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AKT1 Leader Gene and Downstream Targets Are Involved in a Rat Model of Kidney Allograft Tolerance

Vojislav Jovanovic,[1,2,3] Luca Giacomelli,[4] Victor Sivozhelezov,[5] Nicolas Degauque,[1,2,3] David Lair,[1,2,3] Jean-Paul Soulillou,[1,2,3] Eugenia Pechkova,[4,5] Claudio Nicolini,[4,5]** and Sophie Brouard[1,2,3]*

[1]*Institut National de la Santé Et de la Recherche Médicale INSERM U643, Institut de Transplantation et de Recherche en Transplantation ITERT, Nantes F-44093, France*

[2]*Centre Hospitalier Universitaire Hôtel Dieu Nantes, Nantes F-44000, France*

[3]*Université de NANTES, Faculté de Médecine, Nantes F-44000, France*

[4]*Nanoworld Institute and Chair of Biophysics, University of Genoa, Genoa, Italy*

[5]*Fondazione EL.B.A., Rome, Italy*

ABSTRACT

Tolerance is the so-called "Holy Grail" of transplantation but achieving this state is proving a major challenge, particularly in the clinical settings. This tolerance state can be induced in rodent models using a variety of maneuvers. This phenomenon is classically characterized by donor specificity (recipients accept a secondary donor-specific allograft but reject third-party allograft) as well as by the absence of chronic rejection lesion. We previously showed that administration and anti-donor anti-class II serum on the day of transplantation induce tolerance to a kidney allograft in the LEW-1W to LEW-1A strain combination. In this study, we used DNA microarrays to compare gene patterns involved in anti-donor anti-class II tolerated or untreated syngeneic kidney transplants in this strain combination. Statistical and non-statistical analyses were combined with ab initio analysis, using the recently developed leader gene approach, to shed new light on this phenomenon. Theoretical and experimental results suggest that tolerance and rejection outcome may be in large part determined by low expression variations of some genes, which can form a core gene network around specific genes such as Rac1, NFKB1, RelA, AKT1, IKBKB, BCL2, BCLX, and CHUK. Through this model, we showed that AKT1 gene, WNT pathway and NO synthesis are strictly connected to each other and may play an important role in kidney tolerance and rejection processes, with AKT1 gene being the center of this complex network of interactions. J. Cell. Biochem. 111: 709–719, 2010.

KEY WORDS: TOLERANCE; REJECTION; GENE PATHWAY; KIDNEY; ALLOGRAFT

Kidney transplant is the reference treatment for patients with end-stage renal disease. This therapeutic option has notably reduced morbidity and mortality in such patients. Nevertheless, despite important improvements in renal allograft survival over the last decades, chronic injury is poorly influenced by novel immunosuppressors and the half-life of renal allograft has increased only marginally. Moreover, life-long immunosuppression is associated to a higher risk of infection and malignancy [Dantal et al., 1998] and drug-related nephrotoxicity may lead to graft loss [Opelz, 1995; Nankivell et al., 2003; Solez et al., 2007]. Thus, inducing donor-specific tolerance, that is, "indefinite survival of a well-functioning graft in an immunocompetent adult host in the

Abbreviations used: AKT1, thymoma viral proto-oncogene 1; FC, fold change; FDR, false discovery rate; LEW, Lewis; MMP7, matrix metalloproteinase 7; NOS2, nitric oxide synthase 2; REJ, rejected kidney grafts; SYN, syngeneic kidney grafts; TOL, tolerated kidney grafts; WNT, wingless INT (integration).

Vojislav Jovanovic, Luca Giacomelli, Eugenia Pechkova, Claudio Nicolini and Sophie Brouard contributed equally to this work.

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*Correspondence to: Sophie Brouard, INSERM U643, 30 Bd Jean Monnet, 44093 Nantes Cedex 01, France. E-mail: sophie.brouard@univ-nantes.fr

**Correspondence to: Prof. Claudio Nicolini, Nanoworld Institute and Chair of Biophysics, University of Genoa, Corso Europa 30, Genoa, Italy. E-mail: manuscript@ibf.unige.it

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absence of immunosuppression'' [Waldmann and Cobbold, 2001], is the most challenging field of transplantation. Whereas tolerance induction remains elusive in clinical practice [Salama et al., 2007], in rodents, a variety of maneuvers can induce donor-specific allograft tolerance [Soulillou et al., 1976; Gagne et al., 2001; Degauque et al., 2006; Jovanovic et al., 2008]. We previously reported that donor-specific tolerance to an MHC mismatched renal allograft in adult rat can be induced by passive administration of antibodies directed against donor MHC class II on the day of transplantation [Soulillou et al., 1976; Gagne et al., 2001; Degauque et al., 2006; Jovanovic et al., 2008]. Tolerant recipients display normal and stable kidney function and normal histology [Degauque et al., 2006]. Although the importance of MHC II signaling in the regulation of antigen presenting cell has been described [Al-Daccak et al., 2004], the mechanisms responsible for maintaining anti-donors MHC II antibodies-induced tolerant state require further investigation.

DNA microarrays have expanded our ability to study complex biological situations [Alizadeh and Staudt, 2000; Alizadeh et al., 2000] and organ transplantation [Sarwal et al., 2003; Brouard et al., 2007]. Microarray data analysis is usually performed with statistical techniques, such as SAM [Butte, 2002]. However, many concerns have been raised against purely statistical analysis of microarray, that requires the correlation of a very high number of parameters with a very small number of samples (i.e., expression of many genes and few microarray slides analyzed) [Nicolini et al., 2006; Ding et al., 2007; Sivozhelezov et al., 2008]. Therefore, the novel concept of ''expression leaders'' has been introduced [Sivozhelezov et al., 2008]. Expression leaders can be defined as a small set of genes changing expression that are identified with non-statistical techniques (K-means clustering) following a preliminary statistical analysis or even as an alternative [Sivozhelezov et al., 2008].

