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Molecular Characterization of the Regenerative Response Induced by Intrarenal Transplantation of Selected Renal Cells in a Rodent Model of Chronic Kidney Disease

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Key Words
Kidney · Regeneration · Regenerative medicine · Tissue engineering · Stem cell · Renal progenitor · SOX2

Abstract
Dedifferentiation and proliferation of resident tubular epithelial cells is a mechanism of action potentially contributing to repair and regeneration in kidneys presenting with ischemic or chronic disease. To more efficiently develop cell and tissue engineering technologies for the kidney, we have developed molecular assays to evaluate the acquisition of a pluripotent state associated with stem/progenitor cell phenotype during induction of a regenerative response within the kidneys of rats with chronic kidney disease (CKD) following therapeutic intervention. Intrarenal delivery of selected bioactive renal cells leads to significant upregulation of pluripotency-associated SOX2 mRNA within the diseased kidney tissue from 1 to 24 weeks after treatment. The overall regenerative response index was assessed by quantitative composite expression of CD24, NODAL and LEFTY1 proteins, which were induced within 1 week of cell treatment and peaked at 12 weeks after treatment, reaching statistical significance ($p < 0.05$) compared to untreated CKD controls. Molecular assays that incorporate the assessment of SOX2 and the regenerative response index may prove to be valuable tools for the detection and monitoring of the tissue response after the delivery of regenerative treatments for CKD, thereby significantly shortening the developmental timelines associated with such therapies.

Introduction

Regeneration of complex solid organs such as the kidney involves the defined reconstitution of multiple specialized cell types organized within highly complex 3-dimensional microarchitectures. The regenerative response

Abbreviations used in this paper

BUN	blood urea nitrogen
CKD	chronic kidney disease
KSFM	keratinocyte serum-free media
Nx	nephrectomy
OCT	optimum cutting temperature
RRI	regenerative response index
TBS-T	Tris-buffered saline with Tween-20
uKSFM	unsupplemented keratinocyte serum-free media

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of the kidney to acute injury is generally understood to be mediated by dedifferentiation of resident tubular cell populations at the site of injury with concomitant acquisition of a stem/progenitor cell phenotype, followed by proliferation and reacquisition of tubular characteristics [Bonventre, 2003]. Rapid and reliable evaluation of a regenerative response within the adult kidney is critical for the development of tissue-engineered or cell therapy-based therapeutic interventions for chronic kidney disease (CKD). Such interventional methodologies are typically evaluated in small animal preclinical models that recapitulate aspects of CKD. One such model is the 2-step rodent 5/6-nephrectomy (Nx) model; 2-step 5/6 Nx in rats reproducibly generates terminally progressive renal failure with characteristic systemic and histological parameters of CKD (e.g. hypertension, reduced glomerular filtration rate, elevated serum creatinine and blood urea nitrogen, glomerular and tubulointerstitial fibrosis, hyperlipidemia, hyperphosphatemia, and anemia) [Ormrod and Miller, 1980]. The clinically relevant features of the Nx model combined with technical reproducibility and commercial availability provided the basis for its selection as the disease model of choice to evaluate cell-based and tissue engineering strategies for the kidney.

These features notwithstanding, such small animal clinical models require significant time, effort and expense to establish. Furthermore, analysis of therapeutic efficacy has been contingent on the laborious collection and analysis of key renal physiological indices from serum and urine over a 24-week time period [Kelley et al., 2010a, b]. Given this context, alternative, molecular methodologies that monitor the acquisition of key transcriptional and protein markers associated with regenerative outcomes at early time points (<24 weeks post-implantation evaluation typically required) [Kelley et al., 2010a, b] have the potential to considerably streamline renal tissue engineering and cell therapy product development. However, efforts to demonstrate such regenerative response within the kidney have been hampered by the absence of straightforward assays to identify and monitor acquisition of stem cell-like phenotypes indicative of regenerative outcomes. To address this, we have sought to develop and establish simplified molecular assays to better facilitate renal cell therapy and tissue engineering product development.

Typically, strategies currently under consideration for cell therapy of the kidney in response to onset of CKD have focused on leveraging the potential paracrine-mediated therapeutic properties of mesenchymal stem cells and embryonic stem cells [for a review, see Sagrinati et al., 2008]. However, from a manufacturing and product development perspective, primary cell types are preferable when compared to stem and progenitor cell populations, owing to considerable streamlining of isolation, expansion, maintenance and characterization conditions [Basu and Ludlow, 2010; for a review, see Basu and Ludlow, 2011]. To this end, numerous studies on the regeneration of renal architecture and function following acute kidney injury point to tubular epithelial cells as central in the restoration of function [Lin et al., 2005; Guo et al., 2010]. Such tubular epithelial-enriched cell populations may be selected from a primary kidney cell isolate prepared from the medulla, cortex and corticomedullary junction compartments of kidneys based on differential buoyant density centrifugation. These cell populations have been phenotypically characterized in detail at the morphological, transcriptomic and immunohistochemical levels [Kelley et al., 2010a, 2010b; Presnell et al., 2010]. Briefly, these cell populations are principally composed of E-cadherin+, Pan-cadherin+, cytokeratin 8/18/19+, γ-glutamyl transpeptidase+ cells and oxygen-responsive erythropoietin+ subpopulations [Presnell et al., 2010]. Taken together, these selected primary kidney cell isolates are described as being 'therapeutically bioactive' because orthotopic transplantation is associated with increased survival and enhanced renal functionality upon intrarenal administration to rodents with CKD secondary to 2-step 5/6 Nx [Kelley et al., 2010a, 2010b; Presnell et al., 2010]. Therefore, such therapeutically bioactive cell populations may be valuable components of products developed to augment kidney function in patients with CKD.

