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Transcriptomic changes in developing kidney exposed to chronic hypoxia

Dumitru A. Iacobas [a,*], Chenhao Fan [b], Sanda Iacobas [a], David C. Spray [a], Gabriel G. Haddad [c]

[a] *Department of Neuroscience, Kennedy Center, Room #915C, Albert Einstein College of Medicine, 1410 Pelham Parkway South, Bronx, New York, NY 10461, USA*
[b] *Department of Pediatrics, Albert Einstein College of Medicine, New York, NY, USA*
[c] *Department of Pediatrics, University of California, San Diego, CA, USA*

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Abstract

cDNA arrays compared gene expression in kidneys of neonatal mice subjected to 1, 2, and 4 weeks of chronic constant (CCH) or intermittent (CIH) hypoxia with normoxic littermates. Five to twenty percent of genes were regulated in each condition, with greater changes in CCH. Up-regulation of 42% of the solute carriers after 1 week of CCH suggests a strong activation of pH controlling pathways. Significant reduction in expression change of genes important in growth, development, and aging as a function of time indicates reduced maturation rate in CIH and CCH treatments. Regulated genes showed gender dependence in CCH, being higher in females than males at 1 week and higher in males than females thereafter. Transcriptional control was enhanced in CCH but not in CIH. Thus, CCH and CIH both alter gene expression and retard maturation with the more profound changes occurring in CCH than in CIH.

Keywords: Development; Maturation; Renal; Gene expression; Growth factor; Apoptosis; Solute carrier; Gender difference; Heat shock protein

Chronic constant hypoxia (as occurs in pulmonary diseases [1] or at high altitude [2]) and chronic intermittent hypoxia (as occurs in sleep apnea [3]) can lead to major changes in organs such as heart and kidney [4]. Several previous transcriptomic studies (e.g., [5]) have detected prominent gene expression changes in kidneys of animals exposed to oxygen deprivation [6–9]. These expression changes could play a role in the phenotypic alterations similar to those observed in other tissues [10,11]. For instance, a number of initiation and elongation factors were up-regulated in chronic hypoxia of cardiac tissue [11], possibly explaining the basis for increased protein synthesis and hypertrophy in heart. However, in spite of these particular studies, not much is known about the alterations of kidney transcriptome under hypoxic stress. Thus, we do not know, for example, whether hypoxia alters the maturational profile of age-dependent genes in early life. Furthermore, it is not known if hypoxia has different effects on the two genders, or whether the hypoxia paradigm affects differently the changes in gene expression.

We performed a comprehensive study using cDNA microarrays and kidneys of neonatal mice subjected to 1, 2 or 4 weeks of normal atmospheric conditions (normoxia) or to chronic constant (CCH) or intermittent (CIH) hypoxia to identify the significant changes in gene expression. In this work, we chose to focus on the kidney because this organ senses and responds in a major way to chronic hypoxia [9], and its response is important for adaptation of the whole organism (e.g., [12]). Particular attention was given to genes encoding solute carriers because of their role in pH and acid–base regulation as well as to genes encoding proteins involved in growth, development, aging, and apoptosis in order to delineate genomic explanations of physiological and morphological alterations observed in kidneys of hypoxic mice [13,14]

* Corresponding author. Fax: +1 718 430 8594.
E-mail address: diacobas@aecom.yu.edu (D.A. Iacobas).

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and to heat shock proteins because of their potential protective role. Alterations of the expression profile of age-dependent genes over time were also studied for all treatments to test whether hypoxia affects maturation, as might be expected by the smaller body size reported in hypoxic mice [11].

Materials and methods

Hypoxia treatment. Several litters of neonatal CD1 mice (Charles River; MA; USA) were split in nine groups and subjected for 1, 2 and 4 weeks to normal atmospheric conditions, hypoxia in which [O_2] was kept constant at 11% (CCH) and hypoxia in which [O_2] was switched between 21% and 11% every 4 min (CIH). In total, 36 mice were sacrificed according to protocols approved by the AECOM Animal Use Committee and kidneys were removed for total RNA extraction (Trizol, Invitrogen, CA).

Microarrays. Eighteen NIA15K mouse cDNA microarray slides (probing redundantly 6874 genes) were hybridized as described in [11] with Cy5/Cy3 fluorescently labeled cDNA obtained from kidneys of 36 mice, with four mice (two males and two females) profiled individually for each condition (treatment-duration). We have used the "multiple yellow strategy" [11,15] in which each slide was hybridized with Cy5-cDNA from one male mouse and Cy3-cDNA from one female mouse, both animals within the same treatment group. Normalized data as described in [11] were organized into redundancy groups (each group composed by all spots probing the same gene [16]) and the adjusted relative transcript abundances computed as described in [17]. A set of 1,775 distinct genes was selected for further processing in CCH with a subset of 843 genes analyzed also in CIH. Supplementary Fig. 1 presents the hybridization design (A), an example of a hybridized array (B) and the flowcharts of the data processing (C–E).

Data processing. Detection of *significantly regulated genes* when comparing kidney transcriptomes of mice subjected to different treatments for the same duration or to the same treatment for different durations relied on both absolute fold change in expression ratio (>2-fold) and on statistical significance ($p < 0.01$) of the *t*-test for equality of two ratios [18] with a Bonferroni type adjustment [16,19,20]. GenMapp and MappFinder Software (www.genmap.org, Gladstone Institute) were used to determine whether altered gene expressions are significantly different from chance for the overlapping functional and structural pathways, and Cluster and TreeView Software (http://rana.lbl.gov/EisenSoftware.htm) to produce the expression heatmaps.

Denoting by "+" and "−" the ($p < 0.01$) significant increase and decrease of the expression level from one time point to the next one in the same treatment, and by "0" the absence of significant change, we categorized genes into eight *active maturational profiles*: "0/−", "0/+", "−/0", "−/−", "−/+", "+/0", "+/−", and "+/+", and one neutral maturational profile "0/0", and identified those whose maturational profile within the first 4 weeks of life was significantly changed by hypoxia treatment.

The *relative estimated variability* (REV) of the transcript abundance and the *gene expression stability* (GES) [16,21,22] were determined for each gene in all conditions and the very stably (GES > 95) and the very unstably (GES < 5) expressed genes were identified. GES analysis introduces a hierarchy of expression stability with GES = 100 indicating the most stably expressed gene among the animals subjected to the same condition and GES = 0 indicating the most unstably expressed gene.

This study was performed according to the standards of the Microarray Gene Expression Data Society (MGED) and data complying with the "Minimum Information About Microarray Experiments" (MIAME) have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo; platform GPL2828, series GSE3289).