Recently, the use of non-statistical bioinformatics and data-mining techniques has been proposed to ab initio identify the genes involved in a given situation and to make a hierarchy among them according to the number and confidence of interactions as derived from web-available databases [von Mering et al., 2005; Sivozhelezov et al., 2006]. Given the keywords describing the cellular phenomenon in question, the approach identifies the genes most pertinent to the given phenomenon, and ranks them according to the interactions between them pertinent to the given cellular phenomenon. With this method, it is possible to identify a small set of genes with a significantly higher number of interactions than the other genes identified, which can be defined as interaction leaders. The identification of expression and interaction leader genes in kidney transplant tolerance and rejection was recently applied to patients who operationally tolerate a kidney graft since several decades without any immunosuppressive treatment [Braud et al., 2008; Sivozhelezov et al., 2008]. However, detailed molecular mechanisms of kidney allograft tolerance or rejection are very difficult to apprehend in human, mainly due to the limited number of tolerant patients and to the nonaccessibility to the graft itself [Ashton-Chess et al., 2007; Sykes, 2007]. Thus, reductionist animal models represent an ideal system for targeted experimentations, since they make experimental analysis simpler and accurate, because of their inbreed status and the possibility for an easy access to the kidney graft itself. We thus took advantage of genome-wide transcriptome analysis to further explore these mechanisms in our model: an MHC mismatched renal allograft in adult rats after administration of anti-donor MHC II antibodies 100 days after transplantation [Soulillou et al., 1976; Gagne et al., 2001; Degauque et al., 2006; Jovanovic et al., 2008]. Gene patterns were compared in allograft from tolerant recipients and untreated recipients who reject their graft acutely and in syngenic grafts. A non-statistical microarray-based approach has been applied to identify expression and interaction leaders. Results are compared with those of an ab initio analysis, to shed new lights on the complex molecular mechanisms underlying this process.

MATERIALS AND METHODS

ANIMAL MODELS

Animal studies were performed at Institut de Transplantation et de Recherche en Transplantation (ITERT)—Université de Nantes, France. Inbred male adult rats (200–250 g) of the LEW.1A (RT1a) and LEW.1W (RT1u) congenic strains were purchased from Janvier (Le Genest-Saint-Isle, France) and maintained in an animal facility under standard conditions according to the European and Institutional Guidelines. In LEW.W to LEW.A strain combination, orthotopic kidney transplantations and tolerance induction were performed as previously described [Soulillou et al., 1976; Gagne et al., 2001]. At day 100 post-transplantation, kidney grafted animals tolerate a skin graft from the same donor and their kidney graft shows no histological signs of chronic humoral rejection [Degauque et al., 2006]. Controls included a group of LEW.1A recipients of LEW.1A kidneys (syngeneic transplants) at day 100 after transplantation and a group of untreated LEW.1A recipients of LEW.1W kidneys (allogeneic, untreated) rejecting their graft on day 3 after second nephrectomy. Rejection, indicated by the death of the binephrectomized rat, was confirmed by histology. Renal function of the recipient was followed every 2 weeks after transplantation: blood urea <8 mmol/L and blood creatinine <40 mmol/L were considered as normal [Degauque et al., 2006] Modif ications in renal allograft function were monitored in the urine (total protein and creatinine) and serum (urea, creatinine). The protein/creatinine ratio was considered normal when below 0.2 (g/mmol) and pathological (proteinuria) when above this level. Vascular lesions (percentage of obstruction, leukocyte infiltration, and medium lesions) were analyzed in at least 10 medium-size vessels of the graft. Kidney grafts from tolerant recipients did not show pathological changes indicative of chronic rejection, but only slight local fibrosis and lymphoid infiltration, only moderate acute tubular necrosis and no vascular or glomerular lesions. Kidney grafts from tolerant recipients displayed barely detectable levels of IgM and IgG deposition, similar to that observed in syngeneic grafts unlike rejected grafts from untreated recipients harvested on day 7 after transplantation (used as a positive control) [Degauque et al., 2006].

RNA from tolerant allografts, was extracted only from those animals that fulfilled previously mentioned strict criteria. From these reasons and from the fact that data come from inbread animals, bred in the pathogen free environment, we can be confident about immunological status, quality of RNA and reproducibility of

experiments even in the situation when arrays were run on small number of animals.

SAMPLE PREPARATION AND RNA EXTRACTION

Three tolerated kidney grafts (TOL), three acutely rejected grafts (REJ), and three syngeneic grafts (SYN) from rat recipients were used for this study. Organs were harvested at day 100 (TOL and SYN) or 1 day before rejection (REJ). Sections of the kidney graft were snap-frozen in liquid nitrogen for RNA extraction. Blood cell contamination was avoided by perfusing the organ with PBS. All samples were stored at $-70^{\circ}C$ until use. Total RNA from rat kidney grafts was prepared using the TRIzol® (Invitrogen, Cergy Pontoise, France) extraction method.

MICROARRAY EXPERIMENTS

Total RNA (10 μg) was cleaned up using Rneasy columns (QIAGEN). RNA quantity and quality were determined using Nanodrop spectrophotometer and Agilent 2100 bioanalyzer. The Applied Biosystems (Applied Biosystems, Foster City, CA) rat Genome Survey Microarray (P/N 4337467), used in this study, contained 26,857 60-mer oligonucleotide probes representing 27,088 individual rat genes. An additional 3400 control spots were present on the chip to cover various steps in the hybridization process. Digoxigenin-UTP labeled cRNA was generated and amplified from 0.5 μg of total RNA from each sample using an Applied Biosystems Chemiluminescent NanoAmp RT-IVT Labeling Kit (P/N4365715). Array hybridization, chemiluminescence detection, image acquisition and analysis were performed using the Applied Biosystems Chemiluminescence Detection Kit (P/N 436875D), Analyzer (P/N 4338036) and v-1.1 analyzer software (P/N 4336391) according to the manufacturer's protocol.

MICROARRAY ANALYSIS

Microarray assay datasets were analyzed using the R-language and environment. Genes were identified using the Panther™ Protein Classification System Probe ID database. For each spot, three main values were calculated: Fold change (FC), a metric for comparing a gene mRNA-expression level between samples and controls; false discovery rate (FDR), the expected proportion of false positives among the declared significant results, and statistical significance (P value). In order to identify differentially expressed entries (both up- and down-regulated), a triple filtering was applied: $FC > 10\times$, $FDR < 0.15$, $P < 0.05$.

