Supplemental data to

Sirolimus induced phosphaturia is not caused by inhibition of renal apical sodium phosphate cotransporters in rats

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Table S1 Primers and Probes used. List of sequences of primers and probes used in these study.

Gene	Primers	Probe
AQP2	FW: 5'-TGGTGCTGTGCATCTTTGCCT-3' RV: 5'-ACTTGCCAGTGACAACTGCTG-3'	5'-ACCTCCTTGGGATCTATTTCACCGG-3'
NaPi-Iia	FW: 5'-GGAATCACAGTCTCATTCGGATT-3' RV: 5'-ATGGCCTCTACCCTGGACATAG-3'	5'-TGTCAACCAGAGACAAAAGAGGCTTCCACT -3'
NaPi-Iic	FW: 5'-GGGATCGGGATGAATTTCAGA-3' RV: 5'-GGGCCAGCTCACTCAGTCTCT-3'	5'-ACGGCATCTTCAACTGGCTCACAGTGTT-3'
Pit-2	FW: 5'-CCTGCTTTGGGTCCTTTGC-3' RV: 5'-CAGCCACAGGGCCACAAG-3'	5'-ACGGTGGCAACGATGTGAGCAATG-3'
HPRT	FW: 5'-GCTGAAGAT TTGGAAAAGGTGTTTA-3' RV: 5'-ACACAGAGGGCCACAATGTGA- 3'	5'- TTATGGACAGGACTGAAAGACTTGCTGGAGATG-3'
NHE3	FW: 5'-CACCCACCACACGTTGCA-3' RV: 5'-GTGAGCTCGTGCCGACTGT-3'	5'-CTCTACAAGCCTCGGCAGGAGTACAA-3'
Klotho	FW: 5'-TATCTCAAGAAGTTCATAATGGAAAGC-3' RV: 5'-GAGGGACCATGCGGTGTA-3'	5'-TAAAAGCCATCAGGCTGGATGGGG-3'

Table S2 Summary of major results.

	2d		7d	
	Vehicle	**Sirolimus**	**Vehicle**	**Sirolimus**
Functional phosphate studies				
Serumphosphate (mmol/l)	3.3 ± 0.09	2.8 ± 0.05†	3.0 ± 0.08	2.4 ± 0.09‡
Urine phosphate/creatinine ratio	12.4 ± 0.6	17.8 ± 0.7‡	10.4 ± 1.1	15.8 ± 0.9†
TmP/GFR (mmol/l)	3.0 ± 0.1	2.5 ± 0.06‡	2.8 ± 0.06	2.1 ± 0.09‡
BBMV phosphate uptake (pmol/mg)	1091 ± 77	1030 ± 129	1145 ± 126	1158 ± 32
Phosphatonins				
PTH (pg/ml)	403 ± 37	177 ± 35†	334 ± 44	193 ± 31*
FGF 23 (pg/ml)	259 ± 15	267 ± 21	249 ± 20	144 ± 12‡
Klotho (pg/ml)	466.5 ± 134.6	592.6 ± 164.5	1110.8 ± 264.1	809.1 ±177.3
1,25 Dihydroxycholecalciferol (pg/ml)	123 ± 11	155 ± 9	131 ± 12	178 ± 20
Transcription				
qPCR NaPi IIa (2^$^{\text{HPRT-NaPi IIa}}$)	1 ± 0.05	0.9 ± 0.03	1 ± 0.05	0.9 ± 0.03
qPCR NaPi IIc (2^$^{\text{HPRT-NaPi IIc}}$)	1 ± 0.05	0.6 ± 0.05‡	1 ± 0.04	0.6 ± 0.05‡
qPCR Pit-2 (2^$^{\text{HPRT-Pit2}}$)	1 ± 0.09	1 ± 0.08	1 ± 0.1	1.4 ± 0.2
qPCR Klotho (2^$^{\text{18S-Klotho}}$)	1 ± 0.19	0.75 ± 0.19	1 ± 0.13	0.7 ± 0.15
qPCR NHE3 (2^$^{\text{HPRT-NHE3}}$)	1 ± 0.2	1 ± 0.1	1 ± 0.1	1.3 ± 0.2
Translation				
WB NaPi IIa (Actin/NaPi IIa Ratio)	1 ± 0.1	1.1 ± 0.07	1 ± 0.1	1 ± 0.1
WB NaPi IIc (Actin/NaPi IIc Ratio)	1 ± 0.1	0.8 ± 0.07	1 ± 0.2	0.7 ± 0.1
WB Pit-2 (Actin/Pit-2 Ratio)	1 ± 0.1	1 ± 0.08	1 ± 0.2	0.9 ± 0.07
WB Klotho (Actin/Klotho Ratio)	1 ± 0.26	0.6 ± 0.09	1 ± 0.25	0.7 ± 0.15
WB NHE3 (Actin/NHE3 Ratio)	1 ± 0.2	1.3 ± 0.2	1 ± 0.06	0.9 ± 0.1
IHC NaPi IIa		No difference		No difference
IHC NaPi IIc		No difference		No difference
IHC Pit-2		No difference		No difference

Values are means ± SE, n = 6/group. A summary of all major results from rats treated with vehicle and sirolimus for two and seven days is shown. *p < 0.05. †p < 0.01. ‡p < 0.001.