In the current study, we identified a broad panel of markers demonstrated in the literature to be associated with renal stem cell populations, including SOX2, CD24, CD133, UTF1, NODAL and LEFTY1 [Sagrinati et al., 2006; Tabibzadeh and Hemmati-Brivanlou, 2006; Shen, 2007; van den Boom et al., 2007; Sagrinati et al., 2008; Chambers and Tomlinson 2009]. The expression of these markers has been evaluated over a 6-month time period in the rodent 2-step 5/6 Nx model of CKD after treatment with the therapeutically bioactive tubular epithelial cell-enriched population previously shown to be effective in ameliorating aspects of CKD disease phenotype in this rodent model [Kelley et al., 2010a, b]. In this manner, we identified the pluripotency factor SOX2 as a robust transcriptional marker that was induced within 12 weeks of treatment with bioactive renal cells and showed significant upregulation during 24 weeks after treatment. Furthermore, we were able to identify a specific subset of markers whose expression in the host animal, taken to-

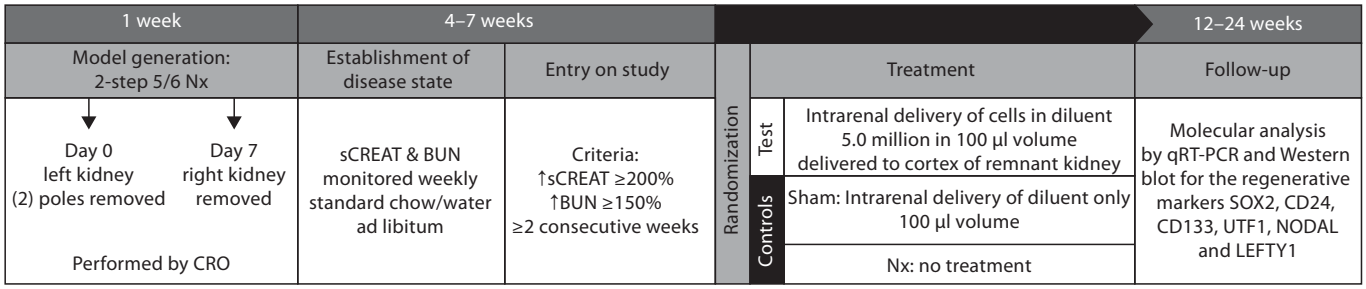

Fig. 1. Summary of the overall experimental design. sCREAT = Serum creatinine; CRO = contract research organization.

gether at the 12-week time point after initiation of the study ('regenerative response index', RRI), is diagnostic of a specific functional response to implantation of these bioactive cell populations. This unique host response was not observed in control animals injected with saline or that remained untreated. We believe such assays may be more broadly leveraged in the future to rapidly assess the therapeutic potential of novel combinations of therapeutically bioactive cells and biomaterials for renal tissue engineering [Basu et al., 2011].

Materials and Methods

Overall Study Design

The overall study design is summarized in figure 1. The study aims to identify molecular markers associated with regenerative outcomes upon treatment of established rodent models of CKD with a therapeutically bioactive cellular therapy. Briefly, 2-step 5/6 Nx was performed on adult Lewis rats (Charles River Labs) and a disease state consistent with stable loss of renal filtration capacity and onset of uremia established over a period of 8 weeks. Rodents with established renal disease (see details below) were randomly assigned to one of three groups: (1) cell-free vehicle controls (n = 5), comprising 100 µl of injection diluent only (sterile PBS); (2) untreated Nx controls (n = 5); (3) cell-treated rodents (n = 11), delivered at 5×10^6 therapeutically bioactive renal cells in 100 µl of sterile PBS. All animals were followed for up to 24 weeks, with individual rodents from each of the three groups harvested at 1, 12 and 24 weeks after study initiation for molecular evaluation of selected regenerative markers by qRT-PCR (transcripts) and quantitative Western blotting (protein). Expression of individual protein markers associated with regenerative outcomes was pooled to form a quantitative RRI. Specific details underlying each of these methodologies are presented below.