IDENTIFICATION OF EXPRESSION LEADER GENES

Expression leader genes [Sivozhelezov et al., 2008] were identified in TOL/SYN dataset and in TOL/REJ dataset both for up-regulated and down-regulated entries by means of K-means clustering, repeated until convergence is achieved in highest cluster. Cluster analysis was performed with FuzMe software (Minasny B, McBratney AB. 2002. FuzME version 3.0, Online. Available at http://www.usyd.edu.au/su/agric/acpa/pag.htm, verified 25 June 2009, Australian Centre for Precision Agriculture, University of Sydney, Sydney, Australia). The IDs of up- and down-regulated entries were checked in Panther database, in order to screen for identified and unidentified entries. Panther database can also provide preliminary information about molecular and cellular function of each entry. When necessary, nomenclature was also checked using a BLASTP search against ENSEMBL database.

AB INITIO ANALYSIS AND PREDICTION OF INTERACTIONS

An ab initio analysis was conducted searching for genes involved in rat kidney transplant tolerance or rejection. Preliminary gene list was identified by a keyword-based query in GenBank (keywords: Rattus Norvegicus [organism] AND kidney AND transplant AND (tolerance OR rejection). Preliminary list was then expanded using direct interaction via STRING database; newly identified genes were cross-checked against PubMed (keywords: rat kidney) [Sivozhelezov et al., 2008]. Only direct interactions (i.e., physical contact between encoded proteins or involvement in the same metabolic pathway) were considered, applying a high-confidence threshold [von Mering et al., 2005]. The expansion was performed until convergence was achieved. Three other genes were added to the list: MMP7, DKK3, and TCF7 (Differential Gene Expression Patterns in Tolerated Versus Syngeneic Kidney Grafts 100 days After Transplantation and Acutely Rejected Grafts Section). For every ab initio gene FC, FDR and P values, as derived from experimental microarray data, were considered.

RESULTS

DIFFERENTIAL GENE EXPRESSION PATTERNS IN TOLERATED VERSUS SYNGENEIC KIDNEY GRAFTS 100 DAYS AFTER TRANSPLANTATION AND ACUTELY REJECTED GRAFTS

The gene expression patterns were analyzed in three tolerated (TOL), three syngeneic (SYN) and three acutely rejected (REJ) kidney allografts using pangenomic Genome Survey Microarrays (P/N 4337467). Among the 27,088 genes present on the microarray, 26,857 genes were expressed. Analysis with R software and subsequent filtering showed that both the entire TOL versus SYN and TOL versus REJ datasets were distributed symmetrically in the log scale. Widths of those distributions differed about one order of magnitude in FC, being larger for the TOL versus SYN than the TOL versus REJ dataset. The triple filtering identified 279 up-regulated and 249 down-regulated entries in TOL versus SYN dataset and only 113 up-regulated and 24 down-regulated entries in TOL versus REJ dataset (1.70% and 1.51% for up- and down-regulated entries in TOL vs. SYN dataset respectively; 0.74% and 0.15% in TOL/REJ dataset, respectively). A significantly higher number of entries was up- or down-regulated in TOL versus SYN dataset, if compared to TOL versus REJ dataset ($P < 0.0001$, Fisher's exact test). FC was comparable in the two datasets, both for up-regulated and down-regulated entries (up-regulated: 35.29 ± 64.67 vs. 39.73 ± 39.72 in TOL vs. SYN and TOL vs. REJ datasets, respectively; down-regulated: 0.06 ± 0.02 vs. 0.07 ± 0.03 in TOL vs. SYN and TOL vs. REJ, respectively; note that $FC < 1$ denotes that the expression was down-regulated 1/FC times). Among up-regulated entries, the gene MMP7 was present. This gene was previously shown to be involved in long term graft outcome in the same model thorough the WNT pathway [Jovanovic et al., 2008]. This prompted us to check if the WNT pathway was compatible with the ab initio dataset. Thus, the MMP7

TABLE I. Expression Up- and Down-Regulated Leader Genes in TOL/SYN Dataset

Panther ID	NCBI or ENSEMBL ID	FC	Panther function
Up-regulated			
21350627	RT1-Bb	721.30	Major histocompatibility complex antigen
21013506	ENSRNOP00000048406	352.51	Immunoglobulin
21398036	ENSRNOP00000039536	345.35	Immunoglobulin
21896072	ENSRNOP00000036083	317.09	Immunoglobulin
22080253	ENSRNOP00000042721	277.09	Immunoglobulin
22058582	ENSRNOP00000049008	252.25	Immunoglobulin
21907618	ENSRNOP00000007211	217.97	Immunoglobulin
Mean FC (±SD)		354.79 ± 168.87	
Down-regulated			
22297483	Spp2	0.006	Other protease inhibitor; other enzyme inhibitor
21329126	Rbp4	0.007	Other transfer/carrier protein
22127763	myl1	0.016	Molecular function unclassified
20811640	cyp2c24	0.016	Oxygenase
21611810	Nat8	0.016	Acetyltransferase
21279172	Rgn	0.017	Other select calcium binding proteins
20860815	Ces1	0.017	Esterase
21269820	RGD1564391	0.017	Molecular function unclassified
22095682	Rdh2	0.018	Dehydrogenase; reductase
21378758	ENSRNOP00000028498	0.018	Receptor
21862395	Pck1	0.018	Decarboxylase
22264541	ENSRNOP00000044432	0.018	Molecular function unclassified
22171277	ENSRNOP00000024455	0.019	Other transporter
22338890	Gc	0.019	Other transfer/carrier protein
21162411	Sah	0.020	Synthetase; other ligase
20782795	ENSRNOP00000014219	0.021	Molecular function unclassified
21409681	ENSRNOP00000024800	0.021	Transporter; apolipoprotein; lipase
21934502	Slc22a9	0.022	Other transporter
Mean FC (±SD)		0.017 ± 0.004	

In this and following tables, ENSMBL nomenclature corresponds to the STRING database version 7.0.

gene and other two genes involved in WNT signaling (DKK3 and TCF7) and also showing high FC were incorporated into the ab initio analysis.

