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Bone Marrow Contribution to Renal Repair Following Ischemic Injury

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ABSTRACT

BONE MARROW CONTRIBUTION TO RENAL REPAIR FOLLOWING ISCHEMIC INJURY. Matthew C. Egalka, Sujata Kale, , Lloyd G. Cantley. Section of Nephrology, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT.

Repair of the mammalian renal tubular epithelium following ischemia/reperfusion injury is largely effected by surviving epithelial cells and other cells resident in the kidney interstitium; however, the precise nature of the contribution of circulating extrarenal cells is unknown. The purpose of this study is to determine whether bone marrow cells have the capacity to differentiate into renal tubular epithelium. This was investigated using both an *ex vivo* transplantation model as well as an attempt to grow epithelial cells from bone marrow in a liquid culture system.

Recipient female C57BL/6 mice were myeloablated via irradiation and transplanted intravenously with lineage-depleted syngeneic male bone marrow. Following this, renal ischemia was induced by surgical interruption of the renal vascular pedicle. Thin sections were assayed for donor contribution by examination by fluorescence *in situ* hybridization (FISH) for the Y-chromosome. Tubules incorporating donor (Y-chromosome positive) cells were only rarely found (< 2%) in the outer medulla of damaged kidneys; far more frequent were donor-derived interstitial cells, which appear not to be entirely inflammatory in phenotype.

For the tissue culture experiments, collagenase-released marrow stromal cells (CR-MSC) were obtained by collagenase treatment of bone chips following mechanical dissociation of bones harvested from C57BL/6 mice. These cells were grown in culture and examined via immunostaining and reverse-transcription polymerase chain reaction for epithelial markers. Unfortunately, the resultant cells, although appearing to form epithelial colonies, were found to not express the epithelial markers cytokeratin and ZO-1 by RT-PCR and immunostaining; instead they appeared to be fibroblasts with a novel morphology. In sum, bone marrow contribution to the

renal epithelium appears to be a rare phenomenon at best and as of now cannot be replicated *in vitro*.

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INTRODUCTION

Acute renal failure

The mammalian kidney is an organ with many roles, including the maintenance of blood pressure, the regulation of pH and other metabolic parameters, the elimination of nitrogenous waste, and the synthesis and release of endocrine factors. The remarkable ability of the "smartest organ in the human body" (Herbert Chase, M.D., personal communication) to maintain homeostasis despite changing conditions has been tested countless times, often by inexperienced medical students entering fluid orders! It is therefore no surprise that conditions where this regulatory behavior is disrupted have been well characterized and extensively investigated.

Acute renal failure (ARF), without reference to proximate cause, describes a rapid decrease in the ability of the human kidney to adequately process and eliminate waste products, manifested by an acute decrease in the glomerular filtration rate (GFR). There is a consequent accumulation of nitrogenous wastes, loss of the ability to concentrate the urine (isosthenuria), as well as a variety of metabolic imbalances. Depending on the cause of ARF, the condition may be entirely asymptomatic until the development of uremic features late in the timecourse; as such, the diagnosis is usually cinched upon gross and microscopic examination of the urine (urinalysis and urinary sediment microscopy) as well as serum markers of kidney filtration (including blood urea nitrogen and creatinine).

Especially in hospitalized patients, ARF is a major cause of morbidity and mortality. Incidence figures vary according to which criteria investigators use to define ARF (currently, no consensus exists) although most are based on either absolute increases in serum creatinine, absolute decreases in calculated creatinine clearance, or oliguria. Prospective cohort studies in the U.K. have reported yearly incidence figures ranging from 486 per million (1) to 620 per million (2) population. Unsurprisingly, the incident rate of ARF among hospitalized patients is significantly elevated, no doubt due in large part to often-multiple comorbid conditions. A recent study

has shown that 7.2% of hospitalized patients develop at least some degree of renal insufficiency (3). A prospective, multi-center analysis of intensive care unit (ICU) patients demonstrated that approximately 25% of medical/surgical ICU patients met diagnostic criteria for ARF, of whom 70% went on to develop multi-organ dysfunction syndrome (4).

The causes of renal dysfunction in patients with ARF can be broadly categorized into three groups. Prerenal failure—representing between 21 and 70% of all cases of acute renal failure (5, 6)—is defined as a decrease in GFR due to hypoperfusion of kidneys, and implies no intrinsic dysfunction of the kidneys themselves. Prerenal failure can be caused by true hypovolemic states such as hemorrhage or dehydration, as well as fluid overload states with decreased effective circulating volume such as those encountered in congestive heart failure and hepatic cirrhosis. Mechanical impediments to renal blood flow (i.e. renal artery stenosis or fibromuscular dysplasia) also cause renal hypoperfusion and can induce a prerenal azotemia. In prerenal failure, the kidneys respond appropriately to what they perceive to be decreased intravascular volume via tubuloglomerular feedback mechanisms which include preglomerular arteriole vasodilation and postglomerular arteriole vasoconstriction. Timely reversal of the hypoperfused states generally results in recovery of renal function. Prolonged prerenal azotemia is a common cause of ischemic intrinsic injury to the kidney tubule, as discussed below.

Postobstructive or postrenal failure occurs when mechanical obstruction impedes the free flow of urine distal to the nephron, causing hydronephrosis and decreased glomerular filtration. The proximal cause of the decrease in GFR is the decreased gradient across which glomerular filtration can occur, due to higher tubular luminal pressures. Again, no intrinsic kidney dysfunction is involved, and resolution of the renal insufficiency usually occurs rapidly following elimination of the distal obstruction.

Intrinsic renal failure certainly represents the gravest etiology of all the causes of ARF, and itself can be further subdivided based on the specific site of injury within the kidney. Glomerular disease—especially that of the rapidly progressive subtype—can lead to fulminant renal

failure as well as the typical signs and symptoms of glomerulonephritis. ARF due to tubular interstitial pathology is usually immune-mediated and is classically due to an allergic reaction to a medication such as an antibiotic or an analgesic. Damage to the renal tubule itself, however, makes up the bulk (5) of ARF due to intrinsic renal injury. This damage can be mediated by direct injury to the tubular epithelium by exogenous or endogenous nephrotoxins. Aminoglycoside antibiotics and myoglobin produced by rhabdomyolysis represent two classical nephrotoxins that respectively conform to the above sub-classes (7). Most commonly, however, tubular injury is caused by a localized ischemic state secondary to hypoperfusion of the kidney. Whatever the cause, tubular injury is characterized by a relatively unchanging set of histological features termed acute tubular necrosis (ATN).

Regardless of the immediate cause of renal failure, the mainstays of therapy for ARF are supportive in nature and include maintenance of appropriate intravascular volume as well as preservation of electrolyte homeostasis (8). Renal replacement therapy—in the form of hemodialysis or peritoneal dialysis—is a treatment of last resort for ARF, and associated with a dismal prognosis (9).

Post-ischemic acute tubular necrosis

The proximate cause of post-ischemic ATN is a reduction in effective oxygen carrying capacity and solute redistribution secondary to a decrease in renal blood flow. This can be due to a myriad of causes. Like prerenal failure, renal ischemia can be caused by decreased intravascular volume due to acute blood loss or third-space displacement of intravascular fluid. Iatrogenic causes, including operative interruption of aortic or renovascular blood flow and administration of vasoactive medications such as non-steroidal anti-inflammatory drugs (NSAIDs), are also commonplace. Small-vessel diseases such as the vasculitides can also adversely affect the renal vasculature. In all of these cases, the end result is decreased effective renal blood flow.

Despite the fact that approximately ¼ of systemic arterial blood flow is directed to the kidneys, the renal tubule is uniquely vulnerable to ischemia during states of hypoperfusion. Most renal blood flow is directed to the glomerulus, and the hairpin loops of the vasa recta that descend into the inner medulla closely apposed to the tubules have characteristically low blood flow, which aids in the synthesis of the countercurrent gradient that aids in urinary concentration (10). The cells of the straight (S3) segment of the proximal tubule, located in the outer stripe of the medulla, have been shown to be particularly sensitive to ischemic injury (11).

Following ischemic insult, the kidney tubule undergoes a series of predictable histopathologic changes. The brush border present on proximal tubule cells is sloughed into the lumen, and cellular swelling is observed. In cases of severe injury, this is accompanied by the incomplete denudation of the epithelial basement membrane. It is apparent that both necrosis and apoptosis play a role in the death and sloughing of these proximal tubular cells (12). These changes are most pronounced in the S3 region of the proximal tubule, and to a lesser extent are detected in the thick ascending limb of the loop of Henlé, as these areas are the most metabolically active areas of the tubule (11). The aggregation of the sloughed material is manifested in the formation of intraluminal casts, which can obstruct the tubule lumen and promote further injury via backleak of luminal contents. Additionally, influx of hematopoietically-derived inflammatory cells is apparent both in the tubular interstitium as well as in the intravascular compartment. These histopathologic features are thought to be due to a complex interplay between microvascular and tubular factors; specifically, endothelial damage, arteriolar/capillary increased vasoconstriction and decreased vasodilation, the production of paracrine factors by sublethally-injured tubular epithelial cells, production of cytokines by inflammatory cells, and hydrostatic effects of backleak all contribute to this complex pattern of injury (13).

It is thought that ATP depletion is the major proximal cause of injury during the ischemic phase of ATN. Paradoxically, however, it is during the reperfusion phase of ATN that the bulk of injury occurs. Reactive oxygen species generated by abruptly-reoxygenated tissue macrophages,

mast cells, endothelial cells, and tubule cells themselves cause significant injury through direct cytotoxic actions, activation of calcium-mediated signaling pathways, and recruitment of further inflammatory cells (14). The exact degree of damage caused by the inflammatory infiltrate is hitherto incompletely characterized, but inflammatory cell depletion as well as inhibition of inflammatory cell adhesion to damaged endothelium has been shown to reduce the severity of renal injury in ATN (15, 16).

The investigation of ischemic ATN is made possible by the induction of kidney injury in animals. Currently, many investigations including our own utilize rodent (mouse or rat) models of renal ischemia induced by operative interruption of the renal artery for a specified length of time. These protocols fairly reliably produce a histopathologic pattern similar to that seen in human kidney biopsies as well as a reproducible decrease in GFR if both renal arteries are clamped. However, there are some doubts as to whether this rodent model is an acceptable substitute for post-ischemic ATN as seen in humans (17). Efforts to generate a more "physiologic" pattern of ATN in rodents by the induction of severe hypotension have been hitherto unsuccessful (18).

