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ORIGINAL ARTICLE

Impaired metabolism in donor kidney grafts after steroid pretreatment

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Summary

We recently showed in a randomized control trial that steroid pretreatment of the deceased organ donor suppressed inflammation in the transplant organ but did not reduce the rate or duration of delayed graft function (DGF). This study sought to elucidate such of those factors that caused DGF in the steroid-treated subjects. Genome-wide gene expression profiles were used from 20 steroid-pretreated donor-organs and were analyzed on the level of regulatory protein–protein interaction networks. Significance analysis of microarrays (SAM) yielded 63 significantly down-regulated sequences associated with DGF that could be functionally categorized according to Protein ANalysis THrough Evolutionary Relationships ontologies into two main biologic processes: transport ($P < 0.001$) and metabolism ($P < 0.001$). The identified genes suggest hypoxia as the cause of DGF, which cannot be counterbalanced by steroid treatment. Our data showed that molecular pathways affected by ischemia such as transport and metabolism are associated with DGF. Potential interventional targeted therapy based on these findings includes peroxisome proliferator-activated receptor agonists or caspase inhibitors.

Introduction

Kidney transplantation is the preferred treatment for end-stage renal disease (ESRD) because it is considerably less expensive than dialysis on an overall basis and allows for an almost normal life. One of the main reasons of graft failure is delayed graft function (DGF), a form of acute renal failure resulting in post-transplantation oliguria, increased allograft immunogenicity and risk of acute rejection episodes, and decreased long-term survival [1]. Roughly one-third of transplant patients receiving an organ from a deceased donor develop DGF and have to be treated by dialysis until the engrafted organ resumes function. The hazard ratio for graft failure is almost twice as high in recipients who experienced DGF when compared with those without initial complications [2]. Factors which contribute to DGF can be divided into donor-related and recipient-related factors. Donor-related factors include donor age, diseases such as hypertension, brain death-associated causes such as hemodynamic instability, massive cytokine release and vasopressor use. A thorough discussion of donor and recipient factors contributing to DGF was published by Schwarz *et al.* [3]. The fact that DGF is a rare exception in live kidney transplantation suggests that donor factors rather than the transplant procedure itself mainly contribute to DGF.

Next to the histopathological examination of renal biopsies the determination of gene expression profiles in

donor organs poses an option to determine graft quality and even predict transplant outcome to a certain extent [4,5]. In a recent study from our group, we reported a number of differentially regulated genes when comparing donor organs from living and deceased donor organs. Up-regulated genes in tissue samples from deceased donors were mainly involved in inflammatory processes, complement activation, apoptosis and cell adhesion [6].

Based on these findings, we initiated a randomized, double-blinded, placebo-controlled trial to elucidate whether pretreatment of deceased organ donors with corticosteroids (1 g methylprednisolone) before organ retrieval will reduce inflammation and subsequently the rate of DGF after engraftment. One main finding of this study with 447 renal allograft recipients was that steroid pretreatment caused a reduction of inflammatory signatures in the donor kidney as monitored on the level of gene expression profiles. However, neither the rate nor the duration of DGF was different in the treatment and placebo group. We therefore hypothesize that additional pathways beyond those related to inflammation are involved in the development of DGF. Thus the analysis of the steroid treatment arm provides a unique opportunity to investigate molecular mechanisms other than inflammation which contribute to DGF.

Brain death is associated with rapid swings in blood pressure, hypo- and hypertension, coagulopathies, pulmonary changes, hypothermia and electrolyte aberrations [7–9]. Therefore, donor brain death not only results in increased inflammation but also leads to hypoperfusion and hypoxia of the donor organ [10].

The main objective of this study was to elucidate molecular causes of DGF that were not abolished by the steroid donor pretreatment. Specifically, we compared the molecular signature of kidney biopsies from steroid-treated donors with primary graft function in relation to kidneys with DGF. We sought to identify potential new targets for intervention that ultimately may reduce the current high rate of DGF.

