
Effects of Chronic Intermittent Hypoxia on Cardiac Rhythm Transcriptomic Networks

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Abstract

This chapter completes a series of four studies analyzing in a mouse model the genomic consequences of chronic obstructive sleep apnea during development from neonatal to puberty. Groups of two male and two female 1-day-old sibling mice each were subjected for 1, 2, or 4 weeks to normal atmospheric conditions or to chronic intermittent hypoxia, and the transcriptomes of their hearts profiled and compared. Our previous papers reported alterations of individual genes, gene ontology categories, translation regulators and responses to stress, and analyzed and quantified the topological changes of the heart rhythm determinant (HRD) genomic fabric, including the ranking of the HRD genes. HRD fabric was defined as the most stably expressed and interconnected gene web that might be responsible for the generation, maintenance, and modulation of the heart rhythm in each condition. Here, we introduce the new analysis of the network landscape to determine the ways by which Ca^{2+} and Wnt signaling pathways, translation initiation, and elongation factors, and SOX (i.e., sex-determining region Y-box) genes control the HRD fabric. We also analyze the changes in the networks by which connexin 43, the main protein that couples the cardiomyocytes by forming intercellular gap junction channels, modulates the HRD fabric during development under normoxic and hypoxic conditions. Remarkably, the amplitude of the transcriptomic alterations diminished from 1 to 4 weeks of hypoxia, indicating activation of certain acclimatization or accommodation mechanisms. In addition to regulation of expression level, our analyses revealed changes in the stability control and interlinking of functional gene networks as well as switch of dominant gene pairs. Thus, we found that *Hif1a-Jup*, *Lmna-Pcdh7*, and *Eef1a2-Gnao1* are the most important pairs at 1, 2, and 4 weeks normoxia, respectively, while *Jup-Slc25a20*, *Cdh16-Vezt*, and *Eif2ak4-Pcdh12* are the controlling pairs at 1, 2, and 4 weeks hypoxia, respectively. The analysis has shown that changes in expression control and coordination had substantial contributions to the overall transcriptomic differences. Moreover, with respect to magnitude, hypoxia transcriptomic effects are comparable to those associated to development. Altogether, these results indicate the profound remodeling of the HRD fabric and regulatory pathways in response to intermittent oxygen deprivation that may explain the cardiac arrhythmias experienced by teenagers suffering by chronic obstructive sleep apnea.

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Abbreviations

CIH	Chronic intermittent hypoxia
HRD	Heart rhythm determinant
NOR	Normoxia (normal atmospheric conditions)
PGA	Prominent Gene Analysis
SIG	Signaling genes
SOX	Sex-determining region Y-box
TRA	Transcription and translation regulators
WNT	Wingless-related MMTV integration site

2.1 Introduction

Snoring is not only unpleasant to those living around you, but it is also bad for your heart. So bad that when chronic it may change the fate of genes responsible for the heart rhythm, generating lifelong arrhythmias. Chronic intermittent hypoxia, such as in obstructive sleep apnea, causes numerous heart problems, including hypertension, coronary artery disease, congestive heart failure, pulmonary hypertension, stroke, and cardiac arrhythmias [1–6]. Lethal cardiac arrhythmias are often associated with altered expression or mutation of key genes [7]. Variants in genes that encode cardiac ion channel α - and β -subunits lead to congenital syndromes such as abnormally long or short QT, Brugada, and catecholaminergic polymorphic ventricular tachycardia [8]. Moreover, inherited mutations in genes related to regulatory pathways of ion channels may also cause cardiac arrhythmias [8–12]. The multitude of arrhythmia forms suggests that normal cardiac rhythm requires appropriate co-expression of numerous and diverse molecular components.

Interestingly, there are substantial differences between effects of chronic intermittent and chronic constant hypoxia (such as in pulmonary disease or living at high altitude) [13–17]. These differences suggest that in addition to oxygen deprivation, the frequency of alternating normal and hypoxic atmospheric conditions may be of importance in developing adaptation mechanisms. Whether caused by constant or intermittent hypoxia, the wide diversity of clinical manifestation indicates high complexity of underlying molecular mechanisms that remain largely unresolved in spite of numerous models that have been proposed (e.g., [18–25]). In the following paragraphs, we focus on the heart rhythm determinant (HRD) gene fabric and signaling networks that modulate it. We have developed methods to select the most relevant genes and quantify the networks responsible for the cardiac function.