[image redacted due to electronic copyright restrictions]

FIGURE 1. Human kidney tubule following ischemic injury, hematoxylin & eosin preparation. Note the large numbers of eosinophilic intraluminal casts inside tubules with sloughed (missing) epithelium. The ongoing acute inflammatory response, as demonstrated by the presence of interstitial neutrophils, is evident. (Image courtesy of the Department of Pathology, University of Alabama at Birmingham, via the *Pathology Education Instructional Resource* Digital Library available online at http://peir.net.)

Role of resident renal cells in tubule repair following ischemic injury

Perhaps the most remarkable feature of post-ischemic ATN is the potential for complete recovery of tubule structure and kidney function following ischemia/reperfusion (I/R) injury. There exists a capacity for complete renewal of the kidney tubule despite extensive cell death. This is in stark contrast to other organs such as the heart and nervous system, which have highly limited potential for regeneration following ischemic injury. The cells responsible for this remarkable regenerative ability have been shown to be sporadic de-differentiated cells that appear or survive following injury and proliferate vigorously to reconstitute the denuded tubule epithelium. Three distinct but not mutually exclusive paradigms have been proposed that can explain the identity of these cells (7).

In experimental models of I/R injury, foci of sublethal injury are observed in addition to regions of frank necrosis, apoptosis and de-epithelialization. These surviving cells undergo de-differentiation into a more primitive (mesenchymal) phenotype, which is accompanied by migration and spreading of these cells into areas of basement membrane denudation. In a manner that recapitulates embryonic renal development, these de-differentiated cells enter the cell cycle and divide, undergoing intense proliferation (19). Eventual differentiation of these cells back into an epithelial phenotype occurs, which results in an intact, functional epithelium. The presence of mesenchymal cells in this paradigm has been verified on a molecular level with the identification of upregulated mesenchymal markers such as fibronectin in the cells undergoing spreading and migration (20).

The second and third mechanisms that have been posited as supporting renal tubular regeneration—at least in part—involve the recruitment of stem cells from either a local or a distant niche. A stem cell is defined as an individual cell that has the twin abilities of broad potential and self-renewal; that is, to undergo mitosis in an asymmetric fashion, producing a daughter cell that can differentiate into many cell types as well as another daughter cell that retains the properties of the initial stem cell (21). Different populations of stem cells have different degrees of plasticity: in particular, pluripotent stem cells have the ability to differentiate into all somatic cells from all three germ layers, and totipotent stem cells can differentiate into all somatic cells as well as extraembryonic tissues. During prenatal life these cells give rise to multipotent, tissue-restricted stem cells, which are physically resident in the parenchyma of the end organs during adult life and can repopulate some or all of the parenchymal cells of the organ in question. Conventional thought holds that these multipotent stem cells are absolutely tissue-restricted in their differentiative capacity and can repopulate only the tissues of the end organ in response to injury, and no other organs (22). Indeed, resident organ-specific stem cells have been shown to be responsible for regeneration of all hematopoietic lineages following bone marrow ablation (21) and reconstitution of liver parenchyma subsequent to partial hepatectomy (23), among several other organ systems.

Precise identification of the tissue-restricted renal stem cell—a slowly-cycling mesenchymal cell present in the adult kidney and capable of asymmetrically dividing to both replenish the stem cell population as well as differentiate into kidney epithelial cells—has been elusive. Two investigators have enumerated populations of cells resident in the kidney that retain the synthetic thymidine analogue 5-bromo-2-deoxyuridine (BrdU) long after incorporation, indicating a slow cycling time. These cells are observed to specifically undergo proliferation in response to ischemic injury (24, 25). Whether these cells represent actual renal stem cells or simply slowly-cycling downstream progenitor (also known as transit-amplifying cells) is unclear; however, their importance in contributing to renal repair following ischemic injury cannot be discounted.

Plasticity of bone marrow-derived cells

A significant amount of investigation in the past 15 years has focused on the enhanced plasticity of bone marrow-derived cells. As briefly mentioned above, convention holds that organ-specific stem cells are restricted in their differentiation potential to the organ in question, and that so-called "trans-differentiation" across lineages does not occur. This dogma has subsequently been disproved, specifically in the case of the plasticity of bone marrow-derived cells following transplantation. Bone marrow-derived cells are uniquely suited to serve as an extra-parenchymal source of stem cells due to the fact that they have the ability to circulate in the bloodstream.

Several investigations have demonstrated the ability of bone marrow-derived cells to cross lineage boundaries and differentiate into functional, non-hematopoietic tissues in a post-natal environment. Gussoni et al. and Ferrari et al. verified the existence of bone marrow-derived cells in regenerating muscle tissue following bone marrow transplant (26, 27). The reconstitution of functional hepatocytes from bone marrow precursors has been shown in both mouse (28, 29) and human (30) experiments. Following myeloablation and stem cell transplantation of a single hematopoietic stem cell, Krause et al. demonstrated the engraftment of donor-derived cells in liver, lung, epithelial tissues, and the GI tract (31). Orlic et al. confirmed that circulating bone marrow-derived cells contributed to myocardium following the induction of mechanical cardiac ischemia (32). Even bone marrow contribution to neuronal cells—traditionally thought to have minimal regenerative capacity—has been demonstrated (33).

One common feature pervading many of these experiments is the induction of a state of regeneration in the end organ to be assayed for bone marrow contribution. Most commonly, this occurs either in the setting of acute injury (e.g. myocardial ischemia) or chronic turnover of differentiated cells (e.g. *mdx* mouse model of Duchenne muscular dystrophy). It is this author's experience, having worked extensively with cross-lineage differentiation of bone marrow into mus-

cle (34), that contribution of bone marrow to quiescent, non-damaged end organs is minimal or absent. This is thought to be due, at least in part, to the release of cytokines and growth factors at the site of injury that cause mobilization from the bone marrow and subsequent chemotaxis of inflammatory and (presumably) stem cells.

Contribution of bone marrow-derived cells to tubules following ischemic injury

Bone marrow contribution to kidney tubule epithelium following ischemic injury has been extensively studied in the near past as well. Evidence of bone marrow contribution to tubule epithelium was first found in human male recipients of female kidneys, which allowed the use of the powerful Y-chromosome fluorescence *in situ* hybridization (FISH) technique to visualize Y-chromosome-containing cells of bone marrow origin. Poulsom et al. and Gupta et al. examined biopsy specimens of male recipients of female kidney transplants via Y-chromosome FISH and found small but significant numbers of recipient-derived cells that co-expressed the epithelial marker cytokeratin upon colocalization by immunohistochemistry (35, 36). Notably, these cells were found only in kidneys that had undergone damage following transplantation.

Several prospective analyses using whole and lineage-depleted bone marrow transplantation in mice, including results from our group, have further supported the conclusion that bone marrow cells contribute directly to tubular epithelium following ischemia/reperfusion injury. Both Lin et al. and Kale et al. used a model involving the transplantation of bone marrow from a mouse expressing the β-galactosidase transgene followed by ischemia/reperfusion injury, with 20-80% of tubules in the outer medulla of the injured kidney containing at least one cell that expressed the transgene (37-39). Other groups using the Y-chromosome FISH technique have found significantly lower numbers of bone marrow-derived cells making up tubular epithelium (36).

Functional changes in laboratory parameters of ARF have also been examined by our group following manipulation of the bone marrow compartment. Unsurprisingly, the typical

laboratory evaluation of a patient suffering from ARF reveals a predictable rise in BUN and creatinine—serum markers of renal failure—that will trend back to baseline following resolution of the renal failure. In mice, radioablation of the bone marrow followed by bilateral ischemia/reperfusion injury to induce acute renal failure resulted in significantly higher elevations of serum markers than seen without the bone marrow transplant. Intravenous infusion of lineage-depleted whole bone marrow cells after radioablation and bilateral I/R injury yields an initial rise in serum markers comparable to radioablation alone, followed by an abrogation of this exaggeration back to control levels (37). This phenomenon of initial injury followed by rescue points away from bone marrow-derived cells contributing directly to tubule regeneration, as one would expect the magnitude of initial injury to be decreased if the cells were able to positively contribute directly. Instead, it appears as though the infused cells interact with the regenerating tubule in a *trans*-acting manner.

Identification, isolation and expansion of highly plastic bone marrow cells

Whole bone marrow consists of two lineages of cells, each with a characteristic stem cell. The hematopoietic stem cell (HSC) can reconstitute all lymphoid, myeloid and erythroid lineages, and has been extensively investigated and fully characterized. The function of the marrow stromal cell or mesenchymal stem cell (MSC), the other stem cell present in the bone marrow compartment, is more elusive. MSC are a heterogeneous population of cells characterized by as well as isolated via adherence to plastic tissue culture vessels. Morphologically, they appear stromal in phenotype, and possess remarkably few stem cell surface markers to aid in immunologic isolation and characterization. That being stated, however, human MSC have been shown to differentiate *in vitro* upon osteoblastic, chondroblastic and adipocytic lineages and are easily maintained in liquid culture for many population doublings (40). They have a broad, active immunosuppressive action mediated by an as-yet-uncharacterized elaborated factor (41, 42); the rodent equivalents of these cells have been shown by several investigators to have renoprotective effects in

models of renal ischemia, which could be postulated to be related to this immunomodulatory action (43-45).

Because of these properties, intense recent investigation into the potential plasticity of MSC has been undertaken. Perhaps the most promising result was published by Johnson et al., who have identified bone marrow-derived cells capable of reconstitution of the mouse ovary with cells that appear histologically to be oöcytes and that express oöcyte markers (46). These cells were expanded from a population of whole bone marrow cells by treating bone chips with collagenase and collecting the resultant single cell suspension. A rare subset of these cells, in turn, was shown to give rise to oöcytes in chemotherapy-sterilized mice. Since *in vivo* contribution of bone marrow to regenerating kidney epithelium is only seen in low numbers, cells that have the ability to be easily isolated, expanded *ex vivo*, and reinjected intravenously in order to reconstitute damaged epithelium would be fantastically important from a clinical standpoint. To this end, we designed a series of *in vitro* experiments that took advantage of Dr. Johnson's bone marrow preparation techniques to determine if a population of bone marrow-derived cells that crossed lineages to differentiate into epithelium could be isolated.