Table S3a Effect of Sirolimus on electrolyte concentration in the urine. P-values of mixed linear models of Glucose, Calcium, Magensium, Sodium, Chloride, Potassium and Phosphate in the Urine. Group refers to the sirolimus effect, day refers to the time effect over seven days and interaction describes effect modification. With the exception of magnesium and sodium, no adjusted group effect could be observed.

Parameter	Group	Day	Interaction Group*Day
Glucose	0.1365	<.0001	<.0001
Calcium	0.0117	0.0003	<.0001
Magnesium	0.0029	0.0025	0.0007
Sodium	0.0068	0.0002	0.0130
Chloride	0.0486	0.1637	0.0044
Potassium	0.6620	0.0157	0.2097
Phosphate	<.0001	<.0001	<.0001

Table S3b Correlation of urinary electrolyte concentration with phosphate in the urine. Correlation coefficients (corr) of calcium, magnesium, sodium, chloride and potassium (Var) to phosphate (phos) in the Urine. Only calcium showed a somewhat stronger correlation with urinary phosphate excretion.

Parameter	estimate Mean (Var)	estimate Mean (Phos)	partial corr	repeated measures corr	overall corr
Calcium	0.8312	11.9963	0.43092	0.78812	0.68137
Magnesium	4.4622	12.4281	0.29622	0.35792	0.52572
Sodium	15.9049	12.4935	0.03887	0.22421	0.37141
Chloride	32.9183	12.3557	0.12278	0.47265	0.40074
Potassium	50.8180	12.4281	0.22331	0.35944	0.10918

Functional Genomics

Methods

Microarray hybridization and bioinformatic workflow

Kidney gene expression analysis was performed according to the NuGEN - recommended protocol using the Affymetrix GeneChip Rat Gene 1.0 ST Array containing probes for 27,342 annotated genes. Total RNA (200 ng per sample) was amplified and labeled using the Applause WT-Amp ST and Encore Biotin Module kit (NuGEN, San Carlos, CA) and hybridized to the arrays as described by the manufacturer (Affymetrix, Santa Clara, CA). The complementary RNA hybridization cocktail was incubated overnight at 45°C while rotating in a hybridization oven. After 18 h of hybridization, the cocktail was removed and the arrays were washed and stained in an Affymetrix GeneChip fluidics station 450. Arrays were scanned on an Affymetrix GeneChip 3000G scanner. All cel files were analyzed by Bioconductor SimpleAffy to assess data quality [1]. Affymetrix data were preprocessed, normalized, summarized and annotated with the robust multi-average (RMA) method, quantile normalization and cdf file implemented in the Bioconductor affy, gcrma and annaffy packages [2].

We used the significance analysis of microarrays (SAM) to determine significant differentially expressed genes (DEGs) between sirolimus and vehicle treatment [3]. All possible permutations (seventy) were used and genes with a fold change over 1.5 and a delta value over 0.5 were assigned as DEGs resulting in a false discovery rate (median) of 10%. DEGs were hierarchically clustered and graphically represented using the MultiExperiment Viewer (MeV) developed at The Institute for Genomic Research (TIGR) [4]. The Pearson correlation and complete linkage were used as distance measure and linkage rule in the hierarchical cluster algorithm, respectively [4, 5].

DEGs were furthermore analyzed with respect to their molecular functions, associated biological processes, and cellular locations using gene ontology terms (GO-Terms) as provided by the Gene Ontology Consortium [6]. Functional grouping of genes was based on GO-Terms and PANTHER (Protein ANalysis THrough Evolutionary Relationships) ontologies [7, 8].

Raw data files as well as the MIAME checklist are available at the GEO Omnibus Database record GSE35048.

Results

A PDF file of tables and figures of further bioinformatical data evaluation is available at the journals website. Gene Expression Omnibus: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=xdyplqkoackcgzs&acc=GSE35048