Material and methods

Donor- and recipient characteristics

Out of the 238 recipients of steroid pretreated donor kidneys, we randomly identified 10 of 52 who developed DGF and matched an equal number of primary graft kidneys. Matching variables of controls were cause of donor death (stroke versus trauma) and caliper-matching of donors' last creatinine and donor age.

The rationale behind the sample size was that based on previous data that 20 biopsies would be sufficient to detect a more-than-twofold difference in the expression of 30 predefined genes at an adjusted P-value of <0.05 using the Bonferroni–Holm method [6,11].

Trial design

Details on the multicenter trial may be found elsewhere (http://www.controlled-trials.com/ISRCTN78828338 and Kainz & Wilflingseder *et al.* [Abstract TTS Sydney 2008 #859, Annals of Internal Medicine submitted 2009] [12]. In brief, 269 donors stratified for age were equally randomized in blocks of 4–1000 mg of corticosteroid or placebo injection 6 h before organ recovery. Before transplantation, kidney wedge biopsies were obtained and subjected to genomics analyses. The post-transplant clinical course was monitored.

The study protocol was approved by the Institutional Review Board (Ethical Committee of the Medical University of Vienna # EK-067/2005, to be found at http://ohrp.cit.nih.gov/search/) and the EUROTRANSPLANT kidney advisory committee (#6021KAC06) at each study site and conducted according to IRB standards at each institution. DGF was defined as the need for more than one dialysis treatment within the first week after transplantation or creatinine values above 3 mg/dl during the first week after transplantation.

Laboratory procedures and biostatistical analyses

Donor kidney biopsy specimen, RNA isolation and amplification

All organs were perfused with a histidine-tryptophan-ketoglutarate (HTK) cold preservation solution at 4 °C during organ procurement [13]. The cold ischemic time was not longer than 24 h. Wedge biopsies of each kidney were taken under sterile conditions at the end of the cold ischemic time right before transplantation. The biopsy specimens were immediately submerged in RNAlater™ (Ambion, Austin, TX, USA) and stored at 4 °C.

Total RNA was isolated and purified using chloroform and trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA yield and quality was checked with the Agilent 2100 Bioanalyzer and RNA6000 LabChip® kit (Agilent, Palo Alto, CA, USA). Stratagene Universal human reference RNA was used as reference (Stratagene, La Jolla, CA, USA).

Two micrograms of isolated total RNA were amplified using the RiboAmp RNA amplification kit (Arcturus, Mountain View, CA, USA). The amplified RNA was inspected on an ethidium bromide stained 1% agarose gel and on the Agilent 2100 Bioanalyzer. For the 20 zero-hour kidney biopsies, the RNA was of sufficient quality to proceed with microarray analysis.

Microarray hybridization and scanning

Complementary DNA (cDNA) microarrays holding 41 421 (batch: SHEO) features were obtained from the Stanford University Functional Genomics core facility. All

microarray experiments were performed as described earlier [14]. The detailed protocols are available at http://genome-www.stanford.edu/. Using a type II experimental setup, 1 μg of sample and standard Stratagene Universal human reference aRNA were labeled with CyScribe cDNA postlabeling kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) in a two-step procedure.

Samples were loaded onto arrays and incubated for 18 h in a 65 °C water bath. After three washing steps, the fluorescence images of the hybridized microarrays were examined using a GenePix 4100A scanner (Molecular Devices, Sunnyvale, CA, USA). The GENEPIX PRO 6.0 software (Molecular Devices, Sunnyvale, CA, USA) was used to grid images and to calculate spot intensities. Arrays were numbered according to the anonymous organ donor ID, and were processed in random order. Image-, grid- and data-files were submitted to the Stanford Microarray Database (http://genome-www5.stanford.edu/MicroArray/SMD/) and followed MIAME guidelines for arrays experiments [15,16]. Raw data files as well as the MIAME checklist are available at our laboratory webpage at http://www.meduniwien.ac.at/nephrogene/data/DGF/.

