Journal of Cellular Biochemistry 103:1693–1706 (2008)

Immunosuppressive Drug-Free Operational Immune Tolerance in Human Kidney Transplants Recipients: Part II. Non-statistical Gene Microarray Analysis

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Abstract Kidney transplant is the reference treatment for patients with end-stage renal disease, but patients may develop long-term rejection of the graft. However, some patients do not reject the transplant, but instead are operationally tolerant state despite withdrawal of immunosuppressive treatment. In this second article we outline a microarray-based identification of key leader genes associated respectively to rejection and to operational tolerance of the kidney transplant in humans by utilizing a non/statistical bioinformatic approach based on the identification of "key genes," either as those mostly changing their expression, or having the strongest interconnections. A uniquely informative picture emerges on the genes controlling the human transplant from the detailed comparison of these findings with the traditional statistical SAM (Tusher et al. [2001] Proc Natl Acad Sci USA 98:5116–5121) analysis of the microarrays and with the clinical study carried out in the accompanying part I article. J. Cell. Biochem. 103: 1693–1706, 2008. © 2007 Wiley-Liss, Inc.

Key words: gene expression; DNA microarray; bioinformatics; kidney transplant; transplant tolerance

Kidney transplant is the reference treatment for patients with end-stage renal disease. The advent of this therapeutic option has notably reduced morbidity and mortality in patients with this disease. Moreover, improvements in the clinical management of transplant recipients have contributed to increase graft survival and to limit the risks of rejection in kidney transplantation by proper pharmacological immunosuppression [Hariharan et al., 2000]. However, such lifelong immunosuppression, which poorly influences long-term chronic transplant dysfunction [Opelz, 1995], may promote tumor growth by a direct effect on tumor cells [Hojo et al., 1999] and may also decrease recipient immune responses to pathogens, including oncogeneic viruses [Dantal et al., 1998; Soulillou and Giral, 2001]. In humans, immunosuppression withdrawal leads, in most cases, to transplant rejection. Nevertheless, certain rare patients ('operationally tolerant') maintain stable graft function despite the absence of treatment, suggesting that a state of nonresponsiveness can be achieved in clinical transplantation [Strober et al., 2000]. This phenomenon can occur in liver transplantation [Thomson et al., 2001;

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

Grant sponsor: FIRB International MIUR (Ministry for Education, University, and Research), Italy; Progreffe foundation, France.

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Received 19 June 2007; Accepted 3 August 2007
DOI 10.1002/jcb.21557

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Mazariegos et al., 2005] and in kidney recipients [Roussey-Kesler et al., 2006]. The understanding of the molecular mechanisms of tolerance in humans is therefore of central importance. Tolerant patients, indeed, offer a unique opportunity to study the clinical and biological characteristics potentially associated with tolerance. DNA microarrays are one of the most promising tools for molecular genomics [Butte 2002; Nicolini et al., 2002, 2006; Nicolini, 2006] capable to draw a picture of a whole genome. Recently, the employment of non-statistical bioinformatics and data-mining techniques has been proposed in order to identify ab initio a set of genes involved in a certain process and to make a hierarchy among them [Sivozhelezov et al., 2006]. The hierarchy is based upon the number of interactions of every single gene with all the other genes of the set, according to web-available databases such as STRING [von Mering et al., 2005] based on physical contact, involvement in the same metabolic pathway or co-citation in abstracts. The leader gene approach [Sivozhelezov et al., 2006] was applied for the first time in combination with microarray technology [Giacomelli and Nicolini, 2006; Nicolini et al., 2006] on human T lymphocytes stimulated to enter cell cycle with PHA [Abraham et al., 1980]. It was interesting to notice how genes with the highest number of interactions were invariably a very low number (only 6 for human T lymphocytes cell cycle) and that those genes, that were defined as 'leader genes,' are actually playing a central role in the process, namely at the transition or progression of cell cycle phases [Modiano et al., 2000; Kawabe et al., 2002; Oster et al., 2002; Baluchamy et al., 2003; Torgler et al., 2004] and inhibiting the whole cyclin complexes [Jerry et al., 2002; Chang et al., 2004].

In this article, we have applied this approach [Nicolini et al., 2006] to a unique situation of spontaneous tolerance of a mismatched kidney graft in human. This article suggests that even applied to a complex in vivo situation, this approach can allow to identify key genes, which may offer new opportunity of monitoring or modulating immune response against allograft. However several open problems and questions still remain open in the application of nanogenomics to medicine [Nicolini et al., 2006] and it is hoped that these studies of human kidney transplant will shed new light and clarifications.

METHODS

Microarray Datasets

The experimental datasets are derived from pangenomic microarrays fully described elsewhere [Braud et al., 2007]. Fifty-one individuals were included in the study: 8 patients tolerating a kidney graft (TOL) without any treatment and 18 patients with chronic rejection (CR) were evaluated against 8 healthy volunteers (HV) using a subset of the pangenomic (more than 35,000 genes) array displaying 6,865 genes (hence, "individual fullchip"). For every patient, two independent DNA amplifications were used. Data were expressed as mean values (log 2) of the relative intensities [Cy3 (grafted patient)/Cy5 (pool of 169 kidney grafted recipients with stable graft function)]. This database emerges from our previous similar studies of original datasets called west-genopole based on different microarrays utilizing different gene nomenclature and obtained from 14 CR, 11 TOL and 6 HV patients (hence, "pool fullchip"). Note that the pool fullchip data will be further used for comparison of various methods of microarray data processing only, while functional and diagnostic conclusions will be drawn from the individual fullchip.

In every patient of both datasets, genes appear distributed in a Gaussian curve, being either upregulated or downregulated in both the CR and the TOL patients (see Fig. 1 for the "pool fullchip" and also Fig. ES1a,b). There is no discontinuity in gene expression-fluorescence intensity distribution. For the fullchip of rejection and tolerance gene subsets, distribution of their expression levels, both in natural and log scale, is analyzed in Figure ES2. In this graph, the genes are numbered according to the fluorescence intensity resulting from the difference (CR–TOL), defined, for each of the given gene, as the difference between "average log 2 (expression) values for rejection cases – aver-average log 2 (expression) value for tolerance cases," the simplest imaginable discrimination criterion. Thus, negative values correspond to pro-tolerance genes, and positive to pro-rejection genes.

