Embryonic Stem Cells Proliferate and Differentiate when Seeded into Kidney Scaffolds

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ABSTRACT

The scarcity of transplant allografts for diseased organs has prompted efforts at tissue regeneration using seeded scaffolds, an approach hampered by the enormity of cell types and complex architectures. Our goal was to decellularize intact organs in a manner that retained the matrix signal for differentiating pluripotent cells. We decellularized intact rat kidneys in a manner that preserved the intricate architecture and seeded them with pluripotent murine embryonic stem cells antegrade through the artery or retrograde through the ureter. Primitive precursor cells populated and proliferated within the glomerular, vascular, and tubular structures. Cells lost their embryonic appearance and expressed immunohistochemical markers for differentiation. Cells not in contact with the basement membrane matrix became apoptotic, thereby forming lumens. These observations suggest that the extracellular matrix can direct regeneration of the kidney, and studies using seeded scaffolds may help define differentiation pathways.

Tissue engineering using stem cells to repair or reconstitute organ function is thought to hold great promise for regenerative medicine. A variety of remarkably different approaches have been proposed, and these range from tissue repair to growth of entirely new organs.¹,² There are many progenitor cell types that could potentially be used, and candidates include stem cells of embryonic origin as well as those harvested from adult organs. Stem cells have the advantage of self-replication and the potential to differentiate into multiple somatic cell types, allowing the maintenance of only a few cell lines for use in a variety of applications. Isolating the signals necessary to deliver organ-specific differentiation is at the crux of contemporary investigation, but this has been hampered because of the cellular heterogeneity, complexity of the tissues targeted for growth, and the limited, albeit evolving, state of knowledge about their nature. It is thought that the proper tissue microenvironment for support of stem cell differentiation and tissue formation would be characterized by a multitude of signals, including matrix proteins, soluble or immobilized growth factors, cytokines, cell-to-cell interactions, and mechanical forces.

To address these issues various scaffolds have been used as a framework for precursor cells. Those that are synthetic, however, typically do not have the intricate architecture needed for whole organs, do not provide signals for differentiation, and thus have had limited success. Scaffolds made from biological tissues would be appealing if they could be prepared to remove all native cells but still retain...
the extracellular matrix (ECM) necessary for signaling. Appropriate choices for the donor scaffold and precursor cells would thus be the basis of achieving the long-term goal of regenerating an organ of limited immunogenicity. Indeed, decellularized tissue scaffolds have been tried for direct implantation (relying on the presence of endogenous precursor cells), but they have been limited to relatively simple structures such as the ureter, esophagus, or heart valves. We believe that even intricate organs can be carefully decellularized to retain the innate matrix constituents needed for promoting cell adhesion, migration, proliferation, differentiation, and regulation of intercellular signaling. It has been postulated that only organ-derived intact ECMs capture the histoarchitecture that best represents the in vivo milieu and are in theory best able to facilitate tissue regeneration. Therefore, our novel approach was to establish an ECM-preserving decellularization method for a complex organ, seed the resultant scaffold ex vivo with embryonic stem (ES) cells and study its ability to induce tissuespecific differentiation. We chose the kidney because of its highly complicated microarchitecture, characterized by basement membranes of diverse protein composition that supports at least 26 different cell types in close apposition. Kidneys are also an appealing model because there is a wildly recognized hope that eventually they can be grown in vitro to address the needs of the growing number of patients suffering from renal failure, the lack of available organs for transplantation, and the inadequacies and expense of dialysis. Specifically, we removed all cells from rat kidneys using sequential gentle detergent, osmotic, and enzymatic steps; seeded the whole organ with murine pluripotent ES cells through the renal artery or the ureter; and incubated the whole kidney in growth media that was either static or perfused through a pulsatile system to simulate the mechanical forces (and potential signals) delivered by a native circulatory system. No prodifferentiation agents were added to the culture so that cell responses to just native heterogeneous ECM could be determined. We believe that this is the first demonstration of both proliferation and differentiation of primitive ES cells in such a complex organ scaffold structure. While being far from the ultimate goal of a functional organ suitable for transplantation, we believe that this approach provides a model for studying the signaling mechanisms for growth and differentiation between precursor cells and their matrix milieu.

