SDC, Material and Methods

Immunosuppressive Treatment

Induction therapy was administered according to established center practices. Recipients at high-risk of rejection (PRA>50%, re-transplantation) received induction therapy with rabbit anti-thymocyte globulin (rATG, Thymoglobulin®, Genzyme Corporation, Cambridge, MA), and recipients with PRA 20-49% received induction therapy with Basiliximab (Simulect®, Novartis Pharma AG, East Hanover, NJ). Low-risk renal transplant recipients received no induction. All patients received maintenance immunosuppressant therapy with tacrolimus (Prograf or Advagraf®; Astellas Pharma, Inc., Deerfield, IL), mycophenolate mofetil (Cellcept®; Roche Laboratories, Nutley, NJ), and tapered prednisone.

Real-time RT-PCR Analysis Using TaqMan Low Density Arrays (TLDA)

RNA extraction and DNA synthesis was carried out as described previously (44). To identify potential gene signatures associated with I/RI and their potential impact on delayed graft function we used real time RT-PCR with a custom-made TaqmanR low density array (TLDA) of 96 genes. A quantitative real-time RT-PCR assay based on TLDA technology was performed as previously described (45) and data were quantified using the SDS 2.4 software package (Applied Biosystems).

Expression Data Analysis

Relative gene expression values were generated from TLDA analysis using the comparative 2-ΔΔCt method for relative quantification (46) implemented by the Applied Biosystems Relative Quantification Manager Software v1.2.1 (47) (Applied Biosystems). To calculate the RQ of target genes in transplanted kidneys, commercially available normal human kidney RNA (Applied Biosystems) was designated as a calibrator. Using the $2^{-\Delta\Delta Ct,}$ the data are

presented as the fold-change in gene expression normalized to an endogenous reference gene relative to the calibrator sample.

Immunohistochemistry (IHC)

To further validate the relevance of molecular predictive markers associated with DGF, respective samples were examined using immunohistochemistry. Immunohistochemical detection of Netrin-1 was performed on 4 Qm-thick paraffin sections of donor wedge and post-implantation biopsies using a two-step indirect method. Slides were deparaffinized in xylene and rehydrated in graded ethanol. Heat-induced epitope retrieval in buffer was used to improve antibody reactivity. Endogenous peroxidase was blocked using 0.3% H2O2 in 70% methanol for 30 minutes. Samples were incubated with primary antibody (polyclonal rabbit anti-Netrin-1, Acris, Herford, Germany, 1:800 dilution) overnight at 4°C in EDTA, pH 8. Primary antibody detection was performed using Simple Stain MAX PO (MULTI) Universal Immuno-peroxidase Polymer anti-rabbit Histofine (Nichirei, Japan). Finally, specimens were stained with Dako Liquid DAB Substrate-Chromogen System (Dako, Glostrup, Denmark) for 2 minutes and counterstained with Harris's hematoxilin.

Statistical analysis

Data analyses were performed using PASW 18.0 statistical software package (SPSS Inc., Chicago, IL, USA) and GraphPad InStat Ver. 3.05 for Windows (GraphPad Software, San Diego, California, USA). Multivariable logistic regression analysis was conducted to evaluate the discriminative power of a set of hypothesized predictors with respect to the outcome variable (DGF). For training the logistic regression model, we considered the hypothesized two variables, Netrin-1 expression and tubular atrophy score (ct) as independent variables and the DGF variable as the dependent variable. No feature selection was thus conducted as part of model training. The "glm" package of the R-project software (with the "logit" option

specifying the model family) was used to fit the three model's parameters (a coefficient of each, NTN-1 and ct, and the model's intercept). We employed the leave-one-out cross-validation procedure to repeatedly (35 times) fit the parameters of the model on a training data set and then tested the model's predictive power on an independent (testing) data set. The average area under the ROC curve (c-statistic) derived from this cross-validation procedure reflects the model's discriminative power. For functional interpretation of genes involved in I/RI, we employed techniques designed for gene-set enrichment analysis that can detect differential expression of entire a priori defined gene groups. These gene groups are associated with known biochemical pathways and Gene Ontology term annotations were sourced from the Molecular Signatures Database (48). Gene groups containing none of the genes included in our assays were deleted. The enrichment score (P-values) for each gene group were computed using the Global test method available as a library in the system R and adjusted for multiple testing (49-50). The principal component analysis (PCA) was used to visualize patterns in the dataset without any a priori sample classification. PCA linearly projects a high dimensional sample space (where point coordinates correspond to individual interrogated genes) into a low-dimensional (typically 2D) space amenable to visualization thereby preserving as much of the sample variance as possible. Therefore, similar gene expression profiles are represented by 2 close points in the PCA projection compared to 2 dissimilar profiles represented by 2 distant ones. PCA was applied on the joint data set of 105 kidney biopsy samples (3 sequential biopsies from 35 kidney allografts), each described by the expression of 71 probe sets obtained from the originally studied 92 probe sets by removing all probe sets with at least one missing value.

