Stem Cell Approaches for the Treatment of Renal Failure

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Abstract——The inadequacy of current treatment modalities and insufficiency of donor organs for cadaveric transplantation have driven a search for improved methods of dealing with renal failure. The rising concept of cell-based therapeutics has provided a framework around which new approaches are being generated, and its combination with advances in stem cell research stands to bring both fields to clinical fruition. This budding partnership is presently in its very early stages, but an examination of the cell-based therapies currently under development clearly shows the magnitude of the role that stem cells will ultimately play. The issue over reports of unexpected plasticity in adult stem cell differentiation remains a focus of debate, and evidence for bone marrow-derived stem cell contributions to renal repair has been challenged. The search for adult renal stem cells, which could have a considerable impact on much of the work discussed here, appears to be narrowing. The use of embryonic tissue in research continues to provide valuable insights but will be the subject of intense societal scrutiny and debate before it reaches the stage of clinical application. Embryonic stem (ES) cells, with their ability to generate all, or nearly all, of the cell types in the adult body and a possible source of cells genetically identical to the donor, hold great promise but face ethical and political hurdles for human use. Immunoisolation of heterologous cells by encapsulation creates opportunities for their safe use as a component of implanted or ex vivo devices.

I. Introduction

Total United States expenditure on end-stage renal disease (ESRD[1]) therapy topped $25 billion in 2002, an increase of 11.5% on the previous year (U.S. Renal Data System, 2004), representative of a trend extending back to the early 1970s and projected to continue into the foreseeable future (Lysaght, 2002). This reflects not only a steady increase in patient numbers (431,284 on December 31, 2002, up 4.6% from 2001) (U.S. Renal Data System, 2004) but also rising costs of treatment and extended therapy periods as survival rates improve. Morbidity and mortality rates associated with maintenance dialysis, however, remain high, with a dialysis patient in their early 20s having the same expected

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[1] Abbreviations: ESRD, end-stage renal disease; ARF, acute renal failure; RPTC, renal proximal tubular cell; ES, embryonic stem; HSC, hematopoietic stem cell; BMSC, bone marrow-derived stem cell; MAPC, multipotent adult progenitor cell; NOD/SCID, nonobese diabetic severe combined immunodeficient; MSC, mesenchymal stem cell; GFP, green fluorescent protein; IHC, immunohistochemistry; CNS, central nervous system; hMSC, human MSC; β-gal, β-galacto- sidase; FA, folic acid; FISH, fluorescent in situ hybridization; UB, ureteric bud; MM, metanephric mesenchyme; β2M, β2-microglobu- lin; EPO, erythropoietin; RAD, renal tubule assist device; IL, inter- leukin; FAH, fumaryl acetoacetate hydrolase.

remaining lifespan as a 70-year-old in the general population (U.S. Renal Data System, 2004). Outcomes are considerably improved following transplantation—a transplant patient in their early 20s can expect to survive as many years as a member of the general population in their early 40s—but organ supply lags far behind demand, with only around one-quarter of the extant ESRD population having benefited from a transplant (U.S. Renal Data System, 2004).

The statistics for patients suffering from acute renal failure (ARF) are even worse. Affecting up to 200,000 people in the United States annually, or approximately 5% of all long-term hospital patients, the current mortality rate of around 50% has remained unchanged since the advent of dialysis 30 to 40 years ago (Thadhani et al., 1996; Lieberthal and Nigam, 2000; Nigam and Lieberthal, 2000). ARF develops predominantly due to the injury and necrosis of renal proximal tubule cells (RPTCs) as a result of ischemic or toxic insult (Lieberthal and Nigam, 1998). The cause of death subsequent to ARF is generally the development of systemic inflammatory response syndrome, frequently secondary to bacterial infection or sepsis, resulting in cardiovascular collapse and ischemic damage to vital organs, culminating in multiple organ failure (Breen and Bihari, 1998).

There is growing recognition that the disease state arising from renal failure is the result of more than just the loss of blood volume regulation, small solute, and toxin clearance that are replaced by conventional dialysis therapy (Humes, 2000). The kidney's role in reclamation of metabolic substrates, synthesis of glutathione, and free-radical scavenging enzymes, gluconeogenesis, ammoniagenesis, catabolism of peptide hormones and growth factors, and the production and regulation of multiple cytokines critical to inflammation and immunological regulation are not addressed by current treatment modalities (Kida et al., 1978; Tannen and Sastrasinh, 1984; Deneke and Fanburg, 1989; Maak, 1992; Frank et al., 1993; Stadnyk, 1994).

Thus, there is considerable drive to develop improved therapies for renal failure with the capacity to replace a wider range of the kidney's functions, thereby reducing morbidity, mortality, and the overall economic impact associated with this condition. Such an ambition lies beyond the reach of conventional medicine, with its mainly monofactorial approach to the treatment of disease. Into this breach steps the nascent and expanding field of cell therapy, which offers the promise of harnessing the native abilities of the cell, endowed to it by a billion years of evolution (Humes, 2003).

Cell therapy, as a blanket term covering the disciplines of regenerative medicine, tissue, and bioengineering, is dependent on cell and tissue culture methodologies to expand specific cells to replace important differentiated functions lost or deranged in various disease states. Central to the successful development of cell-based therapeutics is the question of cell sourcing, and advances in stem cell research have a vital impact on this problem.

"Stem cell" is itself a blanket term that covers a number of separate entities, although, as discussed below, there is at present a great deal of speculation over the extent to which stem cell populations traditionally considered distinct may in fact be interchangeable. As an in-depth treatment of the biology of stem cells and their relationship to more general aspects of regenerative medicine lies outwith the scope of this paper; the reader is directed to several recent reviews (Alison et al., 2002; Rosenthal, 2003; Grove et al., 2004; Rippon and Bishop, 2004).

Briefly, stem cells are characterized by their capacity for self-renewal and ability to differentiate into specialized cell types. Levels of competence form the basis of their classification as totipotent (giving rise to all three embryonic germ layers as well as extraembryonic tissues), pluripotent (able to contribute to all three germ layers of the embryo), and multipotent (with the potential to differentiate into multiple cell types, but not derivatives of all three germ layers). Progenitor cells are more lineage-restricted than stem cells but retain the proliferative capacity lacking in terminally differentiated cells.

ES cells, pluripotent derivatives of the inner cell mass of the blastocyst, are the most primitive cell type likely to find application in cell therapy. Their potential to generate any given cell type of the embryo makes them in some ways the most attractive stem cell for cell therapy but also the one with the greatest challenges to surmount in the laboratory. The political and ethical questions that surround the use of human ES cells have added a further layer of complexity to research aimed at bringing their potential benefits into the clinical arena (Daley, 2003; de Wert and Mummery, 2003; Drazen, 2003; Phimister and Drazen, 2004). These factors have combined to intensify the focus on multipotent adult stem cells such as hematopoietic stem cells (HSCs) and neural stem cells as sources for cell-based therapeutics.

In this review, we consider several potential cell-based therapies for renal failure that are currently under development and which provide a route, direct or indirect, for the application of stem cell technology. The direct route is exemplified by simple administration of stem cells to the diseased or injured organ and relies on their inherent capabilities for differentiation, organization, and integration into existing tissues to restore function. Indirect routes include the bio- and tissue-engineering approaches, which are based on in vitro differentiation of stem cells and the organization of their derivatives within matrices or in association with biomaterials to augment or replace function following implantation or as part of an extracorporeal circuit.