RESULTS

Organ Decellularization

The kidney harvesting process was refined to consistently minimize vasoconstriction and clotting, and the resultant organs were rinsed and securely attached to arterial and ureteral cannuiae (intact and decellularized kidneys; Figure 1, A and B). Both of the detergent-based perfusion protocols successfully produced acellular kidneys that were nearly transparent, yet retained the web-like appearance of the basement membrane architecture (Figure 1B). These organs were easily compressed, followed by a sponge-like re-expansion. Scanning electron microscopy (SEM) showed a continuous three-dimensional extracellular matrix microstructure (Figure 1C) of acellular glomerular, tubular, and vascular structures. The protocol using the SDS ionic detergent was superior to that of sodium deoxycholate, in that, for the vast majority of kidneys, light microscopy showed more complete disruption of intact cells and removal of their debris, yet with preservation of the underlying
ghost-like connective tissue pattern throughout the cortex and medulla (examples of intact and decellularized renal cortex; Figure 1, D and E and F, respectively). In addition, immunohistochemical staining showed a completely contiguous network of laminin (Figure 1G) and collagen IV (Figure 1H). Collagen IV and laminin are primary constituents of kidney basement membrane, and both are involved in cellular viability, migration, and differentiation.

**ES Cell Seeding and Proliferation Patterns**

Intra-arterial ES cell seeding of the acellular whole organ scaffold produced by the SDS protocol showed retention of 95.1 ± 3.6% of the two million cells. Fluorescence microscopy showed that the pattern of distribution of the injected cells was into vascular structures and their associated glomeruli (corresponding views by bright field and fluorescence microscopy; Figure 2, A and B). All three of the incubation modalities yielded images over the first approximately 3 d, consistent with both preservation of the integrity of the underlying renal scaffold and multiplication of the cells, which initially have a doubling time of approximately 12 h. Specifically, low-power fluorescence imaging showed cells in a typical branching vascular pattern leading into glomeruli. SDS-treated scaffolds were characterized by uniform seeding across the vasculature in the renal cortex. The identity of the cells as being of donor (murine) origin was confirmed by anti-green fluorescent protein (GFP) staining. Incubation with static media allowed less reproducible growth in the intact organ model; hence, the culturing of thick sections in wells was necessary to maintain consistent cell viability. Sequential fluorescence images of thick section culture showed proliferation over at least 6 d, in a pattern of progressive migration into the extensive complex vascular networks evenly distributed across the organ (Figure 2, B through G): the cortical afferent, glomerular, and postglomerular vessels, as well as peritubular capillary networks extending deep into the medulla. Similarly, in our preliminary feasibility studies of intact kidneys undergoing pulsatile perfusion of growth media, there was enough cell viability and proliferation that fluorescence microscopy of approximately 1 wk showed migration into glomeruli and other small vessels (Figure 2H). Importantly, light microscopy of both the static and perfused media incubation models confirmed these cellular locations: on injection, the cells were mostly trapped in the glomeruli, with some arteriolar localization. They then migrated into the contiguous vascular networks over the ensuing incubation periods (Figures 3, A through C). The scaffold retained remarkable integrity of the vascular basement membranes because the cells stayed within the vessels and glomerular tufts and did not fill Bowman’s space. Incubations of approximately 6 d showed typical immature ES-like cells completely filling small vessels, so that no lumen was present (Figure 3B). The cells also had a peritubular capillary distribution of growth into the medulla (Figure 3C). By day 10, proliferation in the vasculature of the cortex showed a reticular appearance (Figure 3D), and the donor cells had nearly filled some glomerular tufts and peritubular capillaries. On only rare occasions did the cells seem to escape into what appeared to be tubules in the