IDENTIFICATION OF EXPRESSION LEADER GENES

In TOL versus SYN dataset, 7 up- and 18 down-regulated leaders were identified, whereas 7 up- and 9 down-regulated leaders were identified in TOL versus REJ dataset. Expression of up- and down-regulated leader genes are reported in Tables I and II, for TOL versus SYN dataset and TOL versus REJ dataset respectively. The FC value was significantly larger in TOL versus SYN dataset than in TOL versus REJ, both for up- and down-regulated entries (up-regulated: 354.79 ± 168.87 vs. 168.35 ± 35.71 in TOL vs. SYN and TOL vs. REJ datasets, respectively, $P = 0.0003$; down-regulated: 0.017 ± 0.004 vs. 0.038 ± 0.018 in TOL vs. SYN and TOL vs. REJ, respectively, $P = 0.01$; Mann–Whitney two-tailed test).

VALIDATION OF EXPRESSION GENE DATASETS INCLUDING AB INITIO ANALYSIS

Recognition of entries is similar in the TOL/SYN and TOL/REJ for both annotations available for the Panther database, namely 74.71% of recognized entries in TOL versus SYN dataset and 77.03% in TOL

TABLE II. Expression Up- and Down-Regulated Leader Genes in TOL/REJ Dataset

Panther ID	NCBI or ENSEMBL ID	FC	Panther function
Up-regulated			
22080253	ENSRNOP00000006975	213.67	Immunoglobulin
22055553	ENSRNOP00000042721	203.82	Immunoglobulin
21170620	dnl1	192.28	Endodeoxyribonuclease; non-motor actin binding protein
21013506	ENSRNOP00000048406	154.53	Immunoglobulin
22352996	ENSRNOP00000047195	154.15	Immunoglobulin
21393268	ENSRNOP00000006998	144.69	Immunoglobulin
21196525	ENSRNOP00000049008	115.31	Immunoglobulin
Mean FC (±SD)		168.35 ± 35.71	
Down-regulated			
22369942	ENSRNOP00000013689	0.005	Other receptor
20741316	Arg1	0.011	Other hydrolase
21660968	ENSRNOP00000029845	0.042	Other receptor
22356641	ENSRNOP00000030516	0.043	Molecular function unclassified
21429529	ENSRNOP00000017339	0.043	Other receptor; transporter
21255746	Lasp1	0.044	Non-motor actin binding protein
21877660	ENSRNOP00000033647	0.044	Microtubule binding motor protein
21346518	ENSRNOP00000044053	0.053	Molecular function unclassified
20960205	Fabp7	0.060	Other transfer/carrier protein
Mean FC (±SD)		0.038 ± 0.018	

versus REJ for one annotation and 99.62% and 99.66%, respectively for the other annotation. When only up- and down-regulated entries are considered, recognized entries in TOL versus SYN dataset are 69.53% (up-regulated) and 37.17% (down-regulated) while in TOL versus REJ dataset, recognition percentages are 80.72% and 95.83%, respectively. Molecular functions of identified expression leaders are reported in Tables I and II, for TOL versus SYN dataset and TOL versus REJ dataset, respectively. In both datasets, most up-regulated expression leaders are linked to immunoglobulin (Tables I and II) or are involved in inflammation process. On the other hand, down-regulated expression leaders are mostly enzymes linked to several molecular and cellular processes (Tables I and II).

Ab initio database-based analysis identified 37 genes as involved in tolerant kidney graft (Table III). The microarray-derived parameters and the molecular function according to Panther database are reported in Table III for each ab initio identified gene. Most genes identified from ab initio analysis are up-regulated in the TOL versus SYN dataset (Table IIIA). Nine genes exhibit large positive changes in expression (>5×): CCL2, CYBB, DKK3, MMP7, NOS2, RT1-Bb (also an expression leader), TCF7, TGFb1, VCAM1. The greatest expression change was observed for RT1-Bb (721.30×), and no "ab initio" genes decreased expression in the TOL versus SYN dataset. This is not caused by the trend in the microarray data themselves, since the percentages of up and down-regulated genes in the entire TOL versus SYN dataset are similar (Differential Gene Expression Patterns in Tolerated Versus Syngeneic Kidney Grafts 100 days After Transplantation and Acutely Rejected Grafts Section). In the TOL versus REJ dataset, most "ab initio" genes are down-regulated (Table IIIB). A significantly lower FC was reported in TOL versus REJ dataset when compared to TOL versus SYN, considering overall dataset and only up-regulated genes (24.05 ± 118.04 vs. 0.87 ± 0.56 for all values in TOL vs. SYN and TOL vs. REJ datasets, respectively, $P < 0.0001$; 30.50 ± 133.10 vs. 1.56 ± 0.60 when only genes with $FC > 1$ are considered in TOL vs. SYN and TOL vs. REJ datasets, respectively, $P = 0.016$; Mann–Whitney test). This trend follows the one observed for the entire microarray data, thus being not specific for the "ab initio" identified genes. On the other hand, mean FC in down-regulated genes was similar in the two datasets (Table III). A lower number of "ab initio" genes are up-regulated in the TOL versus REJ dataset, if compared to the TOL versus SYN one (29 vs. 10, respectively). In contrast, a larger number of "ab initio" genes are down-regulated in the TOL versus REJ dataset with respect to TOL versus SYN (8 vs. 27, respectively) (Table IV). Interaction map among ab initio identified genes is represented in Figure 1A. Because of the small number of genes with interactions, rat leaders were not calculated. However, it was possible to preliminarily calculate interaction leaders from their human homologues, identified via the STRING database. Interaction map among human homologues is represented in Figure 1B and interaction leaders derived from this analysis are reported in Table III, marked in bold. In total, eight putative interaction leader genes were identified (RAC1, NFKB1, RELa, AKT1, IKBKB, BCL2, BCLX, CHUK). Intriguingly, AKT1 is the gene with highest number of interactions. Theoretical prediction of interaction via STRING database (human homologues were considered, Fig. 1B) showed that AKT1 is linked, via TNFSF11 to MMP7 and therefore to the WNT pathway. Besides, the gene AKT1 is linked to the TGF-beta signaling pathway via the kinase IKBKB, which is another putative leader gene.

Interactions among ab initio list and expression up- and down-regulated leader genes in TOL versus SYN dataset and TOL versus REJ dataset were also calculated. Noteworthy, no interaction was identified among expression leaders in the two datasets and between expression leaders and ab initio gene list with a single exception: ARG1, also a down-regulated leader in TOL versus REJ dataset, is linked to NOS2. This latter has no interaction when considering rat genes, but is linked to RAC1, which is one putative interaction leader, in human homologues map (Fig. 1B). NOS2 and ARG1 (human homologues) are also linked to AKT1 thorough NOS3 (also known as ENSP00000297494) (Fig. 2).