Results

Gene expression alterations in chronic and intermittent hypoxia

We first compared gene expression levels in hypoxic mice at different time-points with those of their normoxic siblings of the same age. Both hypoxic treatments regulated the expression levels of large numbers of genes (percentages in Fig. 1A and expression heatmaps in Supplementary Fig. 2A) encoding a wide functional variety of proteins, with robustly higher percentages for CCH than for CIH. For instance, at 1 week CCH, 19.9% out of 1775 quantified genes were up-regulated and 5.2% down-regulated. GO analysis using GenMapp software indicated that up-regulated genes are involved in regulating translation and transcription from the RNA polymerase II promoter, carbonate dehydratase activity (carbonic anhydrase II, alcohol dehydrogenase II) and anterior/posterior pattern formation. Down-regulated genes are involved in semaphorin activity, MAP kinase phosphatase activity, caspase activation, negative regulation of transcription from RNA polymerase II, and neurogenesis. By comparison 1 week CIH up-regulated only 2.8% of the quantified 843 genes and down-regulated 3.3%. Supplementary Table 1 presents the results of GO analysis for both hypoxic treatments at all three time-points. The regulated genes are located in all chromosomes (Supplementary Fig. 2B), with the highest numbers on chromosome 11 in CCH and chromosome 16 in CIH, while chromosome 19 harbored the smallest number of regulated genes at all three time-points in both hypoxic treatments.

As illustrated in Fig. 1B, 148 genes were found up- or down-regulated by CCH at all three time-points (UUU or DDD) with a remarkable 100% uniformity of the type of regulation. Table 1 presents the 36 most up- and down-regulated genes at all three time-points in CCH. Of the 176 genes that were regulated at two time-points, 98.3% were regulated in the same direction (e.g., XUU = not regulated (X) at 1 week and up-regulated (U) at 2 and 4 weeks) and 424 genes were regulated at only one time point. Furthermore, all genes which were regulated at two or three time-points by CIH also maintained the same orientation of fold change. Altogether, these findings indicate that both hypoxic treatments regulate expression of genes located on all chromosomes whose encoded proteins are involved in a wide variety of functional pathways.

Interestingly, 3.7%, 7.2%, and 2.9% of the significantly regulated genes at 1, 2, and 4 weeks CCH displayed expression changes that were more than 1.5-fold-change higher in one gender than in the other (Fig. 1C). It is interesting to observe that female mice had a higher fold change in the first week of exposure, in contrast to the latter two time-points (2 and 4 weeks) in which the opposite occurred. Data are not shown for CIH due to the smaller percentage of regulated genes. Supplementary Table 2 presents the regulated genes with significant fold-change difference between the two genders.

Transcriptome maturation

We compared the expression levels at different time-points of the same treatment to determine whether there are age-dependent gene expression patterns and to evaluate the impact of hypoxic stress on the extent and profile (e.g., "+/+", see Materials and methods) of these changes. We assumed then that significant fold changes in these genes as a function of time are responsible for kidney maturation.

We found that on average, 7.2% of the genes in the normoxic kidney, 5.9% in CIH, and 2.3% in CCH kidney significantly ($p < 0.01$) changed expression level by more than 2-fold between successive time-points (Fig. 2A). When the cut-off of fold change was considered 1.5 and that of p-value 0.05, 22.3% of the genes changed expression level in normoxic kidney, 19.4% in CIH, and 9.7% in CCH. Moreover, changes in expression levels in CCH (C) were considerably smaller than those in normoxia (N) as revealed by the slope of the linear regression of log-ratios of the expression levels for two successive time-points in hypoxic mice plotted against the log-ratios of the expression levels in normoxic mice: 1/5.8 for [$\log_2$(C2/C1) vs $\log_2$(N2/N1) (Supplementary Fig. 3A) and 1/5.3 for [$\log_2$(C4/C2) vs $\log_2$(N4/N2)]. Although less consistent than for CCH, the reduction of the fold change was still significant for CIH (1/slope > 3, not shown). Both the significantly reduced percentage of regulated genes and the significant reduction in gene expression change in both CIH and CCH indicate that maturation of the hypoxic kidney is delayed.

Remarkably, the overall fold change of age-dependent genes in male kidneys compared to female kidneys is slightly but significantly higher ($p < 0.01$) in all conditions as seen in Fig. 2B, indicating a faster maturation of the male kidney as compared to the female kidney.

Analysis of the distribution of genes in maturational profiles (Supplementary Fig. 3B) revealed that the overall percentages of genes with significant change between successive time-points were considerably smaller for both CCH and CIH than for normoxic mice (column "ALL"). Interestingly, there are more regulated genes in the profile "−/−" for CCH than for normoxic kidneys, indicating that, in addition to the intensity of the oxygen deprivation, the duration of the hypoxic stress converts some constantly expressed genes in early life into genes with continuous decrease of expression. Fig. 2C presents examples of growth and development genes whose maturation (significant fold change between successive time-points) was practically stopped by CCH. Dazl ("−/−"), Il7 ("0,+"), Ext1 ("+/−"), and Bmp1 ("0,+") in normoxia became "0/0" in CCH. Supplementary Fig. 3C presents examples of

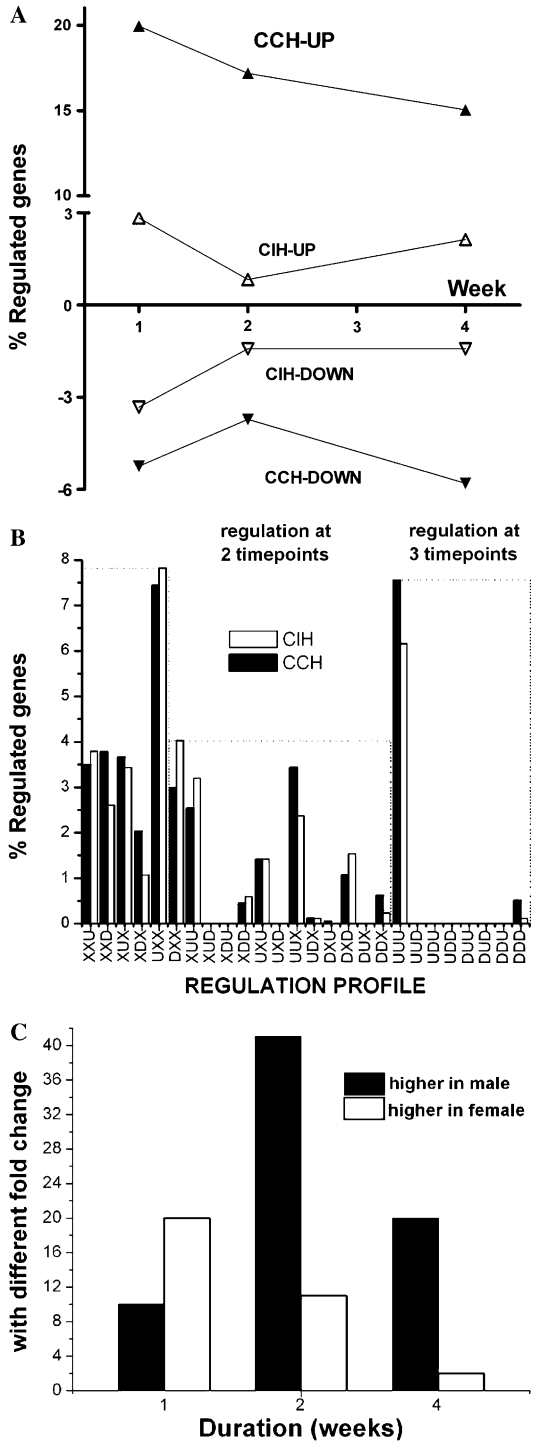

Fig. 1. Gene expression regulation induced by chronic constant (CCH) or intermittent (CIH) hypoxia as compared to normoxia. (A) Percentages of up- and down-regulated out of quantified genes in hypoxic treatment. Note the significantly higher effect of CCH as compared to CIH for both up- and down-regulations at all three time-points. (B) Distribution of regulation profiles in hypoxia. The three letters of the tick labels on horizontal axis indicate the type of regulation (U, up; D, down; X, not significant regulation) at the three time-points, with the first letter indicating the regulation type at 1 week (e.g., XUD, not regulated at 1 week, up-regulated at 2 weeks, and down-regulated at 4 weeks). Note the similar regulation profile (UUU or DDD) for the genes regulated at all three time-points and the small percentages of opposite regulations in two time-points (e.g., UAD). (C) Gender differences in fold regulation for significantly regulated genes in CCH. The height of the columns represents the number of significantly regulated genes whose fold change was over 2-fold larger in one gender than in the other. Note that more genes had higher fold change in females for 1 week CCH than in males while for the other two time-points, significantly larger numbers of genes had higher fold change in males than in females.