Gene Identification

Once the gene set has been identified, the leader gene algorithm allows to identify the genes with maximum connectivity, as fully described elsewhere [Sivozhelezov et al.,

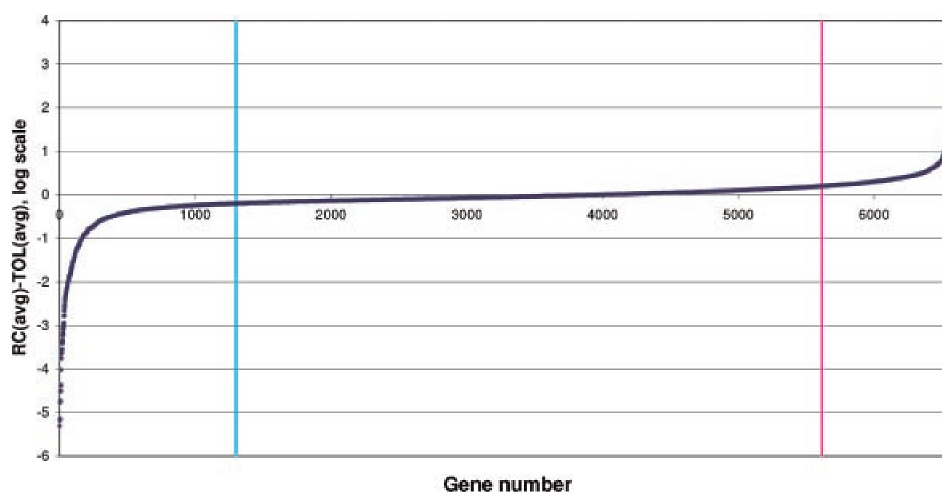

Fig. 1. Genes are numbered according to fluorescence intensity. The fullchip difference in intensity between CR and TOL is thereby giving a measure of their tolerance or rejection propensity. The lines indicate threshold used to select pro-tolerance and pro-rejection genes in the old full-chip database. In the accompanying electronic supplement (Fig. ES1) the frequency distribution is given with respect to healthy volunteers HV of log 2 fluorescence intensity intCR/intHV for gene expression of patients displaying rejection (A) and intTOL/intHV for genes expressing tolerance (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2006]. Interactions between each pair of genes belonging to the set are calculated using STRING database [von Mering et al., 2005], giving a combined association score to each interaction. Scores are based on every kind of links between a pair of genes, for example, physical interaction or involvement in the same metabolic pathway, therefore providing a quantitative approach. Then, the sum of all combined association scores for every gene involved is calculated, and this parameter is defined as weighted number of links. The scores linking genes i and j taken from STRING were summed over j to provide a score for each gene i. Thus, the scores for each gene indicate how strongly it is connected to other genes of the same list. Summation of interaction scores is a feature not existent in the STRING algorithm. The scores are basically connectivity scores but resulting from several interaction types, one of which is co-expression according the microarray data, but others also contribute such as co-evolution, physically detected interactions between proteins encoded by the given genes, and adjacent positions in known metabolic/regulatory networks, but not "textmining", referred to as "co-mentioning" in the literature. Earlier, connectivity scores (however bitwise rather than weighted) were used to evaluate gene essentiality in yeast but with respect to protein interaction networks only [Estrada, 2006]. Scores obtained after summation were clustered using the "K-means" technique [Datta and Datta, 2003; Tassi et al., 2005] to identify the most important genes ("Class A" or "leader genes"), as well as "Class B" genes. The K-means clustering was performed either with the in-house developed MATLAB-based software [Sivozhelezov et al., 2006] or the FuzME program [Minasny and McBratney, 2002], with the fuzzy option switched off. Therefore the algorithm allows predicting key genes responsible for the given cellular process, identified by their connectivity scores.

When we applied the leader gene identification algorithm to the 520 genes subset of pool dataset, and to its pro-tolerance and pro-rejection subsets, the numbers of genes in each class defined leader genes and class B (Fig. ES3). Whenever boundaries between clusters are near, where one gene may belong to several classes, "fuzzy" clustering appears definitely preferential. Such "moving" genes are shown in blue boxes, which characterizes them as doubtful (intermediate) and thereby possibly excluding them from leader genes or Class B genes (Fig. ES3). Performance of MATLAB and FuzME clustering algorithm implementations with respect to the gene expression data was done using the positive and negative subsets from the difference CR−TOL values in the normal (not logarithmic) scale (Figs. 2 and ES4). Besides, we applied the threshold value to reduce the noise. Initially, the threshold was

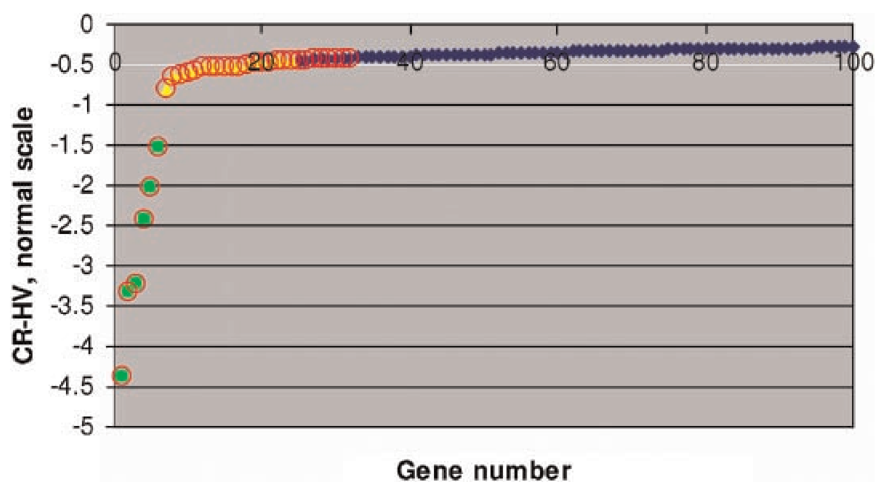

Fig. 2. Divergence of two clustering algorithm implementations on the pro-tolerance subset of reliability-filtered, and amplitude-filtered fullchip genes. Green, Class A genes according to MATLAB; yellow, Class B according to MATLAB; dark blue, the rest of genes. Red circles indicate the 32 top-scoring genes identified by the FuzME implementation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