II. Direct Application of Stem Cells

The main strength of strategies involving the introduction of supplementary cells into a damaged adult kidney to aid in repair and regeneration is that they are rooted in the natural healing process. Numerous studies have shown that renal cell repair and regeneration following ARF follows a program of de-differentiation, migration and proliferation, and restoration of differentiated function (reviewed in Safirstein, 1999; Nony and Schnellmann, 2003). The rationale, therefore, is that accelerating and augmenting this process through cellular supplementation can only improve the prognosis for sufferers of acute renal injury.

This approach has received considerable attention over the last few years, as reports emerged of direct and significant contributions of bone marrow-derived stem cells (BMSCs) to renal tissue by transdifferentiation. The initial excitement generated by these findings has somewhat waned following the publication of results that suggest some revision of the early conclusions may be required.

A. Transdifferentiation

The main attraction of the transdifferentiation approach is that it offers the possibility of avoiding complications from immunogenicity of introduced cells by obtaining the more easily accessible stem cells of another tissue type from the patient undergoing treatment, expanding them in vitro, and reintroducing them as a therapeutic agent. This is based on the findings of a number of groups who showed that adult stem cells, long thought to be highly restricted in their differentiative potential, may possess a considerable degree of plasticity. This posited plasticity of adult stem cell populations has not gone unchallenged, however, with results having been published by other researchers more supportive of the traditional viewpoint. The data have been recently reviewed from both perspectives (Anderson et al., 2001; Weissman et al., 2001; Forbes et al., 2002). The debate centers over the question, as yet unresolved, of whether stem cells are irreversibly committed to a particular lineage during development or whether the apparent restrictions are imposed on them by their environment and are therefore mutable. Compelling evidence supporting the latter possibility has come from work demonstrating that adult stem cells, when exposed to the right environments, are capable of contributing to several embryonic lineages, both in vitro and in vivo.

Making use of neural stem cells derived from ROSA26 mice, which constitutively express bacterial β-galactosidase in all cell types (Friedrich and Soriano, 1991), Clarke et al. (2000) were able to show first in vitro, by coculture with embryoid bodies followed by immunostaining for tissue-specific markers, and subsequently in vivo, by injection into the amniotic cavity of stage 4 chick embryos as well as morula aggregation and blastocyst injection of adult neural stem cell-derived cells, contributions to muscle, mesonephros, notochord, and epithelia of the liver and intestine, representing all three germ layers. Their analysis included the use of clonally derived neural stem cell populations, thus demonstrating that the progeny of a single cell could give rise to all the tissue contributions observed.

Similarly, Jiang et al. (2002), studying a rare subpopulation of bone marrow-derived mesenchymal stem cells designated MAPCs (multipotent adult progenitor cells), were able to demonstrate multilineage differentiation in vitro, following blastocyst injection, and by infusion into adult animals. The progeny of single MAPCs in culture, following the addition of requisite growth factors, displayed characteristics of endothelial, neuronal, and hepatic tissues. Blastocyst injection of single cells gave rise to chimeric animals with donor cell contributions to many tissues, including brain, retina, lung, myocardium, skeletal muscle, liver, intestine, kidney, spleen, bone marrow, blood, and skin. Although infusion of MAPCs into adult nonobese diabetic severe combined immunodeficient (NOD/SCID) (unirradiated or sublethally irradiated) mice led to engraftment of the hematopoietic system, liver, intestine, and gut, no contribution was seen to skeletal or cardiac muscle, skin, kidney, or brain. This serves as a reminder of the unique nature of the embryonic environment, as these tissues do show MAPC progeny contribution following blastocyst injection. Intense interest in the potential of BMSCs to transdifferentiate and contribute to the regeneration or repair of diverse organ/tissue types was generated by publications demonstrating their ability to regenerate infarcted myocardium (Orlic et al., 2003) and rescue a metabolic liver defect (Lagasse et al., 2000).

BMSCs are a many-faceted population made up of (at least) HSCs, marrow stromal cells [or mesenchymal stem cells (MSCs)], MAPCs, and side population cells (Ratajczak et al., 2004). HSCs have been estimated to be present in mouse bone marrow at a frequency of 1 in 10^5 cells (Spangrude et al., 1988; Harrison et al., 1990; Morrison et al., 1997). Crude cell extractions from bone marrow may be enriched for HSCs by sorting based on expression of cell surface markers such as Sca-1 and c-kit, dye exclusion, and depletion of cells expressing numerous lineage-specific markers (Morrison et al., 1997), but functional assays remain an important means of characterizing stem cells. The functional assay for presence of HSCs is their ability to long-term repopulate all the hematopoietic lineages of a lethally irradiated host.

Orlic et al. (2003) experimentally induced an area of infarction in the left ventricle of female mouse hearts and 3 to 5 h later, injected enhanced green fluorescent protein (GFP)-expressing HSCs (two injections of 0.15– 1×10^5 Lin$^-$c-kit$^+$ cells) into adjacent healthy tissue. Nine days following this procedure they were able to show, by GFP expression, presence of the Y-chromo-

some, and expression of tissue specific markers, that donor cells were present throughout the infarcted area and that these cells assumed the morphology and expressed those molecular markers examined consistent with myocyte identity. Hemodynamic assays also revealed improved function in HSC-injected animals and these effects were found to be specific to treatment with HSCs, as the injection of $Lin^- c$-kit^- cells did not produce similar results (Orlic et al., 2003).

Lagasse et al. (2000) used the powerful approach of cell-autonomous rescue from a genetic metabolic liver defect (the $FAH^{-/-}$ mouse) to demonstrate contributions of purified (c-$kit^+ Thy^{lo} Lin^- Sca$-1^+) HSCs to the liver. Lethally irradiated adult female $FAH^{-/-}$ mice were repopulated with HSCs isolated from male ROSA26/BA mice. To select for hepatic engraftment of donor HSC-derived cells 2 months following transplantation, the drug required to treat FAH deficiency was withdrawn twice during a 4-month period and restarted when total body weight had decreased by 30%. The livers of animals surviving this treatment were examined for engraftment of donor-derived cells. By β-galactosidase activity, presence of the Y-chromosome, FAH expression, and expression of hepatocyte-specific molecular markers, the authors were able to show that donor HSC-derived cells had engrafted into the host liver and taken on characteristics of mature hepatocytes (Lagasse et al., 2000).

It has, however, since been shown that this regenerative effect is achieved through fusion of transplanted HSCs with hepatocytes rather than by pure transdifferentiation (Vassilopoulos et al., 2003). Vassilopoulos et al., working with GFP-marked male HSC-repopulated animals, found that GFP-positive, Y-chromosome-containing, FAH-expressing liver nodules actually contained a greater component of FAH^- than FAH^+ alleles. Mathematical analyses revealed the proportions of wild-type versus mutant alleles to be consistent with fusion-based models of engraftment, and this was supported by immunohistochemistry (IHC) showing that FAH^+ hepatocytes coexpressed the host allele of $\beta 2$-microglobulin (Vassilopoulos et al., 2003). The authors also noted that the donor cell nuclei must have undergone epigenetic reprogramming subsequent to fusion with hepatocytes, as the expression FAH and down-regulation of the pan-hematopoietic surface marker CD45 are not characteristics of hematopoietic cells, and that the mechanism of transdifferentiation through cell fusion does not necessarily invalidate the potential for clinical application of BMSCs in this context. These results are consistent with the findings of several groups who demonstrated the ability of neural stem cells and BMSCs to cross-lineage boundaries through cell fusion both in vitro (Terada et al., 2002; Ying et al., 2002; Spees et al., 2003) and in vivo (Alvarez-Dolado et al., 2003).