Table 1
The 36 most up-regulated and down-regulated genes in kidneys of mice subjected to chronic constant hypoxia

Name	Symbol	CHR	X1	P1	X2	P2	X4	P4
Amine oxidase (flavin containing) domain 2	Aof2	4	7.87	0.008	8.57	0.000	7.18	0.000
Aminopeptidase puromycin sensitive	Npepps	11	5.40	0.001	6.09	0.002	8.48	0.001
Anaphase promoting complex subunit 1	Anapc1	2	12.68	0.000	5.09	0.000	6.97	0.002
Angiopoietin 2	Angpt2	8	12.42	0.001	6.49	0.002	12.57	0.001
B-cell CLL/lymphoma 9	Bcl9	3	6.16	0.000	7.55	0.001	9.70	0.004
B-cell translocation gene 4	Btg4	9	6.22	0.003	7.26	0.000	8.89	0.004
Brain glycogen phosphorylase	Pygb	2	3.57	0.004	11.27	0.001	8.67	0.000
Branched chain aminotransferase 1, cytosolic	Bcat1	6	6.88	0.000	11.30	0.000	5.58	0.000
Breast cancer metastasis-suppressor 1	Brms1	19	8.32	0.001	6.33	0.000	9.04	0.004
Cadherin EGF LAG seven-pass G-type receptor 2	Celsr2	3	14.03	0.000	11.12	0.000	20.12	0.000
Calmodulin 2	Calm2	17	11.73	0.000	8.33	0.000	14.91	0.001
Caspase 12	Casp12	9	13.82	0.002	9.34	0.003	15.41	0.001
Cell division cycle and apoptosis regulator 1	Ccar1	10	18.47	0.007	9.28	0.000	13.21	0.001
C-mir, cellular modulator of immune recognition	Mir	6	8.74	0.001	7.30	0.001	7.45	0.001
Complement component 1, q subcomponent, β polypeptide	C1qb	4	5.27	0.007	4.94	0.000	14.00	0.000
Eukaryotic translation elongation factor 2	Eef2	10	7.01	0.000	5.46	0.000	12.02	0.000
Fibronectin type III domain containing 3	Fndc3	14	14.98	0.001	9.16	0.001	13.24	0.002
Growth differentiation factor 9	Gdf9	11	19.80	0.001	15.48	0.001	22.42	0.001
Neuraminidase 1	Neu1	17	18.54	0.000	11.48	0.001	23.27	0.003
NFKB inhibitor interacting Ras-like protein 1	Nkiras1	14	6.63	0.000	5.19	0.001	7.58	0.000
Nudix (nucleoside diphosphate linked moiety X)-type motif 7	Nudt7	8	9.04	0.000	7.23	0.000	10.47	0.001
Peptidylprolyl isomerase C	Ppic	18	13.37	0.001	4.19	0.000	4.82	0.002
Phosphofructokinase, liver, B-type	Pfkl	10	12.19	0.000	10.48	0.002	15.35	0.000
Pleckstrin and Sec7 domain containing 4	Psd4	2	8.44	0.001	8.83	0.000	5.88	0.000
PR domain containing 4	Prdm4	10	3.32	0.001	8.05	0.003	11.53	0.003
Prion protein	Prnp	2	7.17	0.000	5.92	0.003	13.77	0.006
Proteasome (prosome, macropain) subunit, β type 6	Psmb6	11	7.21	0.000	9.58	0.002	14.66	0.003
Pumilio2 (*Drosophila*)	Pum2	12	13.43	0.001	16.19	0.003	10.13	0.000
Rho guanine nucleotide exchange factor (GEF) 1	Arhgef1	7	8.24	0.000	6.34	0.001	7.42	0.000
SLIT-ROBO Rho GTPase activating protein 2	Srgap2	1	9.51	0.000	5.98	0.004	6.83	0.000
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Sdha	13	5.44	0.000	9.89	0.003	6.89	0.006
TBC1 domain family, member 17	Tbc1d17	7	6.72	0.000	11.14	0.000	9.44	0.002
Trinucleotide repeat containing 9	Tnrc9	8	6.21	0.009	5.94	0.000	8.56	0.001
Tripartite motif protein 28	Trim28	7	16.40	0.000	8.53	0.000	17.24	0.000
Tumor protein, translationally controlled 1	Tpt1	6	9.60	0.000	12.83	0.000	10.24	0.000
Vanin 1	Vnn1	10	16.91	0.001	18.48	0.000	21.39	0.000
A disintegrin and metalloprotease domain 23	Adam23	1	−3.64	0.001	−2.63	0.000	−2.74	0.000
Arachidonate lipoxygenase, epidermal	Alox12e	11	−1.95	0.004	−2.10	0.000	−2.08	0.003
Atpase, class VI, type 11C	Atp11c	X	−5.04	0.005	−3.38	0.020	−2.62	0.001
ATP-binding cassette, sub-family F (GCN20), member 1	Abcf1	17	−3.03	0.000	−2.13	0.008	−1.80	0.008
Bardet-Biedl syndrome 4 homolog (human)	Bbs4	9	−2.71	0.002	−1.78	0.026	−2.48	0.004
CDK5 regulatory subunit associated protein 1-like 1	CdkaM	13	−2.27	0.000	−3.54	0.000	−2.21	0.012
Core promoter element binding protein	Copeb	13	−2.42	0.000	−1.90	0.070	−2.34	0.000
Damage specific DNA binding protein 1	Ddb1	19	−3.98	0.001	−3.36	0.020	−4.44	0.003
G patch domain and KOW motifs	Gpkow	X	−3.73	0.000	−2.67	0.000	−3.40	0.005
Golgi autoantigen, golgin subfamily a, 3	Golga3	5	−4.36	0.001	−2.40	0.007	−3.28	0.022
Guanine nucleotide binding protein, α inhibiting 1	Gnai1	5	−2.36	0.006	−2.26	0.002	−1.82	0.003
Guanosine diphosphate (GDP) dissociation inhibitor 3	Gdi3	13	−3.27	0.002	−2.64	0.004	−3.16	0.047
Heat shock 70 kDa protein 5 (glucose-regulated protein)	Hspa5	2	−2.39	0.009	−2.45	0.008	−2.26	0.000
Insulin-like growth factor 2	Igf2	7	−3.32	0.002	−2.36	0.001	−1.41	0.027
Jumping translocation breakpoint	Jtb	3	−2.81	0.000	−3.42	0.001	−1.93	0.031
Kinesin family member 7	Kif7	7	−3.06	0.010	−2.32	0.001	−2.32	0.001
Mitogen activated protein kinase kinase kinase 3	Map3k3	11	−2.30	0.008	−2.31	0.006	−3.65	0.000
Myo-inositol 1-phosphate synthase A1	Isynai	8	−2.44	0.000	−1.84	0.016	−2.10	0.001
Ninein	Nin	12	−5.76	0.001	−1.85	0.365	−3.01	0.002
NK1 transcription factor related, locus 1 (*Drosophila*)	Nkx1-1	5	−2.29	0.000	−1.86	0.005	−2.54	0.000
Phosphoglucomutase 2	Pgm2	4	−2.81	0.000	−2.03	0.005	−2.72	0.104
Protein phosphatase 1, catalytic subunit, β isoform	Pppicb	5	−2.50	0.001	−4.67	0.001	−3.57	0.001
Regulating synaptic membrane exocytosis 2	Rims2	15	−2.16	0.025	−2.23	0.006	−3.27	0.001
Reticulocalbin 2	Rcn2	9	−2.58	0.003	−2.00	0.003	−1.68	0.006
Ribosomal protein L5	Rpl5	5	−2.94	0.006	−2.51	0.001	−3.01	0.001
Secreted phosphoprotein 2	Spp2	1	−2.34	0.001	−2.38	0.011	−2.00	0.009
Smoothelin	Smtn	11	−2.51	0.011	−2.47	0.000	−2.86	0.000