![Figure 2. Distribution of green fluorescence protein (GFP)-labeled murine ES cells delivered into the renal artery of decellularized kidney scaffold after (A through G) SDS protocol, thick section culture, and (H) sodium deoxycholate protocol, whole organ perfusion. Images of ES cells at 1 d of culture showing initial glomerular localization in (A) a bright field micrograph (×40) and the corresponding field of view in (B) a fluorescence micrograph (×40). Fluorescence images showing cell growth and migration patterns in cultures up to 6 d. Outgrowth of cells into the interstitium (C) at 3 d (×40); movement of cells into the medulla (D) at 3 d (×40); further expansion of injected cells into a vascular tree (E) at day 4 (×100); and formation of complex branching networks (F) (×40), including larger vessels (G), (×100), at day 6. Fluorescence micrograph showing periglomerular expansion of cells (H) after 6 d using the pulsatile pressure-controlled perfusion device (×150).](image-url)
Definitive evidence for cell division was provided by the positive staining for KI-67 (Figure 5H). Several initial experiments have been conducted to explore retrograde seeding through the ureter. This seeding route is needed to gain access to the renal collecting system ECM and likely necessary to repopulate a complete organ. Delivery of the ES cells up the ureter thus yielded a seeding distribution into the collecting system: Organs at day 6 of thick section culture showed cells lining the calyxes and into progressively smaller structures consistent with tubules (Figure 3, E and F). Cell retention using this method was greater than 50%, but cells were not uniformly dispersed throughout the kidney on a consistent basis. We believe that the rat kidney’s papillae architecture was a barrier to uniform retrograde cell dispersion, a geometry that would not be present in many other species such as the pig.

**Evidence for Differentiation and Lumen Formation**

Interestingly, the primitive ES cell cytology features (high nuclear/cytoplasmic ratio) were progressively lost with incubations beyond approximately 4 d. An example of this phenomenon is apparent in a whole organ culture sample at day 10: a more mature morphologic appearance in glomerular regions (Figure 4A) or somewhat flattened endothelial-like appearance in vascular structures (Figure 4B). Importantly, the longer incubation periods (i.e., beyond approximately 4 d) permitted by thick section culture more consistently showed apoptosis of the cells within the central core-like regions of the larger vascular and tubular structures, with greater preservation of cells lining the scaffold basement membrane, thus showing lumen formation (Figure 4, C through F). Other unattached cells not in contact with basement membrane structures had a similar apoptotic appearance.

Immunohistochemistry (performed on samples prepared using the SDS protocol and incubated by thick section culture) showed findings consistent with cellular differentiation. The pan-cytokeratin immunostain showed areas of epithelialization on day 10 (Figure 5, A through C), prominently in the cells lining the vascular basement membranes and in some areas of collecting system. Definitive evidence for cell division was provided by the positive staining for KI-67 (Figure 5H).

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**Figure 3.** Morphologic patterns of ES cell growth in acellular kidney scaffold, H&E stains, SDS protocol, thick section culture. After antegrade arterial seeding (A through D): cells at day 4 showing uniform localization within glomeruli and adjacent arterioles (A) (×100); cells at day 6 extending into larger vessel lumens and some peritubular capillary networks (B) (×100) and also growing deeper in the medulla (C) (×100); and by day 10 forming a reticular pattern in the cortex interstitium (D) (×400). After retrograde ureteral seeding (E and F): at day 6, cells lining a calyx (E) and achieving cortical distribution (F).

**Figure 4.** Distinct morphologies of ES cells cultured in the acellular kidney scaffold. After 10 d of whole organ culture (PAS stain, sodium deoxycholate protocol): cells filling the glomerular capillary tufts (A) (×1250) and flat-appearing cells lining vascular structures (B) (×1500). After thick section culture (H&E stain, SDS protocol): at day 6, apoptosis of cells in vessel lumens (C) (×400, a higher magnification of a field in Figure 3B); at day 8, cell aggregates within artery (D) (×400); at day 10, cells lining an artery (E) (×400); and at day 10, solid round cellular aggregates with central apoptosis (F) (×400).
Bowman’s capsule, but not in cells on the tubular basement membrane. Some cytokeratin immunoreactive cells were also present on the kidney capsule. Pax-2 immunoreactivity (which was negative in native ES cells and the unseeded scaffold and positive in loosely organized patterns in the embryoid body controls) was abundant in cells throughout the scaffold, particularly those adherent to vascular and tubular basement membranes and some cells on Bowman’s capsule and lining calyces (Figure 5, D through F). Pax-2 is a critical factor in branching morphogenesis and is expressed in both the ureteric bud and induced metanephric mesenchyme, as well as glomerular and tubular epithelial precursors. Pax-2 is also implicated in adult reparative pathways. In addition, immunohistochemistry for Ksp-cadherin, a cell adhesion protein normally expressed in distal nephron tubular cells at later developmental stages, showed positivity in some peritubular cell aggregates on day 4 (Figure 5G). There was no Ksp-cadherin evident in the rat scaffold preparation before cell seeding, nor in the EB controls. Expression of Pax-2 and Ksp-cadherin mRNAs was confirmed by real-time RT-PCR (Figure 6).