SDC, Table S1. Descriptive statistics stratified by delayed and primary graft function.

	All	DGF (n=9)	PGF (n=26)
N	35	9	26
Donor age (yr)	53 (43-63)	53 (42-66)	55 (43-61)
Donor gender (male)	24 (68.6%)	3 (33.3%)	21 (80.8%)*
Donor BMI (kg/m^2)	26.23 (24.84-27.76)	26.23 (24.92-28.54)	26.27 (24.8-27.76)
Donor CRP (mg/L)	101 (43-174)	100 (59-177)	102 (42-179)
Max. donor S-Cr (µmol/L)	91 (79-106)	66 (48- 128)	91 (80-105)
Donors with hypertension	13 (37.1%)	3 (33.3%)	10 (38.5%)
Donors with DM	5 (14.3%)	1(11.1%)	4 (15.4%)
ECD donor #	14 (40%)	3 (33.3%)	11 (42.3%)
Cold ischemia time (h)	15.2 (14.1-17.4)	16.68 (14.32-19.24)	14.94 (14.1-17.26)
Recipient age (yr)	58 (49-60)	57 (53.5-61.5)	58.5 (48.75-60.75)
Recipient sex (male)	19 (54.3%)	6 (66.6%)	13 (50%)
Recipient PRA (%)	24 (4-66)	28 (5-73)	24 (4-66)
Dialysis vintage(day)	959 (638-1440)	1297 (545-2116)	887 (608-1402)
HLA mismatch	3 (2-4)	3 (2.5-5)	3.5 (2-4)
Graft function onset (day)	0 (0-5.25)	13 (6-17)	0 (0-0)***
S-Cr (µmol/L) Day 7	202 (138-349)	600 (463-600)	155 (123-226)
S-Cr (µmol/L) Month 1	134 (106-174)	180 (143-214)	127 (98-158)
S-Cr (µmol/L) Year 1	126 (100-154)	174 (135-187)	116 (96-138) ***
eGFR (mL/s) Day 7	0.49 (0.24-0.73)	0 (0-0.15)	0.63 (0.44-0.79)***
eGFR (mL/s) Month 1	0.65 (0.47-0.89)	0.46 (0.4-0.67)	0.71 (0.6-0.93)*
eGFR (mL/s) Year 1	0.79 (0.59-0.89)	0.58 (0.44-0.67)	0.81 (0.72-0.97) ***
TAC level (µg/L) day 7	15.8 (13.15-20.35)	15.1 (12.4-19.15)	16.85 (13.18-21.33)
TAC level (µg/L) month 1	13.8 (11.8-17.95)	13.4 (6.45-15.8)	14.1 (11.85-18.6)

Induction treatment n (%)			
None	12 (34.3%)	3 (33.3%)	9 (34.6%)
Basiliximab	7 (20%)	5 (55.6%)	6 (23.1%)
ATG	16 (45.7%)	1 (11.1%)	11 (42.3%)

Continuous data are expressed as median (interquartile range) values and categorical data presented as counts (%). * P<0.05, ***P<0.001 between DGF and PGF grafts. #Expanded criteria donors definition (42): age ≥60 years or older or >50 years with at least 2 of the following conditions: hypertension history, serum creatinine >1.5 mg/dl (132 μmol/L), or cause of death from a cerebrovascular accident.

Abbreviations: ATG, antithymocyte globuline; BMI, body mass index; CRP, C-reactive protein; DGF delayed graft function; ECD, expanded criteria donor; DM, diabetes mellitus; eGFR, glomerular filtration rate estimated with MDRD; HLA, human leukocyte antigen; MDRD, modification of diet in renal disease formula; PGF, primary graft function; PRA, panel reactive antibodies; S-Cr, serum creatinine; TAC, tacrolimus.

SDC, Table S2. List of genes represented on the customized TLDA including gene symbols and Applied Biosystems Gene Expression Assay ID Numbers.