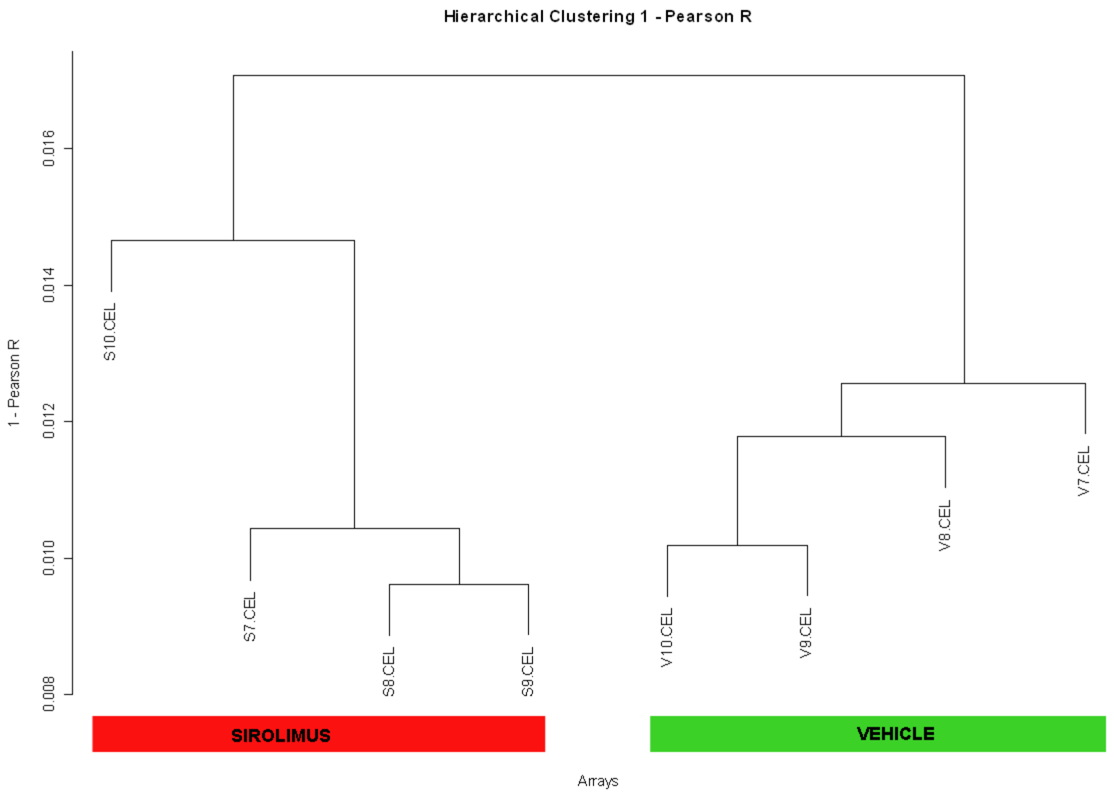

Figure S1 Unsupervised hierarchical clustering. Unsupervised hierarchical clustering, 21131 transcripts, sirolimus treatment day 7 (pearson correlation, complete linkage)

Unsupervised hierarchical clustering of the eight samples showed a perfect separation of sirolimus treatment (figure S1).

In total 154 features were identified as significant differentially expressed when comparing the gene expression profiles of rat kidneys between sirolimus and vehicle treatment with a fold change over 1.5, resulting in 139 down-regulated and 15 up-regulated genes in the sirolimus group (figure S2, table S1).

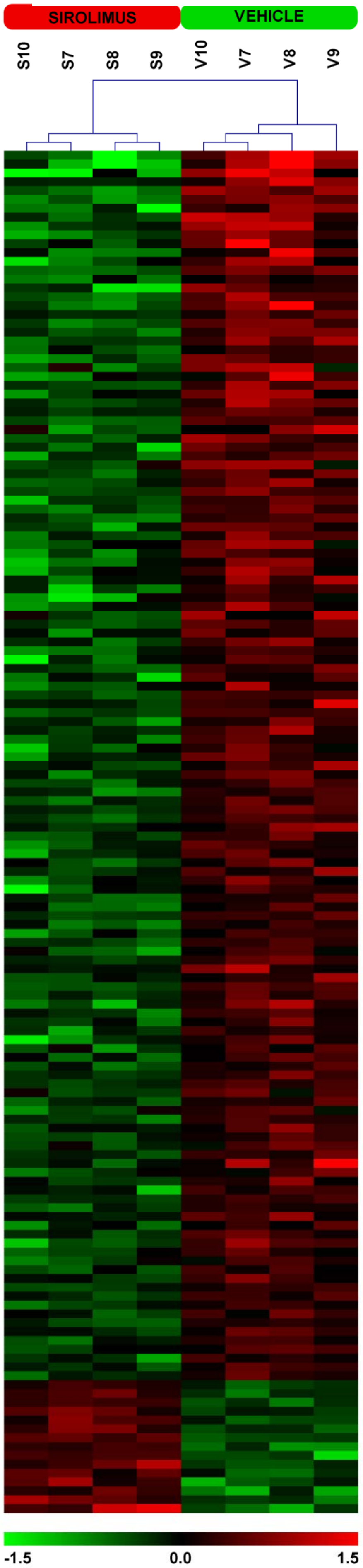

Figure S2 Dendrogram of gene expression profiles. Dendrogram derived by unsupervised hierarchical clustering of gene expression profiles characterizing the sirolimus group (red bar) and the vehicle group (green bar). Red spots ndicate up-regulated transcripts, whereas green spots indicate down-regulated transcripts relative to the other group.

Table S4 Significant differentially expressed genes between sirolimus (S) and vehicle (V) treatment listed by fold change.