Isolation of Selected Bioactive Primary Renal Cell Populations from Rat

The preparation of selected bioactive primary renal cells from whole rat kidney has been previously described [Aboushwareb et al., 2008; Kelley et al., 2010a, 2010b; Presnell et al., 2010]. Briefly, whole kidneys were harvested from 5-week-old male Lewis rats (Hilltop Labs, Scottsdale, Pa., USA), and kidney tissue was dissociated enzymatically in a buffer containing 4.0 U/ml dispase (Stem Cell Technologies, Inc., Vancouver, B.C., Canada) and 300 U/ml collagenase IV (Worthington Biochemical, Lakewood, N.J., USA). Red blood cells and debris were removed by centrifugation through 15% iodixanol (Optiprep®, Axis Shield, Norton, Mass., USA). Primary renal cells were seeded onto tissue culture-treated polystyrene plates (Nunc, Rochester, N.Y., USA) and cultured in 50:50 media, a 1:1 mixture of high-glucose DMEM:keratinocyte serum-free medium (KSFM) containing 5% fetal bovine serum, 2.5 µg epidermal growth factor, 25 mg bovine pituitary extract, 1× insulin/transferrin/sodium selenite medium supplement, and antibiotic/antimycotic solution (all from Invitrogen, Carlsbad, Calif., USA). Prior to post-culture cell separation, primary renal cell cultures were transferred from atmospheric oxygen conditions (21%) to a more physiologically relevant low-oxygen (2%) environment for 24 h to improve cell separation efficiency. Separation of primary renal cell cultures, prepared as 75×10^6 cells in 2 ml unsupplemented KSFM (uKSFM), was performed by centrifugation through a four-step iodixanol (OptiPrep; 60% w/v in uKSFM) density gradient layered specifically for rodents (16, 13, 11 and 7%) in 15 ml conical polypropylene tubes and centrifuged at 800 *g* for 20 min at room temperature (without brake). After centrifugation, cellular subfractions were extracted from the gradient via pipette and collected as 4 distinct bands (B1–B4) and a pellet (B5), as shown in figure 2. All bands were washed 3 times in sterile PBS prior to use. Therapeutically bioactive renal cells were produced by combining B2 and B4 from the density gradient centrifugation step at a ratio of 97% B2 to 3% B4, as illustrated in figure 2. This results in a population of tubular cell-enriched cells previously demonstrated to significantly stabilize serum creatinine and blood urea nitrogen (BUN) and improve survival in the rodent 2-step 5/6 Nx model of CKD described below [Kelley et al., 2010a, 2010b; Presnell et al., 2010].

Creation of the 2-Step Rodent 5/6 Nx Model of CKD

Establishment of the rodent 5/6 Nx model requires surgical removal of one kidney and 2/3 of the contralateral kidney. Rodents were surgically prepared by a contract research organization (Charles River Labs) according to the following 2-stage protocol. Phase 1: a ventral midline incision was made into the abdomen of each rodent and covered with sterile surgical drape. The intestine was retracted laterally to expose the animal's left kidney.

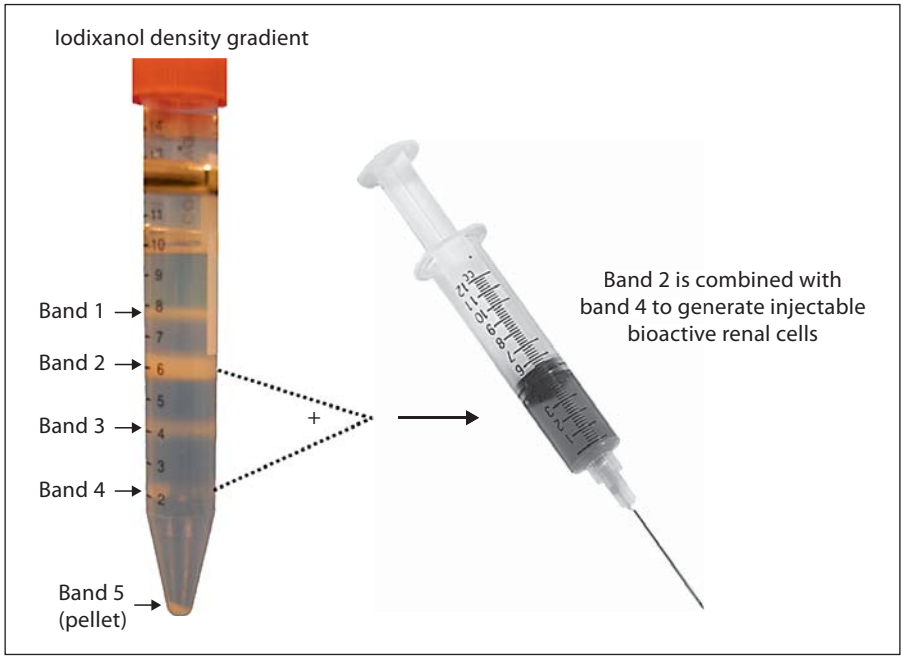

Fig. 2. Preparation of selected bioactive renal primary cell populations. Primary cell isolates from whole kidney are banded over a 4-step iodixinol gradient and selected cell populations combined, as shown, to form therapeutically bioactive primary renal cells.

The kidney was freed from the surrounding tissue. A piece of surgical suture was placed on each pole of the kidney at its 1/3 position. The sutures were gently ligated around the kidney. The 1/3 kidney on each end was excised immediately beyond the ligatures. The abdominal incision was closed with suture and wound clips. Phase 2: 1 week after completion of phase 1, the animal was re-prepped for surgery. The hair on the back of the lumbar region was shaved. A cranial-caudal skin incision was made on the animal's right lateral to the spine with the cranial terminus just behind the rib cage. The kidney was freed from the abdominal cavity and gently removed through the incision. The adrenal gland, which is loosely attached to the anterior pole of the kidney by connective tissue and fat, was gently freed by tearing the attachments and returned to the abdominal cavity. Renal blood vessels and the ureter were cauterized. The kidney was then removed by transecting the vessels and the ureter just distal to the cauterized spot. The incision was then closed with suture and wound clips.

Body weight was recorded weekly. Blood and serum samples were collected biweekly via tail vein or orbital bleed. Serum BUN, serum creatinine, hematocrit and red blood cell number were measured weekly by a qualified commercial vendor (Antech Diagnostics). The enrollment criteria for the current study was defined as an increase in serum creatinine ≥200% and an increase in BUN ≥150% for ≥2 consecutive weeks, diagnostic of a reproducible reduction in renal filtration and the onset of progressive uremia. Importantly, there was no evidence of spontaneous recovery or non-progressive stabilization among nephrectomized rodents – 100% of nephrectomized rodents had progressive elevations in serum creatinine and BUN from the time of model generation until time of death. These observations are consistent with previously published descriptions of disease onset in the rodent 2-step 5/6 Nx model [Ormrod and Miller, 1980].