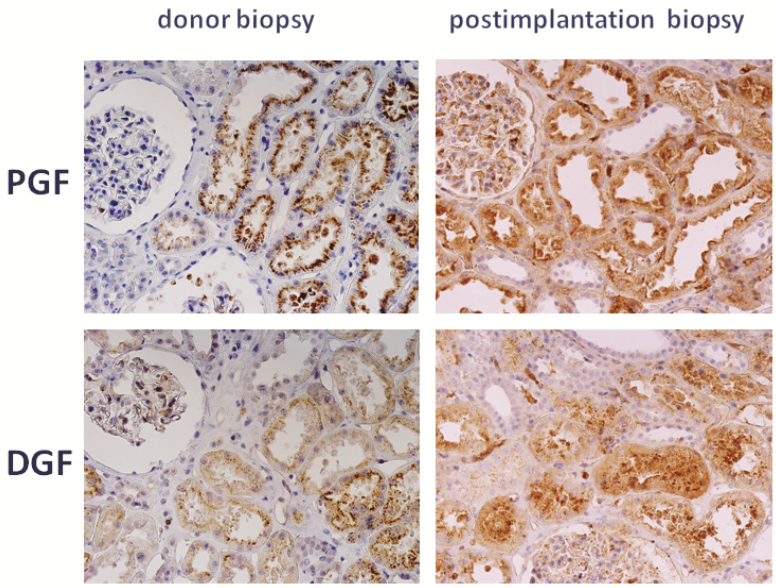
Nr.	Gene symbol	Gene	Assay map	Literature reference
1	A2M	Alpha-2-macroglobulin	Hs00163474_m1	(1)
2	ACTA2	actin, alpha 2, smooth muscle, aorta	Hs00426835_g1	(1, 2)
3	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	Hs01095532_g1	(3)
4	AMBP	alpha-1-microglobulin/bikunin precursor	Hs00155697_m1	(4)
5	ANXA2	annexin A2	Hs01561520_m1	(1, 5)
6	ANXA3	annexin A3	Hs00971411_m1	(1)
7	AQP1	aquaporin 1 (Colton blood group)	Hs00166067_m1	(1)
8	ATF6	activating transcription factor 6	Hs00232586_m1	(6)
9	B2M	beta-2-microglobulin	Hs99999907_m1	housekeeping gene
10	BAD	BCL2-associated agonist of cell death	Hs00188930_m1	(7)
11	BAK1	BCL2-antagonist/killer 1	Hs00832876_g1	(7)
12	BAX	BCL2-associated X protein	Hs01016552_g1	(7-9)
13	BCL2	B-cell CLL/lymphoma 2	Hs00236808_s1	(7-10)
14	BCL2L11	BCL2-like 11 (apoptosis facilitator)	Hs00708019_s1	(10)
15	BID	BH3 interacting domain death agonist	Hs00609632_m1	(11)
16	C3	complement component 3	Hs00163811_m1	(12, 13)
17	C3AR1	complement component 3a receptor 1	Hs00377780_m1	(12, 13)
18	C5	complement component 5	Hs00156197_m1	(12, 13)
19	C5AR1	complement component 5a receptor 1	Hs00704884_s1	(12, 13)
20	CASP3	caspase 3, apoptosis-related cysteine peptidase	Hs00991557_g1	(14)
21	CASP8	caspase 8, apoptosis-related cysteine peptidase	Hs01018151_m1	(14)
22	CASP9	caspase 9, apoptosis-related cysteine peptidase	Hs00609647_m1	(14)
23	CCL19	chemokine (C-C motif) ligand 19	Hs00171149_m1	(15)

