Stem and Progenitor Marker Expression as a Predictor of Renal Regeneration in 5/6 Nephrectomized Rats Treated with Therapeutically Bio-Active Primary Renal Cell Sub-Populations

John W. Ludlow, Christopher Genheimer, Roger llagan, Thomas Spencer, Eric Werdin, Rusty Kelley, Andrew T. Bruce, Sumana Choudhury, Deepak Jain and Joydeep Basu

Summary:

Chronic kidney disease (CKD) is a global public health concern involving progressive loss in renal function. Preservation of renal function is key to reducing morbidity in CKD patients. The disparity between the number of patients on dialysis awaiting kidney transplant and the number of organs actually transplanted highlights the need for new treatments to preserve renal function. Regenerative medicine approaches may offer hope to those CKD patients waiting for a transplant. Using a rat 5/6 nephrectomy model for CKD, we have developed molecular assays to evaluate the mobilization of resident stem and progenitor cells within the rat 5/6 nephrectomized kidney in response to intra-renal injection of defined, therapeutically bio-active primary renal cell populations. We show that treatment was associated with up-regulation of the key stem cell markers CD24, CD133, UTF1, SOX2, LEFTY1, and NODAL at both transcript and protein levels. Up-regulation was detected by 1 week post-injection and peaked by 12 weeks post-injection. Activation of stem and progenitor cell markers was associated with increased survival relative to untreated nephrectomized controls, consistent with the increased survival and preserved renal function observed in multiple, previous studies.

In vivo Study Design:

Detailed descriptions of the isolation of primary renal cell populations (1) and the *in vivo* studies that evaluated the bioactivity of primary renal cell sub-populations in the 5/6 nephrectomized rodent model of CKD (2) were published elsewhere. In the current study, tissues were isolated at necropsy from rats treated with NKA and compared to nephrectomized (Nx) and healthy or sham-operated controls (Control).

This study was a pilot study aimed at investigating the host kidney tissue response to treatment. As such, samples were obtained from a subset of animals in ongoing studies. The data presented in Figure 1 and Table 1 reflect identical animals. Likewise, the data presented in Figures 2 and 3 reflect identical samples.

RNA isolation, cDNA synthesis and qRT-PCR

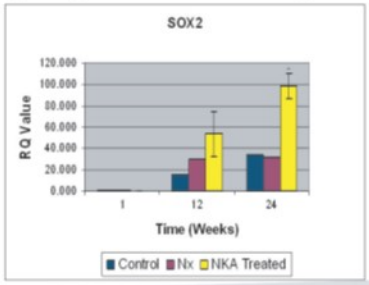
RNA was isolated from tissues embedded in optimum cutting temperature (OCT) freezing media. Aliquoted tissues were pulverized using a pestle and total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen). RNA integrity was determined spectrophotomerically and cDNA was generated from a volume of RNA equal to 1.4 µg using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). The expression levels of target transcripts were examined via quantitative real-time PCR (qRT-PCR) using catalogued primers and probes from ABI and an ABI-Prism 7300 Real Time PCR System (Applied Biosystems). Amplification was performed using the TaqMan® Gene Expression Master Mix (ABI) and peptidylprolyl isomerase B was utilized as the endogenous control.

Western blot

Frozen host kidney tissue embedded in OCT freezing media was utilized for protein sample collection. All tissues were lysed in a buffer consisting of 50mM Tris (pH 8.0), 120mM NaCl, 0.5% NP40, and protease inhibitor cocktail (Roche Applied Science). for 15 minutes at room temperature followed by centrifugation for 10 minutes at 13,000 RPM. Supernatants were collected and protein concentrations determined by Bradford Assay. SDS PAGE Gel 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 200V in MES running buffer (Invitrogen). Proteins were then transferred to nitrocellulose using the I-Blot system (Invitrogen), and blocked with 4% w/v low-fat milk dissolved in Tris Buffered Saline with 0.1% Tween-20 (TBS-T) (Sigma) for 2 hours. The membranes were probed overnight with the indicated specific antibodies before washing and probing with the appropriate HRP-conjugated secondary antibody. Blots were developed using ECL Advance chemiluminescent reagent (GE Healthcare Life Sciences) and visualized using the ChemiDoc™ XRS molecular imager and Quantity One® software (BioRad).

Results:

Figure 1. Expression of SOX2 mRNA in host kidney tissue after treatment of 5/6 NX rats with NKA

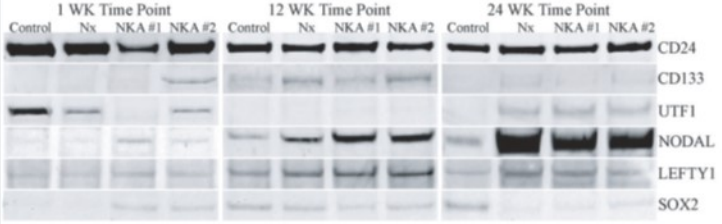


- Temporal analysis of SOX2 mRNA expression showed 1.8-fold increase in SOX2 mRNA expression in NKA-treated rats at 12 weeks post-treatment
- A 2.7-fold increase in SOX2 mRNA expression was observed in NKA-treated rats over Nx control at 24 weeks post-implantation