Microarray data analysis

The microarray dataset consisted of 41 421 cDNA features. 41 025 of those held a UniGene Cluster ID (27 442 unique genes), 396 were expressed sequence tags (ESTs) not assigned to a UniGene Cluster. Mean sector and printing plate ANOVA R^2-values of the microarray experiments were on average 4.5×10^{-2} and 3.1×10^{-2} respectively, suggesting no dependency of results on spatial location or plate printing procedures. In a first preprocessing step a quality filter was applied on the dataset by considering only genes and ESTs with spot intensities of at least 1.5-fold over background in either channel 1 or 2 of the microarray thus leaving 32 588 cDNA features in the dataset. Only genes and ESTs with at least 80% of valid entries were considered for successive analysis steps thus further reducing the dataset to 24 624 cDNA features. The remaining missing data points were substituted applying a *k*-nearest-neighbor algorithm, where the number of neighbors, *k*, was set to 10 [17]. No correction for a putative batch bias was necessary because only one array batch was used in the whole analysis for all arrays. We used the SAM methods as well as the Student's *t*-test in order to find differentially regulated genes (DEGs) between patients experiencing DGF and the control group with primary functioning (PF) grafts [18]. The *P*-value threshold was set to <0.05 with fold-change values >2. The number of permutations in the significance analysis of microarrays (SAM) method was set to 20 000 and a false discovery rate of 2.5% was selected. Differentially expressed genes were hierarchically clustered and graphically represented using the MultiExperiment Viewer developed at The Institute for Genomic Research [19]. The cosine correlation and complete linkage were used as distance measure and linkage rule in the hierarchical cluster algorithm respectively [19,20].

Functional data enrichment

Differentially regulated genes (DEGs) were furthermore analyzed with respect to their molecular functions, associated biological processes, and cellular locations using gene ontology terms (GO-Terms) as provided by the Gene Ontology Consortium [21]. The SOURCE tool from the Stanford Genomics Facility was used for linking GO-Terms to the genes of interest [22]. Functional grouping of genes was based on GO-Terms, Protein ANalysis THrough Evolutionary Relationships (PANTHER) ontologies, and information derived from the protein data retrieval system iHOP [23,24].

Regulatory network analysis

All identified DEGs were mapped on a molecular dependency graph holding about 70 000 annotated human proteins [25]. Each graph node codes for a particular protein and edges between nodes encode pairwise dependencies. Dependencies were computed based on protein–protein interaction information, similarity in gene expression, conjoint regulatory patterns on the level of transcription factors and microRNAs, as well as assignment to functional ontologies. Subnetworks holding at least two DEGs were retrieved and further analyzed on a functional level.

Statistical analysis

Continuous data were analyzed by Wilcoxon rank-sum tests, categorical data by chi-squared tests or Fisher's exact tests when appropriate. A *P*-value <0.05 was considered statistically significant. For all analyses SAS for Windows 9.2 (The SAS Institute, Inc., Cary, NC, USA) was used.

Results

Demographic data on transplant donors and recipients are provided in Table 1.

Molecular signatures separating DGF from primary function (PF) in steroid-treated donor organs

Using the SAM method, 63 transcripts could be identified as significantly differentially regulated. Both gene lists are provided in Tables S1 and S2 sorted by fold-change values.

Table 1. Demographic data of transplant donors and recipients stratified by treatment assignment. Continuous data are provided as median (first, third quartile), categorical data are shown as counts.