STATEMENT OF PURPOSE

The general aim of this project is to further elucidate the contribution of bone marrow cells to the regenerating kidney following the induction of acute tubular necrosis via ischemia/reperfusion injury. It appears that bone marrow-derived cells contribute in some way to kidney tubular regeneration; however, the nature as well as the proportion of this contribution is currently incompletely understood. In this series of experiments, a stem cell transplantation model was used to determine the nature of the interaction between bone marrow-derived cells and the regenerating kidney epithelium, and to discern whether marrow-derived cells have the capacity to differentiate into kidney epithelium itself. Additionally, *in vitro* experiments were conducted to establish whether a selected population of bone marrow-derived cells subjected to specific culture conditions could be induced to differentiate into epithelium.

METHODS

Mice

Inbred C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and the National Cancer Institute (Frederick, MD). Six-to-eight week old male mice were used as stem cell transplantation (SCT) donors, and six-to-eight week old female mice were used as SCT recipients. Four-to-eight week old male mice were used as sources of whole, unfractionated bone marrow for *in vitro* culture.

Ischemia/reperfusion injury and stem cell transplantation

The ischemic injury and transplantation portions of these experiments were carried out exclusively by Dr. Sujata Kale (Yale University School of Medicine, New Haven, CT) and have been described previously (37). Prior approval was obtained from the Yale Institutional Animal Care and Use Committee.

Femurs and tibias were aseptically collected from male donor mice, and whole bone marrow was flushed from the medullary cavity of the bones. The erythrocytes were specifically lysed using BD PharmLyse (BD Biosciences, San Jose, CA) as per the manufacturer's recommendations. Subsequent depletion of cells committed to hematopoietic lineages was accomplished using a cocktail of biotin-conjugated monoclonal antibodies directed against the mouse hematopoietic lineage markers B220, $CD3_{\varepsilon}$, $CD11_{b}$, Gr-1, and Ter119 (BD PharMingen, San Jose, CA), streptavidin-conjugated magnetic beads, and a midiMACS LD depletion column (both Miltenyi Biotec, Bergich Gladbach, Germany).

Recipient female mice were administered a 3.5 Gy dose of gamma-irradiation using a sealed-source ^{137}Cs irradiator. Following this, 5.0×10^5 cells suspended in small volume of PBS were injected intravenously into each mouse via retro-orbital puncture and housed in specific pathogen-free conditions.

Twelve hours following transplantation, the female mice described above were anesthetized using 100 mg/kg ketamine and 10 mg/kg xylazine administered intraperitoneally. Using sterile surgical technique, a midline incision was performed and subsequently the peritoneum was incised. The left kidney was isolated using blunt dissection, and the left vascular pedicle was isolated. Using a vascular clamp (Fine Science Tools, Foster City, CA) the pedicle was completely cross-clamped, and renal ischemia was verified by observation of color change of the kidney. Saline-moistened gauze was placed over the peritoneal incision, and the mouse was maintained at 37°C using a heating pad. After 25 minutes of renal ischemia, the clamp was removed and reperfusion was ascertained by direct visualization.

The mice were kept under specific pathogen free conditions until they were sacrificed at 7 days following renal injury.

Specimen preparation

Both the clamped and unclamped kidneys in each animal were subjected to analysis. Kidneys were dissected from the abdominal cavity immediately following sacrifice, and were immediately fixed in 10% neutral buffered formalin solution (Sigma-Aldrich, St. Louis, MO) for 1 hour. They were then placed in 100% ethanol and transported to the surgical pathology core facility, which performed paraffin embedding and microtome sectioning. Longitudinal 4 μm microtome sections on conventional glass slides were obtained.

Fluorescence in-situ hybridization probe preparation

DNA template and protocol for synthesis of the fluorescence in-situ hybridization (FISH) probe against the Y-chromosome was kindly provided by Dr. Diane Krause (Yale University School of Medicine, New Haven, CT). This probe has been extensively characterized (47) and its preparation has been previously described (29); the most recent version of this protocol is available on the Internet (48). Briefly, a degenerate oligonucleotide-primed Polymerase Chain Reac-

tion (DOP-PCR) reaction was initially performed using previously-amplified mouse Y-chromosome DNA as a template and the 6AI oligonucleotide (CCG ACT CGA GNN NNN NTA CAC C) as primer. 30 total cycles were run using a Peltier thermal cycler (MJ Research, Waltham, MA). Eight reactions were run in parallel and subsequently pooled and precipitated overnight using sodium acetate and excess ice-cold absolute ethanol.

The resulting pellet was washed in cold ethanol and resuspended in distilled, deionized water. This solution was aliquoted and mixed 5:1 by volume with DIG Nick Translation mix (Roche Applied Sciences, Mannheim, Germany) containing the digoxigenin-11-dUTP labeled nucleotide. This mixture was allowed to incubate for 90 minutes at 15°C, after which the size of the now-labeled probe was verified to be between 100 and 500 base pairs via conventional agarose gel electrophoresis. The reaction was stopped with the addition of 0.5 *M* EDTA and the labeled probe was precipitated using ice-cold excess ethanol, sonicated salmon sperm and Cot-1 carrier DNA (both Invitrogen, Carlsbad, CA). The precipitated probe was pelleted in a microcentrifuge and washed with 70% ethanol, following which it was pelleted again and resuspended in deionized formamide (American Bioanalytical, Natick, MA). This was combined in equal volume with a hybridization buffer consisting of 20% dextran sulfate in 4X SSC.

Fluorescence in-situ hybridization

The FISH procedure was performed according to instructions kindly provided by Dr. Diane Krause. Slides of interest were heated to 95°C and deparaffinized in CitriSolv, a xylene substitute (Fisher Scientific, Waltham, MA). Slides were then subjected to rehydration washes in graded ethanol/water solutions and washed vigorously in phosphate-buffered saline (PBS). Antigen retrieval was performed using the citrate-based Retrievagen A (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

Following antigen retrieval and a wash step, slides were incubated for 12 minutes in a solution of 0.2 *N* HCl. After this, they were washed with two sequential changes of PBS and in-

cubated for 20 minutes in a 1 *M* NaSCN solution at a temperature of 72°C. Slides were then taken through three sequential changes of PBS and dehydrated in serial solutions of ethanol/water, and carefully wiped. 12 μL of FISH probe, prepared as above and pre-warmed to 75°C, was applied to each slide, after which they were coverslipped and sealed with rubber cement. Slides were hybridized overnight in a humid chamber at 37°C.

After the overnight incubation, the rubber cement sealant was removed and the slides were immersed in 2X SSC solution to remove coverslips. Following this, the slides were submitted to two further washes of 2X SSC, and then incubated for five minutes in a stringency wash consisting of 0.4X SSC and 0.3% NP-40 detergent at 55-65°C. They were washed twice in 2X SSC and blocked for ten minutes in a blocking solution consisting of 2X SSC, 3% protease and immunoglobulin-free bovine serum albumin (BSA) (Jackson Immuno Research, West Grove, PA) and 0.1% Tween-20 at room temperature. Following this, rhodamine-conjugated anti-digoxin Fab fragments (Roche, Mannheim, Germany) at a concentration of 1 μg/mL was applied to the slides, which where incubated for 45 minutes at room temperature. Slides were then subjected to two final washes in PBS, after which they were counterstained using immunofluorescence techniques as described below.

Immunofluorescence staining of hybridized sections

Primary antibodies used in immunofluorescence colocalization experiments were as follows: rabbit polyclonal anti megalin (considerate gift of Dr. Dan Biemesderfer) used at 1:500 dilution; sheep polyclonal anti-human Tamm-Horsfall protein (Serotec, Raleigh, NC) used at 1:2000 dilution; and biotinylated mouse anti-mouse lineage panel (BD PharMingen, San Jose, CA) used at 1:500 dilution per constituent antibody. Secondary immunoreactants were, respectively, Alexa Fluor 488-conjugated donkey anti-sheep IgG used at 1:200 dilution; Alexa Fluor 488-conjugated goat anti-rat IgG used at 1:200 dilution (both Molecular Probes/Invitrogen, Carlsbad, CA); and fluorescein-Avidin D (Vector Labs, Burlingame, CA) used at 1:250 dilution.

Following *in situ* hybridization as described above, slides were blocked in a blocking solution consisting of 1% BSA or 10% goat or donkey serum (Jackson Immuno Research, West Grove, PA) in PBS for 45 minutes at room temperature. Excess blocking solution was removed from the slide, and primary antibody diluted as above in blocking solution was applied to the section, which was incubated for one hour at room temperature. Slides were then washed in three changes of PBS, and the appropriate secondary antibody diluted in blocking solution was applied was applied to the sections. Slides were incubated for ½ hour. Finally, slides were washed three more times in PBS (the final wash supplemented with 0.1% Tween-20) and mounted in Vectashield plus DAPI (Vector Labs, Burlingame, CA).

Epifluorescent and Hoffman Modulation Contrast microscopy was performed on an inverted Nikon TE200 microscope outfitted with dry 10X, 20X and 40X Plan-Apochromat objectives (Nikon, Melville, NY). Images were obtained using a SPOT RT CCD camera running SPOT Advanced software (Diagnostic Instruments, Sterling Heights, MI). Image analysis was performed using Photoshop 7.0 and Illustrator CS 2(Adobe Systems Inc., San Jose, CA).

Bone marrow isolation and primary culture

To obtain collagenase-released marrow stromal cells (CR-MSC), mice as described above were sacrificed by administration of a lethal dose of isoflurane anesthetic or by carbon dioxide asphyxiation. Femurs and tibias were aseptically collected into a 50 mL conical tube (BD Biosciences, San Jose, CA) filled with 5 mL of Dulbecco's Modified Eagle Medium (DMEM) with 25 mM HEPES buffer (EmbryoMax, Millipore Biosciences, Temecula, CA). Using a sterile, porcelain mortar and pestle (CoorsTek, Golden, CO) the bones were mechanically disrupted for 10-15 seconds. Following this, the resultant cell suspension was aspirated and fresh medium was added. This procedure was repeated for three total iterations. The remaining bone chips were flushed with PBS over a 40 μm nylon mesh cell strainer (BD Biosciences, San Jose, CA) and placed in a 100 mm tissue culture-treated Petri dish (BD Biosciences, San Jose, CA).

In order to dissociate cells from the bone chips, a collagenase solution consisting of 200 activity units/mL crude collagenase CLS-2 (Worthington Biochemical, Lakewood, NJ) was prepared in DMEM and sterilized via 0.22 μm syringe filtration. 20 mL of this solution was added to the bone chips and the dish was incubated for two hours at 37°C in 5% carbon dioxide. This slurry was mechanically triturated via serological pipet 2-3 times during this period.