set at 0.5. This resulted in 114 positive (pro-rejection) and 376 negative (pro-tolerance) genes. Expression leader genes were calculated for both 114 pro-rejection and 376 pro-tolerance gene sets. For both pro-rejection and pro-tolerance Class A genes remain the same for two algorithm implementations, although convergence details vary. For tolerance, an extra Class B gene is observed in the case of MATLAB. Further, algorithms were compared for the dataset unfiltered by threshold. For pro-rejection, both the Class A and Class B lists as well as convergence was the same. For pro-tolerance, however, the results now showed two extra Class B genes, with essential difference in convergence. Different performances of the two algorithm implementations could serve as an indicator of poorer data reliability. The similarity of the two clustering algorithms is however confirmed in most cases both in terms of total number of genes in Classes A and B (Table ES1) and of the actual number of the corresponding genes (Table ES2).

The "fullchip" (both pool and individual) provides an opportunity to separate the "pro-rejection" and "pro-tolerance" genes, and we thereby initially mapped the 507 "ab initio" genes and 520 genes earlier tentatively identified from the "old fullchip" microarray as most varying from CR patients to TOL patients, onto an averaged "rejection-tolerance" graph of the "fullchip" (Fig. ES5). The 507 "ab initio" genes were obtained by the following procedure described in detail in [Sivozhelezov et al., 2006]—Step 1: identification of initial gene list by keyword searches of multiple databases; Step 2: expansion of the list using interaction networks; Step 3: crosschecking the newly found genes against PubMed/Genbank links to delete irrelevant genes; Repeat Steps 2 and 3 until convergence to obtain the final gene list. Interestingly and comfortingly the 507 "ab initio" independently computed genes [Sivozhelezov et al., 2006] are distributed over the pool fullchip plot as evenly as the 520 "microarray-experimentally identified" genes. Consequently, considering the highly flexible nature of FuzME, which is entirely in the public domain, we adopted it for further use in the in-house developed software LEADERGENE (in preparation).

In analyzing microarray-based data, FuzME and MATLAB in most instances gave identical results in identifying Class A and Class B genes yielding occasional divergence of the two clustering algorithm implementations only in such discrimination but not in the total A + B genes identification.

Validity of Microarray Data

The data validity is determined by counting the fraction (%) of valid data, that is, data actually present in the microarray for each gene, separately for tolerance and rejection samples. In the pool fullchip, the total numbers

of the tolerance and rejection samples were 28 and 42, respectively. For example, for gene ABCA1-1A, the fraction is 39/42 = 93% among the "rejection" samples, and 18/28 = 64% among the "tolerance" samples. Clustering analysis according to that parameter showed that about 74% "pro-rejection" genes and 71% "pro-tolerance" genes were classified in the top category, which we termed "reliable." Three more categories were revealed, termed "medium," "unreliable," and "very unreliable." Figure 3(top) shows the presence of the genes in the four categories. This limited validity does not appear however to introduce any bias in the "pool fullchip" data for either pro-TOL or pro-CR genes (Fig. ES6), with the linear regression giving 93% correlation coefficient with the slope of the regression line close to unity.

To further check if unreliability could be related to nomenclature problem, we calculated the fraction of genes not adhering to HGNC nomenclature in each of the four categories. If the nomenclature problems did not affect the reliability, we could expect the same fractions for the "bad" genes as for all genes. This is not the case. Even though the fractions of "bad genes" are close to those for all genes, occurrence of "bad" genes relative to all genes increases from category to category (Fig. 3, bottom). The fact that the observed differences are small is readily explained by the fact that the disagreement with HUGO nomenclature does not necessarily mean that the deposited sample is unreliable. In fact, many of the genes obviously not adhering to the HGNC nomenclature in the "fullchip" microarray can

