**Molecular Pathways Underlying Adaptive Repair of the Injured Kidney: Novel Donation After Cardiac Death and Acute Kidney Injury Platforms**

# DCD and AKI Allografts Offer a Valuable Platform to Study Molecular Pathways Underlying Successful Adaptive Repair of the Kidney after Damage

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RD, SB, and HO conducted detailed biostatistics analysis of the microarray data and detailed interpretation of the results. They accepted the final version of the manuscript.

BB provided expertise in renal regeneration, detailed interpretation of results and helped to draft the discussion. She accepted the final version of the manuscript.

AF, RJS, JR provided input in the study design, were involved in the clinical undertaking of the project and accepted the final version of the manuscript.

BIF provided input in the study design, provided input in the data analysis and interpretation, and accepted the final version of the manuscript.

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**Objective:** To test the hypothesis that gene expression profiling in peripheral blood from patients who have undergone renal transplantation will provide mechanistic insights regarding graft repair and regeneration.

**Background:** The source (and therefore functional status) of kidneys donated for transplantation varies widely. Traditionally, grafts obtained from a living donor (LD) function well immediately following transplantation whereas organs that experience acute damage, e.g. from donation after cardiac death (DCD) or acute kidney injury (AKI) donors, may experience a period of delayed function – after which they function as well as LD kidneys. Our goal is to provide a more complete understanding of the molecular basis for this recovery as it is occurring in the graft recipient within the first 30 days post-transplant.

**Methods:** Peripheral blood was collected from patients prior to surgery, immediately after (for up to 5 consecutive days), twice weekly for the next 3 weeks, and at post-surgery day 28-30 following kidney transplantation (KT) with LD, DCD or AKI donor grafts. Total RNA was isolated from patient samples (representing from 4-9 time points per patient) and assayed on whole genome microarrays. Longitudinal gene expression analysis was performed to identify molecular pathways and processes involved in the recovery phase following KT.

**Results:** Comparison of longitudinal gene expression between LD and AKI/DCD samples revealed two clusters, representing 141 differentially expressed transcripts. A subset of 11 transcripts was found to be differentially expressed in *both* AKI and DCD samples compared to LD. In *all* KT patients, regardless of donor graft source, the most robust gene expression changes were observed in the day immediately

following KT, with a coordinate upregulation of innate inflammatory response pathways (e.g. IL-6, IL-8, IL-17A, and nitric oxide signaling). Beyond day 1, gene expression profiles differed depending upon the source of the graft. In patients receiving LD grafts, the expression of most genes did not remain highly elevated beyond the first day post-transplantation, while in the other two groups much of the elevation in gene expression was maintained for at least 5 days following surgery. In all cases, the pattern of coordinate gene over-expression had ceased by days 28-30.

**Conclusions:** Following kidney transplantation, gene expression in peripheral blood is significantly different in patients receiving LD versus AKI or DCD grafts, with 11 transcripts showing differential expression, over time, in both AKI and DCD recipients compared to LD. Moreover, the *pattern* of gene expression in the peripheral blood of KT patients is different, depending upon the source of donor graft. These markedly different expression patterns, particularly the coordinate elevation hundreds of expressed genes in DCD and AKI recipients beyond post-transplant day 1, provide further insight into potential mechanisms and timing of kidney recovery (i.e. repair and regeneration) that occurs following kidney transplantation.

**Keywords:** renal transplantation, gene expression, peripheral blood, biomarkers, regeneration and repair

As the demand for transplantable kidneys far exceeds the available supply [1], it has long been a clinical imperative to the transplant community to develop strategies that increase the number of transplantable organs or, more recently, reduce the demand by preventing progression to end stage organ failure requiring replacement therapy [2, 3]. And while kidney donation from a living donor (LD) still represents the *gold standard* in kidney transplantation (KT), the usable donor organ pool goes well beyond this resource (www.unos.org, [4]). Among the different categories of renal allografts, kidneys that have experienced acute damage before or during procurement (e.g. acute kidney injury (AKI) renal allografts and donation after cardiac death (DCD)), represent valuable organ sources for KT since the allograft will typically recover and resume normal function in the new host within the initial few weeks post-transplant [5-12].