Following incubation, approximately 30 mL of CR-MSC medium was added to the slurry and mechanically triturated via serological pipet. This medium consisted of DMEM/HEPES + 10% characterized fetal bovine serum (Hyclone, Logan, UT) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (all Invitrogen, Carlsbad, CA). A single-cell suspension was obtained by straining the solution through 40 μm nylon mesh, and the cells were pelleted in a swinging-bucket centrifuge at 400 x *g* for ten minutes. The cells were washed twice with 50 mL volumes of PBS, centrifuging between each wash, and finally resuspended in CR-MSC medium. Cells were plated onto tissue culture-treated surfaces in either 100 mm Petri dish or one or two well LabTek Chamberslides II (Nalge Nunc International, Rochester, NY) configurations, and incubated at 37°C at 5% CO_2.

24 to 36 hours following initial plating, medium and non-adherent cells were aspirated and the remaining adherent cells were vigorously washed with a single aliquot of PBS, after which a complete medium change was performed. Following this, complete media changes were performed every other day. Cells were not passaged until preparation for flow cytometric analysis or RT-PCR.

Cell lines

The immortalized inner medullary collecting duct (IMCD) mouse cell line (49) was obtained from the American Type Culture Collection. Cells were grown at 37°C in 5% carbon dioxide in IMCD medium, which consisted of 50% DMEM / 50% Ham's F12 Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL strepto-

mycin, and 250 ng/mL amphotericin B (all Invitrogen, Carlsbad, CA). Cells were passaged at an approximate ratio of 1:5 every 3 days using 0.05% trypsin-EDTA.

The immortalized mouse fibroblast cell line 3T3 was obtained from the American Type Culture Collection. Cells were grown at 37°C in 5% carbon dioxide in complete medium, which consisted of DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (all Invitrogen, Carlsbad, CA). Cells were passaged at an approximate ratio of 1:3 every 3 days using 0.05% trypsin-EDTA.

IMCD and 3T3 cells were used variously as positive and negative controls for reverse-transcriptase Polymerase Chain Reaction (RT-PCR) experiments as well as immunofluorescence cytology controls.

Immunofluorescence cytology

Adherent cells (including CR-MSC and appropriate controls) to be analyzed using immunofluorescence markers were stained after 7-14 days of growth on Chamberslides. Media was aspirated and the slides were washed once in PBS, and then fixed for exactly five minutes with ice-cold 100% methanol, followed by a second wash in PBS. Afterwards, the protocol described above for immunofluorescent staining following *in situ* hybridization was applied, with exceptions noted below.

Primary antibodies used for staining cultured cells included: mouse monoclonal anti-pan-cytokeratin (clone C-11, Sigma-Aldrich, St. Louis, MO) used at 1:100 dilution; mouse monoclonal anti-ZO-1 (clone R40.76, Chemicon, Temecula, CA) used at 1:200 dilution; and rat monoclonal anti-mouse CD45 (clone 30.F11, BD-PharMingen, San Jose, CA). Alexa Fluor 488 and 594-conjugated goat anti-mouse and goat anti-rat, were used at 1:200 dilution as secondary immunoreactants. Note that the use of mouse primary antibodies on mouse tissues necessitated the addition of a step to block mouse IgG non-specifically binding to mouse tissue. This was ef-

fected by increasing the blocking time, when applicable, to 75 minutes, adding goat anti-mouse Fab fragments (Jackson Immuno Research, West Grove, PA) at a concentration of 5 μg/mL for the final 60 minutes of the blockade step.

RT-PCR

RT-PCR experiments were pursued in order to analyze expression of epithelial and mesenchymal genes on a message level. All RT-PCR experiments were performed with the assistance of Jiankan Guo, PhD. Cells to be isolated for RT-PCR were prepared from CR-MSC, 3T3 and IMCD cells grown as above. Colonies of 100-150 CR-MSC (on day 14 of growth) were isolated using plastic cloning cylinders and detached from the tissue culture surface with 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA). Cells were pelleted via microcentrifuge and then homogenized using QIAshredder columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total cellular RNA was then prepared using RNeasy Mini kits (Qiagen, Hilden, Germany) according to provided instructions, and eluted in 30 μL of buffer.

Following the isolation of total cellular RNA, a reverse transcription (RT) reaction was undertaken. Each reaction was prepared in an 0.8 mL thin-walled microcentrifuge tube, to which were added the following:

- Mixed dNTPs, 2m*M* — 1 μL
- 5X RT-PCR buffer — 4 μL
- Dithiothreitol, 0.1 m*M* — 0.2 μL
- Random primers, 3 μg/μL — 0.5 μL
- Total cellular RNA — 1 μL
- DEPC-treated dH_2O — 13 μL
- Superscript RT, 200 units/μL — 1 μL

After addition of the RT and vortexing to mix, the reaction mixture was incubated at 42°C for one hour, followed by a five minute incubation at 95°C to inactivate the RT enzyme. Resulting cDNAs were frozen at -80°C.

Standard PCR and subsequent gel electrophoresis were performed to amplify and examine specific cDNAs of interest. PCR primers were designed using the ENSEMBL genome

browsing software available on the Internet at http://www.ensembl.org/Mus_musculus/index.html (50). Primers were designed to span adjacent exons so only messenger RNA would be amplified. Sequence uniqueness was verified using the NCBI Basic Local Alignment Search Tool available at http://www.ncbi.nlm.nih.gov/BLAST/ (51). Primer pairs included the following:

	Forward primer	**Reverse primer**
Cytokeratin-8	TCA AGA ATA AGT ATG AGG AT	AGA CTC CAG CCT GCT CTC CT
Vimentin	GCA CGA TGA AGA GAT CCA GG	AGA AAT CCT GCT CTC CTC GC
E-cadherin	CTG GGC AGA GTG AGA TTT GA	CCT GTT GGA TTT GAT CTG AAC C
Fsp-1	CTT GGT CTG GTAC TCA ACG GT	TCT GTC CTT TTC CCC AGG AAG

Forward and reverse primers for the β-actin gene, which served as a positive reaction control, were thoughtfully provided by Jiankan Guo.

PCR reactions were prepared in thin-walled tubes, to which were added the following reagents:

- Sigma REDTaq ReadyMix (Sigma-Aldrich cat. #R2523) 12.5 µL
- Forward primer (0.5 µ*M*) 0.5 µL
- Reverse primer (0.5 µ*M*) 0.5 µL
- Template cDNA 1 µL
- RNAse-free H_2O 10.5 µL

The PCR was run in a Peltier thermal cycler using the following protocol:

step		
1	94°C	2 minutes
2	94°C	30 seconds
3	5°C	30 seconds
4	72°C	30 seconds
5	Steps 2-4	35 cycles
6	72°C	5 minutes
7	4°C	indefinitely

PCR products were subjected to agarose gel electrophoresis on a 2% agarose/TAE gel supplemented with Gel-Star (Cambrex, East Rutherford, NJ) at a dilution of 1:10000 as recommended. Visualization of bands was accomplished using a Kodak Gel Logic ultra-violet transilluminator and computerized gel acquisition system (both Kodak Molecular Imaging, New Haven, CT).

RESULTS

Bone marrow contribution to renal repair

We first set out to determine the contribution to regenerating tubular epithelium by bone marrow cells using the Y-chromosome FISH procedure. We speculate that the number of tubule epithelium cells observed as positive using this procedure will be more representative of the true rate of contribution than the problematic β-galactosidase transgene model, which may not adequately report a true proportion of donor-derived kidney cells.

To quantify the sensitivity and specificity of the Y-chromosome FISH stain, control mouse male kidney sections were subjected to Y-chromosome FISH and examined microscopically (Figure 2). Y-chromosome-positive nuclei were identified by direct visualization of intranuclear signal in the red channel with a corresponding lack of non-specific signal in the green and blue channels.

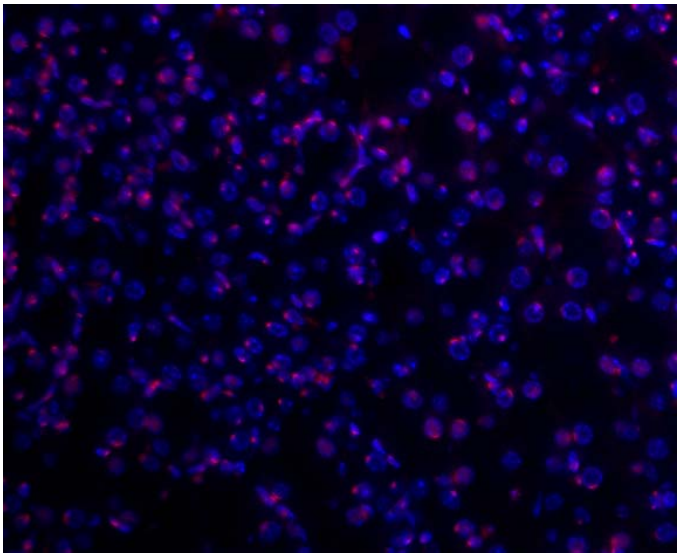

FIGURE 2. Y-chromosome FISH stain, male mouse kidney, 100X total magnification. Approximately 59% of nuclei have a visible Y-chromosome FISH signal. No Y-chromosome containing cells were observed in female kidney sections stained with the FISH probe.

Unsurprisingly, no Y-chromosome-positive nuclei were observed in any of the female kidney sections examined. Examination of approximately 1400 total nuclei across several high powered fields of male kidney revealed that 59% of all nuclei contained visibly staining Y-chromosomes. This corresponds with previously published reports of Y-chromosome FISH efficacy.

To determine the contribution of bone marrow cells to regenerating kidney tubules following ischemic injury, co-localization experiments were performed. Following radiologic bone marrow ablation and unilateral ischemia/reperfusion injury, female recipient mice underwent transplantation via intravenous infusion of 0.5×10^6 lineage-depleted male bone marrow cells. These animals were sacrificed after seven days and their kidneys examined for donor cell contribution. The proximal and distal tubules were identified via positive staining with antibodies against the brush border protein megalin (specifically expressed by proximal tubular epithelium) as well as Tamm-Horsfall protein which is specifically expressed by the TAL. Colocalization of Y-chromosome signal and tubular epithelial markers was noted in both the kidneys subjected to ischemic injury as well as the contralateral (uninjured) kidney (Figures 3-4).