To summarize, expression datasets were largely validated in terms of nomenclature and gene–gene interactions. Considering that the text mining option was switched off in the interaction analysis, expression patterns in this study can be considered properly validated.

Validation of the ab initio data was performed by calculating, for each gene considered, the overall numbers of interactions, and plotting the numbers of interactions relevant to allograft rejection/tolerance to the overall interactions. The resulting plot has shown neither considerable correlation between the two, nor outset of the plot form the origin (not shown). Thus, high interaction scores of interaction leaders were not caused by their general (unrelated to tolerance/rejection) trend to high numbers of interactions.

DISCUSSION

In this work, microarray non-statistical and ab initio analyses were applied to a model of rat kidney allograft tolerance to further investigate the molecular mechanisms underlying this phenomenon. First, we observed that a significantly larger number of entries are up- or down-regulated in TOL versus SYN dataset than in TOL versus REJ dataset. These results can be somehow expected, since both TOL and REJ rats have undergone allogeneic transplantation and both models display an infiltrate of recipient immune cells in the graft [Degauque et al., 2006]. We observe that number of up- and down-regulated leader genes is similar in the two datasets with up-regulated leader genes mainly immunoglobulins in both datasets. An exception is represented by RT1-Bb (MHC class II antigen). Splicing and sequencing of immunoglobulins are not completely understood yet: this may have an effect also on nomenclature, which is not completely defined even in standard NCBI annotation. Considering also nomenclature problems and the finding that most up-regulated genes are immunoglobulins, in this particular model, results of processing of microarray data alone (either statistical or not) are not completely informative. In the same way, down-regulated entries are in most cases enzymes in TOL versus SYN dataset, while in TOL versus REJ dataset, their function is often unclassified. In the microarray system we used, ID numbers are given according to the Panther probe-to-gene annotations system, which has only recently been applied to biomedical research. Nomenclature, including Panther probe-to-gene annotations sys-

TABLE III. FC: *P* and PDR Values in TOL/SYN (a) and TOL/REJ (b) Datasets of Ab Initio Identified Genes Involved in Rat Kidney Transplant Tolerance/Rejection

	FC	*P*-Value	FDR (BH)	Panther molecular function
A: Values in TOL/SYN dataset				
RT1-Bb	721.30	0.00	0.04	Major histocompatibility complex antigen
mmp7	38.62	0.00	0.04	Metalloprotease; other extracellular matrix
Nos2	19.61	0.00	0.04	Synthase; oxidoreductase; calmodulin related protein
Ccl2	15.72	0.07	0.17	Chemokine
tcf7	14.22	0.00	0.03	Nucleic acid binding
dkk3	11.88	0.03	0.11	Molecular function unclassified
Tgfb1	7.64	0.01	0.05	Growth factor
cybb	6.96	0.16	0.28	Oxidoreductase
Vcam1	6.63	0.06	0.16	CAM family adhesion molecule
Ccl3	5.02	0.16	0.29	Chemokine
Itgb2	4.69	0.00	0.02	Other receptor; cell adhesion molecule
tiam1	3.70	0.01	0.05	Guanyl-nucleotide exchange factor
Ccl5	3.34	0.07	0.17	Chemokine
nfkbia	2.46	0.01	0.05	Select regulatory molecule
prp	2.38	0.00	0.03	Peptide hormone
Tnf	2.27	0.16	0.29	Tumor necrosis factor family member
Rac1	1.83	0.04	0.13	Small GTPase
Sell	1.75	0.21	0.34	Other cell adhesion molecule
nfkb1	1.66	0.33	0.47	Other transcription factor
rela	1.62	0.00	0.03	Other transcription factor; nucleic acid binding
Cyba	1.45	0.14	0.26	Molecular function unclassified
AKT1	1.42	0.29	0.43	Non-receptor serine/threonine protein kinase
bcl10	1.41	0.02	0.09	Molecular function unclassified
Hgf	1.29	0.79	0.85	Growth factor; hydrolase; serine protease; defense/immunity protein
Edn1	1.22	0.65	0.74	Peptide hormone
pak1	1.18	0.07	0.17	Protein kinase; transferase
bax	1.16	0.33	0.46	Other signaling molecule
Xdh	1.11	0.56	0.67	Molecular function unclassified
pkcq	1.07	0.56	0.67	Transfer/carrier protein; non-receptor serine/threonine protein kinase
Ikbkb	0.92	0.71	0.79	Non-receptor serine/threonine protein kinase
bad	0.90	0.51	0.63	Molecular function unclassified
Bcl2	0.76	0.14	0.26	Other signaling molecule
Mapk1	0.69	0.08	0.18	Non-receptor serine/threonine protein kinase
Bclx	0.59	0.06	0.15	Other signaling molecule
chuk	0.59	0.01	0.06	Non-receptor serine/threonine protein kinase
Vegfa	0.55	0.09	0.20	Growth factor
Dpp4	0.23	0.01	0.05	Serine protease
Mean FC (±SD)	24.05 ± 118.04			
B: Values in TOL/REJ dataset				
tcf7	2.71	0.01	0.05	Nucleic acid binding
pkcq	2.36	0.00	0.04	Transfer/carrier protein; non-receptor serine/threonine protein kinase
tiam1	2.05	0.01	0.05	Guanyl-nucleotide exchange factor
Vegfa	1.67	0.13	0.22	Growth factor
Bcl2	1.26	0.06	0.14	Other signaling molecule
Tnf	1.23	0.50	0.60	Tumor necrosis factor family member
Tgfb1	1.22	0.12	0.22	Growth factor
nfkb1	1.11	0.75	0.81	Other transcription factor
Cyba	1.02	0.56	0.65	Molecular function unclassified
chuk	1.00	0.93	0.95	Non-receptor serine/threonine protein kinase
Rac1	0.99	0.94	0.96	Small GTPase
prp	0.99	0.83	0.88	Peptide hormone
Ikbkb	0.95	0.71	0.78	Non-receptor serine/threonine protein kinase
RT1-Bb	0.94	0.36	0.47	Major histocompatibility complex antigen
Ccl2	0.87	0.60	0.68	Chemokine
bcl10	0.84	0.07	0.16	Molecular function unclassified
Dpp4	0.84	0.34	0.45	Serine protease
bax	0.81	0.11	0.21	Other signaling molecule
rela	0.79	0.01	0.05	Other transcription factor; nucleic acid binding
Vcam1	0.76	0.06	0.13	CAM family adhesion molecule
Ccl5	0.70	0.42	0.52	Chemokine
Edn1	0.68	0.15	0.24	Peptide hormone
bad	0.67	0.00	0.03	Molecular function unclassified
Bclx	0.64	0.02	0.08	Other signaling molecule
mmp7	0.56	0.00	0.03	Metalloprotease; other extracellular matrix
dkk3	0.56	0.13	0.23	Molecular function unclassified
Mapk1	0.55	0.01	0.06	Non-receptor serine/threonine protein kinase
Itgb2	0.55	0.02	0.08	Other receptor; cell adhesion molecule
Ccl3	0.52	0.11	0.20	Chemokine
Hgf	0.50	0.23	0.33	Growth factor; hydrolase; serine protease; defense/immunity protein
pak1	0.41	0.00	0.03	Protein kinase; transferase