As the mechanisms that underlie adaptive repair in the kidney are still largely unknown [13], we suggest that monitoring DCD and AKI renal allograft recovery in the transplant recipient represents a truly unique platform to study this phenomenon. In fact, due to the very nature of DCD and AKI (DCD/AKI) organ donation, the majority of DCD/AKI allograft recipients will experience delayed graft function (DGF) - which is a form of acute injury unique to the transplant process - but will eventually resume a normal function if donors and recipients are judiciously matched[5, 11]. Very importantly, given that DGF occurs in a tightly controlled scenario where the sequence of events and the evolution of the underlying biological phenomena can be closely monitored, the study of gene expression changes in DCD/AKI allograft recipients provides a highly relevant in-human model to investigate how the kidney repairs and regenerates after injury.

The genome-wide study of temporal variations in gene expression[14] is being widely used in modern medicine to better understand the molecular control of biological processes[15]. Importantly, this approached has been applied to transplant medicine and in particular to kidney transplantation, in

the attempt to better understand the pathophysiology of phenomena like acute rejection[16-20], chronic allograft injury[21], tolerance[22-24] and graft loss[25-27] through the identification of the consequences of static and dynamic genomic variation on allograft outcomes[15]. In this pilot study, we have used a functional genomics approach to investigate changes in peripheral blood gene expression that occur in the days following kidney transplantation. Two groups of patients were examined: (1) those who received a kidney from a related or unrelated living donor and, (2) those who received a kidney from a DCD or AKI donor – both of which, as mentioned above, are grafts that typically experience a transitory delay prior to optimal functioning in the new host.

This study was conceived, designed and performed to test the hypothesis that non-invasive, gene expression-based monitoring of peripheral blood following renal transplantation will provide novel insights into ongoing mechanisms of repair and regeneration responsible for allograft recovery.

## METHODS

Between May 2014 and July 2015 a total of 110 patients preparing to undergo KT at the Abdominal Organ Transplant Center at Wake Forest Baptist Hospital (representing all types of donor grafts: e.g. LD, DCD, AKI, standard criteria donor (SCD) and donation after brain death (DBD)) were recruited into our IRB-approved study (IRB00027118). From these, 16 patients (5 each from those receiving LD or AKI and 5 receiving DCD grafts) were chosen for this pilot study. Table 1 reports essential information on each patient and corresponding donor. Fifteen of the sixteen patients were induced with alemtuzumab 30 mg IV, administered throughout 3 hours during surgery. One patient received basiliximab 20 mg IV, at time 0 and day 4. All patients received a maintenance regimen based on tacrolimus (targeting 8-10 ng/ml during the first three months), mycophenolic acid (360 mg BID for

patients older than 60 years of age, 720 mg BID for younger patients) and prednisone taper. However, sensitized patients as well as patients whose baseline kidney disease was immune-mediated, were kept on prednisone long term. Data gathered for each consented patient included demographic information and a complete medical history, as well as medication usage, pre- and post-transplant clinical laboratory results, and results from outcomes monitoring. Banked biospecimens include peripheral blood samples collected pre-transplant (Day 0) and at up to 11 additional times throughout post-transplant Day 1-30 (Figure 1). The study follow up ended at one month because, in our experience, the chances to resume a decent function (corresponding to a GFR >30 ml/min) for a DGF kidney that, at this time point, still shows a poor function (GFR <20 ml/min), are basically null.

KT patients within our clinic are monitored closely in the initial 30 days following surgery as part of the routine standard of care. Blood is drawn from the graft recipient at admission (day 0), daily until discharge, and then twice weekly up to day 30, for a total of up to 12 blood samplings per patient (Figure 1). For patients who had consented to participate in this study, each time a clinically indicated blood draw was scheduled, an additional blood sample (2.5 ml) was collected, transferred into a PAXgene Blood RNA tube (Qiagen; Valencia, CA) and stored at -20$^{o}$C for later processing. Multiple blood samples (4-9) from each individual transplant patient, representing each of three kidney donor groups (LD, DCD, and AKI), were processed and those RNAs of sufficient quantity and quality were used for gene expression analysis (Table 2).

Total RNA was isolated from peripheral blood in a QIAcube robotic workstation using regents and protocols recommended by the manufacturer (PreAnalytix; Qiagen, Valencia, CA). Total RNA was evaluated for quantity (Nanodrop spectrophotometer) and quality (Agilent Bioanalyzer). Labeled cDNA derived from total RNA samples (RIN $\geq$ 7) was hybridized to whole-genome microarrays (HumanHT-12 v4 Expression BeadChip; Illumina Inc.) using protocols recommended by the manufacturer.