	PF group	DGF group	*P*-value
No. donors	16		na
No. donor organs	10	10	na
Donor age (years)	52.5 (45.0, 58.0)	62.5 (55.0, 72.0)	0.045
Donor gender (female/male)	4/6	7/3	0.370*
Last creatinine of donor (mg/dl)	1.00 (0.71, 1.20)	0.70 (0.60, 1.00)	0.254
Vasopressors used (*n*/year)	2/8	0/10	0.136
Multiorgan donors (*n*/year)	7/3	8/2	1.000*
Cause of death (trauma/intracranial hemorrhage/cardiac arrest/else)	1/8/1/0	0/9/0/1	0.383
No. recipients	10	10	na
Recipient age (years)	57.3 (51.6, 62.2)	59.1 (46.3, 67.1)	0.734
Recipient gender (female/male)	3/7	3/7	1.000
Transplant number (1/2)	9/1	9/1	1.000*
Cold ischemic time (h)	9.9 (7.0, 15.0)	12.7 (10.3, 4.4)	0.308
PRA latest (%)	0.0 (0.0, 2.0)	0.0 (0.0, 2.0)	1.000
Sum of HLA mismatches (0/1/2/3/4/5/6)	0/1/4/1/1/0/0	0/0/1/3/1/5/0	0.076*
No. dialysis treatment (0/1/2/3/4)	10/0/0/0/0	3/5/0/1/1	0.003*
Immunosuppression (CNI/else)	8/2	9/1	1.000*
Induction therapy (none/antiCD25/ATG)	6/4/0	7/3/0	0.639

na, not applicable.
*Fisher's exact test.

In total, 147 features showed fold-change values >2 and *P*-values smaller than 0.05 following a *t*-test. The majority of features were suppressed with only 10 genes being up-regulated in the DGF as compared with the PF group.

An expression profile-based clustering resulted in an almost complete discrimination between DGF and PF samples as given in Fig. 1.

Functional analysis

Thirty-nine out of the 63 transcripts (SAM, 41 unique genes) and 84 out of the 135 down-regulated transcripts (*t*-test, 91 unique genes) could be mapped to PANTHER IDs. Significantly enriched or depleted biological processes with at least two members are given in Table 2 (*P*-value <0.05 given by a chi-squared test when comparing the number of genes associated to the category with the total number of genes belonging to this particular process). Enriched processes mainly include genes involved in transport and metabolism. DGF-associated down-regulated genes include many transcripts encoding solute carriers (ion, amino acid and glucose transporters) in the plasma membrane and other transporters in the cytoplasma and extracellular space. Prominent members are the organic anion transporter (SLC22A8), neutral amino acid transporter (SLC6A19), the sodium/glucose cotransporter (SLC5A12), lipocalin 2 (LCN2), and apolipoprotein D (APOD). Proteins involved in metabolism, including lipid, fatty acid, and steroid metabolism, were predominantly down-regulated in DGF samples. Depleted processes are nucleoside and protein metabolism, mRNA transcription and intracellular protein traffic. Up-regulated transcripts (*t*-test, nine unique genes) were mainly associated with blood clotting as well as immunity and defense.

Interactome analysis

We retrieved in total seven networks holding at least two of the differentially regulated genes (Fig. 2). Members of network cluster 1 holding 13 proteins are mainly involved in blood clotting with fibrinogen gamma (FGG), fibrinogen alpha (FGA), and the frizzled homology 8 being up-regulated in patient samples experiencing DGF. Hypoxia and an older donor age might lead to the activation of fibrotic pathways which contribute to DGF. The central protein of network cluster two is the suppressor of cytokine signalling 3 (SOCS3) that shows higher expression values in the group of patients with DGF post-transplant. The other network clusters contain mainly down-regulated genes with members of cluster 6 being involved in steroid metabolism and members of clusters 4 and 7 being involved in lipid and fatty acid metabolism (Fig. 2).