Ying et al. (2002) cocultured central nervous system (CNS) cells, derived from the forebrain of 14.5 days post coitum mouse embryos, with ES cells either directly or following neurosphere derivation. The donor CNS cells were derived from embryos marked either by constitutive expression of neomycin resistance and β-galactosidase activity or by puromycin resistance and GFP expression driven by the regulatory sequences of the *Oct4* gene. The ES cells carried the hygromycin phosphotransferase/herpes simplex virus thymidine kinase fusion gene, rendering them hygromycin-resistant and sensitive to the thymidine analog ganciclovir. Selection (G418 or puromycin) was applied to eliminate the ES cells from coculture, and following a further 2 to 4 weeks in culture, the authors observed the emergence of proliferating cells with undifferentiated ES cell morphology expressing the markers appropriate to the donor cells (β-galactosidase or GFP). This was particularly interesting in the case of the GFP-marked cells, as marker expression under the control of the Oct4 regulatory sequences is expressed only in germline and pluripotent cells (Pesce and Scholer, 2001) and is thus suggestive of epigenetic reprogramming of the CNS cells. Although these cells possessed ES cell-like characteristics, including the ability to form embryoid bodies generating multiple differentiated cell types and generation of chimeric embryos following blastocyst injection (with contributions to intestine, kidney, heart, and liver), they were also found to be tetraploid and to have arisen from fusion events between ES and CNS cells (Ying et al., 2002). This phenomenon was found to be repeatable with adult brain cells, and in both cases, the frequency of hybrid generation was estimated at 10^{-4} to 10^{-5} per brain cell plated.

Similarly, Terada et al. (2002) found that mouse bone marrow cells, which expressed GFP and puromycin resistance, cocultured with ES cells prior to the application of puromycin selection, could give rise to GFP-expressing ES cell-like populations. These cells were shown to express undifferentiated ES cell markers (Oct3/4 and UTF1) and, following withdrawal of leukemia inhibitory factor, to form embryoid bodies and differentiate into disparate cell types. Genetic analyses, however, revealed that the ES cell-like populations were actually tetraploid or even occasionally hexaploid fusion products of bone marrow and ES cells (Terada et al., 2002). In this case, the rate of spontaneous cell fusion was estimated at 2 to 11 clones per 10^6 bone marrow cells. The relatively infrequent occurrence of these fusion events in vitro (of the order of one event per 10^5 cells) (Terada et al., 2002; Ying et al., 2002) led to objections from the proponents of BMSC transdifferentiation that such a mechanism was unlikely to account for their observations in vivo.

Spees et al. (2003) went some way toward addressing this in their study of bone marrow-derived human mesenchymal stem cell (hMSC) differentiation in response to epithelial injury in vitro. Their work involved the addition of GFP-expressing hMSCs to monolayer cultures of small airway epithelial cells, following heat

shock injury to the monolayer. After 1 week of coculture, 4% (1 in 25) of the GFP$^+$ cells recovered were also found to be positive for the epithelial-specific cell surface marker CD24. Approximately one-quarter of these were observed to be multinucleate, and some mononuclear examples were found to be polyploid, giving an approximate cell fusion frequency of 10^{-2} in the absence of selection pressure (Spees et al., 2003). In this study, differentiation of hMSCs was found to occur in a single-cell manner and via cell fusion at a considerable rate. Differentiated identity was assessed by expression of multiple epithelial-specific markers. Interestingly, hMSCs added to nonheat-shocked cultures showed little sign of transdifferentiation (Spees et al., 2003), highlighting the potential role of tissue injury in the recruitment of heterologous stem cells.

As with all tissue culture-based studies, these come with the caveat that in vitro results may be artifactual and do not necessarily reflect the in vivo situation. However, an in vivo role for cell fusion in the liver is supported by the work of Vassilopoulos et al. (2003) (described above) and additionally in the brain and heart in a study carried out by Alvarez-Dolado et al. (2003). The latter study made use of a simple yet sensitive Cre/lox reporter system to detect fusion events between transplanted Cre-expressing bone marrow cells and somatic cells of β-galactosidase reporter mice (R26R). R26R mice bear the ROSA26 lacZ reporter flanked by a floxed stop cassette (Mao et al., 1999) so that fusion of a Cre-expressing cell with an R26R cell will result in Cre-mediated excision of the stop cassette and expression of β-galactosidase in the fusion product and its progeny. Lethally irradiated R26R mice were injected with Cre-/GFP-expressing whole bone marrow ($1–2 \times 10^7$ cells, intraperitoneally). Two or 4 months later, brain, liver, heart, gut, kidney, lung, and skeletal muscle tissues from engrafted animals were serially sectioned and stained for the presence of β-galactosidase. β-Gal-positive cells were found, but only within the brain, heart, and liver (Alvarez-Dolado et al., 2003). Electron microscopy analyses of cellular morphology and expression of tissue-specific markers confirmed the presence of β-gal-positive hepatocytes in the liver, Purkinje cells in the cerebellum, and cardiomyocytes in the heart. Transdifferentiated cells (i.e., GFP-positive, β-gal-negative, and not of hematopoietic origin) were not observed, although GFP$^+$ β-gal$^-$ cells with characteristics of microglia, Kupffer cells, and macrophages (all of hematopoietic origin) were observed in brain, liver, and heart tissues, respectively, suggesting that cell fusion is the predominant mechanism by which bone marrow cells contributed to these organs (Alvarez-Dolado et al., 2003).

B. Bone Marrow-Derived Stem Cell Contribution to Kidneys

The functional contributions of BMSCs to heart and liver regeneration described above raised hopes that circulating stem cell populations derived from the bone marrow might be capable of widespread organ repair. The studies that have looked at BMSC contributions to renal parenchymal maintenance or repair have, however, generated a good deal of conflicting evidence. As discussed below, some results appear to show that bone marrow-derived cells contribute significantly to daily turnover of renal tissue and to a high degree during recovery from tissue damage, whereas others indicate that this is a rare event, seen only to a very limited extent following injury.

Poulsom and colleagues (Poulsom et al., 2001) examined kidney biopsies from male patients who had received transplants from female donors, as well as female mice that had received a male bone marrow transplant. In both cases, they identified Y-chromosome-containing (and therefore host or donor-derived, respectively) cells within both tubules and glomeruli. Cellular phenotype in human subjects was assigned on the basis of immunostaining for epithelial markers (CAM 5.2 and the lectin *Ulex europaeus*) for tubules and by position and morphology for glomeruli. In murine subjects, this assignation was made based on immunostaining for the tubular epithelial markers *Ricinus communis* and *Lens culinaris* as well as a specific cytochrome P450 enzyme (CYP1A2), to give some indication of functional capacity in the donor-derived cells (Poulsom et al., 2001). To avoid mistakenly identifying cells of macrophage or leukocyte lineages as renal cells, costaining was carried out for the mouse macrophage marker F4/80 antigen and leukocyte common antigen (CD45).

The authors reported that 3.8 to 7.9% of cortical tubular epithelial cells in the female recipients, 13 weeks following male bone marrow transplantation, contained a Y-chromosome (Poulsom et al., 2001). Although recipients were lethally irradiated prior to bone marrow transfer, the authors note that nephric damage induced by this treatment is minimal, and they considered therefore that this figure represents a basal turnover level of contribution by circulating stem cells to the renal parenchyma. Although bone marrow-derived cells were observed within the glomeruli with a position, morphology, and vimentin staining profile consistent with podocyte phenotype as well as in the tubules and glomeruli of the human subjects, percentage contributions were not reported (Poulsom et al., 2001).