In a previous study [13], we have profiled the heart transcriptomes of 36 mice subjected for 1, 2, or 4 weeks of their early life to normal atmospheric conditions or to chronic constant or intermittent hypoxia, with two males and two females profiled in each condition. However, for the unity of

the book, in this chapter, we discuss only the genomic consequences of the chronic intermittent hypoxia. We here introduce new analyses by which to reveal additional possible transcriptomic mechanisms that make chronic intermittent hypoxia an arrhythmogenic condition. They complete the analyses of individual genes and gene ontology (GO) categories that have been regulated [13], alteration of translation regulators and response to stress [16], alteration of HRD gene hierarchy and remodeling of the topology of HRD genomic fabric [17].

2.2 Materials and Methods

Results of this chapter were obtained by analyzing from new perspectives part of the data obtained in a previously described gene expression experiment [13] performed in the laboratory of Dr. Haddad (UCSF). Expression of 4,862 completely annotated unigenes was adequately quantified in all RNA samples (publically available in <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2271>). Of these genes, 54 were assigned to the heart rhythm determinant (HRD) fabric while other 44 genes were included in transcription and translation regulators (TRA) and 35 to signaling (SIG) gene cohorts owing to their influence on the HRD fabric. HRD genes were selected from the quantified genes in this experiment though our prominent gene analysis (PGA) as reported in a previous paper [17]. The selection includes the adrenergic receptor kinase beta 1 (*Adrbk1*), ankyrins (*Ank2*, *Ank3*), ion channels and transporters (*Atp1a1*, *Atp1a2*, *Atp2a2*, *Kcnh2*, *Slc25a20*, *Slc8a1*), regulators of heart rhythm (*Lamp2*, *Lmna*, *Sema3a*, *Ttr*), contraction (*Dmpk*, *Gnao1*), inotropy (*Csrp3*, *Gaa*), and development (*Id2*, *Nfatc3*, *Tbx5*). Direct and indirect epigenetic modulators (*Hand2*, *Hdac5*, *Mef2a*, *Mef2b*, *Mef2c*, *Mef2d*, *Smyd1*) were added because of their relation with various miRNA, short noncoding RNAs involved in posttranscriptional regulation whose altered expression was recently linked to arrhythmias [26–28]. Moreover, since generation of synchronized rhythmic heart contractions requires integration of electrical and mechanical properties, we have also incorporated in this study genes encoding components of the intercalated discs (specialized regions of the plasma membrane that link neighboring cardiac myocytes). Thus, we have profiled several adherens (*Jup*, *Vcl*, *Vezt*), members of the cadherin superfamily (*Cdh13*, *Cdh16*, *Cdh2*, *Cdh22*, *Cdh5*, *Dsc2*, *Dsg2*, *Pcdh12*, *Pcdh7*, *Pcdhgc3*) and cadherin-associated proteins (*Cttna1*, *Cttna11*, *Ctnb1*, *Ctnm1*), plakophilins (*Pkp2*, *Pkp3*, *Pkp4*), and tight (*Cxadr*, *Tjp1*, *Tjp2*) and gap junction proteins (*Gjal*). However, 6 HRD genes (*Gjal*=gap junction membrane channel protein alpha 1; *Itp1l/2*=inositol 1,4,5-triphosphate receptor 1/2; *Ryr1*=ryanodine receptor 1;

Slc8a1; *Ttr*=transthyretin) were included also in SIG cohort. In the TRA cohort, we have considered the effects on HRD fabric of hypoxia inducible factor 1 alpha subunit (*Hif1a*), 5 (eukaryotic) translation elongation (*Eef*), and 38 translation initiation (*Eif*) factors. SIG cohort includes SOX genes and genes involved in Ca^{2+} and Wnt signaling because their reciprocal interactions and interactions with Cx43 and other intercalated disc proteins control cardiomyogenesis [29–35].