**DISCUSSION**

A commonality of many of the diverse strategies for tissue repair and regeneration is that proliferation and differentiation of precursor cells require an appropriate interaction with the ECM milieu. This signaling would be integral to both of the fundamentally different approaches: cell growth *in vivo* for organ repair or *ex vivo* for organ generation. For the former, one possibility is the administration of primitive cells (of autogenous or donor-compatible origin, for example) into a recipient patient’s damaged organs. In this regard, there have been encouraging models using precursor (*i.e.*, mesenchymal) cells for the study of tissue repair. Concerns remain, however, that organ reconstitution might be limited if the donor cells are not truly pluripotent or if the healing is just localized because of paracrine effects, cell fusion, or dedifferentiation of native cells. Similarly, for the tissue engineering approach using ECM scaffolds, the selection of which cells (somatic versus stem) is not straightforward. In the case of the kidney, with a very heterogeneous cell arrangement, the coordination of each of the required somatic cell types would be laborious at best. Therefore, an appealing alternative is the use of pluripotent precursor cells, which could be derived from either normal adult reparative pathways or embryonic tissues. Concerning the former, adult stem cells are linked to the body’s normal maintenance and injury response mechanisms. It has been proposed that some of these populations are resident in mature organs and others are circulating (*e.g.*, extrarenal) stem cells. In addition, somatic epithelial cells may dedifferentiate or transdifferentiate, migrate, proliferate, and redifferentiate.

**Figure 5.** Immunohistochemical analysis of recellularized rat kidneys after SDS protocol and thick section culture. Positive immunoreactivity for pancytokeratin at day 10 in cells within glomeruli and blood vessels (A and B) (×100 and ×200, respectively) and lining arteries (C) (×400). Positive immunoreactivity for Pax-2 at day 6 in glomeruli (D), blood vessels (E), and calyx (F). Positive immunoreactivity for Ksp-cadherin at day 4 in cells in the cortex (G) (×400). Positive immunoreactivity for KI-67 at day 3 in the cortex (H) (×20).

**Figure 6.** Temporal gene expression pattern of Pax-2 and Ksp-cadherin after seeding mES cells in the decellularized scaffold. Total RNA was extracted at the indicated days and subjected to real-time RT-PCR.
ate to repair an injury. At issue is whether any of the proposed adult renal stem cells could yield the totality of adult renal somatic cells. In a related sense, cells such as the mesenchymal stem cell or amniotic fluid stem cell have been successfully integrated into primordial kidney organ culture. They thus show promise as a source for stem cell therapy but have not yet been proven to cover the gamut of adult somatic cells.\textsuperscript{23,24} This also holds true for a cell line derived from metanephric mesenchyme.\textsuperscript{12}