Lin et al. (2003) examined the ability of HSCs, purified by cell sorting on the basis of dye-exclusion, lineage depletion, and expression of Sca-1 and c-kit antigens as well as CD45.2 expression (to exclude bone marrow-derived mesenchymal cells), to contribute to the regeneration of renal tubules following ischemia/reperfusion injury. HSCs (2×10^3) isolated from male ROSA26 mice were administered to lethally irradiated nontransgenic female mice following induction of ischemic injury by clamping of the renal artery for a 15-min period. Their results appear to show a striking contribution of donor-

derived cells to renal proximal tubules, 4 weeks after transplantation, in the recovering kidney and no contribution in animals where transplantation was carried out without concomitant induction of ischemic injury (Lin et al., 2003). In this study, the regenerating kidneys were found to contain 8.3 ± 3.2% of Y-chromosome-positive cells, spread throughout approximately 80% of proximal tubules that contained some cells coexpressing β-galactosidase and proximal tubule-specific markers (Na/Pi2 or Fx1A, which mark the brush border of proximal tubule cells). No β-galactosidase expressing cells were observed within the distal tubules, collecting ducts or glomeruli. The authors considered that donor-derived macrophages and leukocytes did not represent a significant proportion of the β-galactosidase expressing cells found in recovering kidneys (Lin et al., 2003). This assertion was based on comparisons of macrophage (revealed by staining with macrophage-specific antibody F4/80) and leukocyte (by expression of the common leukocyte antigen CD45.2) numbers between uninjured kidneys and kidneys 4 weeks following ischemia/reperfusion injury. Although inflammatory cell infiltration has previously been shown to continue increasing for at least 3 months following severe ischemic injury (Ibrahim et al., 1996), it is conceivable that this may depend on the extent of injury.

These findings are broadly in agreement with those of Kale et al. (2003), who also used the renal ischemia/reperfusion injury model, although with some differences in experimental design. In the study of Lin et al. (2003), mice were lethally irradiated, subjected to renal ischemia/reperfusion injury, and received BMSC transplant all within hours of each other. In the Kale study, mice were subjected only to sublethal radiation, followed by transplantation and a 16-week engraftment period prior to the induction of ischemic injury (Kale et al., 2003). Accordingly, in an examination of sections from kidneys harvested 7 days postinjury, they found that 20.9% of tubules contained some β-galactosidase-positive, donor-derived cells. When the engraftment period was reduced to 2 weeks, this figure dropped to 8.5%, presumably reflecting a correspondingly lower percentage of β-galactosidase-positive cells in the bone marrow at this time point (3.3% versus 24.5 ± 6%). In control animals not subjected to ischemic injury, no β-gal-positive cells were observed in the kidney 3 weeks posttransplantation. When this period was extended to 12 weeks, however, approximately 2 to 5% of tubules were found to contain β-gal-positive cells. These results were obtained following injection of whole bone marrow (5 × 10^6 cells). Repeating the experiment with Lin^-Sca-1^+c-kit^+ cells (5 × 10^3) yielded similar results. Approximately 22% of outer medullary tubules contained some β-galactosidase-positive cells by 1 week after induction of ischemic injury, following a 5-week engraftment period.

In an effort to correlate these results with functional/physiological aspects of ischemic injury, Kale et al. (2003) also examined the numbers of Lin^-Sca-1^+ cells in peripheral blood 24 h after the induction of injury. Interestingly, they found that Lin^-Sca-1^+ cells made up 20 to 24% of the nonerythrocyte circulating population in those subjects that had undergone ischemic injury, although they were undetectable above background levels in sham-operated mice. In addition, a comparison was made of blood urea nitrogen levels in mice that had been lethally irradiated with or without stem cell transplantation prior to the induction of ischemic injury and in a third group that underwent ischemia/reperfusion in the absence of bone marrow ablation. The results appear to show that stem cell transplantation ameliorated the effects of ischemic injury, as blood urea nitrogen levels in the group that received transplantation were indistinguishable from unirradiated controls and lower than those of animals that did not receive stem cells over a 7-day period following injury (Kale et al., 2003). Although these results are suggestive and encouraging, repetition and examination of additional physiological parameters is essential to confirm the conclusions.

The three studies described above, if considered in isolation, would certainly lead one to the conclusion that BMSCs make a significant contribution to the regeneration of renal tubules following acute injury and may even be involved in daily turnover of parenchymal cells. There are, however, several studies that have produced evidence to the contrary.

Szczypka and colleagues transplanted whole bone marrow (5 × 10^6 cells) from male ROSA26 mice into lethally irradiated female recipients and examined the kidneys of otherwise healthy animals in addition to subjects with folic acid (FA)-induced renal injury (Szczypka et al., 2005). Their choice of the FA injury model was based on the rational that this gives rise to chronic as well as acute damage and should therefore maximize the opportunity for stem cells of whatever origin to be recruited and participate in renal regeneration. Mice were FA-treated either 1 or 9 months following transplantation and sacrificed 2, 4, or 8 weeks after injection. Detection and characterization of donor-derived cells was by β-galactosidase activity, Y-chromosome detection by fluorescent in situ hybridization (FISH), and IHC for *Lotus tetragonolobus* lectin (proximal tubule-specific) and the pan-hematopoietic marker CD34. According to these criteria, the authors found no donor-derived tubule cells in kidney sections of experimental animals, although some donor-derived cells were detected in the glomeruli (Szczypka et al., 2005). Most β-gal^+/Y-chromosome^+ cells observed within the kidney were seen to express CD45. In a parallel experiment, Szczypka et al. isolated and cultured proximal tubule cells from control and experimental animals. Of approximately 4 × 10^6 proximal tubule cells isolated, a single cluster of seven β-gal^+ cells were found, which were also CD45^-, AT1^+

(a central cilliary component), and ZO1$^+$ (an epithelial tight junction protein). Taken together, their results indicate that it is possible for bone marrow-derived cells to adopt proximal tubule cell fate but that this is an extremely rare event, even in response to FA-induced injury.

This conclusion is supported by the work of Gupta et al., who examined kidney biopsies from male patients who had received transplants from female donors. They found only rare (affecting <1% of tubules) instances of Y-chromosome-positive cells within the tubular epithelia in patients recovering from acute tubular necrosis and none at all in cases without resolving acute tubular necrosis (Gupta et al., 2002).

Krause et al. (2001) developed a functional purification step to enrich for HSCs by introducing male bone marrow cells, marked with a membrane-bound dye (PKH26), into lethally irradiated female recipients and then reisolating those labeled cells that specifically homed to the host bone marrow over a 2-day period. This led to an estimated 500- to 1000-fold enrichment of long-term repopulated cells. The authors went on to examine engraftment of epithelial tissues by the progeny of single, bone marrow-homed cells. Donor-derived cells in the host were identified by FISH for the Y-chromosome and tissue type determined based on morphological criteria and IHC for epithelial-specific cytokeratin markers. Five months following transplantation, engraftment was observed in the epithelia of all tissues examined (bronchi, alveoli, esophagus, stomach, small intestine, large intestine, skin, and bile duct), with the exception of kidney (Krause et al., 2001).

Wagers et al. (2002) repopulated lethally irradiated hosts with single, GFP-marked, c-kit$^+$Thy1.1^{lo}Lin$^-$Sca-1$^+$ HSCs. The authors looked for GFP-positive, potentially transdifferentiated cells based on any or all of the following properties: morphology, tissue-specific marker expression, and lack of expression of the hematopoietic marker CD45. Of all the tissues examined (brain, liver, kidney, gut, skeletal muscle, cardiac muscle, and lung), cells meeting the authors' criteria for being considered as transdifferentiated were observed only in brain (a Purkinje cell) and liver (hepatocytes) (Wagers et al., 2002). In light of the findings of Alvarez-Dolado et al. (2003) (see above), it seems likely that these instances may have been due to cell fusion events. Nearly all of the GFP-positive cells in all tissues were found to express CD45 (Wagers et al., 2002).