Table 1 (*continued*)

Name	Symbol	CHR	X1	P1	X2	P2	X4	P4
Sortilin-related VPS10 domain containing receptor 2	Sorcs2	5	−3.72	0.000	−2.75	0.018	−3.05	0.000
Sorting nexin 13	Snx13	12	−4.66	0.006	−5.67	0.057	−4.61	0.004
Tachykinin receptor 1	Tacr1	6	−2.99	0.000	−1.64	0.009	−2.44	0.001
TEA domain family member 1	Tead1	7	−2.46	0.000	−1.99	0.042	−2.54	0.002
Topoisomerase (DNA) II α	Top2a	11	−3.09	0.008	−3.10	0.015	−4.34	0.003
Tumor necrosis factor (ligand) superfamily, member 13b	Tnfsf13b	8	−2.36	0.018	−2.88	0.000	−2.59	0.006
Ubiquitin specific protease 16	Usp16	16	−3.25	0.002	−2.05	0.000	−2.46	0.009
Ubiquitin specific protease 28	Usp28	9	−3.03	0.001	−1.87	0.000	−2.43	0.000
Wolf-Hirschhorn syndrome candidate 2 (human)	Whsc2	5	−3.43	0.009	−2.25	0.007	−2.16	0.000

CHR, chromosomal location; X1, X2, X4, fold change (negative for down-regulation) after 1, 2 or 4 weeks chronic constant hypoxia as respect to 1, 2 or 4 weeks normoxia; P1, P2, P4, *p*-value of the indicated comparison. Note: GenBank Accession numbers were not provided since each result is an average of results within the set of spotted clones probing the same gene.

genes whose maturational profile was significantly changed by CCH; for instance Ribosomal protein S3 was turned from an "+/−" into a "−/0" gene.

Variability and control of transcript abundance

The heatmap of 15 genes expressed in all 36 mice is presented in Fig. 3A with each colored square representing the normalized expression level of the gene indicated on the right side of the panel. Note the significant up-regulation in CCH for all three exposure durations (red colors in the columns 1C1:4C4). Note also that there was less significant regulation in CIH mice (columns 1I1:1I4).

Biological variability was very high for some genes (non-uniform color for the expression levels in the four animals subjected to the same condition) and very low for others (uniform color). To examine this issue more quantitatively, we investigated the expression variability within groups of genes encoding functionally similar proteins such as solute carriers (illustrated in Fig. 3B). Thus, we found in each condition genes whose expression level was similar in all four mice (e.g., Slc16a1, GO-F: monocarboxylate porter activity and Slc31a1, GO-F copper ion transporter activity) suggesting a tight control of transcript abundance, while expression of others (e.g., Slc2a8, GO-F: fructose uniporter activity and Slc38a1, GO-F: L-glutamine transporter activity) was widely variable.

The average expression variability was significantly ($p < 0.01$) lower for CCH than for normoxic mice at all time-points, while, for CIH mice, the average REV was not significantly changed (Fig. 3C). This is consistent with data that we have obtained in organs of genetically manipulated mice [16,17]. These findings suggest that CCH increased the average transcription control in early life (reducing the expression variability) while CIH had no effect on transcription control. The most stably and unstably expressed genes during the three treatments are listed in Supplementary Table 3. The stability or instability of some genes (e.g., NK1 transcription factor related, locus 1 and inositol polyphosphate-5-phosphatase E) did not vary significantly during maturation (close values for all three durations in normoxia), while the stability of others significantly increased (e.g., cell adhesion molecule-related/down-regulated by oncogenes) or decreased (isocitrate dehydrogenase 2 (NADP+), mitochondrial).

We found that 55 very stably (GES > 95) or very unstably (GES < 5) expressed genes in normoxia preserved their stability/instability of expression under constant hypoxic stress, while 13 very stably or very unstably expressed genes in normoxia switched their stability as presented in Supplementary Table 4. For example, Cyclin D2 (Ccnd2), an essential mediator of BCR-induced proliferation of mature B lymphocytes [17aa], lost transcription control after 4 weeks CCH while glutaminase and inositol polyphosphate 5-phosphatase E switched from being very unstably expressed to very stably expressed.

Discussion

Methodological aspects

In our "multiple yellow" strategy the expression ratios were obtained by averaging the ratios determined separately for the red-labeled male extracts and green-labeled female extracts. Hence they were not affected by the non-uniform bias toward one tag [23]. This strategy allowed us to compare expression regulations for different treatments with the same duration or for different durations of the same treatment, to identify differential regulations between the two genders as well as to analyze the expression variabilities of different genes among animals subjected to the same condition. Our results showing gender differences in the fold change demonstrate the importance of keeping the same gender composition for all sets of mice that are being compared.

Analysis of expression level

Both CCH and CIH induced major alterations in kidney transcriptome as indicated by the percentages of regulated genes (Fig. 1A), with significantly more consistent effects caused by CCH than CIH. The considerably smaller percentages of significantly regulated genes in the CIH treatment may be partially explained by the higher expression variability observed in CIH than in CCH mice (illustrated in Fig. 3A and quantified in Fig. 3C, presumably due to the

differential adaptation of individual mice to the periodic oscillations of the oxygen concentration).