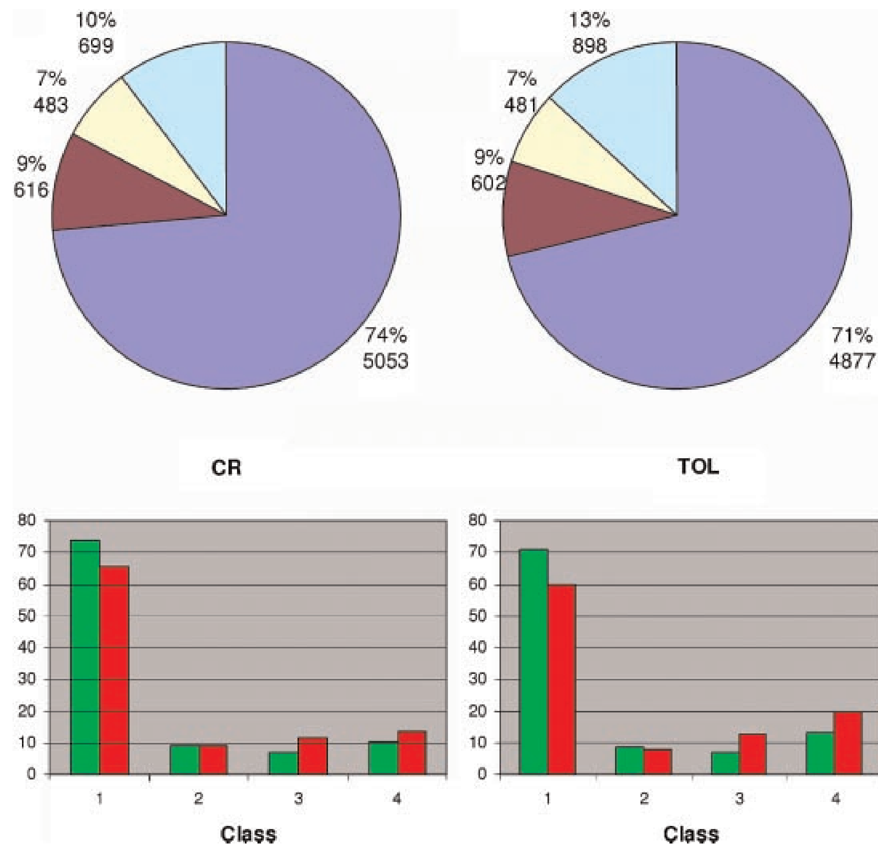

Fig. 3. **Top**: percentages of classes of genes belonging to the four categories with respect to reliabilities in the old "fullchip" raw dataset. Color code is: blue, high reliability; brown, medium reliability; yellow, low reliability, and cyan, very low reliability. **Bottom**: the same values compared to fractions (%) of genes not adhering to the HGNC nomenclature ("bad") in each genes with respect to total "bad" genes. **Left**: CR data. **Right**: TOL data. Color code is: green, fraction of all genes belonging to the class as in pie chart above, for example, 74% for high reliability in CR; red, fraction of "bad" genes belonging to the given class among all "bad" genes. CR dataset consists of 42 samples in total and 6,864 genes, while TOL dataset consists 28 samples in total and 6,864 genes. Reliability is given by the percentage of proven expression data by GENEPIX in such genes for 70 microarray samples. No such distributions are observed in the new fullchip raw dataset where over 98% of the genes show instead reliable data with respect to the 70% reliability criteria. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

be assigned using parsing and database searches, for example, siUNC13Celegans is resolved as the following gene, "Official Symbol: UNC13B and Name: unc-13 homolog B (*C. elegans*) [Homo sapiens]." Similarly, "siRAB11amemberR" is resolved as "Official Symbol: RAB11A and Name: RAB11A, member RAS oncogene family [Homo sapiens]." However, some gene specifications used in the "old fullchip" microarray contain sequences that have been revoked from GenBank presumably by their own authors, in which the GenBank record contains a note that it has been discontinued, one example being siVoltLOC121358. Such nucleotides do not necessarily contain gene sequences, and thus may well be the cause of the entire absence of expression data, as well as in poor reproducibility of the data when present. Our findings are in agreement with the reported generally poor (32–33% correlation coefficient) reproducibility of the microarray data across laboratories [Members of the Toxicogenomics Research Consortium, 2005], which, however, was increased to 56–59% after nomenclature and data handling was standardized. Further increase (in some cases up to 97%) was indeed achieved by standardizing experimental procedures. This is exactly what appears in our individual FullChip, which, in contrast to the pool fullchip, has as much as 98% genes passing the 70% reliability criterium.

RESULTS AND DISCUSSION

Leader genes calculated from interactions of pro-rejection and pro-tolerance genes from both individual and pool microarrays are shown in Table I, filtered in the logscale, and in Table II filtered in the natural scale. From Table II which is normal scale filtered at ± 0.2 for CR, almost all Class A and Class B interaction-based genes are also in the SAM list, while there are only 3 out of 45 interaction-scored Class A and Class B genes in the pool dataset. The drastically increased reliability of the individual microarray dataset compared to the pool dataset is causing improved compatibility of the SAM analysis results with the results of scoring the fullchip genes by interaction (Table II), showing a potential for combined use of expression-based and interaction-based approaches. From Table I, the log scale data appear to make no sense at all with respect to interactions. Not only the occurrence in the SAM-filtered data is nearly zero, but also the Interaction Classes A and B are completely different for individual fullchip derived data compared with the pool fullchip.

Variation of Filtering Parameters for Leader Genes Identification

The microarray genes "0.2 filtered" were ranked according to interaction scores, and the resulting Class A and Class B are shown in Table II. For TOL, 4 out 29 Interaction Class A + B genes are in the SAM list while none was present in the pool dataset. Interestingly, molecular mechanisms of tolerance and rejections appear to be very different. For CR, many genes are changing their expression, but they interact not so strongly as the TOL ones. Instead, TOL genes do not change their expression so much but they are connected stronger. Results on the individual fullchip are simply amazing, namely our non-statistical (expression-based) approach gives almost perfect compatibility with SAM data. The individual dataset is extremely reliable according to our criteria (70% valid samples). Namely, data for 6,743 out of 6,864 genes (98%) are reliable. Moreover, the two TOL genes, namely CCL4L1 and BANK1, most frequently changing their expression, are also the most SAM-significant.

Furthermore for Classes A and B genes: (1) among pro-CR are ubiquitins and proteasomal proteins, which are responsible for protein degradation and are not occurring among pro-TOL interactions; (2) instead among pro-TOL genes, there are many ribosomal proteins and translation factors (both initiation and elongation) genes responsible for protein synthesis; (3) two out of four highly interacting pro-CR genes also occurring in the 343 SAM list are proteasomal proteins. Among TOL, one out of two highly interacting pro-TOL is a ribosomal protein; (4) genes responsible for transcription, namely those encoding different subunits of RNA polymerase, are high-scoring among both pro-CR or pro-TOL genes.

This example shows the potential of combining statistical (SAM) microarray analysis with non-statistical analysis as proposed herein, aided by calculations of gene interaction networks. Obviously the statistical and non-statistical approaches to microarray data analysis should be further optimized since their basic principles are drastically different, and nomenclature issues should be resolved for

TABLE I. "Interaction-Based" Class A and Class B Leader Genes in Kidney Transplant Calculated from the Two Log-Scale, ±0.2 Filtered Pro-Tolerance and Pro-Rejection Datasets, Without Any Reliability Filtering