To overcome the above concerns, ES cells were selected as the cell source in this decellularized kidney model because they have the potential to differentiate into any of the adult renal cell types.\textsuperscript{25} ES cells have established cell lines, are easy to maintain, have a high doubling capacity, and have an intrinsic ability to develop into and form any embryonic organ \textit{in vivo}.\textsuperscript{26–28} Although it is still difficult to generate tissue-like structures from ES cells solely \textit{in vitro}, the cells have a potential to differentiate into all of the cell types required for organogenesis.\textsuperscript{25} Importantly, they have been shown to differentiate into cells of renal lineage and integrate into primordial kidney culture. In teratomas developed from implanted ES cells, branching ducts with nephrogenic morphology and kidney markers have been observed.\textsuperscript{29} Also, ES cells have been transplanted in to murine day 12 and 13 embryos amid the metanephric mesenchyme, and these cells have integrated into the mesenchytic kidney in the organ culture.\textsuperscript{30} ECM proteins have been shown to direct ES cell differentiation and are factors affecting cell behavior during organogenesis.\textsuperscript{32,31–35} Furthermore, differentiation toward a target tissue is improved if the ECM protein architecture closely resembles the \textit{in vivo} ECM of the targeted tissue.\textsuperscript{31,35,36} In addition, three-dimensional cultures have been shown to improve differentiation of ES cells through increased cell–cell and matrix association.\textsuperscript{37} Therefore, it was reasoned that an assessment of ES cell response to our acellular kidney patches, as well as in more shaped forms (e.g., rotator cuff and blood vessel).\textsuperscript{7} These scaffolds promote angiogenesis, recruitment of circulating progenitor cells, and constructive tissue remodeling. Other acellular matrices being explored\textsuperscript{9} have been extracted from heart valve,\textsuperscript{5,40} artery,\textsuperscript{41} skin, skeletal muscle, tendon, ligament, nerve, esophagus, liver, ureter, and even amniotic membrane.\textsuperscript{6,64} There have also been recent advances in seeding an acellular cardiac model with cells already in the differentiated cardiac and endothelial lineages.\textsuperscript{43} Some of these have been used for \textit{in vivo} culture and others tried for direct implantation (relying on the presence of endogenous cells). The greatest success has thus far been limited to less complex organ structures. The primary drawback to using ECM is the potential immunogenic response. However, such xenografts, if properly prepared, have been shown to be immunologically tolerated, without triggering cell-mediated implant rejection.\textsuperscript{9,44} Although our proof of concept work used well-characterized murine cells in a rat matrix, we foresee our future studies involving solely mouse tissue.

The \textit{in vitro} embryonic cell seeding studies reported here showed that decellularized rat kidneys, prepared using our treatment protocols, are effective cellular scaffolds that not only support cell growth and migration, but also induce differentiation of xenotransplanted murine ES cells. We believe that this is the first demonstration of ES cells multiplying (K1-67 positive) and differentiating in a tissue-specific whole organ extracellular matrix in the absence of any autologous cells. Gross morphologic changes suggestive of epithelial matura- tion along basement membrane structures were observed with optical microscopy and were supported by immunohistochemical markers Pax-2,\textsuperscript{13,14} Ksp-cadherin,\textsuperscript{16} and pan-cytokeratin\textsuperscript{19} (reported markers of renal differentiation). Furthermore, RT-PCR confirmed Pax-2 and Ksp-cadherin expression. Because of the emerging understanding of differentiation pathways, the use of these markers is not unambiguous; for instance, Pax-2 is also expressed in the eye, ear, spinal cord, and mid-hindbrain. Expression of Ksp-cadherin, however, is thought to be more consistent with development toward a renal lineage. Overall, the presence of these three markers in cells that were generally well organized within the scaffold is strong evidence of ECM-induced differentiation. With evidence in support of cell-to-cell and (now) matrix-to-cell signaling for differentiation, we believe that possible cell-to-matrix signals warrant study because newly formed basement membrane may be an important mechanism for proper remodeling of the scaffold.

The goal of these initial studies is to assess the ability of just the renal scaffold’s ECM to induce ES cell proliferation and differentiation, without the addition of pro-differentiation agents. However, it is assumed that further steps are needed to advance the cells, in a robust fashion, into functional renal populations. First, protocols have been established to differentiate ES cells into kidney-specific lineages \textit{in vitro}. Two in particular claimed intermediate mesoderm differentiation, from which the kidneys are derived. One used a combination of retinoic acid, activin-A, and bone morphogenetic protein 7 (BMP7) and has been shown to differentiate embryoid bodies according to increased Pax-2 expression.\textsuperscript{45} The other prescribed a strict application of BMP4, BMP2, and BMP7 to ES
cells grown in serum-free media. In addition, yet another study showed renal proximal tubular progenitor cells can be differentiated from ES cells using activin-A and sorted for Brachyury (T). These methods can be used to differentiate the cells before scaffold seeding, providing a more kidney-specific lineage, or integrated into the culture system, allowing the cells to form ECM-mediated structures as they grow and differentiate. Other additives that may be used to enhance the outcome and shape organ development include hyaluronic acid, which stimulates tubule differentiation, or other growth factors such as vascular endothelial growth factor to support endothelialization of the vasculature. Finally, steps should be taken to better approximate the in vivo microenvironment. This concept is highlighted by our feasibility studies using the continuous perfusion system we constructed, which have produced encouraging preliminary results. This apparatus was designed to circulate media through the seeded scaffold, regulating nutrient intake and removing waste, as well as providing a pulsatile waveform that mimics rat circulatory hemodynamics and possibly the associated mechanical shear forces. Whether there can be adequate oxygen delivery by aqueous solutions alone (i.e., without red blood cells) remains to be determined.