In a parallel experiment, Wagers et al. (2002) generated parabiotic pairs of animals (surgically joined with common blood circulation, leading to chimerism of the hematopoietic system) consisting of GFP$^+$ and GFP$^-$ littermates, to study circulating HSC engraftment without the complications of lethal irradiation. Extensive engraftment was observed within the hematopoietic system following a period of several months, but the authors were unable to detect partner-derived nonhemato-

poietic cells in any of the tissues (see above) examined. The results of these two experiments together led to the conclusion that transdifferentiated HSC progeny are unlikely to have a significant role in the maintenance of other tissues, although this does not exclude possible involvement in tissue repair following severe injury (Wagers et al., 2002). Although the results of Krause et al. (2001) and Wagers et al. (2002) are at odds over the extent of the plasticity of HSCs, they agree regarding the lack of contribution to renal tissue, thus supporting the conclusion that BMSCs are not involved in kidney maintenance, but leaving open the possibility of a role in repair.

C. Transdifferentiation Summary

The disparate results generated by the overtly similar studies described above are difficult to reconcile but can likely be ascribed to four main factors: 1) differing detection methods of donor-derived cells, 2) differing methods and/or degrees of rigor in excluding donor-derived hematopoietic cells from analysis, 3) different starting populations of donor cells, and 4) different injury models. In the studies of Poulsom et al. (2001) and Krause et al. (2001), identification of donor-derived cells relied exclusively on Y-chromosome detection by FISH, a method that can lead to the identification of false positives (Szczypka et al., 2005). Although Krause et al. used IHC with a specific marker (CD11b) to exclude macrophages, use of the pan-hematopoietic marker CD34, by Wagers et al. (2002), would appear to be the more thorough approach to identifying hematopoietic cells. Kale et al. (2003) identified donor-derived cells in the kidney by β-galactosidase activity but did not confirm their identity by coexpression of kidney-specific markers, or eliminate from analysis β-gal$^+$ cells expressing hematopoietic markers. Lin et al. (2003) also failed to specifically exclude β-gal$^+$ cells expressing hematopoietic markers, although they did identify some cells with β-galactosidase activity that expressed proximal tubule-specific markers.

Starting populations of donor cells ranged from whole bone marrow (Poulsom et al., 2001; Kale et al., 2003; Szczypka et al., 2005) through Rho^{lo}Lin$^-$Sca-1$^+$c-kit$^+$CD45.2$^+$ (Lin et al., 2003) and Lin$^-$Sca-1$^+$c-kit$^+$ fractions (Kale et al., 2003) to single bone-marrow homed (Krause et al., 2001) and c-kit$^+$Thy1.1^{lo}-Lin$^-$Sca-1$^+$ (Wagers et al., 2002) cells. This leaves open the possibility that subtly different stem cell populations were being evaluated in the different studies. However, it should be pointed out that Kale et al. obtained the same results using whole bone marrow or the Lin$^-$Sca-1$^+$c-kit$^+$ fraction, and they, Poulsom et al., and Szczypka et al. all observed different outcomes when starting with whole bone marrow, suggesting that the main reason for the discrepancies lies elsewhere. Finally, Lin et al. (2003) and Kale et al. (2003) both saw a significant contribution of BMSCs to regenerating renal tubules

following ischemia/reperfusion injury, whereas Szczypka et al. (2005) found only a very limited contribution following FA-induced injury, leaving open the possibility that BMSCs may respond only to specific types of injury.

In any case, the very lack of an emerging consensus during the expansion of this field has served to dampen the initial optimism over results that appeared to indicate a short journey to the clinic for transdifferentiating BMSCs. Further work is certainly required to settle the controversy. One possible experiment would be to use whole bone marrow transplantation in conjunction with ischemia/reperfusion injury and, most importantly, a specific, sensitive, and reliable method to detect transdifferentiated progeny. Donor cells obtained from a double-transgenic mouse for a β-gal or GFP Cre reporter (as, for example, the R26R strain) (Mao et al., 1999) and a proximal tubule-specific Cre expresser (Rubera et al., 2004) would likely satisfy these criteria. Thus, donor bone marrow-derived cells would only express the reporter gene following induction of Cre expression following transdifferentiation into the tissue of interest.

D. Renal Stem Cells

One reason for the intense interest in using BMSCs in cell therapy for renal failure has been the lack of an identified adult kidney stem cell population. This emphasis may be about to change, following the recent discovery of cells residing in the renal papilla with characteristics suggesting that they may represent the elusive adult kidney stem cell (Oliver et al., 2004).

Metanephric development in the mouse is initiated on the 11th day postfertilization as the ureteric bud (UB), an offshoot of the nephric duct, invades the metanephric mesenchyme (MM) and morphogenesis proceeds by reciprocal induction events between these two tissues (Saxén, 1987). Prior to induction by the UB, the MM consists of a few thousand morphologically indistinguishable mesenchymal cells which, together with the UB, give rise to the 26 or so cell types that make up the mature kidney (Al-Awqati and Oliver, 2002). It remains unclear whether these cell types are derived from multiple distinct progenitor populations within the MM or if they are generated by multipotent stem cells. Evidence supporting the latter hypothesis, however, has emerged from several studies.

Single cells within the MM were initially shown to have the potential to generate all the epithelial elements of the nephron, with the exception of the collecting duct (which is UB-derived) (Herzlinger et al., 1992). Subsequently, evidence of a broader differentiation potential for the progeny of individual MM cells was reported, with the expression in vitro of markers suggestive of smooth muscle cell, myofibroblast, and endothelial fates (Oliver et al., 2002).

Most recently, Oliver et al. (2004) identified a population of slow-cycling cells residing in the papilla of the adult kidney that commence proliferating in response to

ischemia/reperfusion injury and may migrate to the medulla. In vitro, these cells were shown to possess similarities to cultured MM cells, with coexpression of epithelial and mesenchymal markers.

Further characterization of this potential stem cell population is essential and will likely intersect with work such as that of Challen et al. (2004) whose approach has been to identify specific genetic markers of the MM by cDNA microarray analysis, comparing gene expression in the MM with that of neighboring uninduced mesenchyme. It is hoped that the identification of a combination of cell-surface markers specific to renal progenitor cells will assist in their isolation and analysis throughout nephrogenesis and provide further clues about the putative adult renal stem cells. The existence of such a cell type, should they prove amenable to in vitro manipulation, would have an immediate impact on the development of cell therapies for kidney disease, both by the direct approach (discussed above) and by the various indirect approaches (discussed below).

III. Tissue and Bioengineering Approaches

The tissue and bioengineering approaches are, in general, based on in vitro manipulation of the cells of interest and their association with biomaterials, which may be either biodegradable or permanent in nature, to produce a device for implantation or incorporation into an extracorporeal circuit. At the more modest end of the scale are strategies such as the implantation of a single differentiated cell type to replace a metabolic or catabolic function, whereas at the far (as yet theoretical) end of the spectrum sits the goal of "growing" a functioning organ for transplantation. These functions range from add-ons aimed at improving conventional therapy to complete renal replacement, which should ultimately make long-term maintenance dialysis and even cadaveric transplants obsolete. The role of stem cells in these technologies is yet in its infancy, but together they will make a powerful combination. The examples discussed below represent promising cell-based therapeutic approaches in the renal field. At present, some of these have more direct stem cell involvement than others, but all have the potential to act as a conduit for the translation of stem cell science into clinical benefits.

Cell-based therapies rely on the expansion of large cell populations that are uniform in activity and pathogen-free. Current tissue/bioengineering methods of satisfying these requirements typically depend on progenitor or transformed cell types, although the emphasis is likely to shift more and more to stem cells, with advantages to be gained in scalable production without the safety concerns attached to transformed cell lines.