Pool fullchip, pro-tolerance (Class A)	Pool fullchip, pro-rejection (Class A)	Individual fullchip, pro-tolerance (Class A)	Individual fullchip, pro-rejection
FYN	HDAC1	RB1	POLR2I
ATM	HSPCA	POLR2B	POLR2D
TP53	STAT3	CREBBP	PLCB3
PIK3R1	CSF3R	GTF2F1	GTF2B
PTPN11	TNFRSF1A	JAK3	HRAS
INSL3		SFRS1	GTF2E2
POLR2D		SF3B1	TAF7
LCK		HNRPH1	GTF2A1
IL6ST		EGFR	IL2RB
IL7R		MAPK14	TAF5
EPOR		PIK3CA	TAF10
AR		HNRPD	LCK
PTPRC		CPSF3	
		HNRPH2	
		CASP3	
		SFRS3	
		MADH4	
		FYN	
		HNRPR	
		SNRPA1	
		SF3A2	
		PIK3R1	
Old fullchip, pro-tolerance (Class B)	**Old fullchip, pro-rejection (Class B)**	**New fullchip, pro-tolerance (Class B)**	**New fullchip, pro-rejection (Class B)**
UBA52	**JAK2**	SHC1	IL4R
JUN	RB1	PIP5K1A	HNRPU
IL7	SYK	ATF2	NME2
BLM	MAP3K7	GTF2H2	RPL5
PTK2	MDM2	GTF2H3	DGKB
SRC	IL13RA1	STAT1	DGKI
RPA3	PRKR	PIK3CB	LIPG
CCND2	FOS	YWHAZ	DGKD
TCEA1	ITGB2	**IFNG**	DGKQ
HNRPA1	HSPA8	IRAK2	VAV1
FRS2	LRP1	MAP3K1	CD4
IL12B	HNRPM	CSNK1G2	EP300
SOCS4	HTATIP		TRAF2
CTLA4	TUBA2		RPL22
INSR	SAP30		RPL35
CDC45L	HNRPK		POLD1
MCM6	PSMD14		SNRP70
AKR1C3	PSMD8		THOC4
SRD5A2	PSMC1		DDB1
PLCB3	PSMB8		CDKN1A
IL11	NT5C3		PRKCQ
GHR	PSME1		IL6
SFRS7	G22P1		TRAF6
	UBE2I		CD38

Genes occurring in SAM selected list are shown in bold.

more efficient scanning of gene interaction databases. In order to identify the most SAM-compatible gene set, we have changed expression threshold and expression versus interaction-based gene clustering (Table III). From Table III, it appears that the number of leader genes interaction-based at 0.2 expression threshold is identical to the SAM derived leaders identified by the same FuzME clustering algorithm. It is worth noting that K-means clustering by FuzME is unfeasible (not converged) for the 56 gene SAM-selected list from the pool fullchip dataset, while perfectly converged for the 343 gene SAM-selected list from the individual fullchip SAM dataset (Table ES3). Furthermore (Table IV) the occurrence of top interaction-scored genes among SAM-derived gene list dramatically increases in the individual with respect to the pool dataset, up to 29 out of 68.

Interestingly to further corroborate the choice of this 0.2 expression filtering, Figure 4

TABLE II. "Interaction-Based" Class A (Leader) and Class B Genes in Kidney Transplant Calculated From the Two Natural Scale, ±0.2 Filtered Pro-Tolerance and Pro-Rejection Datasets, Without Any Reliability Filtering

Gene	Score	Class	Gene	Score	Class	Gene	Score	Class	Gene	Score	Class
CSF3R	12.78	A	JUN	20.31	A	**MGAM**	5.39	A	IRAK2	9.00	A
JAK2	10.24	A	IL6ST	18.60	A	**CD14**	4.78	A	CDC2L2	5.40	A
LYN	8.91	A	PLCB4	18.01	A	**ITGB2**	4.48	A	PAK6	5.40	A
IL13RA1	8.34	A	IL2RB	14.65	A	**CTSG**	4.48	A	PCTK3	5.40	A
CTSB	7.35	A	IL2RA	13.92	A	JAK2	3.76	A	RPS5	3.65	A
IFNG	6.59	A	IL7R	12.86	A	**PYGL**	3.60	A	RPS4Y	3.64	A
ITGB3	6.35	A	IL5RA	12.79	A	**AGL**	3.59	A	RPS4Y2	3.64	A
HCK	6.34	A	PIP5K1A	12.60	A	**F5**	3.49	A	**RPL36**	3.64	A
YWHAE	6.30	A	PIP5K2A	12.60	A	STAT5B	3.44	A	PMAIP1	3.60	A
ITGB2	5.98	A	PTPN11	11.59	A	**CSTB**	2.98	A	IL7R	3.20	A
CTSG	5.97	A	DGKI	10.81	A	**TLR2**	2.80	A	RARA	2.86	A
CD9	5.97	A	CDC2L5	10.80	A	**F2**	2.00	B	CCL20	1.99	B
ASAH1	5.40	A	DCAMKL1	10.80	A	**ANXA2**	1.87	B	**CCR6**	1.99	B
DYRK3	5.40	A	DGKE	10.80	A	**S100A10**	1.87	B	PTPRC	1.90	B
PRKR	5.40	A	DGKH	10.80	A	**CDA**	1.87	B	PTPRCAP	1.90	B
SNF1LK	5.40	A	ENPP2	10.80	A	**NP**	1.87	B	E2F5	1.80	B
MGAM	5.39	A	LIPG	10.80	A	**GLA**	1.80	B	IL1B	1.80	B
STAT5B	5.24	A	PNLIPRP1	10.80	A	**IFNG**	1.80	B	**PTGDS**	1.80	B
GOT2	5.20	A	PRKAA2	10.80	A	**ATP6V1B2**	1.52	B	PTGS2	1.80	B
TRAF2	4.97	A	PLG	10.51	A	ATP6V1D	1.52	B	ATM	1.80	B
TNFRSF1A	4.97	A	HRAS	10.44	A	**CD53**	1.49	B	**IL1A**	1.80	B
CDK5R1	4.83	A or B	IFNG	9.80	A	CD9	1.49	B	IL11	1.60	B
SYK	4.79	A or B	AGPAT1	9.18	B	**ILK**	1.49	B	IL12B	1.60	B
CD14	4.78	A or B	MAPK10	8.70	B	**MAD**	1.49	B	KRAS2	1.60	B
PCTK1	4.52	A or B	IL11	8.37	B	**SAP30**	1.49	B	RASA2	1.60	B
SDC1	4.48	A or B	IL7	8.10	B	**VNN2**	1.49	B	TIF1	1.49	B
CSTB	4.47	A or B	IL20	8.00	B	PPBP	1.49	B	GZMB	1.48	B
KIT	4.43	A or B	IL24	8.00	B	**CAMP**	1.49	B	SERPINB9	1.48	B
TNFSF10	3.65	B	SOCS5	8.00	B	**CTSL**	1.49	B	RXRG	1.37	B
ERCC2	3.60	B	C3	7.94	B	FCER1A	1.49	B			
ACADM	3.60	B	PTK2	7.86	B	IGHE	1.49	B			
PYGL	3.598165	B	IL3	7.39	B	**CTSH**	1.49	B			
F2	3.598	B	AKR1C3	7.20	B	LTF	1.49	B			
AGL	3.592404	B	AKR1C4	7.20	B	NMI	1.48	B			
IL13	3.54872	B	SRD5A2	7.20	B	CARD12	1.48	B			
F5	3.49146	B	GPX2	7.20	B	**CASP1**	1.48	B			
TLN1	3.368252	B	GSTO2	7.20	B	**LGALS1**	1.31	B			
ANXA2	3.365658	B	DUSP10	6.99	B	**LGALS3**	1.31	B			
PLAUR	3.364	B	UGT2A1	6.86	B	**LY96**	1.00	B			
S100A10	3.36362	B	MAP3K7IP1	6.82	B						
CASP1	3.283873	B	IL6	6.76	B						
IL15	3.199415	B									
IL21	3.196838	B									
MADH7	3.094	B									
A2M	3.0717	B									

Gene list: CR (left) and TOL (right). Genes occurring in SAM lists are shown in bold.