TABLE III. (*Continued*)

	FC	*P*-Value	FDR (BH)	Panther molecular function
cybb	0.32	0.00	0.04	Oxidoreductase
Xdh	0.29	0.01	0.06	Molecular function unclassified
Nos2	0.27	0.07	0.15	Synthase; oxidoreductase; calmodulin related protein
Sell	0.27	0.02	0.08	Other cell adhesion molecule
nfkbia	0.24	0.03	0.10	Select regulatory molecule
AKT1	0.20	0.01	0.05	Non-receptor serine/threonine protein kinase
Mean FC (±SD)	30.50 ± 133.10			

Genes in brighter background are derived from interaction-based expansion via STRING database; genes in darker background are suggested by a previous analysis. Genes with no background are derived from initial list obtained from GenBank. Genes in bold are putative interaction leaders. As derived from calculation of interactions among human homologues.

TABLE IV. Number of Up- and Down-Regulated Ab Initio Identified Genes in TOL/SYN and TOL/REJ Datasets

Number of up-regulated	29	10
Number of down-regulated	8	27
Number of up-regulated expression leaders	1	0
Number of down-regulated expression leaders	0	0

Number of ab initio genes also classified as expression leaders is reported.

tem, represents a great hurdle in analyzing molecular genomics and proteomics [Noth and Benecke, 2005; Sivozhelezov et al., 2008].

Another finding is the higher FC in TOL versus SYN than in TOL versus REJ dataset (about 50× vs. 10×). The lower expression variation in TOL versus REJ dataset may suggest that molecular mechanisms underlying tolerance and rejection do not rely upon large variations in gene expression. This trend was confirmed also when considering ab initio identified genes involved in kidney transplant tolerance and rejection processes. Expression-based leaders present almost no known interactions among them. Gene interactions have not been largely studied in rats, while are much more defined in humans [Sivozhelezov et al., 2008]. However, nomenclature issues were circumvented, since all identified genes were properly defined. First, ab initio identified genes are linked by a complex network of interactions, while expression leaders in both datasets are not. This finding suggests that gene expression changes in ab initio identified genes have more immediate effects on molecular mechanisms underlying tolerance process, because of their high number of interactions. Indeed, a small change in expression in a single gene could have an important effect also on the other genes interacting with it or on the encoded proteins. Of note, tolerance seems to be linked to up-regulation of gene expression. In fact, 29 genes are up-regulated but only nine present strong accumulation (>5×): CCL2, CYBB, DKK3, MMP7, NOS2, RT1-Bb (also an expression leader), TCF7, TGFB1, VCAM1, while no gene presented decreased expression. Moreover, some of them are linked by already known interactions such as in neuroprotection where TGFB1 involves activation of NFκB through phosphatidylinositol-3-OH kinase/Akt signaling pathway [Zhu et al., 2004] or abnormal expression levels or patterns MMP7 and TCF7 in tumors that are correlated with beta-catenin/TCF complexes [Hovanes et al., 2001]. Instead, when tolerance was compared to rejection, the opposite was observed, that is, most ab initio identified genes are down-regulated in TOL versus REJ dataset. However, FC of genes identified ab initio was significantly greater in TOL versus SYN dataset, with respect to TOL versus REJ one, as also shown in microarray data analysis. Ab initio identified genes did not largely change their expression in TOL rats with respect of REJ ones and these genes can reflect remodeling that may be more present in tolerant than in rejecting graft or in syngeneic grafts [Jovanovic et al., 2008]. Taken together, these findings suggest that a complex phenomenon such as transplant tolerance may depend upon low variations in expression of some genes, which form the core of the network of genes involved in kidney graft tolerance. These low changes are likely to be underestimated with mass-scale molecular genomics, which relies mostly on magnitude of gene expression variations.

Exploring the AKT signaling pathway in the situation of chronic rejection is a pertinent question and further experiments in this direction would present next phase in our study. However However there is no relevant model of kidney chronic rejection in the rat. We tried to establish a model of chronic kidney allograft rejection in the rat strain combination Fischer to Lewis where graft survival induction was induced using CsA 5 mg/kg IP for ten days following transplantation as described previously in the literature [reviewed by Marco, 2006]. The grafts were analyzed 3 and 6 months after transplantation. Unfortunately, Ficher to Lewis kidney transplant model of chronic allograft nephropathy revealed chronic pyelonephritis with no vascular lesions [Ashton-Chess et al., 2010].

Regarding the microarray array analysis, it is necessary to put in evidence that our theoretical calculations are based upon previous knowledge, In fact, data mining, that is, sorting thorough large amounts of data and picking up relevant information to potentially discover new knowledge was used to conduct this analysis. Of note, we used only direct interactions, that is, those based directly on published experimental observations to calculate the interconnections between the ab initio identified genes. Direct interactions include physical interactions between encoded proteins, such as ligand-receptor contact, microarray gene expression data, and proved involvement in the same metabolic pathways. Moreover, only interactions with a high degree of confidence in the STRING database, that is, those with a stronger experimental evidence, were considered. These choices can represent a validation of our ab initio analysis.