Implantation of Primary Renal Cell Populations from Rat

Once a chronic disease state was established, rats were anesthetized and remnant kidneys were exposed via ventral medial-lateral incision. Selected bioactive renal cells, produced by the density gradient centrifugation protocol described above and consisting of 4.85×10^6 cells from B2 combined with 0.15×10^6 cells from B4, were suspended in 100-μl sterile PBS, loaded into a 1-cm^3 syringe fitted with a 0.5-inch 23-gauge needle (Becton Dickinson) and delivered directly to the kidney through the apical cortex at a depth of approximately 3–5 mm (for a diagram of the injection site, see fig. 3). The cells were delivered to the rats 6–12 h after cell harvest. In this study, the following treatment groups were used (table 1): (1) cell-free vehicle controls (n = 5), comprising 100 μl of injection diluent only (sterile PBS); (2) untreated Nx controls (n = 5); (3) cell-treated (B2 + B4) rats (n = 11), delivered at 5×10^6 cells in 100 μl of sterile PBS. Animals were sacrificed at study endpoints (1, 12 or 24 weeks) or when ordered by the study veterinarian. At necropsy, remnant kidneys were collected for molecular analysis as described below.

RNA Isolation, cDNA Synthesis and qRT-PCR

RNA was isolated from kidney sections at the site of cell injection from tissues embedded in optimum cutting temperature (OCT) freezing media as follows: tissue blocks were placed at room temperature and excess OCT was removed; tissues were placed in PBS to allow complete thawing and removal of residual OCT; tissues were washed three times in PBS, coarsely chopped and aliquoted into microfuge tubes; tissues were pulverized using a mortar and pestle and total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, Calif., USA). RNA integrity was determined spectrophotometrically and cDNA was generated from a volume of RNA equal to 1.4 μg using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Following cDNA synthe-

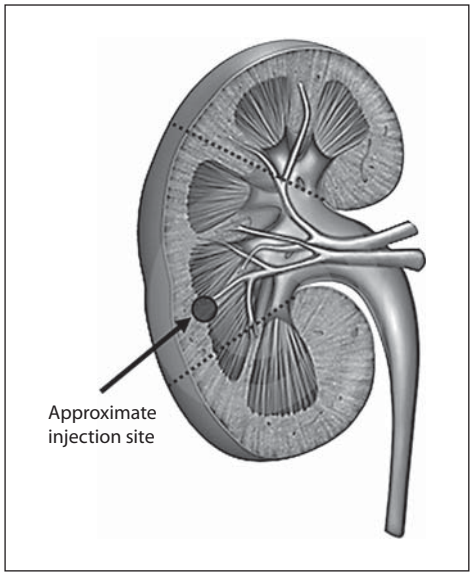

Fig. 3. Delivery of therapeutically bioactive primary renal cells is to the corticomedullary junction. Dashed lines indicate kidney mass removed during nephrectomy.

Table 1. Summary of the study design for analysis of regenerative response induction

Treatment	Total animals
1 week	
Sham control	3
Nx control	3
Cell treatment	3
12 weeks	
Sham control	1
Nx control	1
Cell treated	4
24 weeks	
Sham control	1
Nx control	1
Cell treated	4

sis, each sample was diluted 1:6 by adding 200 μl of diH_2O to bring the final volume to 240 μl. The expression levels of target transcripts were examined via qRT-PCR using catalogued primers and probes from ABI and an ABI-Prism 7300 Real Time PCR System (Applied Biosystems, Foster City, Calif., USA). Amplification was performed using the TaqMan® Gene Expression Master Mix (ABI, Cat. No. 4369016), and peptidylprolyl isomerase B was utilized as the endogenous control. Each reaction was set up as follows using the TaqMan primers and probes listed in table 2: 10 μl Master Mix (2×) + 1 μl primer and probe (20×) + 9 μl cDNA to give a final 20-μl total volume per reaction. For qRT-PCR analysis, study samples were screened as outlined in table 1. Statistical analyses were done using standard 2-tailed Student's t tests assuming equal variance for each sample. A confidence interval of 95% (p value <0.05) was used to determine statistical significance.