Two of nine patients who received successful gene therapy for the correction of X-linked severe combined immunodeficiency disease by retrovirally mediated transfer of the γc gene into their own HSCs, subse-

quently developed acute leukemia (Hacein-Bey-Abina et al., 2003). This was due to the unintended and unforeseen transformation of some of the genetically manipulated stem cells by insertion of the retrovirus near the promoter of a known proto-oncogene (*LMO2*). Even the use of nontransformed cells may carry safety concerns as, for example, in a few patients (5:33) receiving transplantation of embryonic dopamine neurons for the treatment of Parkinson's disease who went on to develop uncontrollable dyskinetic side effects (Dunnett et al., 2001) or in the occurrence of cardiac arrhythmias following implantation of myoblasts into the heart (Menasche et al., 2002). These examples highlight the importance, not only of protecting transplanted cells from possible immune attack, but also of being able to protect the host from implant malfunction.

A. Cellular Implants to Remove Toxins and Deliver Therapeutic Agents

Low molecular weight proteins represent a class of uremic toxins insufficiently cleared from the blood by dialysis or hemofiltration (Clark and Gao, 2002). Examples of this diverse class of molecules include parathyroid hormone, complement factor D, leptin, and β2-microglobulin (β2M) (Clark and Gao, 2002). In the case of β2M, accumulation in ESRD patients at up to 40 times the normal serum concentration often leads to the development of debilitating arthritis, as amyloid deposits of β2M build up in their joints (Gejyo, 2000). Saito et al. (2003) have demonstrated the feasibility of using a cellular implant for continuous degradation of low molecular weight proteins such as β2M. Working in nude mice, they first implanted a collagen sponge impregnated with basic fibroblast growth factor to promote vascularization and subsequently introduced megalin-expressing cells into the newly vascularized structure. The cells (L2), derived from a rat yolk sac carcinoma, were shown to take up and degrade circulating β2M, leading to a 40% reduction in the steady-state level of this uremic toxin in nephrectomized animals (Saito et al., 2003). It should be noted that this result was only achieved with an estimated 7×10^9 cells in the implant, representing approximately 30% of the mouse body weight. This is, however, an issue of cell-sourcing, and cells with higher megalin expression should be able to deliver the same effects at more realistic numbers. The use of a tumor cell line in such a setting is obviously a clinical impossibility, although the fact that stem cells share many features with cancerous cells (Reya et al., 2001) serves as a reminder that their substitution with cells expanded and differentiated in a controlled manner would carry this approach much closer to the clinic.

One strategy to overcome other problems associated with cell implants, such as host immune response (when heterologous tissue is used) and recoverability of the implant (a key fail-safe condition if unwanted events should arise), is encapsulation. Encapsulation within a semipermeable polymeric membrane offers the advantage of immunoisolating allogenic or xenogenic cells and allows a greater degree of control over their destination within the body while still allowing their contact with the bodily fluid required to elicit the desired physiological response (Orive et al., 2004).

Cells may be encapsulated within sodium alginate beads as, for example, in the work of Chang and Prakash (1998) on degradation of urea by orally administered microcapsules containing genetically modified bacteria expressing the urease enzyme. Rats were made uremic by unilateral nephrectomy and partial ligation of the remaining kidney, resulting in substantially increased urea levels without disturbing water and electrolyte balances. Daily oral administration of 11.15 $\pm$ 2.25 mg/kg b.wt. of microencapsulated genetically modified bacteria for a 21-day period led to a reduction in plasma urea level from 52.08 $\pm$ 2.06 to 9.10 $\pm$ 0.71% mg (within the normal range) (Chang and Prakash, 1998). The equivalent dose for a 70-kg human patient would be 4 g of microencapsulated cells per day.

Alternatively, cells may be encapsulated within hollow fibers incorporated into an implantable device. Erythropoietin (EPO) production in response to reduced oxygen tension represents a possible direct application of this technique for treatment of renal failure. Cells of the human hepatoma cell line HepG2 are known to display oxygen-regulated EPO production (Goldberg et al., 1987). Incorporation of such cells within the hollow fibers of an intravascular implanted device (Humes, 1998) represents a promising alternative to the administration of recombinant human EPO (Humes, 2000).

B. Renal Augmentation

In the sense that developing nephrons or whole metanephroi contain/are derived from renal stem cells (see above), transplantation of fetal kidneys may be thought of as a therapeutic stem cell application. In addition, these studies provide insights into the immune response to immature tissue with relevance to transplantation of engineered tissues generally. The work in this field has recently been comprehensively reviewed by Hammerman (2003).

Briefly, it has been shown that the severity of the immune response to implantation of allogenic or even xenogenic renal tissue is ameliorated when prevascularization embryonic donor tissue is used (Foglia et al., 1986; Rogers and Hammerman, 2001b), although, in the latter case, costimulatory blockade (administration of anti-CD154, anti-CD45RB and CTLA4Ig, or anti-CD11a) is still required to prevent rejection (Rogers and Hammerman, 2001b; Rogers et al., 2003). Several mechanisms are thought to account for this observation (Hammerman, 2003), including absence of antigen-presenting cells within the metanephros prior to a certain stage in development (Foglia et al., 1986; Velasco and Hegre, 1989), reduced expression levels of major histo-

compatibility class I and II antigens (Statter et al., 1989; Dekel et al., 1997), and altered expression levels of cytokines, chemokines, and cytolytic effector molecules relative to adult tissue (Dekel et al., 2000). Additionally, vascularization of transplanted metanephroi is accomplished, at least in part, by host blood vessels, resulting in the generation of a chimeric organ shielded to some degree from circulating immune-cell surveillance (Rogers and Hammerman, 2001b).

In rats and mice, metanephroi transplanted beneath the capsule of adult kidneys or into tunnels fashioned in the renal cortex of neonates have been shown to give rise to nephrons with vascularized glomeruli and mature tubules (Woolf et al., 1990; Rogers et al., 1998). By intravascular administration of fluorescently labeled dextran shortly before sacrifice of metanephric-transplant recipients, Woolf et al. (1990) were able to demonstrate glomerular filtration by donor-derived nephrons. It was not, however, determined whether integration with the host's collecting duct system had been achieved.

A more promising approach, from a therapeutic standpoint, has been the transplantation of metanephroi into the omentum (a folded membrane within the abdominal cavity attached to the bottom edge of the stomach and the transverse colon) (Hammerman, 2003). Rogers et al. (1998) and Hammerman (2003) have shown that E15 rat metanephroi transplanted into the omentum of adult hosts grow to approximately one-third the diameter of native kidneys, develop mature glomeruli and tubules, and are vascularized by arteries originating from the omentum. Inulin clearance in bilaterally nephrectomized hosts, following anastomosis between the ureters of the implanted metanephros and native kidney, has been measured at ~0.2% of the mean per single kidney from normal rats expressed as microliters per minute per 100 g of rat weight and 9% of those measured per single kidney of normal rats expressed as microliters per minute per gram of kidney weight (Rogers et al., 2001). Furthermore, it has been shown that exposure of explanted metanephroi, prior to implantation, to a cocktail of growth factors (insulin-like growth factor I and II, transforming growth factor α, hepatocyte growth factor, vascular endothelial growth factor, basic fibroblast growth factor, nerve growth factor, retinoic acid, corticotropin-releasing hormone, Tamm Horsfall protein, prostaglandin E_1, and iron-saturated transferrin) leads to increased urine production and inulin clearance by the implant (Rogers and Hammerman, 2001a).

Dekel et al. (2002) carried out transplantation of 70-day human metanephroi into NOD/SCID mice. Metanephroi implanted intra-abdominally (onto the testicular fat) were found to take up increasing amounts of dimercaptosuccinic acid from the host circulation with the passage of time following transplantation from 3 to 10 weeks, indicative of functional maturation of the implant. Following subcapsular implantation, Dekel et al. also investigated gene expression in the transplanted

metanephroi. cDNA microarray analysis showed the expression profile to be similar (although at lower levels) to that seen during normal kidney development (Dekel et al., 2002).