Whole organ acellular scaffolds are of interest not only because of the potential for having the complete ECM for signaling, but also by virtue of anatomic complexity. Biologic or synthetic scaffolds herebefore have been relatively simple, and we believe it was important to develop methodology for highly complex organs such as the kidney. This is particularly challenging for organs that do not have the capability for regeneration in adults. A functional reconstituted kidney would likely require multiple routes of cell delivery to achieve relatively homogeneous seeding of all of the tissue, antegrade through the vasculature as well as retrograde through the collecting system. Concerning the latter, we believe that nonrodent (i.e., porcine) models would have the benefit of a papillae anatomy that would facilitate more uniform cell seeding. A possible limitation of the use of ES cells is the risk of teratoma formation, and we hope these would not form because of the preservation of the appropriate differentiation signals in the ECM by our decellularization protocols. In this regard, there may also be important cell-to-cell interactions, and we believe this may help explain why in our model unattached cells inside the scaffold become apoptotic rather than form embryoid bodies (EBs). Interestingly, ES cells merely placed on top of the raw cut surface of a scaffold did show growth of EBs, which were not evident when the cells were seeded into (i.e., embedded within) the organized three-dimensional scaffold through the artery or ureter as described here. Thus, future studies are needed to characterize the molecular biology of the scaffold signals, which would thereby allow optimization of the decellularization protocol and control over differentiation. In this regard, we studied the use not only of the previously described ionic detergent sodium deoxycholate but also the stronger SDS. The deoxycholate was comparatively gentle in that it preserved the signal for differentiation (i.e., Figure 4, A and B) but did not clear enough cellular debris to permit uniform organ seeding. Fortunately, the more robust SDS detergent consistently produced pristine scaffolds and yet still retained the appropriate ECM to induce the remarkable cell growth and maturation described above. A tantalizing possibility is that organs that are capable of regeneration might not require the production of uniformly perfect scaffolds and total cellular reconstitution: we pursued seeding of hepatic scaffolds, in hopes that a just partially reconstituted liver could regenerate or repair the rest of the organ spontaneously.

In summary, we showed proliferation and differentiation of pluripotent ES cells in a complex architecture acellular scaffold, derived from a kidney. This approach shows promise for regeneration of whole organs, as well as serving as a model for elucidating the cell signaling mechanisms that could be of use in other approaches to regenerative medicine.

**CONCISE METHODS**

**Organ Preparation: Harvest**

Decellularization protocols originally designed for the morphologic study of glomerular basement membranes were the basis of the procedure developed in this study. All animal work was approved by the Institutional Animal Care and Use Committee (University of Florida, Gainesville, FL). Kidneys were harvested from male 250–350-g Sprague-Dawley rats. Briefly, anesthetized rats were systemically anticoagulated with heparin, and cannulae were inserted in the renal artery and ureter. The kidney was arterially perfused in situ using a saline solution containing a vasodilator (nitroprusside) to remove the blood. The organ was observed to confirm uniform blanching indicative of even distribution of perfusate and was harvested.