2.2.1 Hypoxia Treatment

As described in Fan et al. [13], CD1 mice (Charles River) were placed in a hypoxia chamber (BioSpherix) with their mother starting on the second day after birth (P2) for 1, 2, or 4 weeks. O_2 concentration was switched between 21% and 11% every 4 min, continuously for 24 h/day for the entire period. At the end of each period, mice were anesthetized by inhalation of isoflurane (Baxter Pharmaceutical Products), the hearts removed, and quickly frozen in liquid nitrogen. The surgical procedures and protocols were approved by the Albert Einstein College of Medicine (AECOM) Animal Care and Use Committee.

2.2.2 Microarray Hybridization

Briefly, 60- μg total RNA extracted with TRIzol (Invitrogen) was used to synthesize a fluorescently labeled cDNA probe by direct incorporation of either Cy3 or Cy5 fluorescent dye (Amersham Biosciences). Fluorescent cDNA probes were treated with blocking solution for 1 h before being applied to pretreated and prehybridized microarray slides. Hybridization with 27 k cDNA mouse AECOM microarrays were performed in GeneMachines HybChamber overnight at 50°C . After incubation, each slide was washed to remove unbound cDNAs and SDS, dried, and scanned with a GenePix 4100A scanner (Axon Instruments) at 600 V (635 nm) and 550 V (532 nm). We adopted the “multiple-yellow” hybridization strategy in which each slide was hybridized with Cy5-labeled cDNA obtained from a male heart and Cy3-labeled cDNA from a female heart, both mice being from the same litter and subjected to the same treatment. The multiple-yellow strategy improves intrachip normalization since the mRNA content of the starting total RNA is affected only by the biological variability among animals, matched by gender, age, and condition. It improves also the interchip normalization and the green and red fluorescence signals obtained with the same scanner setting for all slides being compared separately, thus avoiding the inherent nonuniform bias toward one tag. This allowed all possible comparisons among conditions, time points, and sexes.

2.2.3 Scanning, Acquisition, and Normalization

Images were acquired and primarily analyzed with GenePix Pro 4.1 software. A gene was considered as quantifiable if its foreground fluorescence signal was more than twice the background. Any spot that was not quantifiable in all samples was removed from the analysis. The background-subtracted signals were normalized with an in-house-developed iterative algorithm described in [36], alternating intra-array and inter-array normalization until the average-corrected ratio differed by $<5\%$ from the previous one.

2.2.4 Analysis of Expression Regulation

Detection of significantly regulated genes relied on a composite criterion of $>|1.5\times|$ absolute fold change and p value <0.05 with a Bonferroni-type correction for the redundancy groups (set of spots probing the same gene) [37]. GenMAPP and MAPPFinder software (www.genmap.org, Gladstone Institute) were used for the first categorization of the altered genes [13].

2.2.5 Analysis of Expression Variability and Transcript Abundance Control

We have determined the relative expression variability (REV) of each gene as the chi-square statistical estimate of the coefficient of variability (CV) of transcript abundance in the set of biological replicas. This statistical measure was necessary to limit the effect of the nonuniform size of the redundancy group of spots on the gene’s CV. By ordering the genes according to their REVs, we have determined the gene expression stability (GES) as a percentile of transcription control, with $\text{GES}=100$ indicating the most controlled gene and $\text{GES}<1$ indicating the least controlled one [16]. We have also studied whether the strength of the transcription control is age dependent and changes in hypoxia. The transcription control limits the so-called genomic noise (responsible for the expression variability within biological replicas) that we view as a transcriptomic tuning to the variability of local conditions.

2.2.6 Analysis of Expression Coordination and Gene Networking

Transcription variability allows calculation of Pearson correlation coefficient ρ between the expression levels of gene pairs in biological replicas and identification of synergistically (SYN, $\rho>0.90$), antagonistically (ANT, $\rho<-0.90$), and independently