Discussion

In this study, we elucidated molecular mechanisms associated with DGF after renal transplantation in zero-hour

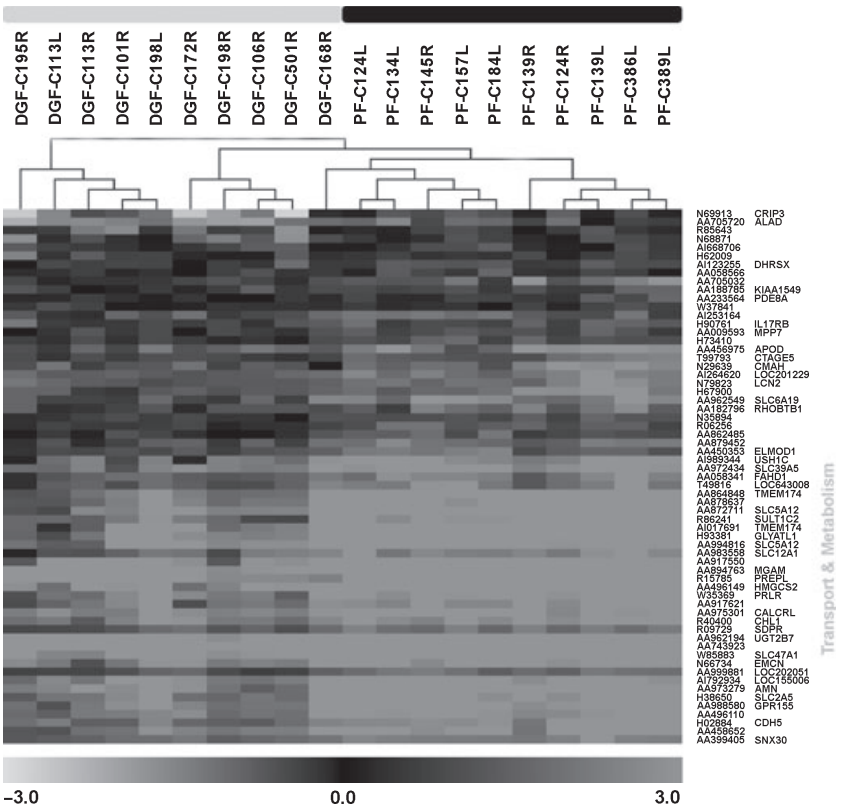

Figure 1 Dendrogram derived by unsupervised hierarchical clustering of gene expression profiles dichotomizing delayed graft function (DGF) group (grey bar) from primary function (PF) (black bar), all received steroid pretreatment. Grey spots indicate up-regulated or down-regulated transcripts relative to the reference RNA used. The differentially regulated genes associated with DGF could be categorized according to GO-terms mainly into transport and metabolism.

donor kidney biopsies pretreated with corticosteroids. Based on our findings, poor initial function can be explained by a partial shutdown of metabolism and transport activity on a molecular level.

One possible explanation of reduced transport and metabolism is hypoxia. In the absence of oxygen, severe energy depletion, i.e. less production of ATP and subsequent activation of number of critical alterations in metabolism, occurs [26]. The effects of limited oxygen supply are aggravated by the higher demand associated with the high tubular oxygen consumption necessary for solute exchange [27] and the high rate of aerobic glycolysis [28]. Hypoxia is also a profibrogenic stimulus for tubular cells, interstitial fibroblasts, and renal microvascular endothelial cells. Hypoxia can also activate fibroblasts and change the extracellular matrix metabolism of resident renal cells [29,30] and has been shown to play a role in the progression of chronic kidney disease [31]. Therefore, the use of effective preservation solutions and reduction of cold ischemia times may improve kidney function after transplantation [32].

The down-regulation of many transporters is probably caused by less oxygen supply and subsequent energy depletion. The solute carrier family 4, sodium bicarbonate cotransporter, member 4 (SLC4A4) built a small cluster with the carbonic anhydrase IV (CA4) and is involved in the regulation of bicarbonate secretion and absorption and intracellular pH suggesting tubular acidosis (Fig. 2). Protein–protein interactions of transporters in the molecular dependency graph are rare suggesting that these pathways are under-represented in the interactome analysis.