TABLE III. Average Number of Pro-Tolerance and Pro-Rejection Genes Respectively With TOL and CR Patients for the Three Different Microarray-Based Estimates, Two FuzME and One SAM With Individual Fullchip Dataset

		CR			TOL			
Set	Analysis	Class A	Class B	Total	Class A	Class B	Total	Total
I nat scale interaction-based T6-I	Unfiltered no thresh	15	65	80	63	27	90	170
I nat scale interaction-based	±0.1 thresh	20	24	44	18	21	39	83
I nat scale interaction-based T6-I	Unfiltered no thresh	15	65	80	63	27	90	170
I nat scale interaction-based	±0.1 thresh	20	24	44	18	21	39	83
I nat scale interaction-based T4	±0.2 thresh no reliab	11	28	39	18	11	29	68
I log scale interaction-based T3	±0.2 thresh	12	13	25	22	12	34	59
II error normalized SAM (similar to logscale) clustered estimates	Weighted by errors no thresh	10	28	38	16	13	29	67
III nat scale expression-based	Unfiltered no thresh	14	17	31	9	13	22	53
III nat scale expression-based	±0.1 thresh	13	13	26	9	8	17	43
III nat scale expression-based	±0.2 thresh	13–14	18–17	31	9	8	17	48

It is worth to notice that (a) since nearly all genes (about 98%) have reliability above 70% in the Individual FullChip, results therefore remain the same as ones for reliability unfiltered Fullchip; (b) since only 15 CR and 12 TOL genes have difference above 0.5 in the Individual FullChip, clustering fails for ±0.5 thresh no reliab.

shows the pro-tolerance and pro-rejection genes belonging to the 56 SAM-based genes derived from the "pool fullchip" dataset mapped onto the same "pool fullchip" plot. We see that a vast majority of the 56 genes fall into the leftmost and the rightmost wings of this distribution. More extreme values are not included into the SAM-based 56 gene set since there are too many missing data for them, and consequently the error is quite large, while SAM normalizes the values by their errors. For example the most "pro-tolerance" gene GPR74 (bottom left point on the diagram) is very unreliable: it is based only on two "rejection" datapoints (−0.04 to 0.28, respectively) and one "tolerance" (6.48) out of 70 possible variants (patients, arrays, hybridization, etc.). Such data are excluded by the SAM software, since statistics used in SAM uses the difference in expression levels divided by error obtained for the particular genes, so such scaled difference will be very low in genes like GRP74. By applying the expression level-based filters that we have introduced for our leader gene identification, we excluded from further consideration the genes that, by themselves, do not appear indeed to discriminate between tolerance and rejection (examples of such genes are shown by arrows in Fig. 4, and the quasi-random nature of their expression profiles are shown in Fig. ES7).

SAM-Compatible Gene Set

For the SAM-compatible gene set, that is, the genes ±0.2 filtered by (CR–TOL) amplitude, interaction map was calculated (Fig. 5). It is immediately visible that the densest network is formed around the cluster of interleukins. The interleukin cascade is tightly connected with two caspase genes, CASP1 and CASP8, which are proteases essential in apoptosis [Danial and Korsmeyer, 2004]. Note that caspase CASP1 is among the pro-CR genes according to the SAM

TABLE IV. Occurrence of Top Interaction-Scored Genes Among SAM-Derived Gene List in the Pool and Individual Datasets

	Pool fullchip dataset		Individual fullchip dataset	
Threshold	Leaders in SAM list	Total leaders	Leaders in SAM list	Total leaders
None	0	59	6	170
0.1	1	73	9	83
0.2	4	86	29	68

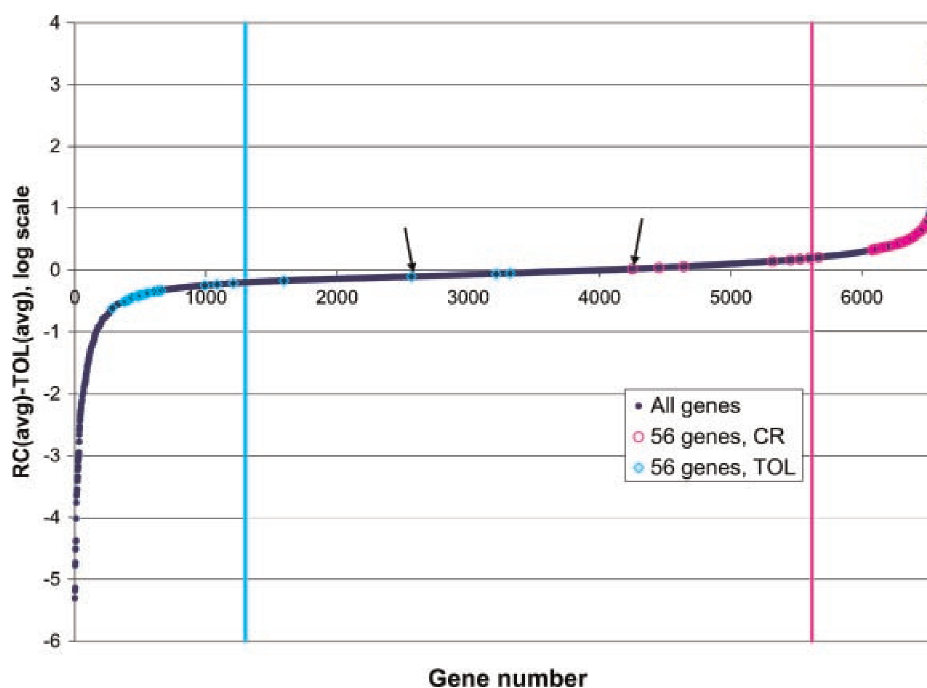

Fig. 4. Genes from the ''fullchip'' plotted according to their tolerance or rejection propensity, that is, difference in expression in log scale between RC and TOL genes, with the 56 SAM-identified genes [Brouard et al., 2005] marked. Arrows indicate genes included in the 56 gene dataset but possibly unable to discriminate rejection/tolerance. Lines indicate thresholds used in selecting genes for ''pro-tolerance'' and ''pro-rejection'' leader gene calculations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