**Organ Preparation: Decellularization**

The perfusion system was gravity based to maintain a constant physiologic fluid pressure of approximately 100 mmHg and was designed to minimize air entrapment. The kidney was suspended and perfused through the arterial cannula, and the effluent fluid was recirculated from a reservoir after passing through a submicron filter. Cells were washed out of the intact kidney using an approximately 5-d stepwise protocol that progressively removed material without causing blockage within the microscopic vascular and tubular networks. The decellularization protocols used nonionic (Triton X-100; Sigma-Aldrich, St. Louis, MO) and ionic detergents. Although each of the two ionic detergents studied had a unique protocol, the common features were (1) initial treatment with Triton X-100 to solubilize hydrophobic cell membranes, lyse cells, and remove their cytoplasmic contents; (2) a solution of 5 mM calcium chloride and 5 mM magnesium sulfate to activate endogenous nucleases and digest bulky nuclear material; (3) 1 M sodium chloride to osmotically disrupt nuclear membranes; (4) deionized water rinses to both exert osmotic pressure on the cells and clear the detergent; (5) enzymatic degradation of nuclear material by 0.0025% deoxyribonuclease 1 (DNase; Sigma) buffered in 50 mM sodium acetate, 10 mM magnesium chloride, and 100 mM sodium chloride at pH 5; and (6) 0.05% sodium azide was added to all of the...
detergent solutions as an antimicrobial agent. Our initial protocol used sodium deoxycholate as the ionic detergent because that reagent had been described in the literature. This method involved arterially perfusing the intact kidney with sequentially increasing concentrations of the Triton X-100 (0.5, 3, 6 and 10% solutions), rinses with deionized water, the DNase, and then 4% sodium deoxycholate. We subsequently explored the use of SDS for better removal of debris, hoping that the stronger detergent would not remove matrix signals for differentiation. Specifically, for the optimized SDS protocol, the sequence was 3% Triton X-100, DNase, repeat 3% Triton X-100, and then the 4% SDS to remove the remaining cellular debris. The method also included multiple rinses and a final step of perfusion with deionized water to remove all of the potentially cytotoxic chemicals.

Scaffold Histology and Histochemistry
Kidneys designated for scaffold analysis were fixed in formalin, and sections were stained with hematoxylin and eosin (H&E) for light microscopy to assess the degree of cell removal and preservation of the ECM architecture. Scaffold morphology was further examined by SEM (JSM-6400; JEOL USA, Peabody, MA) after glutaraldehyde fixation. Additionally, immunohistochemistry was performed to evaluate the retention of the key basement membrane proteins laminin and collagen IV (Abcam, Cambridge, MA).

ES Cell Engraftment and Culture
The murine pluripotent ES (B5/EGFP, courtesy of Andras Nagy) cells have been well characterized, and the GFP allowed tracking by fluorescence microscopy.\(^9\) The cells were maintained in an undifferentiated state on gelatin-coated dishes in Knock-out DMEM (Life Technologies BRL, Grand Island, NY) containing 10% knockout serum replacement (Life Technologies BRL), 1% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES (Life Technologies BRL), 300 μM monothioglycerol, and 1000 units/ml recombinant mouse leukemia inhibitory factor (ESGRO Chemicon, Temecula, CA). To prepare the cells for injection, the undifferentiated ES cells were dissociated using 0.25% trypsin/EDTA (Life Technologies BRL). Then they were suspended in a solution devoid of leukemia inhibitory factor, which was also used as the growth media that would permit differentiation in all three of the incubation protocols described below: IMDM supplemented with 20% fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies BRL), 0.25 μg/ml Fungizone antimycotic (Invitrogen, Carlsbad, CA), and 300 μM monothioglycerol. Notably, because the goal of our studies was to explore the role of the scaffold extracellular matrix, no pro-differentiation agents were added to the media. All cultures in this study were at 37°C in 5% CO₂. The adequacy of the final kidney scaffold rinse for removal of the potentially toxic chemicals was verified by the survival of ES cells placed on a cut surface of an acellular organ. This also allowed preliminary observations of growth patterns when the cells were not in full contact with the ECM, as was the case when seeded inside the whole organ.