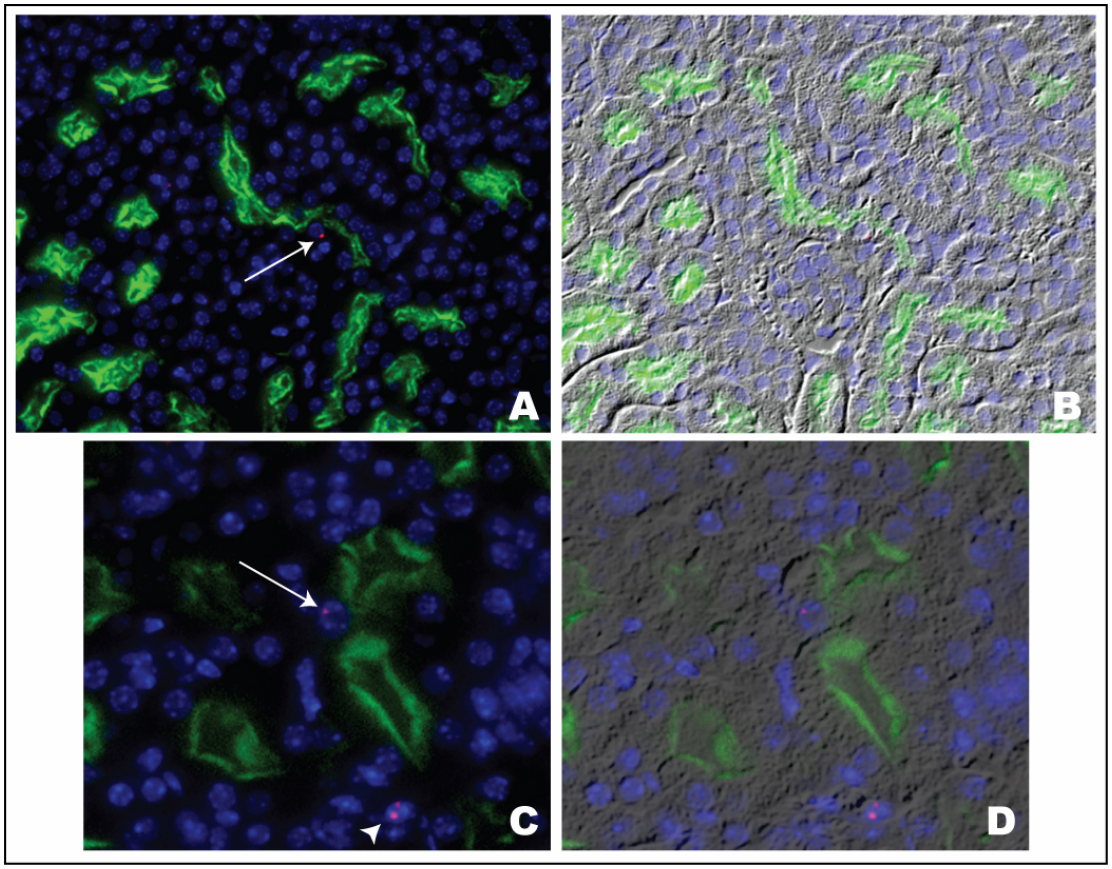

FIGURE 3. **A.** Megalin and Y-chromosome FISH colocalization micrograph, uninjured kidney, 100X. Y-chromosome FISH signal is shown in red; megalin in green, and nuclei (DAPI) in blue. The arrow denotes a rare Y-chromosome positive nucleus that appears to lie within the basement membrane of proximal tubular epithelium. **B**. Same micrograph as 3**A**. with Hoffman Modulation Contrast image overlaid, more clearly showing tubular basement membrane designating the borders of the tubular basement membrane. **C.** Megalin and Y-chromosome FISH colocalization micrograph, injured kidney, 200X. ; The arrow denotes a rare Y-chromosome positive nucleus that appears to lie within the basement membrane of proximal tubular epithelium, and arrowheads denote the more frequent Y-chromosome positive nuclei that lie within the interstitium. **D.** Same micrograph as 3**C**. with Hoffman Modulation Contrast image overlay.

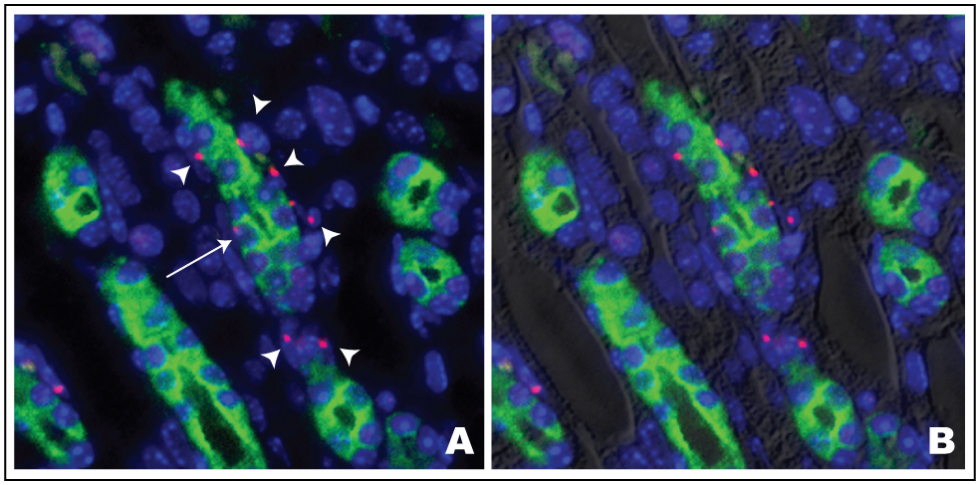

FIGURE 4. **A.** Tamm-Horsfall protein and Y-chromosome FISH colocalization, injured kidney, 200X. Y-chromosome FISH signal is shown in red, Tamm-Horsfall protein staining in green, and nuclei (DAPI) in blue. The arrow denotes a Y-chromosome positive nucleus that appears to lie within the margins of THP staining. Arrowheads denote Y-chromosome positive cells lying immediately subjacent to but definitively outside THP-positive tubules. **B.** Same photomicrograph as Figure 4**A** overlaid with Hoffman Modulation Contrast image to better delineate the tubular basement membrane.

Infrequently, donor bone marrow-derived cells were found within the tubular epithelial layer, primarily in the outer stripe of the medulla. It is apparent from the included representative photomicrographs that colocalization of the Y-chromosome and tubular markers was a relatively rare finding in these analyses, even in the kidneys subjected to ischemia/reperfusion injury. The contralateral, uninjured kidneys in some cases contained Y-chromosome positive cells, but even more infrequently were cells found that also appeared within the tubular epithelium. In both instances, we quantitated the number of tubules that contained at least one Y-chromosome positive tubular epithelial cell. The analysis was conducted across several randomly-chosen high-powered fields located in the outer medullary stripe in both injured and control kidneys from two different timepoints post-transplantation (Figure 5).

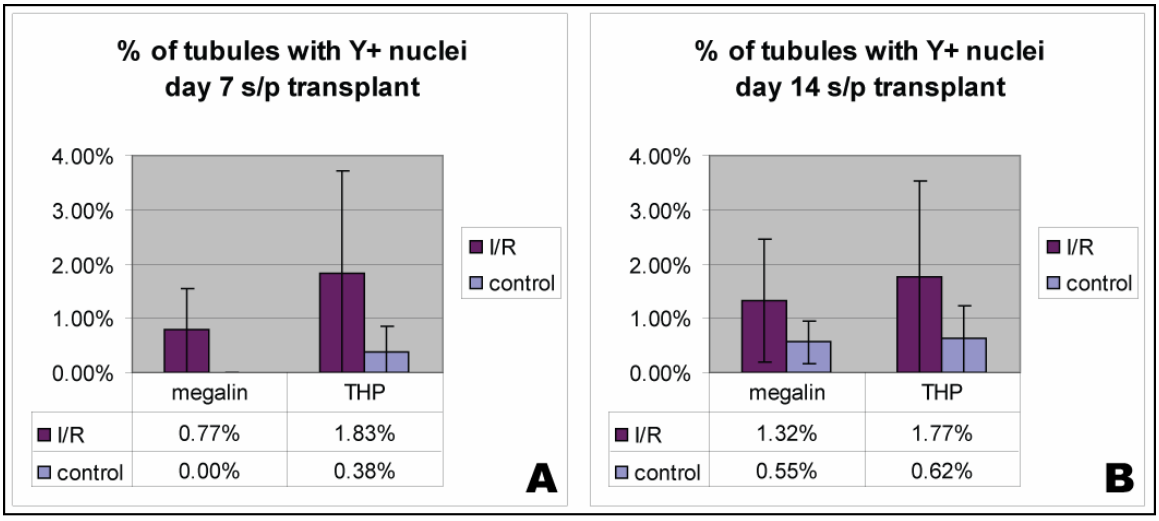

FIGURE 5. Proportion of stained tubules containing a Y-chromosome positive cell within the tubule epithelium at seven (**A**) or 14 (**B**) days post-transplant. The number of "double positive" tubules in both series was extremely low, and no significant difference was observed between proximal (megalin-positive) and distal (THP-positive) tubules, nor between injured and uninjured (control) sections.

The proportion of tubules containing Y-chromosome positive cells was considerably less than the percent donor-derived tubules in our group's previous experiments utilizing transplantation with β-galactosidase-positive bone marrow. The total (megalin-positive or proximal and THP-positive or distal, combined) number of tubules containing Y-chromosome positive nuclei was 2.6% to 3.1% of all outer stripe tubules visualized. When this number is corrected for the assay's inherent sensitivity of 59%, approximately 4.4 to 5.3% of all tubules were estimated to contain cells of donor origin. Determination of whether a particular nucleus containing a Y-chromosome FISH signal could be localized to the inside or outside of the tubule basement membrane was extremely difficult by epifluorescence microscopy, and these figures represent a generous assessment of all possible nuclei that appeared to lie within the basement membrane.

There appeared to be no significant difference between contribution to proximal and distal tubules. Although control kidneys had overall fewer Y-chromosome positive nuclei resident in tubules than injured kidneys, this difference is also not significant. Anecdotally, donor contri-

butions to kidney epithelium were highest in the outer stripe of the medulla, and were not visualized in the inner medulla or cortex.

From this series of experiments we concluded that bone marrow contribution to tubule epithelium could likely occur, but that it was a rare phenomenon. We estimate that less than 0.01% of total kidney epithelial cells in injured kidneys were derived from bone marrow (7). This finding contrasts with our group's previous data showing up to 20% of tubules contain bone marrow-derived cells. This discrepancy is likely explained by the non-specific nature of the β-galactosidase reporter gene as an effective screen of donor vs. recipient cells in this context. We also speculate that this sparse contribution of bone marrow cells is unlikely to produce the functional/laboratory amelioration of the acute renal failure observed following bilateral ischemia/reperfusion injury.