data. A distinct network of signal transduction responsible for immune response is visible spanning the entire map (Fig. 5), which could be a starting point for further analysis of molecular mechanisms of kidney graft tolerance and rejection. This network contains kinases, receptors, and other signal transducers. One of those transducers, STAT5B, activator of transcription, is connected, via N-myc contacting protein (NMI, top left) to a network of genes encoding proteasomal proteins not shown on the diagram since their difference in expression is slightly below 0.2, but still present among SAM proCR genes. Proteasomes, as well as caspases, participate in protein degradation and both are pro-CR. Ribosomal proteins RPL36, RPS4Y1, and RPS5 are also present (bottom right of the diagram). Notably, these proteins are present in the SAM gene list as pro-tolerance. Both CR and TOL genes tend to group together apparently forming pro-CR and pro-TOL sub-networks, respectively. Such sub-networks likely indicate gene sets that are upregulated or downregulated in a concerted manner, specifically to tolerance or rejection. Thus, the sub-network centered on the RARA gene (top left of Fig. 5) is pro-TOL with the exception of the pro-CR PIN1. Similarly, the network around the genes ITGB2 and TLR2 (bottom left) is pro-CR, again with an exception of TLR10 (pro-TOL). Similarly, the already mentioned networks involving caspases and interleukins are pro-CR with the exception of the pro-TOL IL1A (bottom center of Fig. 5).

One example of the opposite pattern is also observed, namely a pro-TOL IL7R gene at the very center of a pro-CR network, denoting its strong regulatory role in the changes in gene expression incurred during tolerance and rejection. Taken together, the data indicate that the regulation mechanism of tolerance and rejection may involve networks of genes all showing completely pro-CR or pro-TOL expression patterns. However, these sub-networks are connected to each other and pro-CR networks can be regulated by a pro-TOL gene as in the example of IL7R. Involvement of caspases in cell death is widely known, so appearance of a pro-rejection caspase network among the genes differentially expressed during tolerance/rejection is in agreement with the finding that cell death-related genes are among those changing

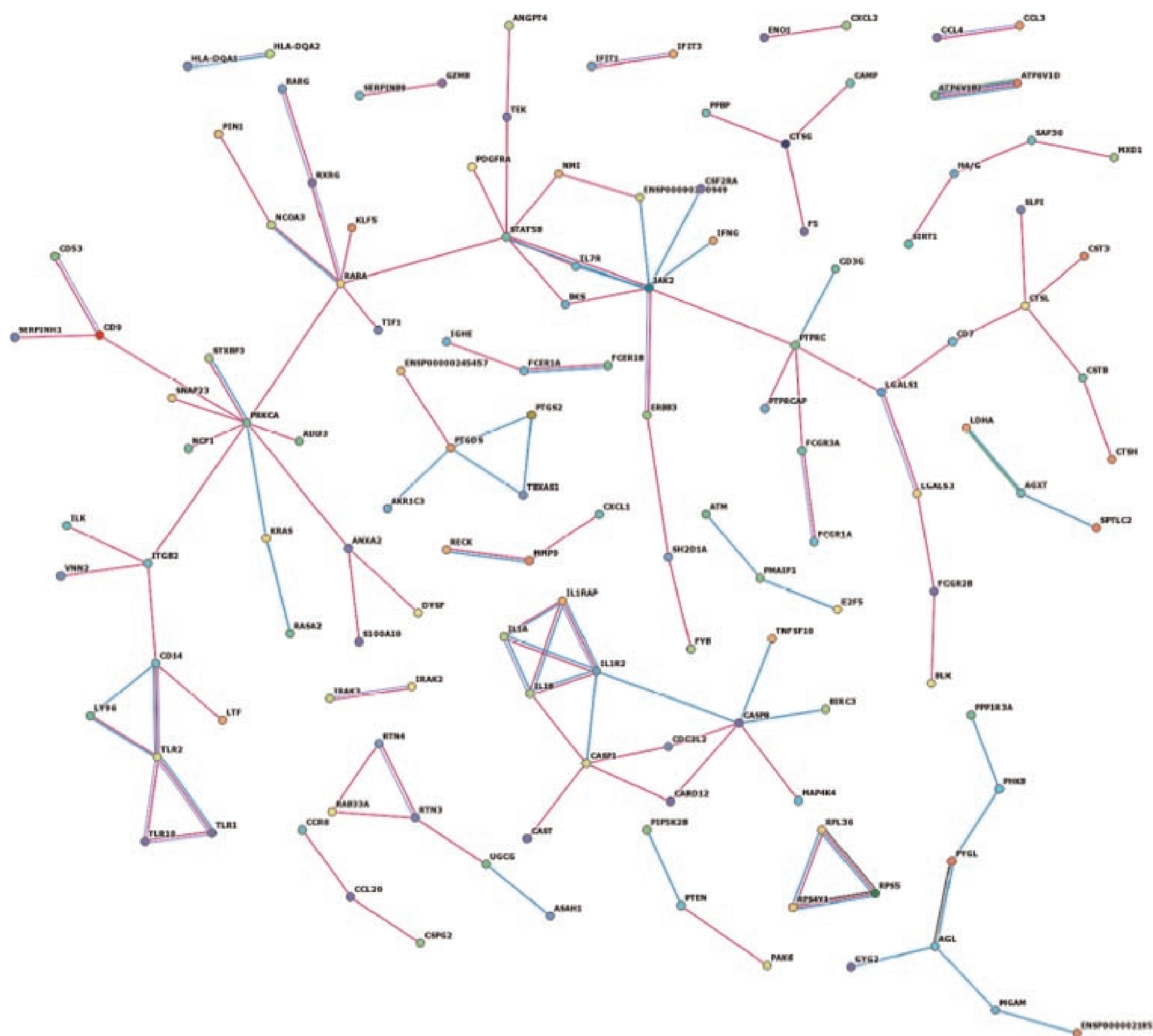

Fig. 5. Complete interaction map "no textmining" interaction map calculated for the "Class 1" reliable genes and filtered by (CR–TOL) amplitude, as obtained from the new fullchip microarray datasets (see Table II for their names and ranking according to their number of interactions). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

their expression similarly in kidney and liver graft. The interleukin networks seen in Figure 5 and especially the special role of IL7R observed herein can be a part of the molecular basis of the known role of interleukin 7 in graft survival [Wang et al., 2006].