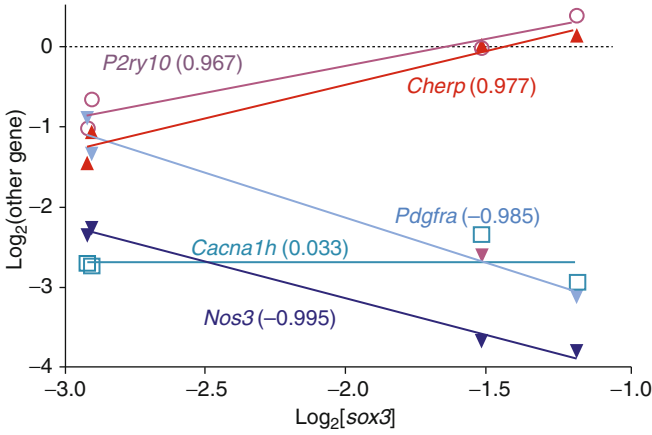


Fig. 2.1 Examples of synergistically (*Cherp*, *P2ry10*), antagonistically (*Nos3*, *Pdgfra*), and independently (*Cacna1h*) expressed partners of *Sox3* in hearts of 1-week-old mice grown under normal atmospheric conditions. Genes: *Cacna1h*=calcium channel, voltage-dependent, T-type, alpha 1 H subunit; *Cherp*=calcium homeostasis endoplasmic reticulum protein, *Nos3*=nitric oxide synthase 3; endothelial cell, *P2ry10*=purinergic receptor P2Y; G-protein coupled 10, *Pdgfra*=platelet-derived growth factor receptor; alpha polypeptide. Note that for synergistically expressed partners, the expression levels go in the same direction among biological replicas; for antagonistically expressed, they go in opposite directions, while no correlation can be established for independently expressed genes

(IND, $|\rho| < 0.05$) expressed partners (examples in Fig. 2.1). Expression of each gene is expected to be tied to that of other genes so that amounts of encoded proteins respect the “stoichiometry” of the biological processes [38]. Expression coordination may occur between genes from the same GO category (examples in [17]) or between genes from different GO categories (as illustrated in Fig. 2.1 where the transcription factor *Sox3* is coordinately expressed with certain Ca^{2+} -signaling genes). This expression coordination between genes involved in different functional categories suggests that the functional pathways can modulate each other.

2.2.7 Prominence Gene Analysis

The most relevant HRD genes were selected from the adequately quantified genes in all conditions through our Prominence Gene Analysis (PGA), a variant of the principal component analysis (PCA). The essential difference between PGA and PCA is that genes with the largest variance among distinct conditions are no longer considered as “centroids.” Instead, PGA selects the genes that can form the most interconnected and stably expressed network with respect to a particular biological process, molecular function, or cellular location. Thus, the interest is shifted from the most alterable genes in a series of (not necessarily causally related) conditions to the most influential ones in each condition. A gene is considered as prominent if it is (1) coordinated with that of numerous other genes (to control the fabric) and (2) resistant

to the variation of the local conditions (to protect the fabric) [17, 39, 40].

2.2.8 “Gene Network Landscape”

In order to “visualize” and compare the topology of gene networking in different conditions, we here introduce a new measure in which each gene pair ($\gamma\epsilon$) is represented by a “mountain” whose height is proportional to the contribution of that gene pair to the coordination and stability of the gene network in condition c (e.g., CIH-2=2 weeks hypoxia). The “mountain height” H increases with the expression coordination of the two genes and decreases with their relative expression variability within the cohort(s) they belong to:

$$H_{\gamma,\omega}^{(\alpha)} = \frac{\langle X \rangle_{\Gamma}^{(\alpha)} \left| \rho_{\gamma,\omega}^{(\alpha)} \right| \langle X \rangle_{\Omega}^{(\alpha)}}{X_{\gamma}^{(\alpha)} X_{\omega}^{(\alpha)}}$$

$$\gamma \in \Gamma, \omega \in \Omega; \quad \Gamma, \Omega = \text{HRD}, \text{SIG}, \text{TRA}$$

$$\alpha = \text{NOR} - 1/2, \text{CIH} - 1/2, 4$$

where $X_{\gamma/\omega}^{(c)}$ is the chi-square estimate of the individual gene expression variability and $\langle X \rangle_{\Gamma/\Omega}^{(c)}$ the average expression variability of gene cohorts Γ and Ω in condition α .