Ensembl_Gene	Gene Symbol	Name	Mean S (n=4)	SEM S	Mean V (n=4)	SEM V	t-test	Fold change
ENSRNOG00000013552	Scd1	Stearoyl-Coenzyme A desaturase 1	6.74	0.34	8.88	0.42	0.007	0.227
ENSRNOG00000012404	Thrsp	Thyroid hormone responsive	7.30	0.42	9.41	0.33	0.007	0.232
ENSRNOG00000015308	Pbk	PDZ binding kinase	5.50	0.46	7.56	0.31	0.010	0.240
ENSRNOG00000001796	Dgkg	Diacylglycerol kinase, gamma	7.57	0.10	9.00	0.12	0.000	0.371
ENSRNOG00000015850	Rbp7	Retinol binding protein 7, cellular	5.30	0.14	6.69	0.32	0.008	0.381
ENSRNOG00000039103		Transcribed locus, moderately similar to XP_002725225.1 PREDICTED: mCG140493-like [Rattus norvegicus]	6.36	0.32	7.74	0.18	0.010	0.385
ENSRNOG00000037198	Usp18	Ubiquitin specific peptidase 18	5.29	0.10	6.63	0.09	0.000	0.394
ENSRNOG00000023272	Rrm2	Ribonucleotide reductase M2	6.33	0.17	7.60	0.13	0.001	0.417
ENSRNOG00000031211	Acsm5	Acyl-CoA synthetase medium-chain family member 5	6.61	0.31	7.87	0.16	0.012	0.418
ENSRNOG00000015550	Ptgds	prostaglandin D2 synthase 21kDa	7.76	0.10	9.01	0.22	0.002	0.421
ENSRNOG00000003202			4.89	0.16	6.12	0.26	0.007	0.427
ENSRNOG00000032404	Top2a	topoisomerase (DNA) II alpha 170kDa	7.14	0.28	8.35	0.25	0.018	0.433
ENSRNOG00000012640	Dpp7	Dipeptidylpeptidase 7	7.26	0.05	8.45	0.08	0.000	0.439
ENSRNOG00000037304	Casc5	cancer susceptibility candidate 5	4.88	0.08	6.05	0.16	0.001	0.446
ENSRNOG00000026440	RGD1559891		4.73	0.21	5.88	0.21	0.008	0.449
ENSRNOG00000019542	MGC108823;RGD1559715	Similar to interferon-inducible GTPase	4.99	0.19	6.13	0.14	0.003	0.453

Ensembl_Gene	Gene Symbol	Name	Mean S (n=4)	SEM S	Mean V (n=4)	SEM V	t-test	Fold change
ENSRNOG00000001821	Adipoq	Adiponectin, C1Q and collagen domain containing	6.69	0.17	7.82	0.31	0.019	0.456
ENSRNOG00000009086	Apcs	Amyloid P component, serum	9.41	0.11	10.53	0.11	0.000	0.460
ENSRNOG00000003959	Rgs18	regulator of G-protein signaling 18	5.86	0.16	6.97	0.11	0.001	0.463
ENSRNOG00000017539	Mmp9	Matrix metallopeptidase 9	6.24	0.12	7.35	0.14	0.001	0.465
ENSRNOG00000028137	RGD1566252		6.63	0.22	7.74	0.22	0.012	0.465
ENSRNOG00000015411	Apobec1	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	7.46	0.09	8.55	0.18	0.002	0.472
ENSRNOG00000000045	Cd244	Cd244 molecule, natural killer cell receptor 2B4	4.09	0.22	5.15	0.31	0.032	0.481
ENSRNOG00000034190	LOC678701	Transcribed locus, moderately similar to XP_002729795.1 PREDICTED: mCG129376-like [Rattus norvegicus]	7.34	0.13	8.39	0.39	0.042	0.483
ENSRNOG00000014938	Gabrb3	Gamma-aminobutyric acid (GABA) A receptor, beta 3	5.76	0.12	6.81	0.40	0.047	0.483
ENSRNOG00000018505	Cidea	Cell death-inducing DFFA-like effector a	7.32	0.25	8.35	0.31	0.041	0.490
ENSRNOG00000038738			7.35	0.25	8.38	0.09	0.008	0.490
ENSRNOG00000000168	Gatm	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	9.95	0.06	10.97	0.04	0.000	0.494
ENSRNOG00000002925	Tsx	Testis specific X-linked gene	7.07	0.05	8.08	0.18	0.001	0.495
ENSRNOG00000006731	Spc25	SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae)	6.35	0.19	7.36	0.23	0.015	0.495
ENSRNOG00000038960	RGD1309362	Similar to interferon-inducible GTPase	7.57	0.29	8.58	0.14	0.020	0.496