Following hybridization, washing, and scanning, data were extracted from scanned images using Genome Studio Software (Gene Expression module; Illumina Inc.) and processed for upload to gene expression analysis software.

Statistical Analysis: Following normalization (quantile) and log transformation of the raw data, unsupervised hierarchical clustering and analysis of variance (ANOVA) were performed, using Qlucore Omics Explorer (Qlucore, Lund, Sweden), to generate principal component analysis (PCA) plots and heat maps.

Gene ontology and pathway analyses were performed by evaluating lists of differentially-expressed transcripts in Ingenuity Pathway Analysis software (IPA, Qiagen).

## RESULTS

Gene expression data were generated from peripheral blood samples taken at multiple times from each of 15 KT patients representing three donor kidney types: LD, AKI and DCD (Table 2). The majority (9/15) of the KT patients were Caucasian, 4 were African-American, and 2 were "other", with a nearly equal number of males and females (8M:7F). The total number of blood samples processed was 100. Following quality check, a total of 91 (28 LD, 31 AKI, and 32 DCD samples) were determined to be suitable for microarray analysis (Table 2).

Initially we performed a comparative analysis of longitudinal peripheral blood gene expression (using *all* sample data) between recipients of LD grafts versus those with AKI/DCD grafts. This was accomplished using maSigPro, a statistical procedure specifically designed to identify genes that show different gene expression profiles across groups in time-course experiments[14]. The analysis revealed two significant gene clusters ($p < 0.01$), representing a total of 141 genes that show a different

expression profile between AKI/DCD and LD samples (Figure 2). Within the first two weeks post-transplant there appear to be several small individual groups of transcripts that show differential expression between the LD and AKI/DCD groups (Figure 2; highlighted in blue boxes). Although the number of samples at these time points does not provide sufficient power to draw meaningful conclusions, the transcripts populating these mini-clusters may warrant further investigation if confirmed in a larger sample.

The 77 transcripts that comprise cluster 1, most of which are downregulated over time compared to day 0, show a significant over-representation of genes in several key biological pathways including *mTOR signaling* (p=3.98E-05), *Granzyme B Signaling* (p=1.13E-03), and *Th1 and Th2 Activation Pathway* (p=2.73E-03) (Table 3). *Immunological Disease* (p=1.25E-02 - 3.76E-05) is the top diseases and disorders category over-represented in this cluster.

The second cluster of differentially-expressed genes (N=64; mostly upregulated) in the comparison between AKI/DCD and LD are significantly over-represented in *Toll-like Receptor Signaling* (p=6.61E-05), *Adrenergic signaling* (p=1.12E-04), and *fMLP Signaling in neutrophils* (p=4.24E-04) pathways (Table 3). The most significant diseases and disorders category over-represented in the set of transcripts is *Inflammatory Response* (p=8.54E-03 –1.41E-06).

The functional clustering of the 141 genes using PANTHER (http://pantherdb.org) identifies numerous relevant pathways. Of particular interest is the Wnt/beta catenin signaling pathway, known to play a positive role in the control of T-cell development, cell proliferation rate and differentiation. Three genes - transforming growth factor beta receptor type 3 (TGFBR3), gamma-secretase subunit (APH1B) and beta catenin (CTNNB1) - involved in Wnt/beta catenin signaling and in its immunomodulatory role were found to be altered in AKI/DCD patients compared to LD.

The 141 transcripts that are differentially expressed in the longitudinal comparison between LD and AKI/DCD include genes found to be different in: (1) LD vs AKI *only* (N=65) and, (2) LD vs DCD *only* (N=63). In the comparison of LD vs AKI + DCD (i.e. differentially expressed in *both* DGF groups) 11 transcripts were identified: LPCAT2, MAL, DDX52, LMNB1, CYP1B1-AS1, RHOC, ID2, KLHDC4, GNG10 and GNl1 (Table 4).

Next we performed gene expression analysis for each of the three donor types (LD, AKI and DCD) *individually* to determine if there were observable time-dependent and graft type-dependent patterns of gene expression discernible in peripheral blood. Due to the paucity of usable data for samples collected in weeks 2 and 3, these analyses were confined to samples from the early times points (day 0-5) and the latest time point (day 28-30) only. For the heatmap depictions of the ANOVA results, the time points were arranged from day 0 to day 30 (Figure 3). We expected to see the greatest differences in blood gene expression between day 0 (before the kidney was transplanted to the recipient) and the first days following transplant, and then another a return to baseline at day 30 after the kidney function had stabilized in the new host.