Should it prove possible through growth factor treatments (Rogers and Hammerman, 2001a) and/or optimization of transplantation methodology (Dekel et al., 2002) to increase the functionality of transplanted metanephroi to physiologically significant levels, this approach may become clinically useful. Safety concerns over xenotransplantation, however, would likely restrict possible sources of donor tissue to aborted human fetuses. This, of course, would have considerable political implications, although an ethical/legal framework for the use of human embryonic tissue in the treatment of neurodegenerative diseases has been worked out (Boer, 1994). Even assuming these hurdles could be overcome, the extent of embryonic donor organ availability would be highly limiting, although the development of techniques for expansion and propagation of metanephroi in vitro may mitigate, or ultimately overcome, this factor (Steer et al., 2002). It seems probable, therefore, that not until stem cell technology has progressed to the point where renal precursor cells can be generated, differentiated, and organized into a functional kidney precursor in vitro, will this strategy have a serious impact on the treatment of renal failure.

C. Full Renal Replacement

There are currently two main bioengineering programs with the aim of producing a device providing full renal replacement therapy in the short to medium term. Both employ biomaterial scaffold structures to overcome the as yet insurmountable difficulties inherent in marshalling cells into organized three-dimensional structures capable of coordinated filtration, resorption/meta-/catabolism/secretion, collection, and disposal of waste. Atala and coworkers have approached this problem by expanding renal cells in culture and seeding them onto collagen-coated cylindrical polycarbonate membranes, relying on the cells' innate morphological/organizational properties to reconstitute functional nephron units (Amiel et al., 2000; Lanza et al., 2002; Koh and Atala, 2004).

Initial experiments involved adult rabbit renal cortex harvested and fractionated into glomeruli, distal, and proximal tubules, expanded separately in vitro, and seeded onto biodegradable polyglycolic acid sheets for subcutaneous implantation into syngenic hosts (Amiel et al., 2000). Retrieval and histological examination of the scaffolds at 1 week, 2 weeks, and 1 month postimplantation revealed evidence of vascularization and identifiable nephronal elements. Glomeruli and tubular segments of various identities were observed, including proximal tubules, distal tubules, loops of Henle, collecting tubules, and collecting ducts, identified on the basis of morphology and IHC with nephron segment-specific

lectins (Amiel et al., 2000). It does not, however, appear that these elements were continuous, or that they displayed any higher-order organization into potentially functional nephrons.

In an attempt to address the issue of functionality, further experiments were carried out with cells seeded onto a section of cylindrical polycarbonate membrane connected at one end to a silastic catheter, which terminated in a reservoir (Amiel et al., 2000). Devices were implanted in athymic mice and subsequently retrieved following 1, 2, 3, 4, or 8 weeks for histological and immunocytochemical examination. The investigators reported "extensive vascularization and the formation of glomeruli and highly organized tubule-like structures" (Amiel et al., 2000), although it is not clear what factors are responsible for the increased level of structural organization relative to their earlier experiments. It was noted that the tubular structures stained positively for osteopontin (secreted by proximal and distal tubule cells, as well as the thin ascending loop of Henle) and that proximal tubule-like structures stained positively for alkaline phosphatase. It was not specified whether these structures were contiguous (Amiel et al., 2000). A yellow fluid collected from the reservoir was shown to have uric acid and creatinine levels of 66 mg/dl and 27.91 ± 7.56 mg/dl (versus normal plasma concentrations of 2 mg/dl and 4.49 ± 0.08 mg/dl), respectively, raising the possibility that the reconstituted nephronal units may possess some level of functional capacity.

These results were confirmed and extended by subsequent experiments in which Lanza et al. (2002) further explored the potential of their renal construct. Of particular interest, embryonic renal cells for the device were derived from the metanephros of a 56-day-old bovine embryo generated by nuclear transfer (also referred to as therapeutic cloning), to provide donor tissue histocompatible with the host. Nuclear transfer enables the generation of cells genetically identical to an adult animal by the transfer of a somatic cell nucleus (in this case from a skin fibroblast) into an enucleated oocyte to generate a cloned embryo (reviewed by Hochedlinger and Jaenisch, 2003). The cells of this cloned metanephros were dissociated and expanded in culture where they were shown to possess normal renal cell characteristics, including expression of renal-specific proteins (synaptopodin, aquaporin-1, aquaporin-2, and Tamm-Horsfall protein) and production of 1,25-dihydroxyvitamin D_3 and EPO (Lanza et al., 2002). EPO production was further shown to be responsive to changes in oxygen tension.

The devices used in these experiments were similar to that described above (Amiel et al., 2000) but with three collagen-coated (coating thickness 2 mm) cylindrical polycarbonate membranes (3-cm long, 10-μm thick, 2-μm pore size, 1.4-mm internal diameter) catheterized at one end to a 2-ml collecting reservoir and sealed at the other (Lanza et al., 2002). The cultured cells were seeded onto the coated membranes (at a density of approximately 2×10^7 cm^{-2}) and the constructs implanted into the flank subcutaneous tissue of the same animal from which the cloned fetus was derived, followed by recovery and analysis after 12 weeks. As controls, units seeded with cells isolated from an allogenic fetus as well as cell-free units were implanted on the contralateral side.

Upon recovery, the reservoirs were found to contain small amounts of fluid, with the greatest volumes seen in the cloned-cell devices (600 ± 40 versus 100 ± 10 and 130 ± 40 μl for the allogenic and unseeded control groups, respectively) (Lanza et al., 2002). Unique to the cloned-cell devices, this fluid was yellow in color with elevated urea and creatinine and a profile of physical/chemical properties (electrolyte levels, specific gravity, pH, glucose concentrations, and mineral content) similar to that of bovine urine (Lanza et al., 2002). These observations support the conjecture that the implanted renal cells may possess filtration, reabsorption, and secretory functions.

Examination of the tissue itself revealed extensive vascularization and the presence of glomeruli and tubule-like structures which expressed aquaporin-1, aquaporin-2, synaptopodin, and Factor VII (confirmed by IHC and reverse transcription-polymerase chain reaction) (Lanza et al., 2002). No evidence of immune rejection was observed in the cloned-cell devices, whereas the allogenic-cell controls were observed to be extensively necrotic. In at least some cases, continuity was noted between glomeruli, their tubules, and the polycarbonate membrane (Lanza et al., 2002). It will be fascinating to learn with what frequency this remarkable level of spontaneous organization occurs and what its physiological relevance may be. A demonstration of inulin clearance from the circulation into the reservoir and/or fluorescently labeled dextran into the reconstituted tubules, for example, would be most welcome. The results reported so far are encouraging and more is to be hoped for in the future from this approach.

The strategy adopted by Humes and colleagues has been to start with the clinically proven method of hemofiltration as a working substitute for glomerular filtration and add to it specifically the functionality of proximal tubule cells in the form of the renal tubule assist device (RAD) to increase the scope and efficacy of extracorporeal renal replacement therapy. This approach has shown considerable promise following testing in animal models and in Phase I/II clinical trials on human patients with ARF. Progress has recently been reviewed by Humes and Szczypka (2004) and Fissell and Humes (2003).