2.2.8.1 Overall Transcriptomic Differences

In our opinion, it is not enough to present a list of differently expressed genes to know how much the compared conditions differ from each other from transcriptomic point of view. Therefore, we have developed a comprehensive computational procedure by which to evaluate the *overall transcriptomic differences* $\Delta_{\text{tot}}^{(\alpha,\beta)}$ between conditions α and β ($= \text{NOR}-1, \text{NOR}-2, \text{NOR}-4, \text{CIH}-1, \text{CIH}-2, \text{CIH}-4$):

$$\text{Card}_{\Gamma} \Delta_{\text{tot}}^{(\alpha,\beta)} = \underbrace{\sqrt{\sum_{\gamma \in \Gamma} \left[\left(1 - p_{\gamma}^{(\alpha,\beta)} \right) \left(x_{\gamma}^{(\alpha,\beta)} - 1 \right) \right]^2}}_{\text{Change of expression level}} + \underbrace{\sqrt{\sum_{\gamma \in \Gamma} \left(\frac{X_{\gamma}^{(\beta)}}{\langle X \rangle_{\Gamma}^{(\beta)}} - \frac{X_{\gamma}^{(\alpha)}}{\langle X \rangle_{\Gamma}^{(\alpha)}} \right)^2}}_{\text{Change of transcription control}} + \underbrace{\sqrt{\sum_{\gamma \in \Gamma} \left(\frac{\text{Card}_{\Gamma}}{\text{Card}_{\Gamma} - 1} \sum_{\epsilon \in \Gamma, \epsilon \neq \gamma} \left(\rho_{\gamma,\epsilon}^{(\beta)} - \rho_{\gamma,\epsilon}^{(\alpha)} \right)^2 \right)}}_{\text{Change of networking}}$$

where $x_{\gamma}^{(\alpha,\beta)} = \text{expression ratio}$ (negative for downregulation) and $p_{\gamma}^{(\alpha,\beta)} = p$ value of the significant regulation. In addition to quantifying the magnitude and statistical

significance of expression regulation (expression ratio x , negative for downregulation), it considers also the changes in the transcription control and networking. This new measure that is not affected by the arbitrary cutoffs in expression regulation and significant coordination was used to quantify the global alterations induced by hypoxia as well as transformations during the development.

2.3 Results

2.3.1 Expression Regulation

Table 2.1 presents the genes (from the 127 analyzed in this chapter) that were significantly regulated by chronic intermittent hypoxia. Overall, hypoxia regulated expression of

Table 2.1 Significantly regulated genes in hearts of mice subjected for 1, 2, or 4 weeks of their early life to chronic intermittent hypoxia with respect to their normoxic counterparts