24	CCL2	chemokine (C-C motif) ligand 2	Hs00234140_m1	(16, 17)
25	CCL21	chemokine (C-C motif) ligand 21	Hs00171076_m1	(15)
26	CCL5	chemokine (C-C motif) ligand 5	Hs99999048_m1	(17, 18)
27	CD28	CD28 molecule	Hs00174796_m1	(19)
28	CD40	CD40 molecule, TNF receptor superfamily member 5	Hs01002913_g1	(17)
29	CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	Hs00892618_m1	(20)
30	CD68	CD68 molecule	Hs00154355_m1	(1, 21)
31	CD69	CD69 molecule	Hs00156399_m1	(18)
32	CD80	CD80 molecule	Hs00175478_m1	(17)
33	CD86	CD86 molecule	Hs00199349_m1	(17)
34	CD8A	CD8a molecule	Hs01555600_m1	(18)
35	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Hs99999142_m1	(1, 22-24)
36	CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	Hs00923894_m1	(23)
37	CFB;C2	complement factor B	Hs00156060_m1	(12)
38	CFD	complement factor D (adipsin)	Hs00157263_m1	(25)
39	CLU	clusterin	Hs00971651_m1	(1, 26)
40	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Hs00605382_gH	(1, 27)
41	CXCL10	chemokine (C-X-C motif) ligand 10	Hs01124251_g1	(28)
42	CYCS	cytochrome c, somatic	Hs01588973_m1	(10)
43	CYR61	cysteine-rich, angiogenic inducer, 61	Hs00155479_m1	(29)
44	DAXX	death-domain associated protein	Hs00985567_g1	(30)
45	EGF	epidermal growth factor	Hs00153181_m1	(1)
46	EGR1	early growth response 1	Hs00152928_m1	(1, 31)
47	ENG	endoglin	Hs03986114_s1	(32)
48	FABP1	fatty acid binding protein 1, liver	Hs00155026_m1	(33)
49	FADD	Fas (TNFRSF6)-associated via death domain	Hs00538709_m1	(30)

50	FOS	FBJ murine osteosarcoma viral oncogene homolog	Hs01119266_g1	(1)
51	FOSL1	FOS-like antigen 1	Hs00759776_s1	(1)
52	GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1	housekeeping gene
53	HAVCR1	hepatitis A virus cellular receptor 1, also known as kidney injury molecule (KIM1)	Hs00273334_m1	(1, 34)
54	HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	Hs00900066_m1	(35)
55	HMOX1	heme oxygenase (decycling) 1	Hs01110251_m1	(26, 36-39)
56	HSPA1A	heat shock 70kDa protein 1A	Hs00359163_s1	(39-41)
57	ICAM1	intercellular adhesion molecule 1	Hs00277001_m1	(42-45)
58	IFNG	interferon, gamma	Hs99999041_m1	(46)
59	IL10	interleukin 10	Hs00961620_g1	(46-48)
60	IL12A	interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	Hs99999036_m1	(49)
61	IL17A	interleukin 17A	Hs99999082_m1	(50, 51)
62	IL2	interleukin 2	Hs00914135_m1	(48)
63	IL2RA	interleukin 2 receptor, alpha	Hs00907779_m1	(52)
64	IL6	interleukin 6 (interferon, beta 2)	Hs02621719_u1	(46, 48, 53)
65	IL8	interleukin 8	Hs00174103_m1	(54)
66	JAK1	Janus kinase 1	Hs00233820_m1	(55)
67	LAMA5	laminin, alpha 5	Hs00245699_m1	(55)
68	LCN2	lipocalin 2, also known as neutrophil gelatinase- associated lipocalin (NGAL)	Hs00194353_m1	(1, 56, 57)
69	MAPK8	mitogen-activated protein kinase 8	Hs00177083_m1	(58)
70	MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	Hs00957555_m1	(59)
71	MYD88	myeloid differentiation primary response gene (88)	Hs00182082_m1	(60)
72	MYO5A	myosin VA (heavy chain 12, myoxin)	Hs00165309_m1	(1)
73	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells	Hs00231653_m1	(16)