Western Blot

Frozen whole-kidney tissue embedded in OCT freezing media was utilized for protein sample collection. OCT was removed as described above, and all tissues were lysed in a buffer consisting of 50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP40, and protease inhibitor cocktail (Roche Applied Science, Indianapolis, Ind., USA). Lysis proceeded for 15 min at room temperature with rocking followed by centrifugation for 10 min at 13,000 rpm. All supernatants were collected and protein concentrations were determined by Bradford Assay. SDS-PAGE was carried out by adding 30 μg of protein per sample to each well of NuPAGE® Novex 10% Bis-Tris Gels (Invitrogen). The gels were electrophoresed for 40 min at 200 V in 2-(N-morpholino)ethanesulphonic acid running buffer (Invitrogen). The proteins were then transferred to nitrocellulose membranes using the I-Blot system (Invitrogen) and blocked with 15 ml of 4% w/v low-fat milk dissolved in Tris-buffered saline with 0.1% Tween-20 (TBS-T; Sigma, St. Louis, Mo., USA) for 2 h at room temperature. The membranes were probed overnight at room temperature with the antibodies shown in table 3, each diluted in 5 ml TBS-T with 2% w/v low-fat milk. The membranes were washed 3 times/10 min each with TBS-T, then probed with the appropriate horseradish peroxidase-conjugated secondary antibody (table 3) diluted in TBS-T with 2% w/v low-fat milk for 1.5 h at room temperature. The membranes were washed 3 times/10 min each in TBS-T, followed by two 10-min washes in diH_2O. The blots were developed using ECL Advance chemi-luminescent reagent (GE Healthcare Life Sciences, Piscataway, N.J., USA) and visualized using the ChemiDoc™ XRS molecular imager and Quantity One® software (BioRad, Hercules, Calif., USA). Quantitation of Western blot data was performed as follows. Band intensity was calculated from each Western blot using Image J version 1.4 software (National Institutes of Health). Intensity units were normalized per unit area for each protein marker. Average intensity was determined for the sham control group, the Nx control group and the renal cell treatment group by compiling the three (CD24, NODAL and LEFTY1) stem markers used in the Western blot analysis. This process was repeated for each time point (1, 12 and 24 weeks). The average intensity for each group was plotted over time to highlight the trends in stem marker protein expression. Statistical analysis was done using standard 2-tailed Student's t test assuming equal variance for each sample. A confidence interval of 95% (p value <0.05) was used to determine statistical significance.

Results

Expression of the Pluripotency-Associated Transcription Factor SOX2 mRNA Is Associated with Effective Treatment of CKD Rats with Selected Renal Cells

Kidney tissue harvested from CKD rats that had been treated with renal cells (n = 3 for 1 week and n = 4 for 12 and 24 weeks) [Kelley et al., 2010a, 2010b] was evaluated

Table 2. TaqMan primers used for renal stem and progenitor marker analysis

Gene	Abbreviation	TaqMan Cat. No.
POU class 5 homeobox 1	POU5F1/Oct4A	Rn01532129_g1
Nanog	Nanog	Rn01462825_m1
RNA exonuclease 1	Rex1	Rn01408442_g1
SRY (sex-determining region Y)-box 2	Sox2	Rn01286286_g1
v-myc myelocytomatosis viral oncogene	c-Myc	Rn00561507_m1
Musashi	MSI1	Rn00596059_m1
Podocalyxin	PODXL	Rn00593804_m1
Telomerase reverse transcriptase	TERT	Rn01409452_g1
GATA binding protein 4	GATA4	Rn00595169_m1
Undifferentiated embryonic cell transcription factor 1	UTF1	Rn01498190_g1
Nodal homolog from mouse	NODAL	Rn01433623_m1
Snail homolog 2 from drosophila	SNAI2	Rn00709370_m1
Left-right determination factor 1	LEFTY1	Rn01412531_g1
v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene	KIT/CD117	Rn00573942_m1
Melanoma cell adhesion molecule	MCAM/CD146	Rn00576900_m1
Prominin 1	PROM1/CD133	Rn00572720_m1
Nerve growth factor receptor	NGFR/CD271	Rn00561634_m1
CD24	CD24	Rn00562598_m1
Cadherin-11	CDH11	Rn01536921_g1
Retinoic acid receptor alpha	RARA	Rn00580551_m1
Peptidylprolyl isomerase B*	PPIB	Rn00574762_m1

* Used as internal control.

Table 3. List of antibodies used for renal stem and progenitor marker analysis

Vendor	Cat. No.	Ab description	Ab, μg/ml	MW, kDa
Primary Abs				
R&D Systems	MAB7461	mouse anti-human LEFTY-A long and short isoforms	1	40
Abcam	AB19898	rabbit anti-human, mouse and rat CD133	1	110
Millipore	MAB4337	mouse anti-human and rat UTF1	1	36
Abcam	AB55676	mouse anti-human NODAL	1	40
Cell Signaling	2748	rabbit anti-human, mouse and rat SOX2	1	78
Becton Dickinson	551133	mouse anti-rat CD24	1	78
Secondary Abs				
Vector Labs	PI-2000	peroxidase horse anti-mouse IgG antibody	Ab dilution 1:60,000	
Vector Labs	PI-1000	peroxidase goat anti-rabbit IgG antibody	Ab dilution 1:60,000	

Ab = Antibody; MW = molecular weight.

for expression of a panel of markers associated with acquisition of a stem/progenitor cell phenotype using the primer set from table 2 at 1 week, 12 and 24 weeks after implantation of selected renal cells. No significant trends in expression either with time or by treatment group was observed for this marker set with the exception of SOX2. Temporal analysis of SOX2 mRNA expression showed a 1.8-fold increase in SOX2 mRNA within the cell treatment group over Nx control by 12 weeks after implantation. A statistically significant ($p < 0.05$) 2.7-fold increase in SOX2 mRNA expression was observed in the cell treatment group over untreated Nx controls by 24 weeks after implantation (fig. 4). CD24, UTF1, NODAL and CD133 (PROM1) showed a trend towards overexpression with cell treatment relative to Nx, but were not statistically significant (data not shown).