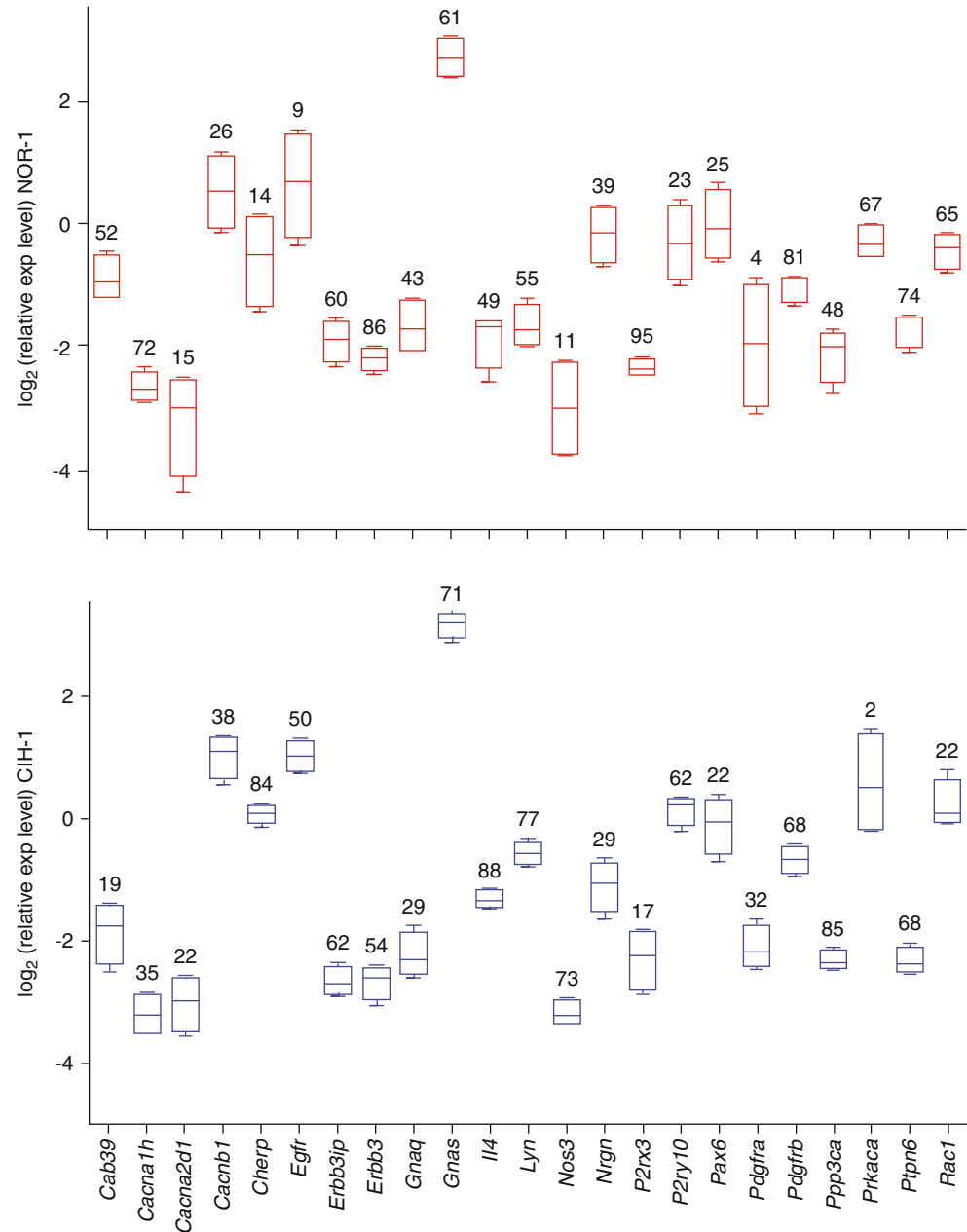
Gene	Symbol	CAT	CHR	1 week	2 weeks	4 weeks
Eukaryotic translation elongation factor 1 alpha 1	<i>Eef1a1</i>	TRA	9	2.21		
Eukaryotic translation elongation factor 1 alpha 2	<i>Eef1a2</i>	TRA	2		2.60	
Eukaryotic translation elongation factor 1 beta 2	<i>Eef1b2</i>	TRA	1		-2.11	1.66
Eukaryotic translation initiation factor 1A, Y-linked	<i>Eif1ay</i>	TRA	X	-1.62		
Eukaryotic translation initiation factor 1B	<i>Eif1b</i>	TRA	9	-1.87		
Eukaryotic translation initiation factor 2 alpha kinase 4	<i>Eif2ak4</i>	TRA	2		-1.60	
Eukaryotic translation initiation factor 2, subunit 1 (alpha)	<i>Eif2s1</i>	TRA	12	-2.34		
Eukaryotic translation initiation factor 2, subunit 2 (beta)	<i>Eif2s2</i>	TRA	2	-2.49		
Eukaryotic translation factor 3, subunit 12	<i>Eif3s12</i>	TRA	7	-3.93	1.89	
Eukaryotic translation factor 3, subunit 2 (beta)	<i>Eif3s2</i>	TRA	4		-1.93	
Eukaryotic translation factor 3, subunit 3 (gamma)	<i>Eif3s3</i>	TRA	15		2.36	
Eukaryotic translation factor 3, subunit 6 interacting protein	<i>Eif3s6ip</i>	TRA	15	-3.03		
Eukaryotic translation factor 3, subunit 9 (eta)	<i>Eif3s9</i>	TRA	5		-1.86	
Eukaryotic translation initiation factor 4E	<i>Eif4e</i>	TRA	3	-1.86	-4.07	
Eukaryotic translation initiation factor 5	<i>Eif5</i>	TRA	12		2.24	
Eukaryotic translation initiation factor 5A	<i>Eif5a</i>	TRA	11	-1.55	-2.23	-1.51
Wingless-related MMTV integration site 3A	<i>Wnt3a</i>	SIG	11	-1.94		
Wingless-related MMTV integration site 5B	<i>Wnt5b</i>	SIG	6	1.58		
SRY-box containing gene 11	<i>Sox11</i>	SIG	12		-2.35	
SRY-box containing gene 3	<i>Sox3</i>	SIG	X	2.39		
Calcium binding protein 39	<i>Cab39</i>	SIG	1	-1.89		
Calcium channel, voltage-dependent, alpha2/delta subunit 1	<i>Cacna2d1</i>	SIG	5		-1.72	
ErbB2 interacting protein	<i>ErbB2ip</i>	SIG	13	-1.69		
Yamaguchi sarcoma viral (v-yes-1) oncogene homolog	<i>Lyn</i>	SIG	4	2.15		
Nitric oxide synthase 3, endothelial cell	<i>Nos3</i>	SIG	5		1.65	
Neurogranin	<i>Nrgn</i>	SIG	9	-1.90		
Protein tyrosine phosphatase, non-receptor type 6	<i>Ptpn6</i>	SIG	6		-2.53	
RAS-related C3 botulinum substrate 1	<i>Rac1</i>	SIG	5		-3.11	
Inositol 1,4,5-triphosphate receptor 1	<i>Itpr1</i>	HRD	6	-2.21		
Ankyrin 2, brain	<i>Ank2</i>	HRD	3	1.55		
Cadherin 22	<i>Cdh22</i>	HRD	2		1.76	
Guanine nucleotide binding protein, alpha o	<i>Gnao1</i>	HRD	8		-2.88	
Inhibitor of DNA binding 2	<i>Id2</i>	HRD	12	-1.85		
Junction plakoglobin	<i>Jup</i>	HRD	11	1.97		
Potassium voltage-gated channel, subfamily H (eag-related), member 2	<i>Kcnh2</i>	HRD	5	1.93		
Protocadherin gamma subfamily C, 3	<i>Pcdhgc3</i>	HRD	18	1.90		
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	<i>Sema3a</i>	HRD	5		2.40	
Solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20	<i>Slc25a20</i>	HRD	9	-2.32	-2.41	
Vezatin, adherens junctions transmembrane protein	<i>Vezt</i>	HRD	10	-1.62	-1.56	2.28