- 1-week: n= 3 each for Control, Nx, and NKA Treated groups.
- 12 weeks: n=1 each for Control and Nx groups; n=4 for NKA Treated group
- 24 weeks: n=1 each for Control and Nx groups; n=4 for NKA Treated group

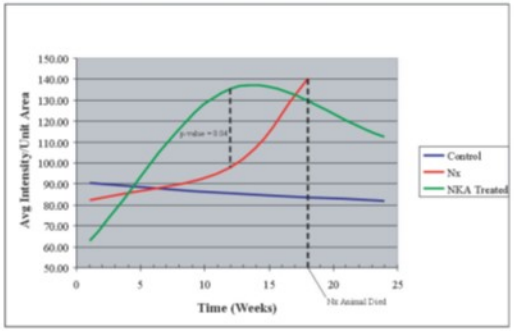
* Indicates p-value = 0.023 or < 0.05

Figure 2. Western blot showing time course of expression of CD24, CD133, UTF1, SOX2, NODAL and LEFTY in Control, Nx, and 2 NKA-treated rats (NKA #1 and NKA #2) at 1, 12 and 24 weeks post-treatment



- Protein samples were prepared from the host kidney tissue of 1 Control, 1 Nx, and 2 NKA-treated animals at each time point
- Lanes were normalized by total mass protein loaded
- CD24 protein expression in NKA-treated kidneys was elevated relative to Control or Nx rats at the 12 and 24 week time points
- CD133, UTF1, NODAL, LEFTY and SOX2 protein levels in NKA-treated tissues were elevated relative to Control or Nx tissues at all time points

Figure 3. Time course of regenerative response index (RRI)



- Densitometric analysis of individual protein expression (**Figure 2**) was used to generate a quantitative index of regenerative marker protein expression, or regenerative response index (RRI)
- Average intensity (normalized per unit area) was determined for sham, Nx, and NKA treatment groups (NKA#1 + NKA#2) by compiling the 5 markers used in the western blot analysis for each time point
- Plot shows XY scatter with smoothed line fit generated from 1,12,and 24 week time points

- The average intensity for each group was plotted over time to highlight the trends in the host tissue response of stem cell marker protein expression
- Statistical analysis was performed using standard two tailed Student's t-test assuming equal variance for each sample
- Confidence interval of 95% (p-value < 0.05) was used to determine statistical significance
- NKA treated group n=2; Control (sham) n=1; Nx (control) n=1
- In sham control animals, RRI shows only a slight reduction from 90.47 at 1 week post-treatment to 81.89 at 24 weeks post treatment
- In contrast, kidney from 5/6 Nx control presents essentially the opposite response, with RRI increasing from 82.26 at 1 week post-treatment to 140.56 at 18 weeks post-treatment, at which point the animal died
- Interestingly, in NKA-treated animals, the RRI increased sharply from 62.89 at 1 week post-treatment to 135.61 by 12 weeks post-treatment and fell to 112.61 by 24 weeks post-treatment.

Table 1. Animal Survival data

	Animals surviving to scheduled sacrifice from Figure 1		
	1 week	12-13 weeks	23-24 weeks
Control	3/3	2/2	1/1
Nx	3/3	2/2	0/1*
NKA Treated	11/11	8/8	4/4

* Unscheduled death at 18 weeks post-treatment

- Survival profile of these animals is consistent with that observed in multiple studies of NKA treatment in 5/6 nephrectomized rats (2)

Conclusions:

- NKA treatment is associated with up-regulation of the key stem cell markers CD24, CD133, UTF-1, SOX-2 and NODAL at transcript and protein levels in the host kidney tissue
- Up-regulation of these progenitor cell markers in host kidney tissue was detected by 1 week post-treatment and the regenerative response index peaked by 12 weeks post-treatment
- NKA treatment has consistently been associated with increased survival and preservation of renal function in multiple studies using the 5/6 nephrectomy rodent model of progressive CKD. These data show that up-regulation of stem cell markers in the host kidney tissue is also associated with NKA treatment, suggesting that such up-regulation may be a component of the NKA mechanism of action.

References:

1. Presnell SC, Bruce AT, Wallace SM, Choudhury S, Genheimer CW, Cox B, Guthrie K, Werdin ES, Tatsumi-Ficht P, Ilagan RI, Kelley RW, Rivera EA, Ludlow JW, Jayo MJ, and Bertram TA. Cells with Therapeutic Potential Can be Isolated and Propagated from Normal and Chronically-Diseased Kidney Tissue: Translation from Rodent to Canine and Human. Tissue Engineering, Part C, *in press, 2010.*
2. Kelley R, Werdin ES, Bruce AT, Choudhury S, Wallace SM, Ilagan, RM, Cox BR, Tatsumi-Ficht P, Rivera EA, Spencer T et al. A tubular cell-enriched subpopulation of primary renal cells improves survival and augments kidney function in a rodent model of chronic kidney disease. Am J Physiol Renal Physiol *in press, 2010*

Presented at the 2010 World Stem Cell Summit, held in Detroit, MI, Oct 4-6, 2010