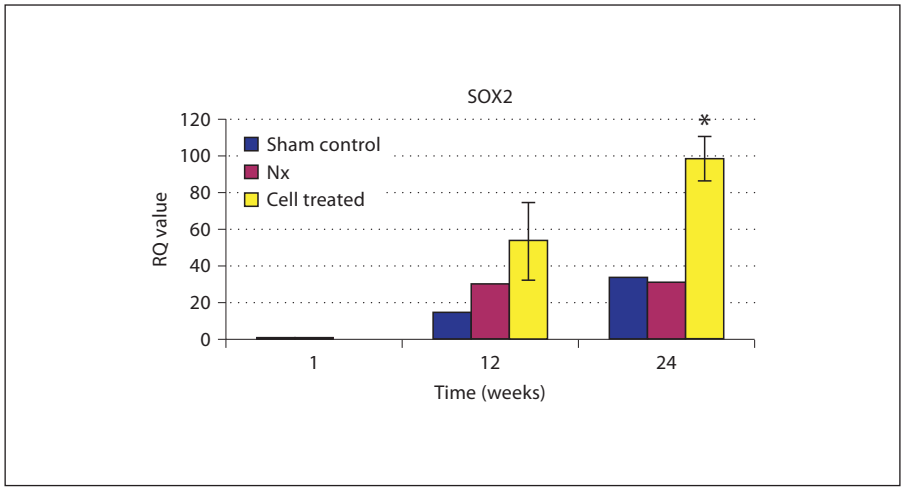

Fig. 4. SOX2 is a transcriptional marker of regenerative response induction. Time course for transcription of SOX2, a marker associated with maintenance of the pluripotent state in sham control, 5/6 Nx kidney and 5/6 Nx kidney with cell-based treatment. Error bars indicate standard error. * $p < 0.05$.

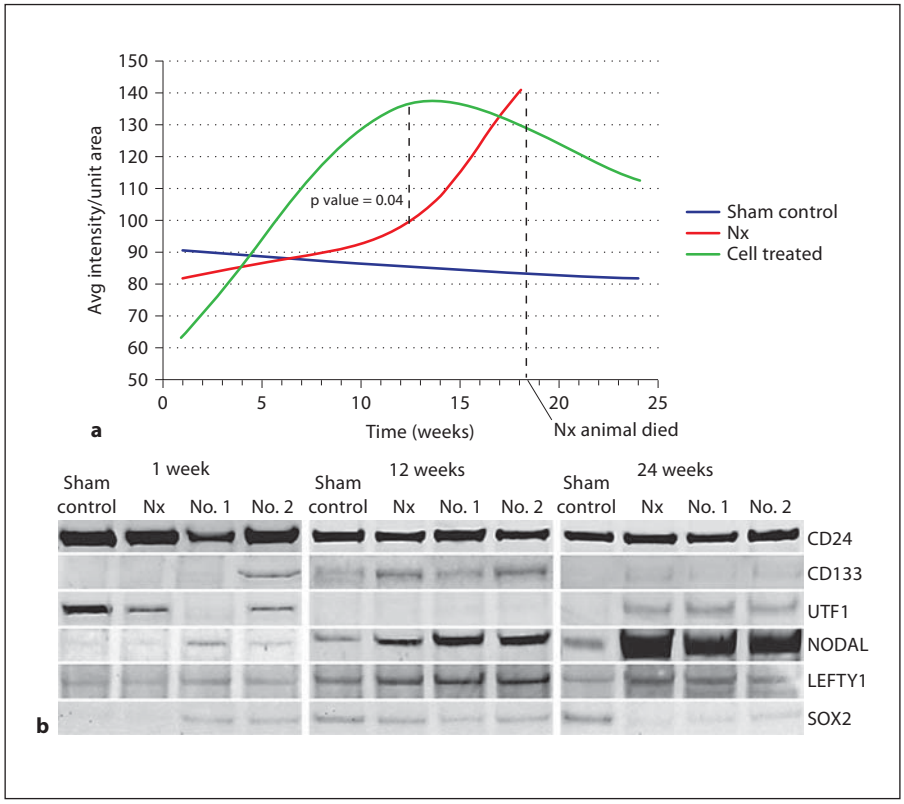

Fig. 5. Time course of acquisition of stem cell phenotype during regenerative response induction. **a** Time course of the RRI over 24 weeks in sham control, 5/6 Nx kidney and 5/6 Nx kidney with cell-based treatment [average (Avg) of cell-based treatment No. 1 and No. 2]. **b** Western blot showing the time course of expression of CD24, CD133, UTF1, NODAL, LEFTY1 and SOX2, in sham control, Nx control and cell-based treatment rats (No. 1 and No. 2) at 1, 12 and 24 weeks after treatment. Lanes were normalized by total mass protein loaded.

Expression of the Pluripotency-Associated Proteins CD24, CD133, UTF1, NODAL, LEFTY1 and SOX2 Is Associated with Implantation of CKD Rats with Selected Renal Cells

Based on the qRT-PCR analysis described above, the regenerative proteins CD24, CD133, UTF1, NODAL, LEFTY1 and SOX2 were selected for temporal analysis of protein expression to assess induction of a regenerative response to cell-based treatment of CKD rodents. Protein expression was evaluated by Western blot analysis of renal protein extract isolated from kidneys of individual animals treated with renal cells, untreated Nx controls, or sham controls. As with qRT-PCR analysis, animals were evaluated at 1, 12 and 24 weeks after implantation with renal cells. At 1 and 12 weeks after treatment, CD133, UTF1, NODAL, LEFTY1 and SOX2 protein expression