Lipid metabolism, fatty acid metabolism and steroid metabolism are down-regulated in DGF samples and are the most enriched functional categories next to transport function (Fig. 2, network clusters 4, 6, 7). Although the hydroxyprostaglandin dehydrogenase 15-(NAD) (HPGD), the sulfotransferase family, cytosolic, 1C, member 2 (SULT1C2), and the three glucuronosyltransferase 2 family polypeptides UGT2B15, UGT2B4, UGT2B7 are members of the steroid metabolism, they cannot be linked directly to methylprednisolone treatment. Another

Table 2. Functional classification of DEGs using PANTHER ontologies: Enriched or depleted biological processes Separating DGF and PF as derived on the level of differential gene expression by *t*-test and SAM. Categories are ranked by the *P*-value (comparison of expected number of genes and observed number of genes in each biological process) indicating the relevance of a particular process.

	t-test (n = 84)		SAM (n = 39)	
Biological process	No. genes	*P*-value	No. genes	*P*-value
DEGs down-regulated in DGF enriched processes				
Transport	20	<0.001	8	0.001
Lipid, fatty acid and steroid metabolism	12	<0.001	5	0.006
Amino acid metabolism	7	<0.001	2	0.049
Steroid hormone metabolism	4	<0.001	2	0.002
Steroid metabolism	6	<0.001	3	0.003
Ion transport	9	<0.001	–	–
Coenzyme and prosthetic group metabolism	5	<0.001	3	0.003
Amino acid transport	3	0.001	–	–
Carbohydrate metabolism	8	0.001	–	–
Fatty acid metabolism	4	0.004	–	–
Other amino acid metabolism	2	0.005	–	–
Cation transport	6	0.005	–	–
Electron transport	4	0.010	–	–
Vitamin/cofactor transport	2	0.011	–	–
Other polysaccharide metabolism	3	0.012	–	–
Cell adhesion	6	0.017	–	–
Homeostasis	3	0.028	–	–
Extracellular transport and import	2	0.028	–	–
Anion transport	2	0.034	–	–
Sulfur metabolism	2	0.035	–	–
Proteolysis	7	0.036	–	–
Other developmental process	2	0.042	–	–
Depleted processes				
Nucleoside, nucleotide and nucleic acid metabolism	5	0.042	–	–
Intracellular protein traffic	0	0.043	–	–
mRNA transcription	2	0.047	–	–
DEGs up-regulated in DGF-enriched processes	*t*-test (n = 9)		SAM (n = 0)	
Blood circulation and gas exchange	2	<0.001	–	–
Blood clotting	2	<0.001	–	–
Immunity and defense	3	0.009	–	–

prominent gene, the suppressor of cytokine signaling 3 (SOCS3), belongs to a family of negative-feedback regulators of cytokine signaling. This regulator is induced by its corresponding cytokines leading to the subsequent shutdown of the respective signaling cascade [33]. SOCS3 is involved in the JAK/STAT-dependent cytokine signaling pathways and is linked to the down-regulated prolactin receptor. On the other side, SOCS3 is linked over IRS2 (insulin receptor substrate 2) to the down-regulated insulin receptor (Fig. 2, cluster 2).

Reduced transport activity and metabolism indicating poorer quality of renal grafts was also reported by other trancriptomics studies of donor kidney biopsies developing DGF [6,34,35]. Approximately one-third of reported down-regulated genes by Mueller *et al.* were also identified in our study, thus strengthening the validity of obtained results. The common theme of inflammation and immune response in the context of DGF was delineated in all three studies. The suppression of inflammation with corticosteroids in our study led to the identification of novel molecular mechanisms besides inflammation and complement activation associated with the development of DGF, namely limited transport capabilities and decreased metabolic activity of the renal organ. However, one cluster in the dependency graph with the down-regulated major histocompatibility complex, class II, DR beta 3 (HLA-DRB1) and the up-regulated CD3d molecule, delta (CD3-TCR complex) (CD3D) belongs to immunity response.