Thus a pattern emerges whereby increased protein degradation is related to chronic rejection while increased protein synthesis is related to tolerance. Generally, the microarray data in combination with interaction analysis allow identifying functional networks of co-expressed proteins, which is not feasible using each of the methods separately.

CONCLUSIONS OF PART I AND PART II

The overall conclusion is that there are many genes in common in the highest interaction genes derived from individual fullchip and the 343 SAM-gene list. Notably, convergence between the SAM approach and our non-statistical approach becomes much better for the individual dataset, in which the CR/TOL fold changes are much lower, compared to the old dataset. The primary reasons are (1) we use expression levels in normal scale whereas SAM uses expression levels normalized by their errors (thus practically reducing the signal to

noise level) and (2) SAM uses permutation (random shuffling) of the data, and then extracts significant genes by comparing the permuted and non-permuted set. The SAM-derived genes typically have shown small differences of expression levels explained by the fact that SAM package operates with "relative difference" d which is the actual difference D divided by a sum of its standard deviation s with an arbitrary constant s_0, $d = D/(s + s_0)$, which should make equally significant the small but highly reproducible change and the large but poorly reproducible change in gene expression. Since physical grounds of such an approach are unclear, we separated the two parameters, that is, the magnitude and the reliability, but using two independent filters, one based on percentages of valid samples, and the other on amplitude threshold. In this respect, our approach is less arbitrary because we calculate them using objective clustering and the actual experimental fluorescence distribution in the microarray. When proper microarray reliability and proper expression threshold are applied, we reach the conclusion that it was necessary to acquire and analyze a new, individual dataset, which proves quite adequate to the task. Poor compatibility between our approach and the SAM approach in the pool dataset is apparently caused by the essential difference between the two approaches in that our two filtering parameters are addressing the reliability and the amplitude of the expression levels independently. Indeed, two thresholds are present: one by amplitude, and the other by reliability. Instead, both the SAM denominator parameter and the SAM significance threshold are related to reliability and amplitude in a complicated manner. Similarly to our approach, SAM has two adjustable parameters, namely the above-described arbitrary constant in the denominator $d = D/(s + s_0)$ for relative difference, and the significance threshold. In this respect, our approach is less arbitrary because we calculate them using objective clustering and the actual experimental fluorescence distribution in the microarray. When proper microarray reliability and proper expression thresholds are applied, the compatibility between the two approaches is very good (Table IV). Furthermore, the final leader genes map shed new light in the molecular mechanisms controlling human kidney transplant. Microarray experimentation becomes indeed much more targeted and significant, by comparing gene expression analysis with the analysis of gene networks and interactions. In this context, we successfully applied different variants of the leader gene identification algorithm, in order to identify the ones best representing real gene networks.

All the findings described here, regarding kidney transplant tolerance but also possibly being extended to other systems, confirm the existence of a small set of genes, having a higher number of interactions among all the genes involved in the cellular process and therefore playing a central role. The identification of most interacting genes can be of great importance in the systematization and analysis of data, since leader genes, considering also those largely changing expression in different patients, form a unique network: the mere changing in the expression of a particular gene is not significant by itself, but only if it is put in a proper framework. This change can be often considered as a consequence of a more complex network of events, starting from leader genes, identified with bioinformatics predictions, which often do not vary their expression so much to be identified as significant using pangenomic arrays. However, microarray technology is a necessary confirmation of every prediction made by theoretical network analysis. On the other hand, statistically processed microarray data can serve as the starting point for network analysis.

We introduce a non-statistical approach to processing microarray data, in which we apply K-means clustering to microarray data only after independent filtering by both amplitude and reliability that we define as percentage of valid data for each gene. The need for non-statistical treatment, in addition to statistical treatment of microarray data, has been recognized time ago [Affymetrix Inc., 2004] because "microarrays are the unusual statistical case where the number of tests greatly exceeds the number of samples, so standard statistical methods for multiple comparisons are pushed to their limit." To our knowledge this is the first step in that direction. Results of the non-statistical approach of microarray data interpretation are widely different from the statistical (SAM) approach for the pool dataset, but are similar for the individual dataset. At the moment, none of the three approaches (Tables IV and ES3), namely the "ab initio"

approach, the microarray-based statistical approach, and the microarray-based non-statistical approach, has proved superior in identifying the key genes responsible for kidney graft rejection and/or tolerance, and showing that those approaches must be used in a complementary manner, considering also that reasons for the divergence of those approaches have been identified. Moreover, Table IV of this article showing average number of pro-tolerance and pro-rejection genes, respectively, with TOL and CR patients for the three different microarray-based estimates provides a basis for combined sets of genes to be used in such forthcoming studies. Besides, identification of a pathway possibly important in controlling mechanisms of tolerance and rejection has demonstrated a high potential for the combination of approaches used herein. Genomics does however suffer many pitfalls [Nicolini et al., 2006] and only proteomics [Ramachandran et al., 2006] represents the long range answer to the basic molecular understanding and to the clinical control of the human kidney transplants.

ACKNOWLEDGMENTS

This project was supported by an FIRB International Grant on Proteomics and Cell Cycle (RBIN04RXHS) from MIUR (Ministero dell'Istruzione, Università e Ricerca) to CIRSDNNOB-Nanoworld Institute of the University of Genova and by MIUR grant for "2007 Funzionamento" to Fondazione Elba. This project was also supported by Progreffe foundation from Nantes (France).

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