`The vast majority of Y-chromosome positive cells we visualized were located between tubules, in the interstitial spaces of the kidney parenchyma. Often, these cells were found closely apposed to the basolateral surface of the tubule, just outside the basement membrane. These cells were always present in far greater numbers than Y-chromosome positive tubular epithelial cells. It is speculated that the functional amelioration of acute renal failure alluded to above might be due in part to bone marrow cells engrafting in the interstitium and exerting a paracrine or *trans*-acting effect on regenerating tubular epithelium. To rule out a simple inflammatory reaction, we examined kidney sections of mice sacrificed twelve weeks following transplantation (to allow the vigorous inflammatory response provoked by ATN to subside). Sections were subjected to Y-chromosome FISH followed by indirect immunofluorescent staining with a cocktail of antibodies directed against hematopoietic lineage antigens, to enumerate inflammatory cells (Figure 6).

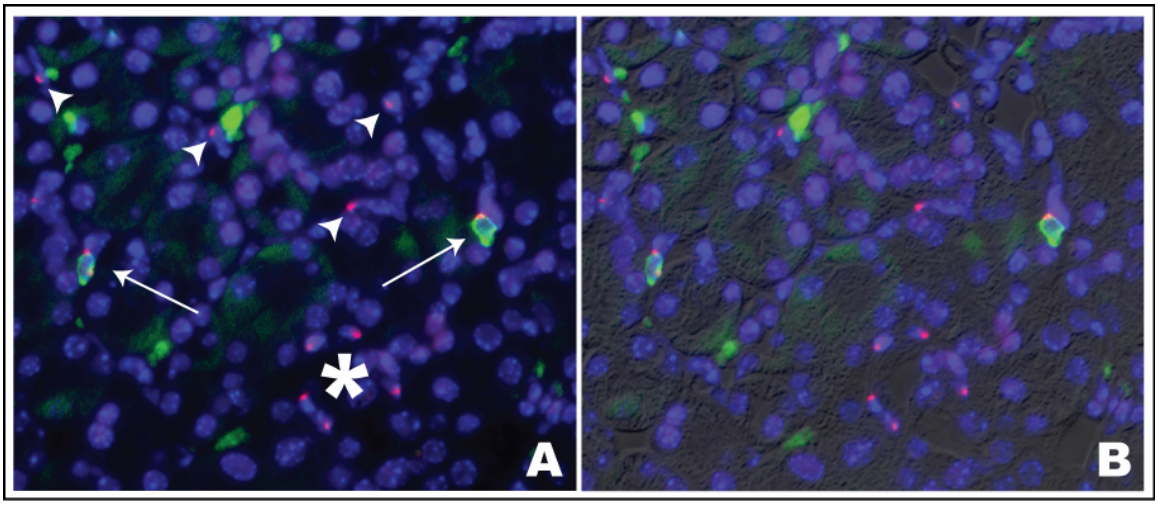

FIGURE 6. **A.** Injured kidney 12 weeks status-post bone marrow transplantation, 200X. Y-chromosome staining is shown in red; lineage markers in green; nuclei (DAPI) in blue. Sporadic lineage-positive donor-derived cells are observed (arrows) but the majority of donor-derived cells lie clearly in the tubule interstitium (arrowheads). The asterix denotes a glomerulus. **B.** Same photomicrograph as 6**A.** but with Hoffman Modulation contrast image overlay.

After the inflammatory reaction is allowed to subside, the majority of interstitial donor-derived cells are lineage-negative. As expected, no lineage-positive tubular cells were observed. Lineage-positive interstitial inflammatory cells are unlikely to mediate the renoprotective effect of infusion of bone marrow cells, so it is postulated that another type of bone marrow-derived resident cell is responsible for this effect.

In vitro *expansion of epithelial cells derived from bone marrow*

A second series of experiments undertaken by our group attempted to expand and characterize epithelial cells *in vitro* from a bone marrow preparation. A member of our group had serendipitously grown a colony of epithelioid cells from bone marrow previously (Figure 7) but these results were not pursued. This was intriguing because, apart from endothelium, there are no epithelial cells located within bone or bone marrow (stromal elements are mesenchymal and appear fibroblastic in liquid culture, and hematopoietic cells are generally round mononuclear or polynuclear cells). The concept of epithelial cells able to be easily expanded from accessible

specimens would also give further insight into whether the sparse bone marrow contribution to regenerating tubular epithelium could be further augmented.

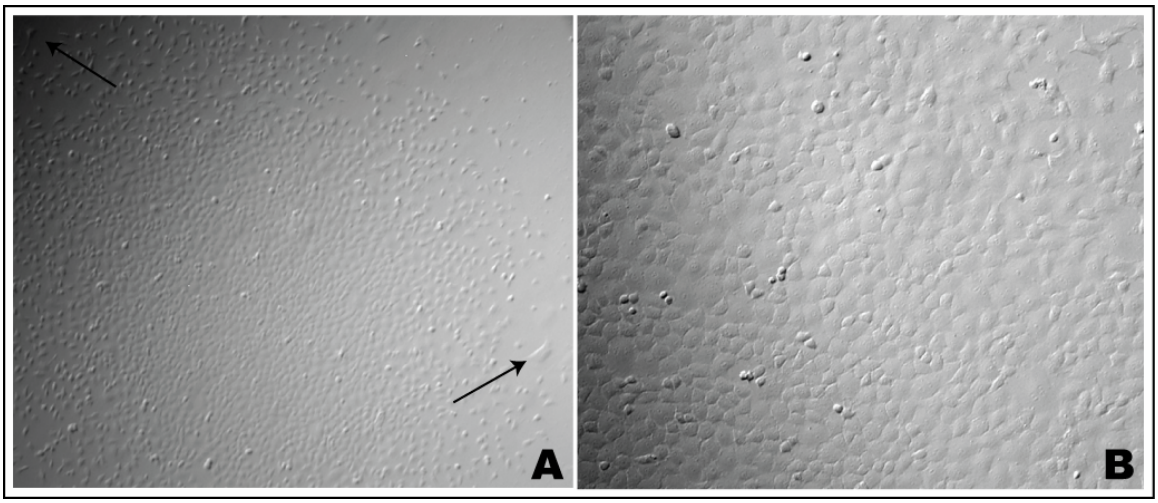

FIGURE 7. **A.** and **B.** Epithelioid colonies via Hoffman Modulation Contrast microscopy, 40X and 100X total magnification. While some cells appear to be fibroblastic and thus similar to mouse MSC (arrows) the majority of the cells are epithelioid, polygonal-shaped cells that make what appear to be tight junctions with adjacent cells.

These cells appear to form a contiguous epithelial layer with apparent tight junctions between adjacent cells, with occasionally interspersed triangular or fibroblast-like cells (with pseudopod extension). It was unclear whether these cells represented a clonal population derived from a single undifferentiated bone marrow cell, or whether this was simply a novel, undescribed shape taken on by the normally-triangular fibroblast cells grown from mechanically-dissociated bone.

Using these results as a guideline, we empirically created and optimized a protocol for isolating these collagenase-released MSC (CR-MSC), described in detail in this report. In brief, bones from juvenile mice were mechanically dissociated using a mortar and pestle, and the resulting bone chips were subjected to crude collagenase digestion for two hours. The cells were then washed and plated in fetal bovine serum-containing medium for 5+ days without passaging.

From these preparations, we were routinely able to identify colonies of epithelioid cells among plates containing largely groups of fibroblastic cells. The colonies were first visualized on day #3 of culture. Attempts to subculture the colonies via trypsinization and passage were unsuc-

cessful, as only fibroblastic cells and no epithelioid colonies could be identified on plates following passage. As previously, these cells were polygonal and closely apposed to adjacent epithelial cells; also appreciated were fibroblastoid cells at the periphery of the colony (Figure 8).

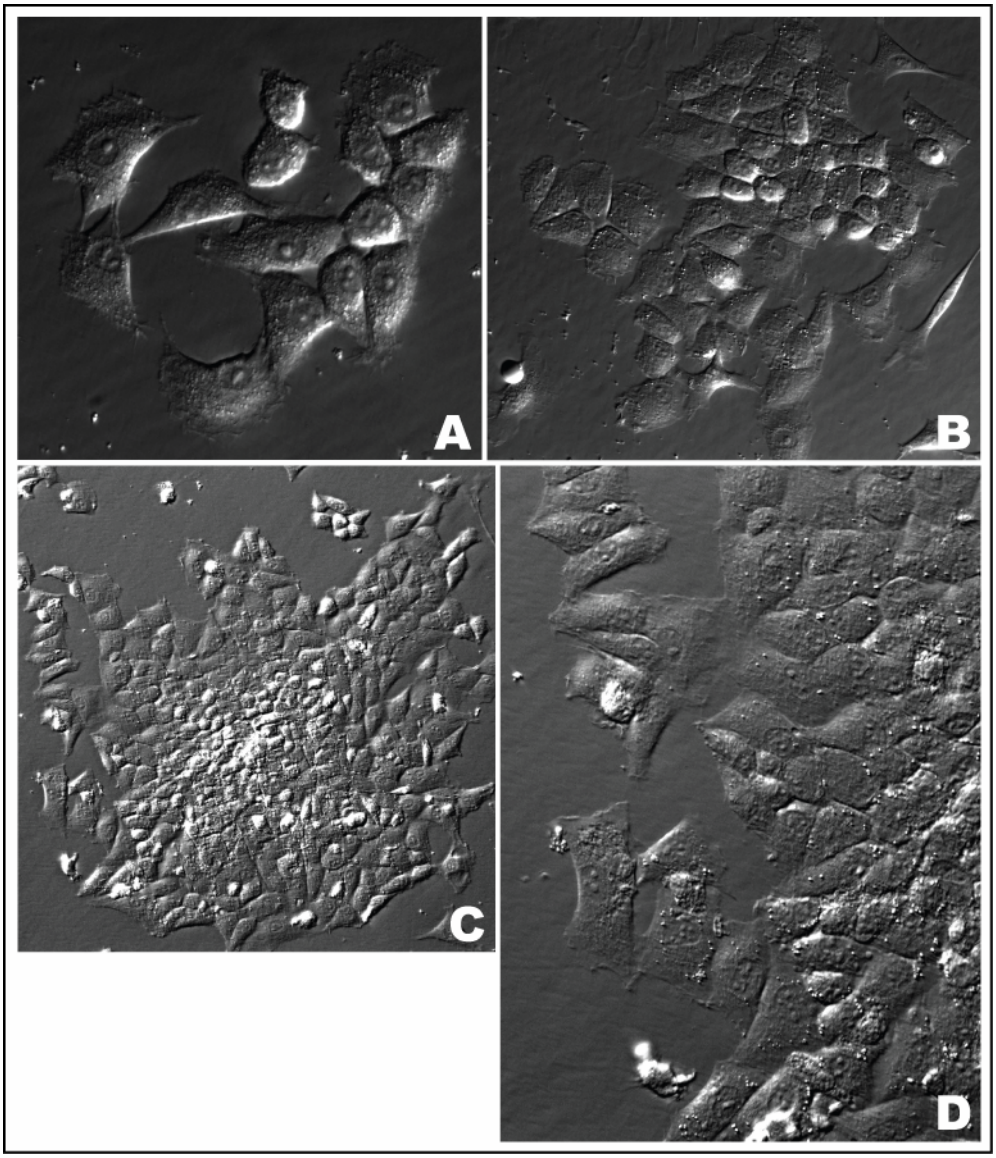

FIGURE 8. Hoffman Modulation Contrast photomicrographs of CR-MSC at various stages of growth (day #3, day #5, day #5, day #5). Rapid growth of large colonies of relatively homogeneous cells was observed, with the notable presence of fibroblast-like cells at the periphery of the colonies. The highly refractile cells present in image **C** may represent non-specific binding of contaminant hematopoietic cells such as macrophages to the free surface of these cells.

