth factors and other mediators in the surrounding environment after injury. These originate from both resident and infiltrating cells [7]. EMT is facilitated by the sequential loss of epithelial cell adhesion [24], degradation of basement membrane, *de novo* SMA expression, and migration into the interstitial space [21, 25]. In fibrotic kidney disease, this culminates in increased deposition of extracellular matrix and a consequent destruction of renal architecture and loss of function [4, 21].

In vitro experiments that have been used to demonstrate EMT to date have provided valuable insights into various mechanisms governing EMT and have highlighted the complexity in bringing about a complete change in cellular phenotype and function. However, although they are able to provide more steadfast evidence of the sequential events involved, *in vitro* experiments typically use immortalised cell lines or isolated cells in which the relationship between the tubular epithelium and surrounding matrix environment, one of which is integral to EMT, cannot be studied [13, 14]. Our work was therefore aimed at taking a more pathophysiologically relevant approach to examine the potential of EMT to occur under *ex vivo* circumstances.

The explant model described here resembles the spontaneous EMT that has been shown to occur with other tissue explants, in particular the cornea [26]. Initial outgrowths are cytokeratin positive with cells then changing their phenotype as they grow outward from the tissue fragments. The fact that only peripheral cells undergo EMT is consistent with

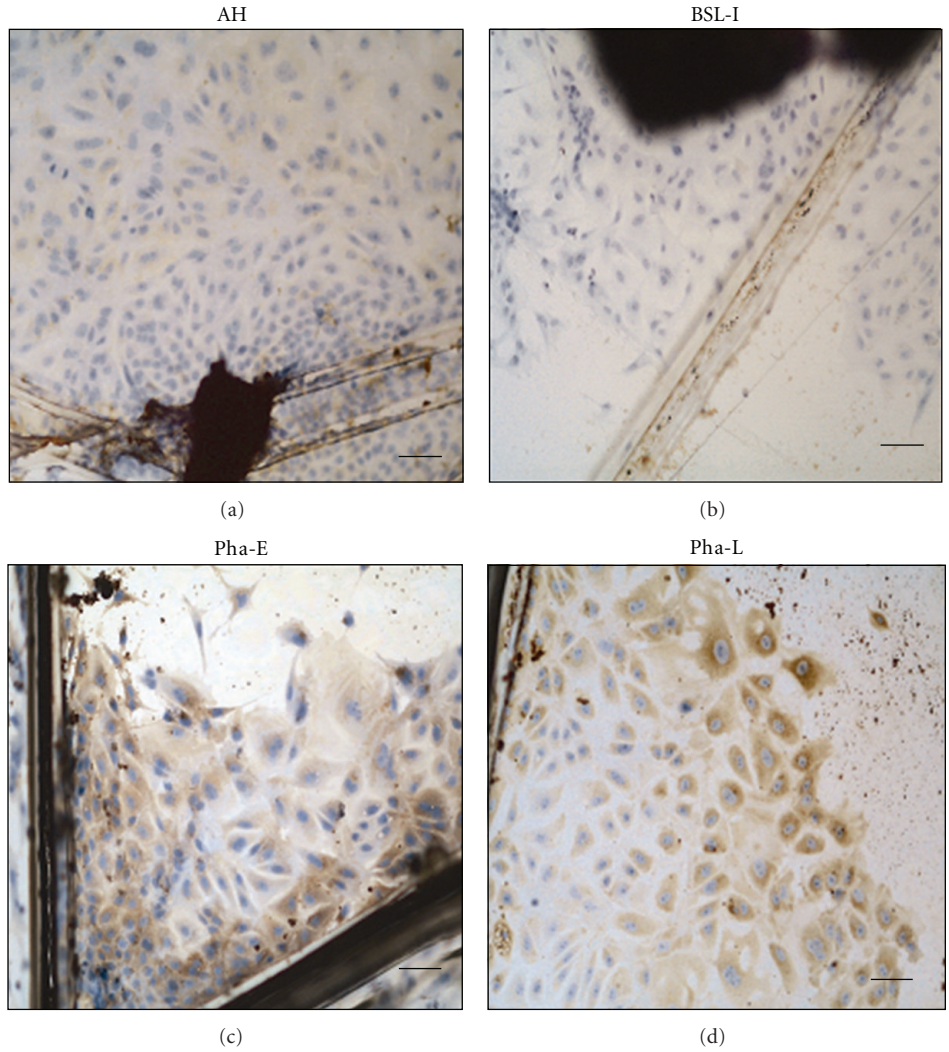

Figure 4: Lectin staining characteristics of cell outgrowths, 10 days after explanting. The majority of cells stain for Pha-E and Pha-L, lectins specific for the proximal tubular epithelium. (a) *Arachis hypogaea* (AH); (b) *Bandeiraea simplicifolia* I (BSL-I); (c) *Phaseolus vulgaris* erythroagglutinin (Pha-E); (d) *Phaseolus vulgaris* leukoagglutinin (Pha-L). Scale bar = 25 μm.

Masszi et al.'s postulate that injury or absence of intracellular contact is a key priming factor for EMT [27, 28]. As indicated by our results, cells subsequently lose their staining for cytokeratin and express SMA only. To confirm that these cells were not autonomous cell populations but rather that these cells were once epithelial in phenotype, double labelling was used. This demonstrated that 50% of cells in the population coexpressed SMA and cytokeratin. Although this study only provides a snapshot of EMT at one given time point, it supports previous evidence of EMT's important contribution to renal fibrogenesis [7].

Several tubule segments have been shown to give rise to myofibroblasts [29–32], with our study supporting a proximal tubule origin for myofibroblasts. However, given that we specifically excised renal cortex for explanting, the contribution of other nephron segments to EMT cannot clearly be determined.

In conclusion, our study highlights that explanting of normal rat renal tissue is a useful *ex vivo* model to study EMT. Given the significance of EMT in the pathogenesis of end-stage renal disease, this model is a valuable tool for the study of this important process.

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