Table 4. RRI in 5/6 Nx rats at 1–24 weeks after treatment with NKA

a Sham control

Sample/detector	1 week	12 weeks	24 weeks
CD24	**173.32**	**131.10**	**156.88**
CD133	29.91	53.85	44.65
UTF1	141.78	26.10	31.34
NODAL	**40.52**	**51.95**	**44.12**
LEFTY1	**57.57**	**73.32**	**44.67**
SOX2	16.34	23.91	31.74
RRI	90.47	85.46	81.89

b Nx

Sample/detector	1 week	12 weeks	18 weeks
CD24	**159.67**	**109.93**	**179.25**
CD133	34.62	61.96	49.33
UTF1	62.37	26.34	50.09
NODAL	**37.11**	**94.47**	**166.03**
LEFTY1	**50.00**	**89.21**	**76.41**
SOX2	15.04	21.23	19.98
RRI	82.26	97.87	140.56

c Cell treated

Sample/detector	1 week	12 weeks	24 weeks
CD24	**129.82**	**125.41**	**170.10**
CD133	43.25	64.17	41.59
UTF1	39.68	27.07	48.24
NODAL	**35.17**	**152.00**	**135.69**
LEFTY1	**53.14**	**110.29**	**57.27**
SOX2	18.20	19.27	19.95
RRI	62.89	135.61	112.61

NKA = Neo-kidney augment.

differentiated the renal cell treatment animal from both the untreated Nx control and the sham control animals. Densitometric analysis of individual protein expression was performed as described in Materials and Methods and used to generate a quantitative index of regenerative marker protein expression, or RRI representing the averaged expression of CD24, NODAL and LEFTY1 (representing the minimal subset of markers found to generate indices of statistical significance) presented as average band intensity per unit area of gel (table 4). This index was used to derive the time course of regenerative response induction presented in figure 5. Other markers, with a trend towards significance, did not enhance the significance of the RRI as defined above. In the sham control animal, RRI showed a slight reduction from 90.47 at 1 week after treatment to 81.89 at 24 weeks after treatment. In contrast, the untreated Nx control presented essentially the opposite response, with the RRI increasing from 82.26 at 1 week after treatment to 140.56 at 18 weeks after treatment, at which point the animal died. In Nx animals treated with renal cells, the RRI increased sharply from 62.89 at 1 week after treatment to 135.61 by 12 weeks after treatment and fell to 112.61 by 24 weeks after treatment. As shown in figure 5, the difference in RRI between renal cell-treated and Nx control animals is statistically significant, with $p < 0.05$.

Discussion

Within adult mammals, responses of solid or tubular organs to damage may principally be either reparative or regenerative in nature. Reparative outcomes are associated with extensive fibrosis and scar tissue formation, absence of significant reconstitution of tissue/organ level architectures and no recovery of tissue/organ-associated functionality. In contrast, regenerative outcomes present minimal evidence of fibrotic damage while showing reassembly of tissue and organ structures and demonstrating rescue of key aspects of organ functionality [for a review, see Basu and Ludlow, 2010]. Regeneration of the liver in response to lobectomy is a well-established example of organ regeneration in adult mammals [for a review, see Zaret and Grompe, 2008]. Efforts to develop cell-based and tissue engineering therapies targeting solid organs must therefore focus on suppressing reparative healing pathways while simultaneously promoting regenerative outcomes [for a review, see Basu and Ludlow, 2011]. For the kidney, host response to remnant kidney damage associated with disease in the rodent 2-step 5/6 Nx model is well established to be principally fibrotic in nature, i.e. characterized by the presence of extensive fibrosis and scarification, and therefore, serves as an excellent small animal model system for evaluation of regenerative therapies for chronic diseases of the kidney that are associated with extensive fibrotic pathologies [Yuen et al., 2010]. These observations notwithstanding, accurate evaluation of the impact of cell-based therapies in the rodent 2-step 5/6 Nx model of CKD requires an assessment of the extent of endogenous regenerative mechanisms. To this end, there have been no reports in the literature documenting a spontaneous regenerative response within the remnant kidney of 2-step 5/6 Nx rats. Furthermore,

we have not documented reversion of an established disease phenotype in any 5/6 Nx animal utilized in this or our previous studies (our unpublished observations).

However, the current study is designed to specifically evaluate the extent of innate regenerative outcomes in the absence of therapeutic interventions at time frames close to the induction of the initial lesion. To recapitulate briefly, 3 groups of rodents were examined (fig. 1): (1) cell-free vehicle controls (n = 5), comprising 100 μl of injection diluent only (sterile PBS); (2) untreated Nx controls (n = 5); (3) cell-treated rodents (n = 11), delivered at 5×10^6 therapeutically bioactive renal cells in 100 μl of sterile PBS. Each of these groups was examined at 1, 12 and 24 weeks after study initiation. Molecular analysis of regenerative response from group 2 animals serves to specifically identify any innate regenerative response inherent to the host in absence of any mock or therapeutic interventions, at time frames close to that of the initial, disease-generating lesion. Group 3 animals may then be compared to group 2 and group 1 animals to isolate any regenerative responses unique to the bioactive cell population being examined (fig. 1), at time frames close to (1 week) or distinct from (24 weeks) the induction of the initial injury.