We attempted to verify the epithelial nature of these cells using immunofluorescent cytometry against the epithelial marker cytokeratin, an intermediate filament cytoskeleton protein present in

all epithelial cells, as well as the tight junction protein ZO-1. Since macrophages are also known to assume an epithelial conformation when undertaking a granulomatous response, staining against the pan-hematopoietic marker CD45 was performed as well (Figure 9).

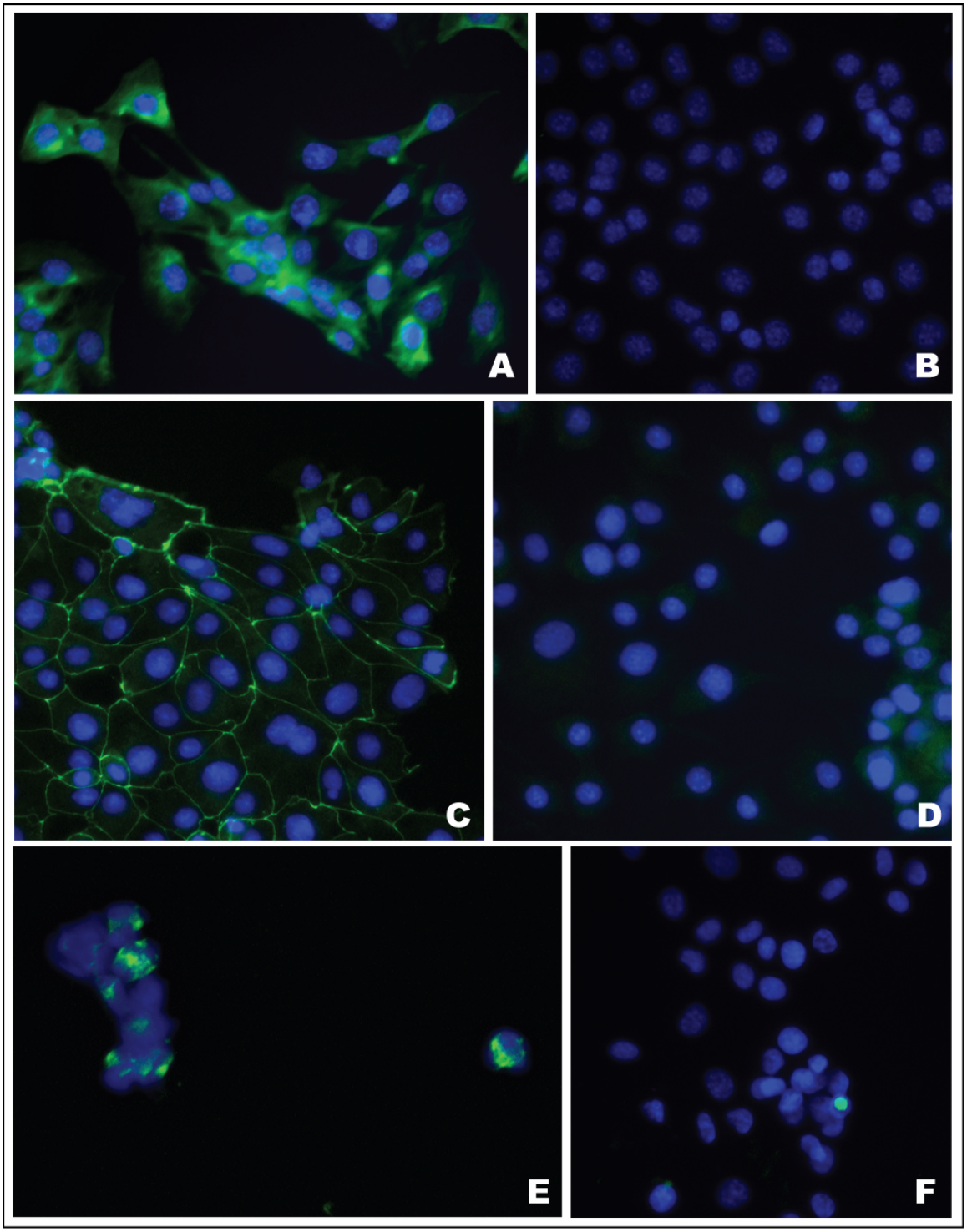

FIGURE 9. Immunofluorescence cytology. IMCD cells and CR-MSC versus pan-cytokeratin (**A** and **B**); IMCD cells and CR-MSC versus ZO-1 (**C** and **D**); whole mouse bone marrow cells and CR-MSC versus CD45 (**E** and **F**). No positive, specific staining was seen for either of the two epithelial markers despite clear staining in the positive-control IMCD cells. Likewise, CR-MSC were revealed to be CD45 negative.

This surprising set of negative results suggested that despite the epithelial appearance, CR-MSC may not be fully-differentiated epithelial cells and instead could be an odd physical conformation of mesenchymal cells, possibly brought upon by artificial (collagenase treatment) manipulation. To test this theory, we performed expression analysis using RT-PCR to assay for expression of common epithelial and mesenchymal markers. We chose the epithelial cytoskeleton protein cytokeratin-8 and the zonula occludens constituent E-cadherin, which are universally expressed in epithelial cells; as well as the mesenchymal intermediate filament protein vimentin and the fibroblast-specific protein FSP-1 (52). Total cellular RNA was isolated from control (IMCD epithelial cells and 3T3 fibroblasts) cells as well as CR-MSC, and a reverse transcription reaction was performed to synthesize cDNAs. PCR primers were specifically chosen to span adjacent exons in order to amplify only messenger RNA. The resulting PCR products were subjected to agarose gel electrophoresis (Figure 10).

Despite the inconsistent expression of the control IMCD cells in these samples which may be due to hitherto uncharacterized de-differentiation of this immortalized cell line, it is clear that CR-MSC have an expression profile quite similar to that of fibroblasts and dissimilar to an epithelial phenotype. No assayed epithelial markers were expressed, and the mesenchymal marker vimentin as well as the fibroblast-specific marker fsp-1 were both expressed. This effectively verifies our immunofluorescence cytology data in that these cells are likely fibroblasts with an altered appearance on plastic tissue culture dishes rather than a novel population of epithelial cells.

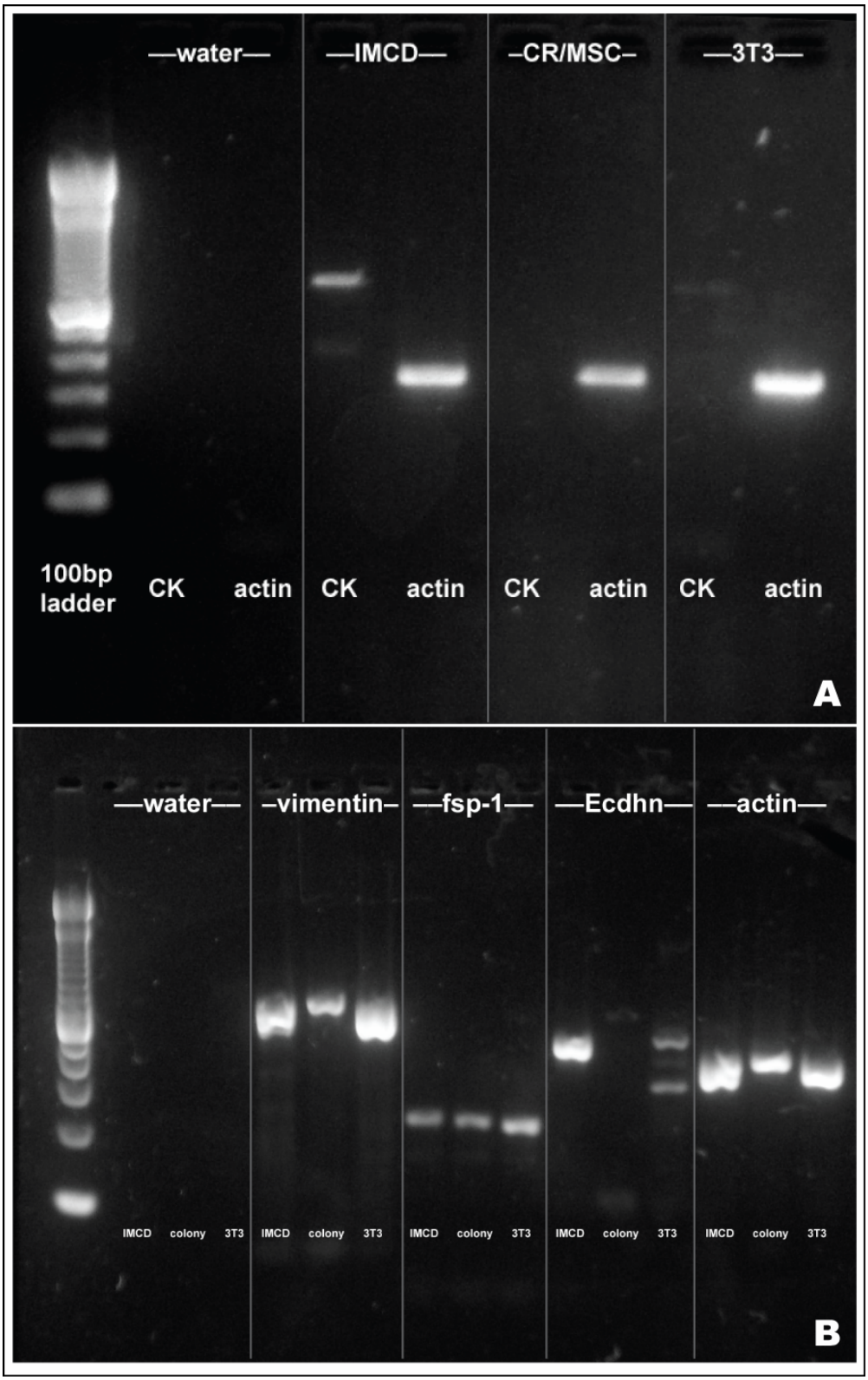

FIGURE 11. **A.** RT-PCR versus cytokeratin (and the housekeeping gene actin) on IMCD, CR-MSC and 3T3 RNA. Only IMCD cells express cytokeratin; CR-MSC and 3T3 cells do not. **B.** RT-PCR versus vimentin, fsp-1, E-cadherin (Ecdhn), and actin on