It is interesting to note that practically all genes significantly regulated at two or all three time-points maintained the orientation of the expression change (Fig. 1B). For example, among the up-regulated genes by constant hypoxia at all three time-points were growth differentiation factor 9 (Gdf9), a member of the transforming growth factor-β superfamily [7] (19.8×, 15.5×, 22.4×), and Cd44 antigen (1.5×, 1.8×, 3.2×), a glycoprotein predominantly expressed by damaged tubular cells and inflammatory cells involved in inflammation and cell–cell/cell–matrix interactions [24]. We found also an up-regulation of the sodium/hydrogen exchanger member 1 (Slc9a1), a major regulator of intracellular pH that is activated by low pH and exchanges extracellular Na^+ for intracellular H^+ to maintain cellular homeostasis [10,25]. Whether its up-regulation is related to an initial drop in intracellular pH (pHi) by hypoxia that compensated for by an up-regulation of Slc9a1 is not known at present. The high up-regulation of the prion protein, Prnp (7.2×, 5.9×, 13.8×), is also very interesting as this is a soluble protein anchored to the cell surface by glycosylphosphatidylinositol (GPI) and is involved in the phagocytosis of apoptotic cells [26]. Mutations or altered expression of Prnp is associated with several diseases including Gerstmann–Straussler disease [27], Creutzfeldt–Jakob disease [28], familial fatal insomnia [29], and even Alzheimer's disease [30].

A striking uniform down-regulation at all three time-points $(-2.4 \pm 0.1)\times$ was detected for the heat shock 70 kDa protein 5 (Hspa5), a glucose-regulated protein involved in the response to the overload of the endoplasmic reticulum. Although heat shock proteins have been generally found to be up-regulated in most other hypoxic and ischemic tissues, these up-regulations were not uniform in all hypoxic animal models [31]. Furthermore, it is clear that even if there was an up-regulation of Hspa5, it is possible that this would not have been sufficient for tissue protection [32]. It is interesting to speculate that the up-regulation of the heat shock protein 1 (chaperonin 10), (Hspe1) in the chronic constant hypoxic kidney which diminished in time (2.5×; 2.4×; 1.3×) might be an indication of adaptation of the organism to the hypoxia stress. Another important finding regarding heat shock proteins relates to our observation that heat shock proteins are over-expressed in the immature as compared to mature kidney [33]. Thus, Hspa5 is 2.25× more expressed at 1 week and 1.68× more expressed at 2 weeks than at 4 weeks normoxia. The same effect was observed also in CCH where Hspa5 is 2.13× more expressed at 1 week and 1.55× at 2 weeks than at 4 weeks. The decrease of over-expression during maturation occurs also in the case of the transforming growth factor, β recep-

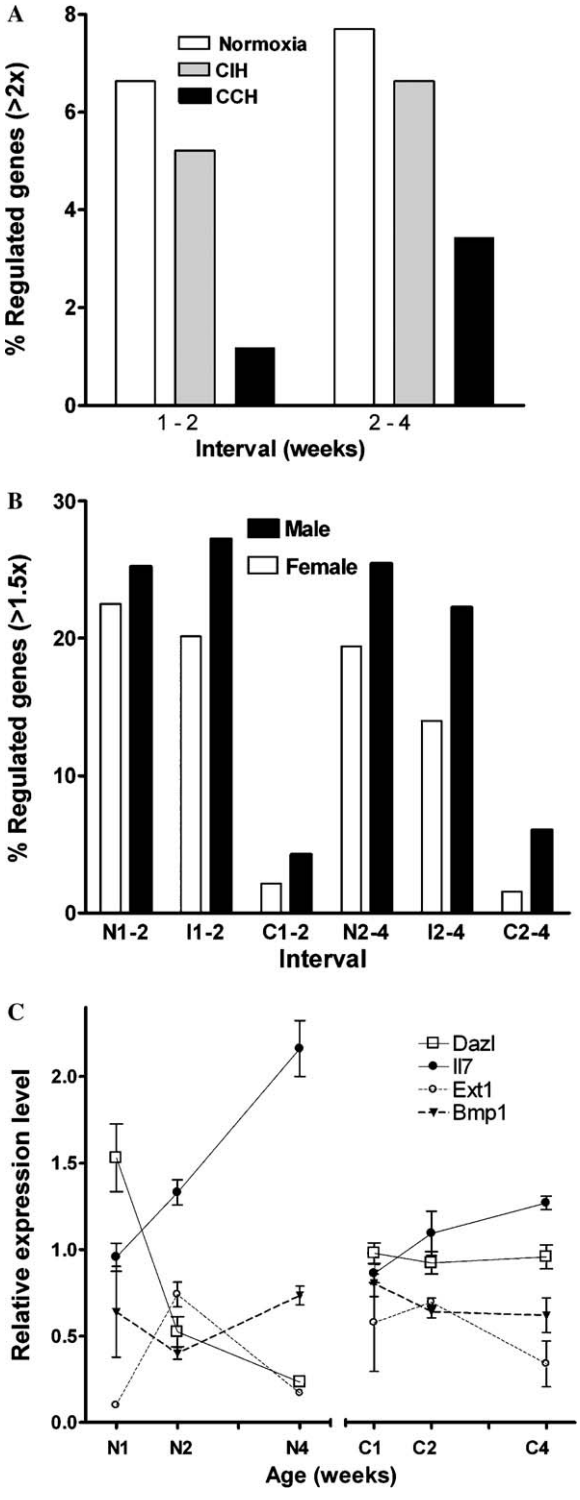

Fig. 2. Analysis of the maturation of the kidney transcriptome. (A) Percentages of significantly ($p < 0.01$) regulated genes (more than 2-fold absolute fold change) when comparing expression levels at successive time-points for each treatment. Note the reduction of the percentage of regulated genes in the hypoxic treatments as compared to normoxia and that CCH is much more effective in reducing the number of regulated genes. (B) Percentages of over 1.5× fold absolute fold change genes for the two genders in each time interval (1w–2w and 2w–4w) for mice exposed to normoxia (N), CCH (C), and CIH (I). Note the robust higher percentage for males than for females in all treatments and both intervals. (C) Examples of growth and development genes whose maturation (significant fold change between successive time-points) was practically stopped by CCH. Bmp1, Bone morphogenetic protein 1; Dazl, Deleted in azoospermia-like; Ext1, Exostoses (multiple) 1, Il7, Interleukin 7.