Ensembl_Gene	Gene Symbol	Name	Mean S (n=4)	SEM S	Mean V (n=4)	SEM V	t-test	Fold change
ENSRNOG00000015945	Cd3g	CD3g molecule, gamma (CD3-TCR complex)	4.86	0.07	5.86	0.18	0.002	0.500
ENSRNOG00000028288	Clrn3	Clarin 3	6.51	0.19	7.51	0.09	0.003	0.501
ENSRNOG00000009785	Cdkn3	cyclin-dependent kinase inhibitor 3	6.02	0.31	7.01	0.11	0.023	0.502
ENSRNOG00000010149	Cmc1	COX assembly mitochondrial protein homolog (S. cerevisiae)	7.35	0.20	8.33	0.09	0.004	0.508
ENSRNOG00000014407		Transcribed locus, moderately similar to XP_224795.4 PREDICTED: similar to C28H8.3 [Rattus norvegicus]	5.28	0.09	6.25	0.18	0.003	0.512
ENSRNOG00000031078	RGD1566137		3.26	0.02	4.22	0.25	0.008	0.512
ENSRNOG00000004601	Hao1	Hydroxyacid oxidase (glycolate oxidase) 1	5.20	0.20	6.15	0.13	0.007	0.516
ENSRNOG00000036701	Actg1;LOC295810	Actin, gamma 1	3.85	0.23	4.80	0.31	0.050	0.518
ENSRNOG00000026501	Slc6a19	Solute carrier family 6 (neutral amino acid transporter), member 19	8.31	0.12	9.25	0.08	0.001	0.519
ENSRNOG00000028626			6.51	0.24	7.46	0.09	0.010	0.520
ENSRNOG00000028870	Acot1	acyl-CoA thioesterase 1	8.03	0.11	8.97	0.11	0.001	0.522
ENSRNOG00000014541	Ddx60;LOC684383	Transcribed locus	5.41	0.20	6.35	0.11	0.007	0.523
ENSRNOG00000015763	Cml3	Camello-like 3	8.40	0.03	9.34	0.12	0.000	0.523
ENSRNOG00000013727	Ndc80	Transcribed locus	5.54	0.15	6.46	0.26	0.023	0.526
ENSRNOG00000000632	Cdc2	Cell division cycle 2, G1 to S and G2 to M	4.95	0.11	5.87	0.19	0.005	0.526
ENSRNOG00000004921	Nusap1	Nucleolar and spindle associated protein 1	6.45	0.32	7.37	0.14	0.039	0.529

Ensembl_Gene	Gene Symbol	Name	Mean S (n=4)	SEM S	Mean V (n=4)	SEM V	t-test	Fold change
ENSRNOG00000003802	Pttg1	Pituitary tumor-transforming 1	6.50	0.12	7.41	0.15	0.003	0.533
ENSRNOG00000037267	Renbp	Renin binding protein	6.48	0.05	7.39	0.06	0.000	0.533
ENSRNOG00000026378	Casc5	cancer susceptibility candidate 5	6.20	0.25	7.10	0.23	0.038	0.535
ENSRNOG00000038035	Kif4	Kinesin family member 4	5.92	0.21	6.82	0.20	0.022	0.539
ENSRNOG00000032778	Bub1	Budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)	5.46	0.17	6.34	0.26	0.029	0.542
ENSRNOG00000005348	Pamr1	peptidase domain containing associated with muscle regeneration 1	7.53	0.27	8.40	0.13	0.027	0.544
ENSRNOG00000018454	Apoe	Apolipoprotein E	8.02	0.07	8.90	0.08	0.000	0.545
ENSRNOG00000013794	Rbp1	Retinol binding protein 1, cellular	9.48	0.11	10.35	0.12	0.002	0.546
ENSRNOG00000008165	Tpx2	TPX2, microtubule-associated, homolog (Xenopus laevis)	6.73	0.15	7.60	0.07	0.002	0.550
ENSRNOG00000030689	Ms4a6b	Membrane-spanning 4-domains, subfamily A, member 6B	6.60	0.16	7.46	0.24	0.025	0.550
ENSRNOG00000001959	Mx1	Myxovirus (influenza virus) resistance 1	5.78	0.13	6.63	0.18	0.008	0.556
ENSRNOG00000000925	Psph	Phosphoserine phosphatase	7.75	0.10	8.59	0.12	0.002	0.561
ENSRNOG00000013057	Prc1	Protein regulator of cytokinesis 1	6.49	0.26	7.32	0.16	0.034	0.562
ENSRNOG00000018503	Bcl2l1;LOC293190;LOC684140		6.16	0.34	6.99	0.10	0.061	0.565
ENSRNOG00000000903	Asl		8.49	0.06	9.32	0.03	0.000	0.565
ENSRNOG00000009597	Cyp4a10;Cyp4a1	Cytochrome P450, family 4, subfamily a, polypeptide 1	8.61	0.15	9.43	0.31	0.052	0.566
ENSRNOG00000010252	Hexa	Hexosaminidase A	9.24	0.06	10.06	0.03	0.000	0.566

Ensembl_Gene	Gene Symbol	Name	Mean S (n=4)	SEM S	Mean V (n=4)	SEM V	t-test	Fold change
ENSRNOG00000021735	Akr1cl1	Aldo-keto reductase family 1, member C-like 1	5.54	0.18	6.36	0.14	0.012	0.568
ENSRNOG00000016561	Ns5atp9	NS5A (hepatitis C virus) transactivated protein 9	6.72	0.15	7.53	0.10	0.004	0.571
ENSRNOG00000007314	Slc26a4	Solute carrier family 26, member 4	8.49	0.18	9.30	0.07	0.006	0.572
ENSRNOG00000039098	Vom2r64	Vomeronasal 2 receptor, 64	4.84	0.19	5.64	0.15	0.015	0.574
ENSRNOG00000020480	Fads1	Fatty acid desaturase 1	8.52	0.16	9.31	0.14	0.010	0.578
ENSRNOG00000005556	Snrpf	Small nuclear ribonucleoprotein polypeptide F	7.05	0.16	7.83	0.24	0.035	0.581
ENSRNOG00000029369	Olr1673	Olfactory receptor 1673	3.64	0.12	4.42	0.12	0.003	0.583
ENSRNOG00000031335	Ankrd37	Ankyrin repeat domain 37	7.77	0.13	8.54	0.12	0.005	0.585
ENSRNOG00000026605	Ifi27l2b		7.51	0.09	8.28	0.06	0.000	0.586
ENSRNOG00000037626			6.23	0.22	6.99	0.13	0.023	0.587
ENSRNOG00000038407			3.01	0.05	3.78	0.19	0.008	0.588
ENSRNOG00000010721	Dlgap5	discs, large (Drosophila) homolog-associated protein 5	6.19	0.17	6.96	0.10	0.009	0.589
ENSRNOG00000002711	Nuf2	NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)	5.74	0.06	6.50	0.12	0.001	0.591
ENSRNOG00000009513	Akr1b1;Akr1b1-ps2	Aldo-keto reductase family 1, member B1 (aldose reductase)	9.92	0.09	10.68	0.20	0.013	0.593
ENSRNOG00000007139	Ttpa	Tocopherol (alpha) transfer protein	6.49	0.10	7.24	0.12	0.003	0.596
ENSRNOG00000038686	Ap1s2	Adaptor-related protein complex 1, sigma 2 subunit	6.17	0.19	6.92	0.09	0.013	0.598
ENSRNOG00000032417	Gabrp	gamma-aminobutyric acid	8.45	0.07	9.19	0.27	0.037	0.598