A potential important role of some genes in the process of kidney transplant tolerance and rejection is suggested from these results. AKT1 is identified as the gene with the highest number of interactions when considering human homologues. AKT1 is also linked, via TNFSF11, to MMP7 and therefore to the WNT pathway. The involvement of this pathway and the role of MMP7 in

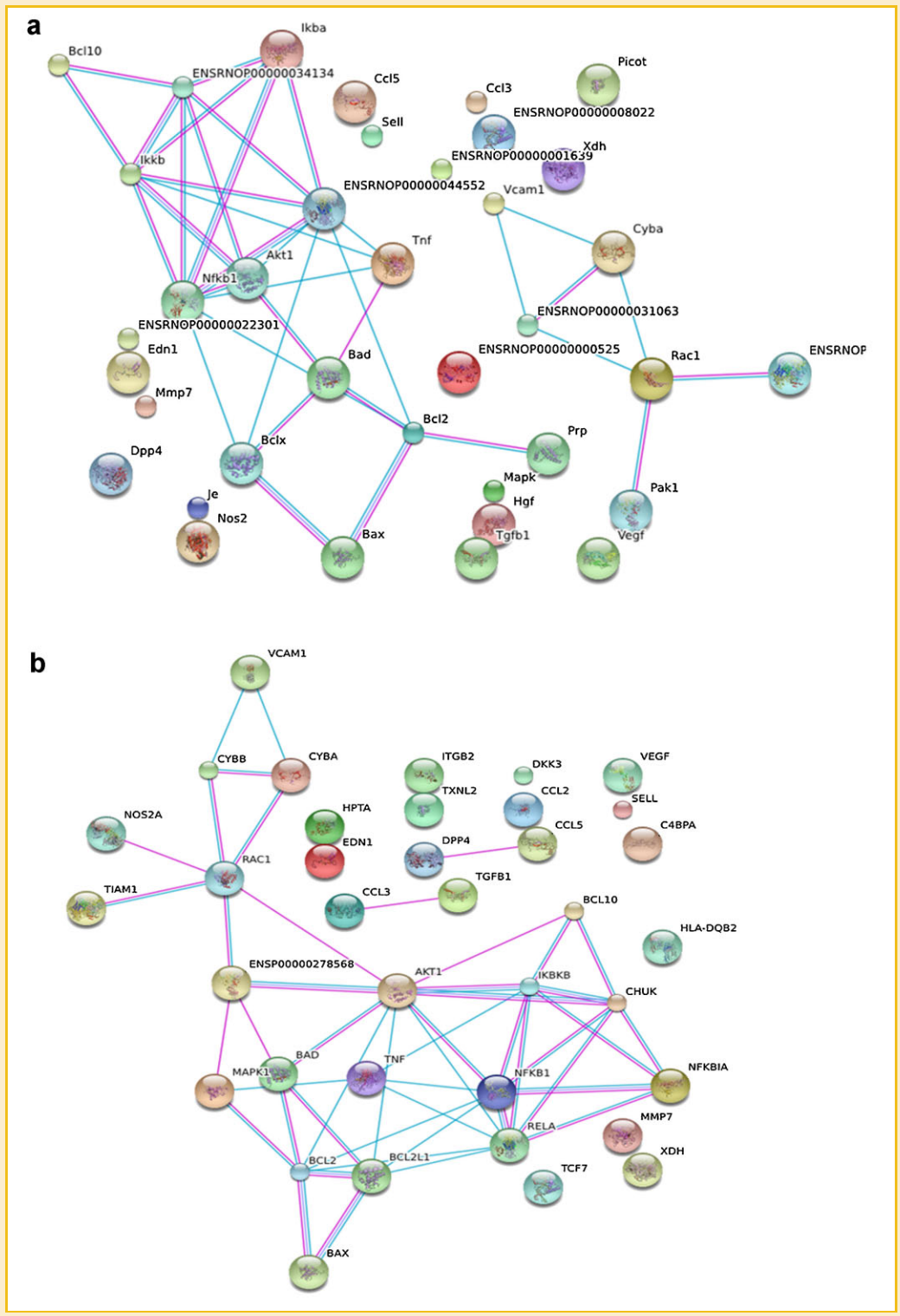

Fig. 1. A: Interaction map among ab initio list of rat genes involved in kidney transplant tolerance/rejection, as calculated via STRING. B: Interaction map of their human homologues. Light blue lines represents involvement in the same metabolic pathway; dark blue lines homology between encoded proteins; magenta lines physical interaction between encoded proteins or between one encoded protein and one DNA sequence. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

maintaining kidney transplant tolerance were recently described by our group, with MMP7 highly expressed in tolerated kidney in the same allograft model [Jovanovic et al., 2008]. Noteworthy, the up-regulation of TNFSF11, also known as TRANCE, was shown to play a role in acute allograft rejection [Guillonneau et al., 2004]. Therefore, the strong direct interaction between AKT1 and WNT pathway via TNFSF11 suggests a central role of this network in the mechanism of kidney transplant tolerance. The analysis of interaction maps shows