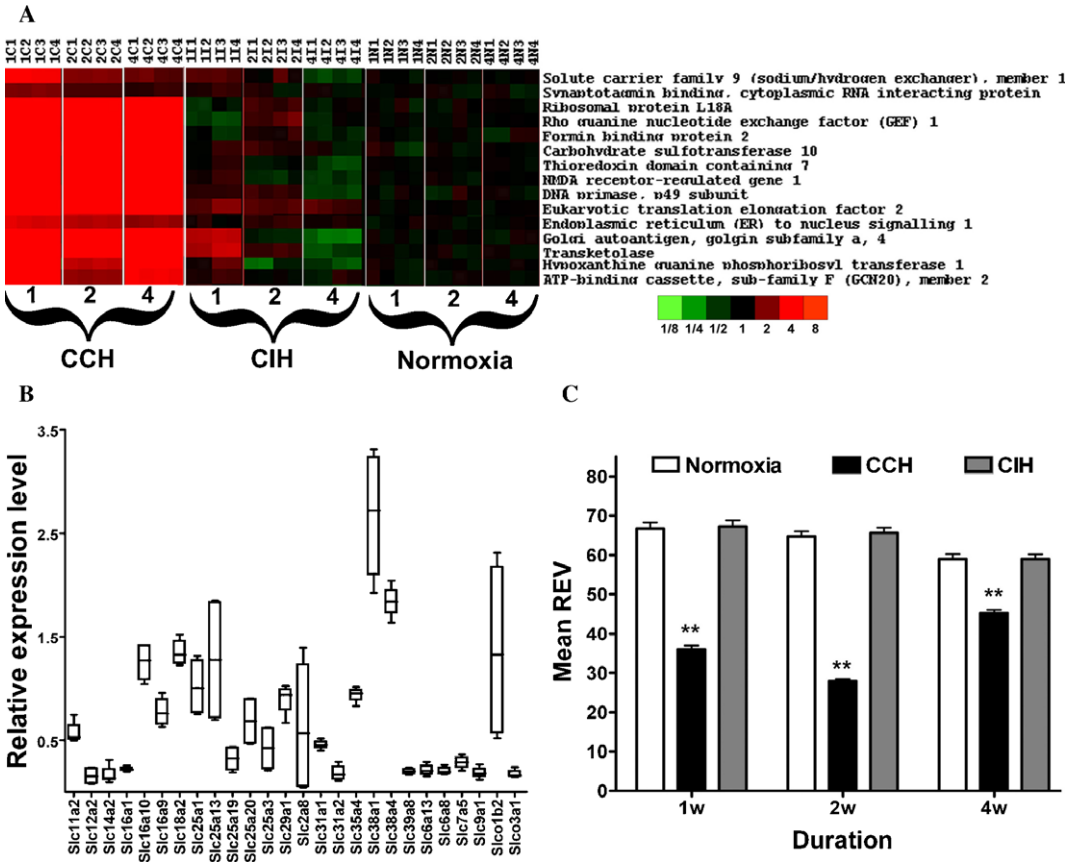

Fig. 3. Analysis of the relative expression variability (REV). (A) Expression heatmap of a small group of genes. Note the uniform up-regulation in CCH and the higher expression variability of CIH and normoxic kidneys. (B) Relative expression levels of genes encoding solute carriers in 1 week normoxia. Values on the vertical axis represent the ratio between the normalized background subtracted fluorescence signal of the probed gene transcript within the redundancy group for both slides and both channels, and the average signal of all 1775 probed gene transcripts. Note the non-uniform dispersion of the expression levels among the genes. Genes such as Slc16a1, Slc39a8 had close expression levels in all four mice while the expression levels of genes such as Slc25a13 and Slc2a8 are widely dispersed with the set of animals. (C) Average values of the relative expression variability (REV) in normoxia, chronic constant (CCH) and intermittent (CIH) hypoxia at all three time-points. Note the significant ($^{**}p < 0.01$) reduction of the average REV in CCH and the non-change in CIH for all durations and that the reduction in CCH is significantly less consistent for the 4 weeks exposure.

tor associated protein 1 (2.3×, 1.7×, 1.1×), a member of a multifunctional protein family involved in cellular proliferation and differentiation [34]. This higher level of expression of such factors as hsp in the young (as compared to the mature animal) and the down-regulation of such proteins with maturation has been shown to play a role in the higher protection of the newly born to hypoxia in the kidney [35].

Eleven of the 26 solute carrier genes investigated were significantly up-regulated and only two down-regulated, indicating a strong activation of pH regulating pathways, presumably thereby limiting the effects of hypoxia on acid–base balance. Considering the average expression ratio at all three time-points, five out of the 17 investigated apoptotic genes were significantly up-regulated and none down-regulated, suggesting an overall accentuation of programmed cell death. We also found significant regulation of several genes involved in kidney development [36] such as bone morphogenetic protein 2 (4-fold up-regulation by 1 w CCH), transforming growth factor-β 2 (3-fold down-regulation by 4 w CCH), and empty spiracles homolog 2 (2-fold down-regulation by 1 w CCH).

Maturation analysis

We have identified a number of age-dependent genes in all three treatments. The set of genes whose expression level changed significantly during maturation in normal atmospheric conditions (therefore presenting the normal tendency, not affected by the hypoxic stress) include regulators of growth (Bmp1, Csf1, Ddx3x, Eps15, Fgf10, Gas6, Gdf3, Gdf9, Grb7, Hdgf, Igf2, Il7, Ogfr, Pdgfrb, Socs3, and Tgfb2), development (Abr, Angpt2, Cops3, Cfdp1, Dab2, Dazl, Dkk1, Dppa3, Emx2, Ext1, Fxr1h, Gata4, Hoxb3, Idb1, Idb2, Odag, Onecut1, Ovol1, Pkp2, Psen1, Tpm4, and Utrn), insulin//IGF (insulin-like growth factor) signaling pathways indicating a possible connection to metabolism [37] (Crim1, Igf2, Igf2bp3, and Irs1), and aging (Mif, Morf4l2). Our finding that hypoxic stress significantly diminishes the average amplitude of the expression changes between successive time-points (i.e., within the intervals 1w–2w and 2w–4w) (Fig. 2A, Supplementary Fig. 3A) may be interpreted as a slowing-down of the maturation of the age-dependent genes. This conclusion is sup-

ported by the fact that maturation of 76% of genes involved in growth, development, insulin/IGF signaling cascade, and aging (Bmp1, Csf1, Ddx3x, Eps15, Fgf10, Gas6, Grb7, Hdgf, Il7, Ogfr, Pdgfrb, Socs3, Tgfb2, Abr, Angpt2, Cops3, Dab2, Dazl, Dkk1, Dppa3, Emx2, Ext1, Fxr1h, Gata4, Hoxb3, Idb1, Odag, Onecut1, Ovol1, Tpm4, Utrn, Crim1, Igf2, Igf2bp3, Irs1, and Mif) was stopped by CCH (i.e., the "active" maturational profiles (e.g., "−/−") were turned into the neutral one, "0/0"), (examples in Fig. 2C).

Moreover, the maturational profile itself may be altered by the hypoxic stress as is illustrated in Supplementary Fig. 3B (examples in Supplementary Fig. 3C). The proteins encoded by these genes perform various functions and one may ask about potential mechanisms that may be involved and the consequences of this change of maturational profile. Thus, Dyt1 acts as a chaperone cofactor dependent protein folding in ATP binding, Eif3s2 is a translation initiation factor and Rsp3 a ribosomal protein both involved in protein biosynthesis, and Pbef1 is an apoptotic gene responsible for cell surface receptor linked signal transduction. Other genes whose maturational profile was significantly altered by CCH include: Anp32a, a component of the nuclear matrix with the function of protein binding, Api5, an anti-apoptotic gene, Gpr149, a membrane protein with receptor activity, Aof1, an amine oxidase involved in electron transport, Tnsf13, a tumor necrosis factor located on the external side of plasma with cytokine activity involved in humoral defense, Rest, a negative regulator of transcription, and Ehd3, a calcium ion binding protein. Moreover, genes without age-dependency in normoxia (profile "0/0") such as: mitochondrial ribosomal protein L13, myosin light chain regulatory B, rho guanine nucleotide exchange factor 17, fibronectin leucine-rich transmembrane protein 2, growth arrest-specific 2 like 3, OCIA domain containing 1, polymerase (RNA) II (DNA directed) polypeptide A, and ATPase family, AAA domain containing 1.