Ensembl_Gene	Gene Symbol	Name	Mean S (n=4)	SEM S	Mean V (n=4)	SEM V	t-test	Fold change
		(GABA) A receptor, pi						
ENSRNOG00000011598	Slc15a1	Solute carrier family 15 (oligopeptide transporter), member 1	7.14	0.22	7.88	0.13	0.027	0.598
ENSRNOG00000003927	Cd55	Transcribed locus	6.86	0.16	7.60	0.07	0.006	0.600
ENSRNOG00000005037	Kif18a	Kinesin family member 18A	6.62	0.19	7.36	0.17	0.028	0.600
ENSRNOG00000038792	Olr1662;LOC682056	Olfactory receptor 1662	4.03	0.16	4.76	0.12	0.010	0.602
ENSRNOG00000028185	LOC500420	Similar to CG12279-PA	7.41	0.18	8.14	0.10	0.012	0.602
ENSRNOG00000005180			6.02	0.12	6.75	0.11	0.004	0.605
ENSRNOG00000021357	Slfn3	Schlafen 3	6.75	0.15	7.47	0.20	0.026	0.607
ENSRNOG00000010283	Cd28	Cd28 molecule	5.68	0.08	6.40	0.23	0.025	0.608
ENSRNOG00000001926	Cldn1	Claudin 1	8.59	0.09	9.31	0.06	0.000	0.609
ENSRNOG00000015857	Ctsa	Cathepsin A	11.03	0.05	11.74	0.08	0.000	0.611
ENSRNOG00000022505	Slc17a4;LOC684568	Solute carrier family 17 (urate transporter), member 4	7.96	0.08	8.66	0.19	0.015	0.614
ENSRNOG00000006395	RGD1561343	Similar to C20orf118	6.16	0.12	6.87	0.06	0.002	0.614
ENSRNOG00000039801	RGD1563091		6.72	0.15	7.42	0.08	0.006	0.615
ENSRNOG00000030250			3.92	0.15	4.62	0.09	0.007	0.617
ENSRNOG00000004405	Pigr	Polymeric immunoglobulin receptor	8.56	0.17	9.25	0.12	0.017	0.622
ENSRNOG00000001333	Azgp1	alpha-2-glycoprotein 1, zinc-binding	8.25	0.26	8.94	0.08	0.048	0.622
ENSRNOG00000001295	S100b	Transcribed locus	5.93	0.05	6.61	0.12	0.002	0.622
ENSRNOG00000012804	C1qc	Complement component 1, q subcomponent, C	7.27	0.08	7.95	0.11	0.002	0.624

Ensembl_Gene	Gene Symbol	Name	Mean S (n=4)	SEM S	Mean V (n=4)	SEM V	t-test	Fold change
		chain						
ENSRNOG00000023093	LOC689296	Similar to expressed sequence C79407	5.34	0.09	6.02	0.12	0.004	0.627
ENSRNOG00000018812	Rpp25	Ribonuclease P 25 subunit (human)	7.66	0.13	8.33	0.10	0.006	0.628
ENSRNOG00000011622	Echdc1	Enoyl Coenzyme A hydratase domain containing 1	6.63	0.14	7.30	0.17	0.024	0.629
ENSRNOG00000014224	LOC684509;Ndufa3		8.61	0.20	9.28	0.06	0.017	0.629
ENSRNOG00000038572	RGD1562646	Similar to chromosome condensation protein G	5.51	0.05	6.17	0.11	0.001	0.631
ENSRNOG00000029924	Klk1l	Kallikrein 1-like peptidase	10.77	0.11	11.43	0.20	0.026	0.632
ENSRNOG00000003388	Cenpf	centromere protein F, 350/400kDa (mitosin)	6.78	0.20	7.44	0.15	0.038	0.632
ENSRNOG00000033996	RGD1565900	Transcribed locus, strongly similar to NP_990668.1 60S ribosomal protein L27 [Gallus gallus]	9.71	0.20	10.37	0.14	0.037	0.634
ENSRNOG00000004280	Tcn2	Transcobalamin 2	8.91	0.05	9.56	0.07	0.000	0.635
ENSRNOG00000029778	Maob	Monoamine oxidase B	6.32	0.04	6.97	0.13	0.003	0.635
ENSRNOG00000022657	Tmem97	Transmembrane protein 97	7.23	0.12	7.89	0.11	0.007	0.637
ENSRNOG00000019716	Ntf3	Transcribed locus	7.19	0.04	7.84	0.13	0.004	0.640
ENSRNOG00000029370	Abhd3		7.39	0.20	8.04	0.13	0.037	0.641
ENSRNOG00000039696	Olr1547	Olfactory receptor 1547	2.70	0.13	3.34	0.17	0.024	0.642
ENSRNOG00000002382	Mfap4	Microfibrillar-associated protein 4	7.16	0.20	7.80	0.14	0.043	0.643
ENSRNOG00000029055	Ttk	Ttk protein kinase	4.92	0.10	5.56	0.10	0.004	0.643
ENSRNOG00000024159	Fcer1g	Fc fragment of IgE, high affinity I, receptor for;	9.97	0.18	10.61	0.12	0.024	0.644