Missing values indicate that no significant regulation was detected for that gene

CAT category, CHR chromosomal location

Negative numbers (grey background) indicate down-regulation

Fig. 2.2 Expression variability of some SIG genes in the heart of 1-week-old mice raised in normal atmospheric conditions (*NOR-1*) or chronic intermittent hypoxia (*CIH-1*). The rectangles represent the 95% confidence intervals of the binary logarithms of the relative (to the average gene) expression level within four biological replicates. Numbers above the rectangles are the GES scores. Note the nonuniform variability among the genes and the change in expression control induced by hypoxic conditions. Genes: *Egfr*=epidermal growth factor receptor, *ErbB3*=v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian), *Gnaq*=guanine nucleotide-binding protein, alpha q polypeptide, *Gnas*=(guanine nucleotide-binding protein, alpha stimulating) complex locus, *Il4*=interleukin-4, *Pax6*=paired box gene 6, *Pdgfrb*=platelet-derived growth factor receptor, beta polypeptide; protein phosphatase 3, *Ppp3ca*=catalytic subunit, alpha isoform, *Prkaca*=protein kinase, cAMP-dependent, catalytic, alpha



16.2% TRA, 11.6% SIG, and 8.6% HRD genes, the percentages decreasing from 1 to 4 weeks of oxygen deprivation. The reduction in number of regulated genes indicates activation of adapting mechanisms. Interestingly, after 1 week hypoxia, expression of *Wnt5b* (which promotes adipogenesis) was increased (as also reported in arrhythmogenic right ventricular cardiomyopathy [41]), while that of *Wnt3a* (required for cardiac differentiation in (human) embryonic stem cells [42]) was decreased.

2.3.2 Change in Transcription Control

Figure 2.2 illustrates the expression variability of certain SIG genes in hearts of 1-week-old mice subjected to normal atmospheric conditions or to chronic intermittent hypoxia and their GES scores in each condition. Note how hypoxia switched the controlling priorities of the cardiomyocytes. For instance, the tight control of *P2rx3* (= purinergic receptor P2X, ligand-gated ion channel, 3) in normoxia (GES=95) was relaxed in