Before receiving cells, the acellular scaffolds were arterially perfused with DMEM. Then, under sterile conditions, approximately 2 × 10⁶ murine ES cells were manually injected through either the arterial or ureteral cannulae. Dispersion of cells was assessed through fluorescence microscopy (IX70; Olympus, Tokyo, Japan). The whole organ scaffolds with the engrafted cells were initially cultured for a day in a deep well to allow for cell adherence. Samples were taken at this point to evaluate the effectiveness of the luminal delivery methods. There were three separate approaches for incubation of the tissue. First, there were “static” studies where intact organs were cultured in media changed every 48 h, and histologic studies were done at various time points. Significant apoptosis occurred, and this approach did not consistently maintain viability beyond approximately 4 d. Because it was anticipated that differentiation would take longer than this period of time, our second protocol involved transverse sectioning of the preseeded organ and culturing the slices in a multiwell dish. The sections were incubated over a range of time intervals (including 3, 4, 6, and 10 d) to gauge cellular response and capture transient differentiation patterns. Gross cell localization and migration tendencies were monitored through GFP expression by fluorescence microscopy. Completed tissue cultures were formalin-fixed. Finally, because our long-term goal was repopulation of whole organs, for our third approach, we devised an automated whole kidney perfusion system. A peristaltic pump and appropriate tubing were selected to deliver a physiologically normal pressure profile that was transduced for continuous monitoring of the waveform. A pressure-relief mechanism was also incorporated, and the fluid circuit could thereby be maintained at a steady range as high as approximately 120/80 mmHg with a periodicity of 270 to 300 beats/min. The perfusion was accomplished in a temperature-controlled incubator supplied by regulated medical-grade sterile gases, and the CO₂ content was adjusted as necessary to keep the media pH at 7.4.

Preparation of EB Control Tissue
EBs were cultured to act as a control for ES cell differentiation. Using the differentiation medium described above, ES cells were cultured for 2 d by the hanging-drop method (2 × 10³ ES cells/30 μl in each drop). The resultant EBs were transferred to suspension culture dishes and incubated at intervals that included 3, 6, and 10 d. HistoGel was used to encapsulate EBs before formalin fixation for microscopy.

Microscopic and Histochemical Evaluation of Cultured Scaffolds
H&E and periodic acid-Schiff staining were performed for light microscopy to evaluate the morphology of the engrafted ES cells, including an assessment of distribution, migration, and grouping tendencies, as well as cell–ECM interactions. Localization of developmental and epithelial protein markers was used to assess any patterns of differentiation. EBs at equivalent time points, embryonic kidneys (E17.5), and unseeded scaffolds were used as controls. To test for evidence of differentiation, we stained for the developmental proteins Pax-2 (Zymed Laboratories, South San Francisco, CA) and Ksp-cadherin (Zymed Laboratories). The tissue was also tested for pan-cytokeratin (Ventana Medical Systems, Tucson, AZ), a marker of epithelialization. We also stained for a marker of cell division, Ki-67. An anti-GFP antibody (Abcam) was used as a control to verify murine GFP cell origin and loss of endogenous rat cells.
Real-Time RT-PCR

Total RNA was extracted with the RNA aqueous kit (Ambion, Austin, TX) and subsequently treated with Turbo DNA-free kit (Ambion) to remove genomic DNA contamination. cDNA was synthesized using the HiCapacity cDNA Archive kit (Applied Biosystems, Foster City, CA) using random primers. Quantitative PCR was performed with SYBR Green PCR Mater mix (Applied Biosystems; 40 cycles of 95°C, 15 s and 60°C, 1 min) on the DNA Engine Opticon 2 (MJ Research/Bio-Rad, Hercules, CA). Gene expression analysis was performed using the comparative cycle threshold (ΔCt) method using β-actin for normalization. The relative transcript level was calculated by the formula, 2^(-ΔΔCt), where ΔΔCt = (average Ct of target gene: Pax-2 or Ksp-cadherin) – (average Ct of housekeeping gene: β-actin) and ΔΔCt = (average ΔCt of indicated day of sample) – (average ΔCt of Day 3 sample). Primer sequences are β-actin (5’-TGACAGAGTCCAGAGGAGA-3’, 5’-GCACCGATCCACAGAGTA-3’), Pax-2 (5’-CAAA-GTCCAGAGGGCTTTCC-3’, 5’-GTTAGAGGCGCTGGAAACAG-3’), and Ksp-cadherin (5’-CTTGGAGTCTGAGGATGGA-3’, 5’-TAGATGGC-CTGGGAGATTG-3’).

ACKNOWLEDGMENTS

We thank Maribel C. Ibanez for technical assistance. The SEM images are courtesy of Bradley Willenberg at Major Analytical Instrumentation Center, University of Florida, Gainesville, Florida. This work was funded in part by the University of Florida Research Foundation.

DISCLOSURES

None.

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