IMCD, CR-MSC (colony) and 3T3 RNA. IMCD and 3T3 cells appear to express both vimentin and fsp-1 in addition to the CR/MSC sample; this likely represents accidental contamination of the IMCD sample as these are known to have an epithelial phenotype. Likewise, the weak expression of E-cadherin in 3T3 cells may be non-specific amplification. Most importantly, CR-MSC express the mesenchymal proteins vimentin and fsp-1 and lack expression of E-cadherin.

In order to verify our suspicion that this population of cells freely exchanges with fibroblasts in liquid culture, we photographed the cells using time-lapse photography for 14 consecutive hours while growing at 37°C in 5% CO_2. These data are available in the included supplemental data on CD-ROM and in the Yale Medicine Thesis Digital Library located at http://ymtdl.med.yale.edu/. In this time-lapse clip, an epithelioid colony is shown. At the periphery of the colony, fibroblastic cells are clearly shown migrating toward the colony and retracting their pseudopods, eventually becoming closely apposed to the epithelioid cells. Likewise, epithelioid cells are seen to extend pseudopods and migrate rapidly away from the colony. This free interplay between epithelioid and fibroblastic cellular morphology is further support for a single, mesenchymal origin of this cell population as opposed to a novel epithelial population from this mesenchymal tissue.

DISCUSSION

To assay for bone marrow contribution to renal repair following ischemia/reperfusion injury, we transplanted female mice with male bone marrow prior to injuring the kidney with mechanical interruption of the renal vascular pedicle. After 7-14 days we examined thin sections of the kidneys using colocalization of immunofluorescence and *in situ* hybridization signals. Our experimental results indicate that bone marrow contribution to the regenerating kidney tubular epithelium is rarely observed, and that the majority of donor-derived cells in injured kidneys are actually localized to the interstitium. This leads to the conclusion that the cells actually responsible for renal regeneration are likely to be intrinsic renal cells, and that contribution from the bone marrow constitutes a definite but small and likely insignificant component of the regenerative response.

We believe that the use of *in situ* hybridization to report rates of donor cell engraftment in the kidney is more accurate than the use of β-galactosidase (ROSA) mice, which relies on the expression of an enzyme capable of catalyzing a histochemical reaction. To date, two groups including our own have found a significantly higher number of donor-derived cells via the β-galactosidase reporter system than via their own analysis of similarly transplanted mice using the Y-chromosome FISH system (37, 38, 53). Notably, our previous results found no evidence of β-galactosidase-positive interstitial cells, which sharply contrasts with our significant donor-derived interstitial infiltrate seen via Y-chromosome FISH.

It is currently unclear why the Y-chromosome FISH technique is superior in these transplantation and renal injury models over the β-galactosidase technique. The β-galactosidase assay may have proven problematic in renal tissue due to a fixation or staining artifact. Alternately, diffusion of the enzyme itself might have occurred out of inflammatory cells and into damaged tubules (39). More unlikely possibilities include the β-galactosidase product itself entering the

vasculature and being freely filtered at the glomerulus (39), or increased intrinsic β-galactosidase activity of the injured tubule itself (54).

Perhaps most interestingly, a recently-published study of bone marrow-derived cells incorporating into intestinal epithelium authored by Rizvi et al. has shown the potential for cell fusion to occur between donor cells expressing β-galactosidase and recipient cells expressing the Y-chromosome (55). A significant number of enterocytes both expressing β-galactosidase and containing a Y-chromosome were observed. Several cells were seen to express β-galactosidase but did not contain a Y-chromosome, a phenomenon which was not observed when β-galactosidase and enhanced green fluorescent protein were used as donor and recipient markers, respectively. The authors concluded that the Y-chromosome might have been extruded subsequent to the fusion event, and that because of this activity Y-chromosome analysis may have its own shortcomings as a marker for transplanted cells.

Regardless, our current results are consistent with recent scholarship in this area. Using transplantation of male bone marrow followed by I/R injury, Lin et al. found only 8.3% of tubules (1% of tubular epithelial cells) contained a Y-chromosome positive nucleus (53). This contrasts with previous results from this group using the β-galactosidase system, which revealed that up to 80% of tubules had some donor contribution (38). The majority of donor-derived cells in the injured kidney were interstitial cells, as we observed. Another study by Herrera et al. has reported labeled cells making up the renal tubular epithelium in a glycerol-induced renal injury model followed by infusion of GFP-labeled MSC (56). Similarly, Morigi et al. demonstrate Y-chromosome positive cells as constituents of a regenerating tubule in cisplatin-induced renal injury followed by MSC infusion (45).

A common limitation to all these studies is the use of conventional (inverted or upright) microscopy and staining with tubular epithelial markers to verify donor nucleus or cell localization on the tubular aspect of the tubule basement membrane. Personal experience has revealed to this author that discerning the precise location of tubule epithelial cell nuclei is difficult and often

uncertain. Definitive ascertainment of the location of these rare intra-tubular donor nuclei will likely require three-dimensional deconvolution microscopy rather than conventional epifluorescent techniques. It is entirely evident that conventional or epifluorescent light microscopy is an incomplete and inappropriate mechanism for adequate analysis of donor contribution to kidney epithelium.

The question of whether bone marrow contributes to regenerating tubular epithelium at all remains controversial. In a recent study, Duffield et al. studied Y-chromosome positive cell contribution to regenerating tubules in a similar experiment to ours but using three-dimensional deconvolution microscopy. They found a small number of donor-derived cells that appeared to constitute tubular epithelium, but when examined with ultrathin deconvolution microscopy and staining for the endothelial antigen von Willebrand Factor the nuclei were clearly shown to belong to endothelial cells (54). Likewise, a recent investigation performed by Szczypka et al. using a folic acid-induced model of renal injury yielded no evidence of donor-derived renal tubular cells by *in situ* hybridization against the Y-chromosome, although the group was able to culture an extremely rare population of donor-derived cells expressing the epithelial markers AT1 and ZO-1 (57). It is unlikely that given these conflicting sets of data that this question will be definitively resolved in the near future; it is apparent, however, that direct engraftment of bone marrow cells into renal tubules is not the prevalent mechanism for renal repair.

Given the functional amelioration seen by our group following infusion of whole bone marrow cells, an interesting question—hopefully to be answered by future experiments—involves the effect of MSC infusion on renal function. Previous data from our group demonstrated a functional improvement in experimentally-induced ischemic renal failure following infusion of fractionated bone marrow. In two subsequent studies as well as unpublished data from our own group, this effect has been reproduced following the infusion of expanded MSC populations following kidney injury. Togel et al. infused approximately 10^6 MSC intra-arterially into rats with recent bilateral I/R injury, and found significant improvements in laboratory parameters of renal

function as well as decreased apoptosis and injury on a histologic basis (43). Purified fibroblasts were unable to reproduce this effect. Donor-derived cells were observed only in transit through the kidney vasculature, and from this the authors concluded that MSC exert their renoprotective effect in a paracrine, *trans*-acting manner. They postulate that due to the incompletely-characterized immunomodulatory activity of MSC, a soluble anti-inflammatory factor may mediate the renoprotective effect. Similarly, Morigi et al. found significant histologic improvement after MSC were injected into mice whose kidneys were injured by cisplatin; this effect was not seen with the injection of purified HSC (45). As mentioned above, donor-derived cells were found occasionally within the tubule epithelium in this study. Most recently, a manuscript accepted for publication by Togel et al. has shown that complex paracrine interactions between endothelial cells and MSC are at least in part responsible for the renoprotective effect of MSC (44).

Even if transdifferentiation of bone marrow cells into renal tubular epithelium is a rare event *in vivo*, the clinical value of a population of expandable, easily obtained cells from bone marrow stroma that are capable of transdifferentiating into epithelium *in vitro* is unquestionable. To explore this possibility, we investigated a population of collagenase-released marrow stromal cells that appeared to assume an epithelial morphology when plated onto tissue culture vessels. Unfortunately, immunofluorescent and RT-PCR analysis proved that these cells expressed no markers of epithelial differentiation, and on time-lapse photography were seen to freely exchange with cells with fibroblast morphology at the edges of the colonies. Thus we draw the conclusion that these cells likely represent a subpopulation of MSC that assume a serendipitously epithelioid phenotype without any evidence of transdifferentiation.

Further characterization of these cells, especially to determine whether they can be induced to differentiate into other mesenchymal-origin tissues and whether they too have a renoprotective effect when infused into mice with kidney injury, would be enlightening. However, given our negative results it is unlikely that these cells represent a population of bone marrow cells with enhanced plasticity as described by Johnson et al. (46).

Certainly, since we only investigated cells derived from male mice, it could be postulated that the differentiative capacity of CR-MSC could be increased in females compared with males, but there is currently no reason to believe that there should be any difference in the makeup of these cells solely based on sex difference.

In summary, using a transplantation model we found positive evidence of rare bone marrow contribution to regenerating kidneys, although to such a small degree that it is unlikely to represent a significant source of epithelial cells during the regenerative process. We also characterized a population of *ex vivo* bone marrow stromal cells that appeared to form epithelial colonies when cultured, but upon further analysis had minimal evidence of an epithelial phenotype.

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