How might this or other cellular therapies operate mechanistically to achieve regenerative outcomes? Proliferation of dedifferentiated resident tubular epithelial cells following induction of injury and reacquisition of a mature epithelial tubular phenotype has been proposed as a mechanism of action for renal regeneration [Duffield and Bonventre, 2005]. As we have discussed, such regenerative response induction may be innate, representing natural attempts by the damaged organ to reorganize and reassemble itself, or may be triggered by introduction of a defined, exogenous therapeutic agent. For rodent models of acute kidney disease or CKD, this hypothesis specifically predicts that regenerative response induction within the kidney and concomitant renal physiologic functionality will be associated with a temporary but significant acquisition of a stem or progenitor cell phenotype. Any innate regenerative response (as monitored by group 1 and group 2 animals in the current study design) will be associated with preliminary mobilization of stem and progenitor markers, but is typically inadequate to promote auto-regeneration, especially during the initial recovery phase (1–12 weeks after Nx). However, treatment with effective cell-based regenerative therapies (group 3 animals) is specifically predicted to enhance the native regenerative response above and beyond that observed from the disease model alone. Such regenerative response may be especially evident during the later (24 weeks after Nx) recovery phase of the regenerative process. Therefore, the timing of the regenerative response is predicted to become the critical differentiator between treatment groups (group 3 animals relative to groups 1 and 2). As shown in figures 4 and 5, these predictions are confirmed experimentally.

Such cell-based therapies may focus on niche-specific populations of stem and progenitor cells that are zonally localized throughout the mammalian kidney [Sagrinati et al., 2008; Basu et al., 2010]. The targeted identification, purification, expansion and reintroduction within damaged kidney of these and other classes of adult and embryonic stem cells has been investigated as a potential strategy for amelioration of ischemic and chronic renal disease [Humphreys and Bonventre, 2008]. Outcomes have been generally mixed, with little if any evidence supporting site-specific engraftment and directed differentiation as a mechanism of action for therapeutic efficacy. In one such study, the parietal epithelium of Bowman's capsule was identified as a niche for localization of multipotent progenitor cells characterized in part by coexpression of the markers CD24 and CD133/PROM1. Intravenous injection of this population within severe combined immunodeficient mice with acute kidney injury led to tubular and glomerular regeneration [Sagrinati et al., 2006]. Such stem cell-based therapeutics are currently believed to mediate regenerative response induction through paracrine mechanisms that may involve signaling by growth factors or exosomes/microvesicles [Togel et al., 2007; Caplan, 2009; Togel et al., 2009; Camussi et al., 2010]. These data notwithstanding, commercialization and process development of multi-potent cell types for applications in regenerative medicine is associated with numerous technical and regulatory challenges [Basu and Ludlow, 2010, 2011]. Therefore, we have selected therapeutically bioactive primary renal cells for process development into a cell-based product for applications in renal regenerative medicine. These bioactive primary renal cell populations have been phenotypically and functionally characterized in detail and have been shown to positively impact disease-related outcomes in rodent 5/6 Nx models of CKD [Kelley et al., 2010a, 2010b; Presnell et al., 2010].

In the current study, we have developed molecular assays to evaluate the hypothesis that regenerative response induction in a rodent model of CKD as triggered by implantation with selected bioactive renal cells is associated with temporary acquisition of a stem/progenitor cell phenotype by resident renal cell populations. Screening of

the transcriptional expression profile of a broad panel of markers associated with regenerative potential (table 2) led to the identification of SOX2 as a potential qRT-PCR marker directly correlating with regenerative response induction as a consequence of cell-based treatment (fig. 4). SOX2 functions as a key transcription factor mediating the pluripotent state and may, in the appropriate context, induce pluripotency within fully differentiated cell types [Chambers and Tomlinson, 2009]. Furthermore, SOX2 has previously been shown to be significantly upregulated in kidney isolated from adult human CKD patients compared to healthy controls [Basu et al., 2010]. Although variation in gene expression across samples was generally too large to permit unambiguous trends to be established for most transcriptional markers screened, SOX2 transcript was exceptional, and tissue-based monitoring of SOX2 transcription may serve as the basis for rapid detection of regenerative response induced by cell-based treatment of kidney or other solid organs.

At the protein level, analysis of the temporal expression of CD133, UTF1, NODAL, LEFTY1 and SOX2 showed a distinct pattern of expression induced by cell-based treatments as early as 1 week after implantation. Based on this initial observation, we developed the RRI as a quantitative measure of the mean expression of the key protein factors associated with regeneration: CD24, NODAL and LEFTY1 [Sagrinati et al., 2006; Tabibzadeh and Hemmati-Brivanlou, 2006; Shen, 2007; van den Boom et al., 2007; Sagrinati et al., 2008; Chambers and Tomlinson, 2009]. Although expression of protein and transcript levels do not show a linear correlation, differences in post-transcriptional regulation between transcripts imply that such straightforward relationships are unlikely to be observed. RRI is maintained in PBS-treated Nx control animals, with a slight decrease in expression levels over time, potentially a consequence of aging of these animals [Wilson et al., 2010]. Conversely, massive organ trauma triggered by 5/6 Nx is associated with continued increase in the RRI until time of death, suggesting mobilization of a robust yet functionally insufficient regenerative response. Treatment with bioactive renal cells leads to significant ($p < 0.05$) upregulation in the RRI above Nx control at 12 weeks after treatment before plateauing at 24 weeks after treatment, consistent with induction of a regenerative response as a consequence of bioactive renal cell treatment by 12 weeks after treatment. We recognize that animal numbers in control populations are small ($n = 1$); nevertheless, taken together, our data are consistent with cell-based treatment functioning to mediate renal regeneration in part through activation of resident progenitor cell populations or through dedifferentiation of resident tubular cell populations, both established mechanisms of action for kidney regeneration in acute models.

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Disclosure Statement

The authors declare an equity interest in Tengion, Inc.

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