The method has its foundation in the ability to isolate and expand in culture tubule cells from adult kidneys (Humes and Cieslinski, 1992; Humes et al., 1996), which are then grown along the inner surface of the fibers in a standard hemofiltration cartridge (MacKay et al., 1998; Humes et al., 1999b; Nikolovski et al., 1999). Cell at-

tachment to the membrane is promoted by first coating it with extracellular matrix molecules. Matrix selection varies, depending on the source of tubule cells. For porcine RADs, either proNectin-L (a laminin-derivative) or murine laminin have been used and for the human RADs, either murine laminin or bovine collagen type IV are used (Humes et al., 2002). The high-flux hemofiltration membrane and associated extracellular matrix molecule coating thus act as scaffold and immune-barrier for the cells of the bioartificial tubule.

In vitro testing of this device, seeded with porcine RPTCs, demonstrated differentiated active transport properties, metabolic activities, and endocrine processes of the RAD (Humes et al., 2002). High-flux hemofiltration cartridges containing polysulfone hollow fibers with surface areas of either $97 \ cm^2$ or $0.4 \ m^2$ were seeded with RPTCs at a density of 3×10^7 cells per milliliter and perfused with culture media for 14 days, by which time cells had reached confluency as assayed by inulin leak rates and electron microscopy. Cell numbers were determined by lactate production at approximately 3.4×10^7 cells in each $97 \ cm^2$ RAD and 1.4×10^9 cells per $0.4 \ m^2$ RAD. This number is of the same order of magnitude as the proximal tubule cell mass within a mammalian kidney, estimated at 5×10^9 cells (Humes et al., 2002).

Testing of these units revealed properties of vectorial fluid transport from intrato antiluminal space, active transport of bicarbonate, glucose, and secretion of *para*-aminohippurate. Glutathione catabolism, ammonia production, and conversion of 25-hydroxyvitamin D_3 were also demonstrated, and the cells were shown to remain viable up to the latest point tested at 6 months (Humes et al., 2002).

The bioartificial kidney setup consists of a conventional hemofilter followed in series by the tubule RAD unit (Humes et al., 1999a, 2002; Fissell et al., 2002). Blood is pumped out of a large animal using a peristaltic pump and ultrafiltrate, generated as the blood passes through the hemofilter, and is then delivered into the tubule lumens of the RAD. Direct contact between the ultrafiltrate and the tubule cells lining the membrane allows them to carry out their metabolic and regulated transport functions. Processed ultrafiltrate exiting the RAD is collected and discarded as "urine". The filtered blood exiting the hemofilter enters the RAD through the extracapillary space port and disperses among the fibers of the device. The direct bathing of the tubule cells within the RAD by ultrafiltrate and transmembrane contact with the filtered blood delivers the metabolic substrates, low-molecular weight growth factors, and oxygen required to maintain their viability while preserving immunoisolation of the heterologous tissue. Processed blood exiting the RAD passes through a third pump required to maintain hydraulic pressures within the unit and is delivered back into the animal. Heparin is delivered continuously into the blood before entering the RAD to diminish clotting within the device, and it is

temperature-controlled at a steady 37°C for optimal functionality of the cells.

Ex vivo animal testing has borne out the results of the in vitro study outlined above. RADs containing either porcine or human cells have been evaluated on uremic dogs following bilateral nephrectomy (Humes et al., 1999a, 2002). RAD treatment of these animals, for either 7 or 9 h on 3 successive days or for a single 24-h period, resulted in improvements in multiple physiological parameters compared with treatments with cell-free sham RAD controls. Plasma levels of HCO_3^-, P_i, and K^+ were maintained nearer to normal values; glucose, HCO_3^-, and K^+ active transport as well as ammonia excretion, glutathione processing, and 25-hydroxyvitamin D_3 conversion were demonstrated (Humes et al., 1999a, 2002). Furthermore, in canine and porcine models of ARF with septic shock, RAD treatment was shown to modulate plasma cytokine levels, maintain better cardiovascular performance (as determined by cardiac output and renal blood flow), and increase survival times (Fissell et al., 2002, 2003; Humes et al., 2003).

The preclinical animal data briefly sketched above have been followed up by a Food and Drug Administration-approved Phase I/II clinical trial on 10 critically ill patients with ARF and multiple organ failure (and predicted hospital mortality rates averaging above 85%) receiving continuous venovenous hemofiltration (Humes et al., 2004). The devices used in this study were seeded with human kidney cells isolated from organs donated for cadaveric transplantation but which could not be used for this purpose owing to anatomic or fibrotic defects. The results demonstrated that RAD treatment can be safely delivered under study protocol guidelines in this critically ill patient population for up to 24 h and that the device retains viability, durability, and functionality throughout. Glutathione degradation and 25-hydroxyvitamin D_3 conversion by the RAD tubule cells were maintained, and for the subset of patients who had excessive proinflammatory levels, significant declines in granulocyte-colony stimulating factor, IL (interleukin)-10, and IL-6/IL-10 ratios were observed, suggesting that RAD therapy produced dynamic and individualized responses. Of the 10 patients treated, six survived past 30 days, one expired within 12 h following treatment due to the family's request that ventilatory life support be withdrawn, and three others died due to complications unrelated to ARF or RAD therapy (Humes et al., 2004). Supported by these encouraging initial clinical results, a randomized, controlled Phase II clinical trial is currently under way, and a clinical trial of RAD treatment for ESRD patients is in preparation.

IV. Conclusions and Future Perspectives

The potential impact of advances in stem cell technology on all the prospective cell-based therapeutic approaches for the treatment of renal failure discussed

above is enormous. It is through methodologies such as these that the promise of stem cells will be carried forward to the clinic. As the understanding of basic ES cell biology deepens and protocols are developed by which their differentiation may be robustly and reproducibly guided toward specific cell fates in vitro, they will increasingly become the answer to the sourcing question on which all cell therapies ultimately stand or fall.

Directed-differentiation protocols for ES cells in culture are emerging, as reviewed recently by Heng et al. (2004). In vitro differentiation into numerous lineages, including neural (Zhang et al., 2001; Rathjen et al., 2002; Wichterle et al., 2002; Nakayama et al., 2003), hematopoietic (Kaufman et al., 2001), osteo/chondrogenic (Buttery et al., 2001; Hegert et al., 2002; Sottile et al., 2003; Sui et al., 2003), myogenic (Muller et al., 2000; Kehat et al., 2001; Mummery et al., 2003; Zandstra et al., 2003), and endothelial (Yamashita et al., 2000; Kaufman et al., 2004) has been reported. For organ and tissue engineering, the intersection between directed-differentiation by chemical means, such as growth factors and cytokines (Schuldiner et al., 2000), and innovative 3-dimensional culture techniques within biodegradable scaffolds and matrices (Kim and Mooney, 1998; Richardson et al., 2001; Chen et al., 2003; Levenberg et al., 2003; Liu et al., 2003) is likely to be particularly fruitful.

Clinical applications will require the use of human ES cells (Gerecht-Nir and Itskovitz-Eldor, 2004), and although the political climate in many countries is presently unfavorable for human ES cell research, progress continues to be made (Brivanlou et al., 2003; Cowan et al., 2004). The acceptability of nuclear transfer as a method of generating nonimmunogenic cells for the treatment of a particular individual, as illustrated by the work of Lanza et al. (see above), is an issue that must be decided. Lanza et al. (2002) achieved their results working with bovine tissue, and cloned embryos were allowed to develop well into pregnancy prior to their termination for the harvest of metanephric tissue—a practice that could never be acceptable in humans. The generation of cloned human ES cells by somatic cell nuclear transfer has, however, been recently reported (Hwang et al., 2004), and continued societal debate will determine whether this or other (Drukker and Benvenisty, 2004) techniques for creating nonimmunogenic cell implants will prevail. In the meantime, it is the approaches that protect cell from host and host from cell by incorporating immunoisolation barriers (such as the RAD, described above) that are most likely to advance the treatment of renal failure and other complex disorders, providing a platform for the greater technological achievements that lie ahead.

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