A fair number of induced genes in DGF samples could be linked to blood clotting with fibrinogen gamma and fibrinogen alpha being two prominent members. This

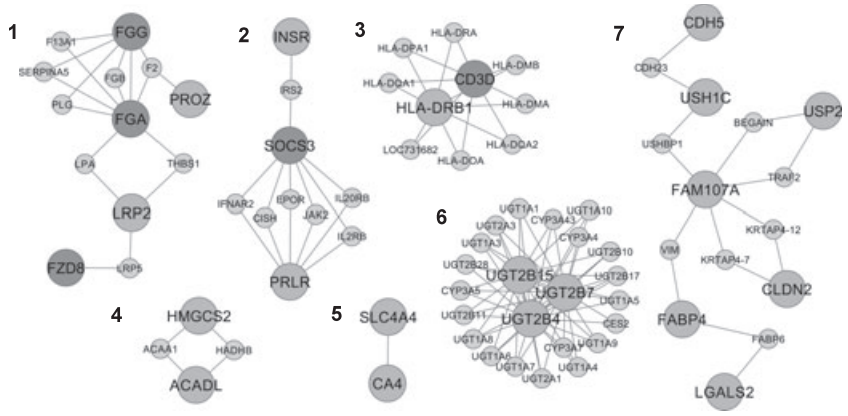

Figure 2 Seven identified networks with at least two differentially regulated genes between delayed graft function (DGF) and primary function (PF) samples. Dark grey nodes depict up-regulated genes in DGF samples whereas light grey nodes depict down-regulated genes. Differentially expressed proteins showed a high connectivity in these networks, thus indicating concerted interaction and relevance in the development of DGF.

might in part be explained by the advanced age of the donors in the DGF group. Donor age is a well known risk factor of DGF but not all grafts from old donors have necessarily poor graft function. Determination of the graft quality based on demographic/clinical and molecular risk factors probably provides a much better forecast model [4]. Especially the shortage of donor organs makes an expansion of donor criteria to include older and non-heart-beating donors necessary with the risk of higher rates of DGF. Therefore a better understanding of molecular mechanisms leading to DGF is of great interest and new strategies and better donor management is of great importance for the prevention of this disease.

A limitation of this study is probably the use of cDNA arrays which cannot discriminate between different splice variants in the measurement of expression levels. Nonetheless, we could identify genes mainly involved in transport and lipid, glucose metabolism associated with DGF in renal transplants.

Based on these results, it can be hypothesized that the activation of lipid and glucose metabolism may prevent the graft from developing acute renal failure. One possible treatment strategy is the administration with peroxisome proliferator-activated receptor (PPAR) agonists. The PPARs are ligand-activated transcription factors that control lipid and glucose metabolism. Activation of PPARs negatively regulates the expression of genes induced by cerebral ischemia/reperfusion injury and was shown to prevent post-ischemic inflammation and neuronal damage in several *in vitro* and *in vivo* models [36].

Another possible strategy to revert the effects of hypoxia is the treatment with caspase inhibitors. The administration of caspase inhibitors *in vivo* was demonstrated to protect against cell death in animal models of ischemic acute renal failure [37]. The pancaspase inhibitor Q-VD-OPH prevents the rise in caspase activity and apoptosis [38]. Therefore PPAR-agonists and caspase inhibitors may be adopted in the donor pretreatment to prevent ischemic/reperfusion injury in the kidney. Donor pretreatment has great advantages for the recipient because improved long-term survival could thus be achieved cost-efficiently and without great effort or side-effects.

In summary, our analyses provide novel insight into the biological processes that are associated with postischemic DGF. Based on our findings prospective trials with targeted therapy, including PPAR-agonists or caspase inhibitors, may be designed to elucidate the causal inference of these risk markers of DGF.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Sixty-three differentially regulated transcripts computed with the significance analysis of microarrays (SAM) method sorted by fold-change values. The number

of permutations in the SAM method was set to 20 000 and a false discovery rate of 2.5% was selected.

Table S2. One hundred and forty-seven differentially regulated transcripts computed with the Student's *t*-test sorted by fold-change values. The *P*-value threshold was set to <0.05 with fold-change values >2.

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