Gender analysis

We identified several genes with significant gender dependence (Fig. 1E presents the numbers of differently regulated genes in the two genders). Thus, 1 week of CCH up-regulated Vanin1 (that hydrolyzes pantethein to cysteamine (a powerful endogenous anti-oxidant in renal repair after ischemia–reperfusion injury [39]) and pantothenic acid) by 12.7× in male and by 26.4× in female kidney. Similarly, growth arrest-specific gene 6 (Gas6), an important mediator in experimental nephritis [38], was more up-regulated in female than in male kidney. By contrast, choline dehydrogenase (Chdh), an important oxidoreductase, was more up-regulated after 2 weeks hypoxic exposure in male than female kidneys.

An interesting robust finding in all three treatments and for both time intervals was the significantly ($p < 0.01$) faster maturation of males as compared to females (Fig. 2C). In addition, the significantly more regulated genes in female than in male after 1 week of hypoxia and the significantly more regulated genes in males than in females at later weeks suggest that females responded more briskly than males with the imposition of hypoxia but adapt shortly after in contrast with males.

Expression variability analysis

In previous studies [16,17,21,22], we found that certain genes have an expression variability among four similar samples less than 1% of the average value while for others it exceeded 100%. Low variability of transcript abundance indicates high stringency of transcription control making gene expression less sensitive to local conditions, while high variability is the result of low control. As illustrated for solute carriers after 1 week normoxia (Fig. 3B), observation valid for all groups of genes in all conditions, the control stringency of transcript abundances is not uniform among genes encoding proteins with similar molecular functions. An enhanced control of gene transcription protects the system against alterations of functional pathways that are responsible for cell survival and phenotypic expression, while a reduced control may allow the system to adapt to various conditions. Therefore, the overall decrease of variability in the hypoxic kidney may be considered as an effort to limit the alterations induced by the stress of oxygen deprivation. The expression variability was significantly smaller for CCH mice at all three time-points than for normoxic and CIH mice.

We interpret the switch of gene stability between very stably expressed and very unstably expressed as a strong indication of a profound alteration of transcription control mechanisms. For instance, the significantly increased transcription control of inositol polyphosphate-5-phosphatase E (Inpp5e), the gene whose mutation may produce the oculocerebrorenal syndrome of Lowe, a rare X-linked disorder characterized by major abnormalities of eyes, nervous system, and kidneys [40], may be a preventive measure of the kidney to strictly control the expression level of a gene that may cause major damage. In addition, considering their roles in the Ca^{2+} cell-signaling system [41], the observed very high increase of the control of glutaminase and inositol polyphosphate-5-phosphatase E may hypothetically compensate for the loss of expression control in the case of Cyclin D2. Interestingly, the intermittent hypoxia had no effect on the average REV values for any duration.

Conclusion

In summary, both chronic constant and intermittent hypoxia are major stresses that affect the expression level, stability, and maturation of several genes whose protein products are involved in a large panel of functional pathways. Although females responded more briskly than males with hypoxia, they seem to adapt faster to the stress. We found transcriptomic evidence that hypoxia can slow down the maturation of kidney. The study of transcriptom-

ic alterations under hypoxic conditions in early life may lead us to a better understanding of mechanisms potentially involved in adaptation or survival of the organism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.08.056.