The pattern of gene expression in the LD group showed two distinct profiles (clusters) over time (Figure 3; left panels). In the first cluster, genes were upregulated at Day 0 and also at Day 1 and then expression trended downward in the majority of samples out to Day 30. In the second cluster, all of the transcripts had a large spike (increase) in expression on the first day following transplantation, and then trended downward from the 2$^{nd}$ day onward. The canonical pathways overrepresented in LD recipients (Table 5) are representative of cellular stress response (e.g. NF-κB signaling and p38 MAPK signaling) and a pro-inflammatory cellular response (e.g. role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis and LXR/RXR activation).

Differential gene expression over time in AKI graft recipients was uniformly high from Days 1-5 following surgery compared to both pre- and 30 days post-transplantation (Figure 3; middle panels). The molecular pathways overrepresented in AKI recipient blood are involved in host defense (e.g. Fcγ receptor-mediated phagocytosis and actin cytoskeleton remodeling), leukocyte migration from blood to tissue during inflammation, and the production of nitric oxide and reactive oxygen species (Table 5).

The longitudinal expression pattern gene expression in the blood of DCD recipients differs from patterns seen in both the LD and AKI recipients. A large number of genes are upregulated from the pre-transplantation time point through most of the initial 5 days post-transplant. By Day 28-30 most of the transcripts' expression levels have decreased significantly (Figure 3; right panels). Pathways that are upregulated throughout pre- and post-transplant (up to at least Day 5) time points are involved with innate and adaptive immunity including in B cell development, Th1 and Th2 pathway activation, antigen presentation, and apoptosis (e.g. the protein ubiquitination pathway; Table 5).

## DISCUSSION

The study of DCD and AKI renal allograft recipients offers a truly unique platform for understanding how the acutely damaged kidney recovers (i.e. repairs and regenerates) in the recipient. Moreover, the use of human samples is arguably the most relevant approach, bypassing the need for animal studies that may not be able to reliably model the human response[28]. The most important finding from this study is significant, biologically relevant, and time-dependent gene expression changes in peripheral blood from KT patients receiving LD, AKI and DCD grafts in the initial day(s) following surgery and both the magnitude and duration of these changes is specific to the source of allograft. In general, once the donor graft has been implanted into the recipient there is an activation of the immune

system - counterbalanced by immunosuppressive therapy - and a resultant transient increase in the expression of apoptosis genes, genes involved in the immune response and in multiple cell signaling pathways. This is followed by a decrease, over time, in the expression of immune response genes, among others, until the recipient blood gene expression profile returns to baseline. The finding that gene expression remains elevated, at least through the first week following surgery, in those who have received AKI or DCD donor grafts suggest that there may be additional repair and regeneration activities occurring in these patients.

The comparison of blood gene expression profiles between LD and AKI/DCD graft recipients revealed two clusters of differentially expressed genes. Among the 141 transcripts that populate these clusters, three genes involved in the Wnt-β-catenin signaling pathway (transforming growth factor beta receptor 3 (TGFBR3), catenin beta 1 (CTNNB1), and aph-1 homolog B, gamma-secretase subunit (APH1B)) were identified. These findings are of particular interest because this pathway is known to be involved both in kidney development[29, 30] and also in kidney repair and regeneration[31, 32]. Indeed, Lin and coworkers, using the kidney as a model, showed that the Wnt pathway ligand Wnt7b is produced by macrophages to stimulate repair and regeneration in the injured kidney[31]. Since Wnt7b is known to stimulate epithelial responses during kidney development, their data suggest that macrophages are able to rapidly invade injured tissue and reestablish a developmental program to facilitate repair and regeneration. Although the current pilot study did not specifically find a significantly increased expression of Wnt7b, there was a significant overrepresentation of genes in pathways involving macrophage activity during the post-transplant period (Table 5). Further analyses in a large patient cohort are required to validate genes within the Wnt-β-catenin signaling pathway as possible blood-based markers predictive of tissue repair and regeneration.