Ensembl_Gene	Gene Symbol	Name	Mean S (n=4)	SEM S	Mean V (n=4)	SEM V	t-test	Fold change
		gamma polypeptide						
ENSRNOG00000017332	Dapk2	Death-associated kinase 2	6.72	0.02	7.35	0.25	0.043	0.645
ENSRNOG00000007089	Lgmn	Legumain	10.90	0.12	11.53	0.05	0.003	0.646
ENSRNOG00000028746	Gsto1	Glutathione S-transferase omega 1	9.08	0.08	9.71	0.11	0.004	0.646
ENSRNOG00000029057	Olr898	Olfactory receptor 898	4.28	0.16	4.91	0.17	0.039	0.647
ENSRNOG00000004667	Gen1	Gen homolog 1, endonuclease (Drosophila)	5.23	0.14	5.85	0.17	0.026	0.647
ENSRNOG00000019662	Tm6sf1	Transmembrane 6 superfamily member 1	8.31	0.05	8.94	0.05	0.000	0.648
ENSRNOG00000013215	Dctd	dCMP deaminase	7.11	0.19	7.73	0.08	0.025	0.651
ENSRNOG00000000137	Ly86	Lymphocyte antigen 86	7.50	0.11	8.12	0.17	0.022	0.651
ENSRNOG00000002253	Wdr5b	WD repeat domain 5B	5.91	0.13	6.53	0.12	0.014	0.651
ENSRNOG00000017259	Tacc3	Transforming, acidic coiled-coil containing protein 3	6.15	0.13	6.77	0.08	0.006	0.652
ENSRNOG00000018815	Plk1	Polo-like kinase 1 (Drosophila)	6.28	0.13	6.90	0.13	0.015	0.653
ENSRNOG00000000853	Aif1	Allograft inflammatory factor 1	8.36	0.08	8.97	0.06	0.001	0.653
ENSRNOG00000018899	C5	Complement component 5	8.74	0.09	9.35	0.09	0.003	0.655
ENSRNOG00000019189	Acat2;RGD1561787	Transcribed locus	9.78	0.14	10.38	0.08	0.010	0.656
ENSRNOG00000014064	Ctsh	Cathepsin H	11.50	0.10	12.11	0.03	0.001	0.656
ENSRNOG00000036674	Cd7	Cd7 molecule	6.76	0.14	7.36	0.14	0.024	0.660
ENSRNOG00000037940			4.83	0.08	5.43	0.18	0.025	0.660
ENSRNOG00000016717	Gas2	Growth arrest-specific 2	7.38	0.09	7.97	0.16	0.016	0.662
ENSRNOG00000006859	Insig1	insulin induced gene 1	9.09	0.12	9.68	0.13	0.014	0.663

Ensembl_Gene	Gene Symbol	Name	Mean S (n=4)	SEM S	Mean V (n=4)	SEM V	t-test	Fold change
ENSRNOG00000036402			5.03	0.03	5.62	0.17	0.015	0.664
ENSRNOG00000028623	Agpat5	Transcribed locus	5.88	0.11	6.47	0.11	0.008	0.665
ENSRNOG00000013001			6.42	0.12	7.01	0.12	0.013	0.666
ENSRNOG00000033056			6.17	0.09	6.76	0.13	0.010	0.666
ENSRNOG00000038319	Akr1c19	Transcribed locus	9.70	0.06	9.10	0.09	0.002	1.512
ENSRNOG00000001809	Hrg;LOC681544	Histidine-rich glycoprotein	9.17	0.10	8.40	0.12	0.003	1.701
ENSRNOG00000007923	Cgref1	Cell growth regulator with EF hand domain 1	8.75	0.14	7.99	0.07	0.002	1.701
ENSRNOG00000033527	Pappa		7.95	0.04	7.16	0.11	0.001	1.721
ENSRNOG00000011184	Slc13a4	Solute carrier family 13 (sodium/sulfate symporters), member 4	7.15	0.14	6.32	0.10	0.003	1.780
ENSRNOG00000023465	LOC500300	Similar to hypothetical protein MGC6835	7.10	0.13	6.26	0.14	0.004	1.785
ENSRNOG00000018693	Asgr1	Asialoglycoprotein receptor 1	7.44	0.15	6.57	0.09	0.002	1.835
ENSRNOG00000017944	Tbx10	Transcribed locus	7.35	0.06	6.45	0.05	0.000	1.876
ENSRNOG00000019260	Ceacam20		8.30	0.20	7.35	0.07	0.004	1.930
ENSRNOG00000036754			9.59	0.04	8.63	0.14	0.001	1.939
ENSRNOG00000028616	Pck1	Phosphoenolpyruvate carboxykinase 1 (soluble)	11.53	0.02	10.53	0.22	0.004	2.009
ENSRNOG00000015992	Ccl20	Chemokine (C-C motif) ligand 20	8.03	0.16	7.01	0.11	0.002	2.026
ENSRNOG00000029066			7.30	0.18	6.27	0.19	0.008	2.035
ENSRNOG00000006367	Slc5a8	Solute carrier family 5 (iodide transporter), member 8	9.46	0.11	8.40	0.21	0.004	2.086
ENSRNOG00000031231	Kng1;Kng2	Kininogen 1	7.27	0.28	5.85	0.17	0.005	2.681