References

[1] E. Zakynthinos, E. Douka, Z. Daniil, K. Konstantinidis, V. Markaki, S. Zakynthinos, Anuria due to acute bilateral renal vein occlusion after thrombolysis for pulmonary embolism, Int. J. Cardiol. 101 (1) (2005) 163–166.
[2] R.L. Naeye, Organ and cellular development in mice growing at simulated high altitude, Lab. Invest. 15 (4) (1966) 700–706.
[3] K. Ehlenz, Regulation of blood volume-implications for cardiovascular pathophysiology in sleep apnoea, J. Sleep Res. 4 (S1) (1995) 30–33.
[4] V.H. Haase, The VHL tumor suppressor in development and disease: functional studies in mice by conditional gene targeting, Semin. Cell Dev. Biol. 16 (4–5) (2005) 564–574.
[5] M. Liang, A.W. Cowley Jr, M.J. Hessner, J. Lazar, D.P. Basile, J.L. Pietrusz, Transcriptome analysis and kidney research: toward systems biology, Kidney Int. 67 (6) (2005) 2114–2122.
[6] D.P. Basile, K. Fredrich, M. Alausa, C.P. Vio, M. Liang, M.R. Rieder, A.S. Greene, A.W. Cowley Jr, Identification of persistently altered gene expression in the kidney after functional recovery from ischemic acute renal failure, Am. J. Physiol. Ren. Physiol. 288 (5) (2005) F953–F963.
[7] M. Kusaka, K. Yamada, Y. Kuroyanagi, A. Terauchi, H. Kowa, H. Kurahashi, K. Hoshinaga, Gene expression profile in rat renal isografts from brain dead donors, Transplant. Proc. 37 (1) (2005) 364–366.
[8] U. Meissner, C. Hanisch, I. Ostreicher, I. Kerr, K.H. Hofbauer, W.F. Blum, I. Allabauer, W. Rascher, J. Dotsch, Differential regulation of leptin synthesis in rats during short-term hypoxia and short-term carbon monoxide inhalation, Endocrinology 146 (1) (2005) 215–220.
[9] A.A. Eddy, Progression in chronic kidney disease, Adv. Chronic Kidney Dis. 12 (4) (2005) 353–365, Review.
[10] D. Zhou, J. Xue, O. Gavrialov, G.G. Haddad, Na+/H+ exchanger 1 deficiency alters gene expression in mouse brain, Physiol. Genomics 18 (3) (2004) 319–331.
[11] C. Fan, D.A. Iacobas, D. Zhou, Q. Chen, J.K. Lai, O. Gavrialov, G.G. Haddad, Gene expression and phenotypic characterization of mouse heart after chronic constant or intermittent hypoxia, Physiol. Genomics 22 (2005) 292–307.
[12] T. Mushiroda, S. Saito, Y. Tanaka, J. Takasaki, N. Kamatani, Y. Beck, H. Tahara, Y. Nakamura, Y. Ohnishi, A model of prediction system for adverse cardiovascular reactions by calcineurin inhibitors among patients with renal transplants using gene-based single-nucleotide polymorphisms, J. Hum. Genet. 50 (9) (2005) 442–447.
[13] J.S. Jung, R.H. Lee, S.H. Koh, Y.K. Kim, Changes in expression of sodium cotransporters and aquaporin-2 during ischemia–reperfusion injury in rabbit kidney, Ren. Fail. 22 (4) (2000) 407–421.
[14] J.M. Thurman, P.A. Royer, D. Ljubanovic, B. Dursun, A.M. Lenderink, C.L. Edelstein, V.M. Holers, Treatment with an inhibitory monoclonal antibody to mouse factor B protects mice from induction of apoptosis and renal ischemia/reperfusion injury, J. Am. Soc. Nephrol. 17 (3) (2006) 707–715.
[15] E. Brand-Schieber, P. Werner, D.A. Iacobas, S. Iacobas, M. Beelitz, S.L. Lowery, D.C. Spray, E. Scemes, Connexin43, the major gap junction protein of astrocytes, is down regulated in an animal model of multiple sclerosis, J. Neurosci. Res. 80 (2005) 798–808.
[16] D.A. Iacobas, S. Iacobas, M. Urban-Maldonado, D.C. Spray, Sensitivity of the brain transcriptome to connexin ablation, Biochim. Biophys. Acta 1711 (2) (2005) 183–196.
[17] D.A. Iacobas, S. Iacobas, W.E. Li, G. Zoidl, R. Dermietzel, D.C. Spray, Genes controlling multiple functional pathways are transcriptionally regulated in connexin43 null mouse heart, Physiol. Genomics 20 (2005) 211–223.
[18] D.A. Iacobas, Medical Biostatistics, third Eng ed., Bucura Mond, Bucharest, 1997, pp. 96–98, 183–195.
[19] S. Draghici, Data Analysis Tools for DNA Microarrays, Chapman and Hall, Boca Raton-London-New York, Washington, DC, 2003, pp. 223–224.
[20] D. Stekel, Microarray Bioinformatics, Cambridge University Press, Cambridge, 2003, pp.133–134.
[21] D.A. Iacobas, S. Iacobas, D.C. Spray, Use of cDNA arrays to explore gene expression in genetically manipulated mice and cell lines, in: S. Dhein, F.W. Mhr, M. Delmar (Eds.), Practical Methods in Cardiovascular Research, Springer-Verlag, Berlin-Heidelberg-New York, 2005, pp. 907–915.
[22] D.A. Iacobas, M. Urban-Maldonado, S. Iacobas, E. Scemes, D.C. Spray, Array analysis of gene expression in connexin-43 null astrocytes, Physiol. Genomics 15 (3) (2003) 177–190.
[23] D.A. Iacobas, M. Urban, A. Massimi, S. Iacobas, D.C. Spray, Hits and misses from gene expression ratio measurements in cDNA microarray studies, J. Biomol. Tech. 13 (3) (2002) 143–157.
[24] K.M. Rouschop, M.E. Sewnath, N. Claessen, J.J. Roelofs, I. Hoedemaeker, R. van der Neut, J. Aten, S.T. Pals, J.J. Weening, S. Florquin, CD44 deficiency increases tubular damage but reduces renal fibrosis in obstructive nephropathy, J. Am. Soc. Nephrol. 15 (3) (2004) 674–686.
[25] J. Xue, R.M. Douglas, D. Zhou, J.Y. Lim, W.F. Boron, G.G. Haddad, Expression of Na+/H+ and HCO_3-dependent transporters in Na+/H+ exchanger isoform 1 null mutant mouse brain, Neuroscience 122 (1) (2003) 37–46.
[26] C.J. de Almeida, L.B. Chiarini, J.B. da Silva, P.M. e Silva, M.A. Martins, R. Linden, The cellular prion protein modulates phagocytosis and inflammatory response, J. Leukoc. Biol. 77 (2) (2005) 238–246.
[27] J.A. Mastrianni, M.T. Curtis, J.C. Oberholtzser, M.M. Da Costa, S. DeArmond, S.B. Prusiner, J.Y. Garben, Prion disease (PrP-A117V) presenting with ataxia instead of dementia, Neurology 45 (1995) 2042–2050.
[28] Y. Nishida, N. Sodeyama, Y. Toru, S. Toru, T. Kitamoto, H. Mizusawa, Creutzfeldt–Jakob disease with a novel insertion and codon 219 Lys/Lys polymorphism in PRNP, Neurology 63 (10) (2004) 978–1979.
[29] P. Gambetti, P. Parchi, Insomnia in prion diseases: sporadic and familial, (Editorial) New Engl. J. Med. 340 (1999) 1675–1677.
[30] R. Cacabelos, Genomic characterization of Alzheimer's disease and genotype-related phenotypic analysis of biological markers in dementia, Pharmacogenomics 5 (8) (2004) 1049–1105.
[31] E.A. Ozer, O. Yilmaz, M. Akhisaroglu, B. Tuna, A.R. Bakiler, E. Ozer, Heat shock protein 70 expression in neonatal rats after hypoxic stress, J. Matern. Fetal Neonatal Med. 12 (2) (2002) 112–117.
[32] M.A. Turman, S.L. Rosenfeld, Heat shock protein 70 over expression protects LLC-PK1 tubular cells from heat shock but not hypoxia, Kidney Int. 55 (1) (1999) 189–197.
[33] A.G. Vicencio, B. Bidmon, J. Ryu, K. Reidy, G. Thulin, A. Mann, K.M. Gaudio, M. Kashgarian, N.J. Siegel, Developmental expression of HSP-72 and ischemic tolerance of the immature kidney, Pediatr. Nephrol. 18 (2) (2003) 85–91.
[34] A.G. Vicencio, C.G. Lee, S.J. Cho, O. Eickelberg, Y. Chuu, G.G. Haddad, J.A. Elias, Conditional overexpression of bioactive trans-

forming growth factor-beta1 in neonatal mouse lung: a new model for bronchopulmonary dysplasia? Am. J. Respir. Cell Mol. Biol. 31 (6) (2004) 650–656.

[35] M. Riordan, R. Sreedharan, S. Wang, G. Thulin, A. Mann, M. Stankewich, S. Van Why, M. Kashgarian, N.J. Siegel, HSP70 binding modulates detachment of Na-K-ATPase following energy deprivation in renal epithelial cells, Am. J. Physiol. Renal Physiol. 288 (6) (2005) F1236–F1242.

[36] K. Schwab, L.T. Patterson, B.J. Aronow, R. Luckas, H.C. Liang, S.S. Potter, A catalogue of gene expression in the developing kidney, Kidney Int. 64 (5) (2003) 588–1604.

[37] H.B. Fraser, P. Khaitovich, J.B. Plotkin, S. Paabo, M.B. Eisen, Aging and gene expression in the primate brain, PLoS Biol. 3 (9) (2005) e274.

[38] A. Fiebeler, J.K. Park, D.N. Muller, C. Lindschau, M. Mengel, S. Merkel, B. Banas, F.C. Luft, H. Haller, Growth arrest specific protein 6/Axl signaling in human inflammatory renal diseases, Am. J. Kidney Dis. 43 (2) (2004) 286–295.

[39] T. Yoshida, M. Kurella, F. Beato, H. Min, J.R. Ingelfinger, R.L. Stears, R.D. Swinford, S.R. Gullans, S.S. Tang, Monitoring changes in gene expression in renal ischemia–reperfusion in the rat, Kidney Int. 61 (5) (2002) 1646–1654.

[40] M. Addis, M. Loi, C. Lepiani, M. Cau, M.A. Melis, OCRL mutation analysis in Italian patients with Lowe syndrome, Hum. Mutat. 23 (5) (2004) 524–525.

[41] C. Erneux, C. Govaerts, D. Communi, X. Pesesse, The diversity and possible functions of the inositol polyphosphate 5-phosphatases, Biochim. Biophys. Acta 1436 (1–2) (1998) 185–199.