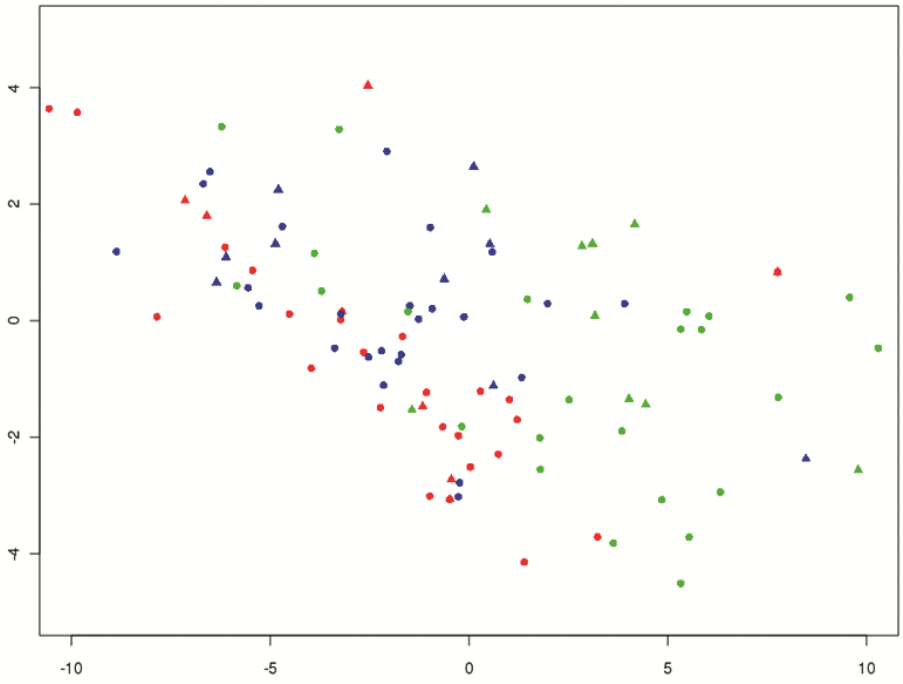
		1		
74	NTN1	netrin 1	Hs00924151_m1	(61, 62)
75	PDGFB	platelet-derived growth factor beta polypeptide	Hs00234042_m1	(55, 63)
76	PECAM1	platelet/endothelial cell adhesion molecule	Hs01065279_m1	(64)
77	PGK1	phosphoglycerate kinase 1	Hs99999906_m1	housekeeping gene
78	PPIA	peptidylprolyl isomerase A (cyclophilin A)	Hs99999904_m1	housekeeping gene
79	PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	Hs00188149_m1	(15, 65)
80	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Hs01573471_m1	(66)
81	PTPRC	protein tyrosine phosphatase, receptor type, C	Hs00236304_m1	(67)
82	S100A6	S100 calcium binding protein A6	Hs00170953_m1	(5)
83	SAT1	spermidine/spermine N1-acetyltransferase 1	Hs00971735_g1	(68)
84	SELE	selectin E	Hs00950401_m1	(39, 53, 69)
85	SELL	selectin L	Hs01046459_m1	(64)
86	SELP	selectin P (granule membrane protein 140kDa, antigen CD62)	Hs00356351_m1	(42, 64, 70, 71)
87	STAT1	signal transducer and activator of transcription 1, 91kDa	Hs01014002_m1	(72)
88	TGFB1	transforming growth factor, beta 1	Hs99999918_m1	(39, 43, 73)
89	TGIF1	TGFB-induced factor homeobox 1	Hs00820148_g1	(1)
90	TLR2	toll-like receptor 2	Hs01872448_s1	(74, 75)
91	TLR4	toll-like receptor 4	Hs01060206_m1	(60, 74, 75)
92	TNF	tumor necrosis factor	Hs01113624_g1	(43, 46, 47)
93	TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A	Hs01042313_m1	(1)
94	TP53	tumor protein p53	Hs01039245_m1	(76)
95	TUBB	tubulin, beta	Hs00763510_gH	(1)
96	VCAM1	vascular cell adhesion molecule 1	Hs00365486_m1	(44, 45, 77)

SDC, Figure S1. Immunohistochemical evaluation. Differences between donor and post-implantation biopsies of grafts with primary function (PGF) compared to delayed graft function in intensity and number of positive (mainly proximal) tubules stained with anti-NTN1 antibodies is shown (magnification x40).



SDC, Figure S2. Principal component analysis calculated on the entire data set of 105 kidney biopsy samples [3 sequential biopsies (T0, T1, T2) in 35 kidney allografts] based on the expression of 71 probe sets shows no separation of DFG from PGF samples and showed the heterogeneity within the groups. PCA also suggests that sample heterogeneity within biopsy groups grows over time as was also confirmed on pre-PCA data including all 92 probe sets.

Circles (●) represent kidneys with primary graft function (PGF), triangles (▲) represent kidneys with delayed graft function (DGF). Colors indicate the biopsy sequence: red color- T0 biopsy, blue color- T1 biopsy, green color- T2 biopsy.



SDC Figure S3. The identification of the most suitable housekeeping genes by geNorm.

For the purpose of housekeeping gene selection, 16 candidate genes were investigated in 12 isolated renal allograft tissues at T0, T1, T2 biopsies using the Human Endogenous Control Array (Applied Biosystems, Foster City, CA). The geNorm program identified 4 genes to be the most suitable for normalization within kidney ischemia reperfusion injury samples (Figure S1). These 4 candidate housekeeping genes: peptidylprolyl isomerase A (cyclophilin A), beta-2-microglobulin (*B2M*), phosphoglycerate kinase 1 (*PGK1*), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were included in the customized TLDA card. Finally, based on geNorm/NormFinder analyses of 150 samples and on evidence presented in the literature, *GAPDH* was used as an endogenous reference gene.