The longitudinal analysis also resulted in the identification of eleven genes that were differentially expressed in *both* DCD and AKI samples compared to LD. These transcripts may represent the most biologically relevant gene targets differentiating the two groups and warrant further investigation. Among them, MAL, a T cell differentiation protein was previously described among the most relevant modulated genes in peripheral blood of immunosuppressive drug treated patients[33]. In fact, the differential expression in AKI/DCD vs LD patients may underlie a differential drug response and inflammatory activation of these patient populations and may possibly represent useful a prognostic marker.

Traditionally, kidney biopsies have been used for gene expression profiling to gain insights regarding mechanisms of acute injury, as well to identify biological pathways involved in tolerance, immunosuppression, rejection and graft survival following KT. The primary limitation with this strategy is that, due to the invasive nature of procuring kidney biopsy tissue, it is not possible to do a multi time point investigation into the molecular events that are occurring within the initial recovery period (i.e. within the first 30 days). Profiling gene expression in peripheral blood offers a viable alternative strategy. In fact, in a recent study that performed a parallel profiling of peripheral blood and biopsy tissue samples from three groups of KT patients ([1] stable transplant, [2] subclinical acute rejection, and [3] clinical acute rejection) the authors report the equivalent predictive performance of the two analyses (blood and biopsy), so supporting the relevance of the results obtained in peripheral blood for detection of tissue dysfunction[34]. They further showed that comparisons between microarray and RNA sequencing signatures demonstrated a strong correlation between the blood and biopsy compartments regardless of the platform used.

While the data generated in this pilot study are suggestive of important gene expression differences, over time, revealed in peripheral blood of patients who have undergone KT, there are

several limitations that must be noted. First, the original study goal was to generate gene expression profiles in a total of 10-12 blood samples per KT patient, covering the entire first 30 days post-transplant, however this was not possible due to: (1) missing samples for some of the time points and, (2) samples for which the RNA quality was inadequate for further analysis. Although the number of samples in the 2nd and 3rd weeks post-transplant was uneven across individual sample sets and across groups, *all* data collected for *all* time points (Table 2) were used for the comparison of longitudinal gene expression differences between LD and AKI/DCD samples (Figure 2). And in the evaluation of gene expression profiles for each individual donor graft type, only samples from the first week (Day 0-6) and the final time point (Day 28-30) were used (Figure 3). This was done to maximize the power to detect relevant genes and biological pathways that are impacted in the critical time following KT. A more comprehensive understanding of genes and pathways involved in kidney repair and regeneration will need to include analysis of a more complete set of samples collected across all time points from Day 0 to 30.

Another limitation with this study was the inability to adequately match samples both within and between groups. The most obvious example is the fact that nearly all of the recipients of DCD grafts were African American whereas most of the other patients were Caucasian. Ethnicity can impact gene expression analysis and may explain at least some of the unusual pattern seen in the heatmap for gene expression in DCD samples (Figure 3; right panels).

Finally, using peripheral blood gene expression analysis to derive mechanistic insight regarding processes occurring elsewhere in the body (e.g. the kidney) is not optimal. Serial kidney biopsies, taken in the days following KT, would likely yield the most relevant information regarding processes that underlie tissue repair and regeneration however, as noted earlier, this is not practical. For this reason, profiling of peripheral blood has become the method of choice and there is at least some encouraging

evidence that this approach can yield results similar to those achieved by profiling the target tissue, at least where the kidney is concerned[34].

In conclusion, our data show that the pattern, magnitude, and duration of gene expression changes following KT are donor graft specific and provide information regarding molecular pathways and specific transcripts that are involved in the host response following this type of surgery. These results provide further evidence that measuring gene expression in an easily accessible surrogate tissue (whole blood) represents a convenient and biologically relevant approach. This has important implications for understanding the molecular mechanisms occurring in the host relative to the organ quality and to the consequent immunological activation. Additional analyses, involving increased sample numbers (number of patients *and* blood collection points), will be needed to validate candidate genes that underlie tissue repair and regeneration in patients receiving DCD and AKI kidney grafts. The discovery and understanding of the molecular pathways underlying successful kidney adaptive repair after damage will provide a unique opportunity to design targeted molecular- or cell-based therapies to accelerate the induction of repair and regeneration mechanisms in damaged kidneys. Such therapies would be broadly applicable across a myriad of clinical settings characterized by an acute or chronic impairment of renal function

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