Table S5 Functional classification of DEGs using PANTHER ontologies. Enriched biological processes separating sirolimus from vehicle group as derived on the level of differential gene expression by SAM. Categories are ranked by the p-value (comparison of expected number of genes and observed number of genes in each biological process) indicating the relevance of a particular process.

Enriched Biological Processes		
down-regulated genes by Sirolimus treatment		
biological process	**number of genes (n=76)**	**p-value**
response to stimulus	21	<0.001
immune system process	23	<0.001
lipid metabolic process	13	<0.001
immune response	11	<0.001
cellular process	35	<0.001
anion transport	4	0.001
cell cycle	14	0.003
Transport	18	0.003
B cell mediated immunity	6	0.004
Mitosis	7	0.005
cellular amino acid and derivative metabolic process	5	0.006
metabolic process	40	0.008
cellular defense response	6	0.009
cellular component morphogenesis	9	0.014
anatomical structure morphogenesis	9	0.014
complement activation	3	0.015
signal transduction	22	0.016
developmental process	17	0.017
primary metabolic process	37	0.020
cell communication	22	0.024
vitamin metabolic process	2	0.027
induction of apoptosis	4	0.027
ion transport	6	0.028
defense response to bacterium	2	0.028
vitamin transport	2	0.035
coenzyme metabolic process	2	0.036
Cytokinesis	3	0.039
cell-cell signaling	8	0.040
chromosome segregation	3	0.042
up-regulated genes by Sirolimus treatment		

biological process	**number of genes (n=8)**	**p-value**
response to external stimulus	3	<0.001
blood coagulation	3	<0.001
response to stimulus	4	0.005
immune system process	4	0.011
regulation of biological process	1	0.020
regulation of vasoconstriction	1	0.020
anion transport	1	0.046

According to PANTHER classification down-regulated transcripts belong to response to stimulus, metabolic processes, immune system, transport and signal transduction. Up-regulated transcripts belong also to response to stimulus, immune system and transport but also to blood coagulation and regulation of vasoconstriction (table S2).

Table S6 Na+/K+ -ATPase subunits expression levels between sirolimus and vehicle group measured on the GeneChip expression array. None of the polypeptides forming Na+/K+ -ATPases are differentially expressed between the groups.

Gene	Ensembl_Gene	EntrezGene	Description	mean S (n=4)	mean V (n=4)	fold change	p-value
Atp1a2	ENSRNOG00000007290	24212	ATPase, Na+/K+ transporting, alpha 2 polypeptide	6.49	6.98	1.40	0.077
Atp1a3	ENSRNOG00000020263	24213	ATPase, Na+/K+ transporting, alpha 3 polypeptide	5.98	5.97	1.00	0.972
Atp1b4	ENSRNOG00000007059	84396	ATPase, (Na+)/K+ transporting, beta 4 polypeptide	5.11	5.25	1.10	0.296
Atp1b2	ENSRNOG00000011227		ATPase, Na+/K+ transporting, beta 2 polypeptide	7.25	7.17	1.06	0.727
Atp1b3	ENSRNOG00000011501	25390	ATPase, Na+/K+ transporting, beta 3 polypeptide	9.53	9.67	1.11	0.287

References

1. Wilson, Miller. Simpleaffy: a BioConductor package for Affymetrix quality control and data analysis. *Bioinformatics*. 2005.
2. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003; **4**: 249-64.
3. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A*. 2001; **98**: 5116-21.
4. Saeed AI, Sharov V, White J, et al. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques*. 2003; **34**: 374-8.
5. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A*. 1998; **95**: 14863-8.
6. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet*. 2000; **25**: 25-9.
7. Mi H, Lazareva-Ulitsky B, Loo R, et al. The PANTHER database of protein families, subfamilies, functions and pathways. *Nucleic Acids Res*. 2005; **33**: D284-8.
8. Hoffmann R, Valencia A. A gene network for navigating the literature. *Nat Genet*. 2004; **36**: 664.