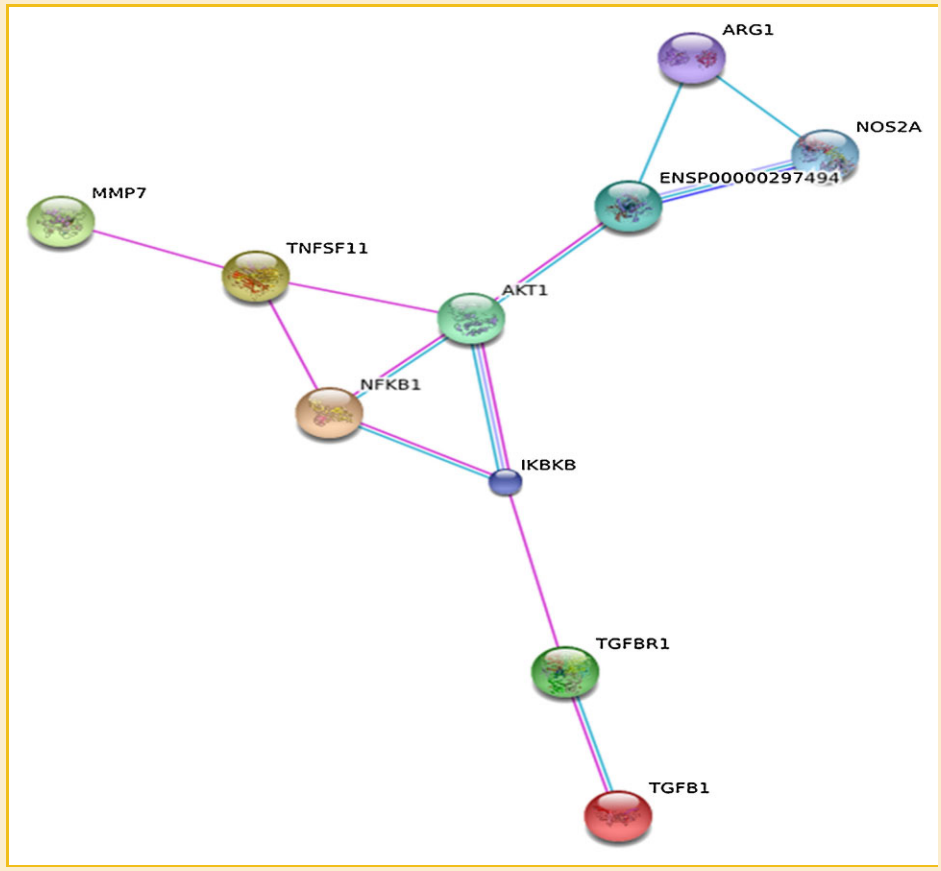

Fig. 2. Map of interactions among AKT1, MM7, and ARG1, that is, some genes suggested to play an important role in kidney transplant tolerance and rejection from experimental and theoretical results. Light blue lines represent involvement in the same metabolic pathway; dark blue lines homology between encoded proteins; magenta lines physical interaction between encoded proteins or between one encoded protein and one DNA sequence. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

also that AKT1 is linked, via IKBKB kinase, to TGF-beta signaling, which is associated to some complications in solid organ recipients, such as post-transplant lymphoproliferative disorder [Babel et al., 2007]. Moreover, TGF-beta pathway mRNA is increased in human kidney chronic rejection [Zegarska et al., 2006]. AKT1 is also linked, via NOS3 (eNOS), to ARG1 and NOS2A. Our theoretical analyses reveal that ARG1 and NOS2A are also connected with another putative interaction leader, that is, RAC1 [Kuncewicz et al., 2001]. Endothelial integrity, especially the expression of protecting vasoactive agents, such as NO, may be a key factor in resistance or sensitivity to transplantation-mediated injury [Vos et al., 2004]. Taken together, these experimental and theoretical findings support the involvement of WNT pathway and NO synthesis into kidney transplant tolerance.

AKT1 is thus implicated in a diverse range of cellular functions and our understanding of cell-type specific and time-dependent AKT signaling is far from complete. Some previous reports bring more information about AKT1 role in kidney biology. Indeed, AKT1 was identified among other up-regulated genes in a model of ischemia-reperfusion injury in kidney [Grigoryev et al., 2006]. Organ ischemia is important because it leads to delayed graft function, increased acute rejection, enhanced chronic allograft nephropathy and reduces long-term allograft survival. Some other studies pointed out positive effect of AKT1 up-regulation in different tolerance induction protocols. In rat, where liver allograft tolerance was induced by immunomodulator FTY720, small-for-size liver graft injury was attenuated by activation of cell-survival AKT signaling pathway [Zhao et al., 2004]. In another experimental model, gene transfer and constitutive activation of AKT1 in human beta-cells improved human islet transplant survival in diabetic severe combined immunodeficient (SCID) mice [Rao et al., 2005]. AKT may play also a role in neovascularization, which is one of the most important aspects of tissue remodeling [Somanath et al., 2006]. Several studies suggest that the level of active AKT1, as well as its short-term and long-term activation states, in vascular cells can regulate various signaling pathways and affect the balance of pro- and anti-angiogenic factors [Chen et al., 2005]. eNOS, a downstream target of AKT1 in ECs has been shown to be important for adaptive angiogenesis following hind limb ischemia in $Akt1^{-/-}$ mice [Ackah et al., 2005] and AKT signaling coordinates blood vessel recruitment with normal tissue growth [Ouchi et al., 2008]. Overall, controversial conclusions about role of AKT1 should be seen in a way that balance between signaling pathways under different conditions determines pro- or anti-angiogenic phenotype [Somanath et al., 2006].

Some recent studies focused on AKT involvement in regulatory T cells (Tregs). In study where BALB/c skin was grafted on C57BL/6,

Wei et al. [2010] reported that enhanced IFN-γ produced by CD4+ CD25+ Foxp3+ Tregs from mice tolerized to alloantigen in vivo can ligate IFN-γ receptors on the Tregs themselves to up-regulate STAT1 activation and decrease PKB/AKT activation. Moreover, Liu et al. showed that loss of Sphingosine 1-phosphate (S1P1) function resulted in enhanced thymic differentiation and suppressive activity of Treg cells. Conversely, greater S1P1 signaling led to diminished development and function of Treg cells in vitro and in vivo and to the development of spontaneous autoimmunity due to defects in Treg cells. Further experiments showed that the function of S1P1 in Treg cells is mediated by the ''downstream'' Akt-mTOR pathway [Liu et al., 2009]. These data suggest that AKT-mTOR signaling pathway are important for the capacity of tolerized Tregs to prevent allogeneic skin graft rejection in vivo.

Although the results reported herein still leave unexplained points, they might contribute a further confirmation of some previously identified issues in mass-scale molecular genomics. Recently, we have proposed the need to build ad hoc arrays instead of sometimes misleading pangenomic ones [Giacomelli and Nicolini, 2006]. In fact, the identification of gene networks can be of first importance in the systematization and analysis of data, since the mere changing in expression of a particular gene is not significant by itself, but only if it is put in a proper framework, for example, that of gene–gene interactions [Sivozhelezov et al., 2008]. This change can be often considered as a consequence of a more complex network of events, starting from leader genes forming the actual core of gene interactions network and often varying their expression so much to be detected using pangenomic arrays [Sivozhelezov et al., 2006, 2008].

In conclusion, this study suggests that transplant tolerance or rejection may be determined by very low expression variations of some key genes. Moreover, these experimental and theoretical results support the connections among AKT1, WNT pathway and NO synthesis; these pathways may play an important role in kidney tolerance and rejection processes. On this basis, further targeted studies may provide more details on these complex molecular mechanisms.

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