Table 2.2 The most stably expressed gene within the selection in each condition

Gene	Symbol	NOR-1	CIH-1	NOR-2	CIH-2	NOR-4	CIH-4
<i>Most stable expressed</i>							
Eukaryotic translation initiation factor 4, gamma 2	<i>Eif4g2</i>	99.20	41.24	24.68	26.10	76.92	51.65
Myocyte enhancer factor 2D	<i>Mef2d</i>	28.73	99.75	95.66	22.32	23.14	69.42
Protocadherin 7	<i>Pcdh7</i>	48.95	83.98	99.94	26.86	97.96	85.68
Vezatin, adherens junctions transmembrane protein	<i>Vezt</i>	47.10	93.29	81.00	98.64	26.53	32.25
Eukaryotic translation elongation factor 1 alpha 2	<i>Efla2</i>	3.85	87.66	67.44	57.84	98.91	31.16
Purinergic receptor P 2Y, G-protein coupled 10	<i>P2ry10</i>	22.79	61.50	44.01	73.49	69.05	98.91
<i>Most unstably expressed</i>							
SET and MYND domain containing 1	<i>Smyd1</i>	0.80	86.24	7.51	20.65	41.73	4.42
Eukaryotic translation elongation factor 2	<i>Eef2</i>	88.63	1.09	3.19	51.21	83.34	1.69
Gap junction membrane channel protein alpha 1	<i>Gjal</i>	17.89	8.17	0.93	15.08	34.97	7.01
Catenin (cadherin associated protein), alpha 1	<i>Ctnna1</i>	1.28	34.04	28.42	0.14	33.81	47.39
Solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20	<i>Slc25a20</i>	61.46	98.64	23.78	90.11	2.34	40.39
Yamaguchi sarcoma viral (v-yes-1) oncogene homolog	<i>Lyn</i>	55.16	76.94	37.76	27.05	75.95	0.41

The numbers are the GES scores (bold numbers on gray background for the most stably/unstably expressed genes). Note the differences among conditions and that chronic hypoxia switched genes such as *Smyd1*, *Eef2*, and *Lyn* between stably (GES > 75) and very unstably (GES < 5) expressed (compare normoxic and hypoxic values for the same duration)

hypoxia (GES=17), while the loose control of *Cherp* (GES=14) was significantly tightened in hypoxia (GES=84).

The most stably and unstably expressed genes in each condition are presented in Table 2.2 together with their GES scores in all conditions. One may note that expression variability is highly influenced by both development (compare values at 1, 2, and 4 weeks) and oxygen deprivation (compare normoxic with hypoxic values). Although the analysis was limited to only 127 genes profiled in six conditions, Table 2.2 shows how large is the interval of expression control from <1 in the case of *Gjal* (at 2 weeks normoxia), *Ctnna1* (2 weeks CIH), and *Lyn* (4 weeks CIH) to >99 for *Eif4g2* (1 week normoxia), *Mef2d* (1 week CIH) and *Pcdh7* (2 weeks normoxia).

2.3.3 Alteration of Gene Networking

Figure 2.3 presents hypoxic alteration of part of the *Gjal*-dependent network that controls the HRD fabric by direct expression coordination or through coordination with its Ca²⁺-signaling partners at 1, 2, and 4 weeks. We found a substantially lower number of significant coordinations in hypoxia. When all SIG and HRD genes are considered, the percent of the coordinately expressed genes in hypoxia decreases by 59% at 1 week, by 40% at 2 weeks, and only by 11% at 4 weeks with respect to the normoxic counterparts. The dynamics of the coordination differences suggests again activation of certain acclimatization or accommodation mechanisms. Although cardiomyocytes proliferate rapidly

during fetal life, the heart growth shifts from hyperplasia to hypertrophy after birth, with most cardiac cells gradually ceasing to undergo DNA replication [43]. Interestingly, as Fig. 2.3 shows, the expression coordination increases also between the first and the second week of life both in normoxic and hypoxic conditions but decreases between the second and the fourth week.

2.3.4 Remodeling of the Network Landscape

Figure 2.4 presents the landscapes of HRD fabric and of SIG-HRD and TRA-HRD networks in hearts of 1-week-old mice regrown in normoxic or chronic intermittent hypoxic conditions. One may observe the profound remodeling of these “landscapes” as well as the change of dominant gene pairs for each network. Thus, the dominant pairs from normoxia (*Cdh16-Id2*, *P2rx3-Cdh16*, *Hif1a-Jup*) are replaced by: *Jup-Slc25a20*, *Wnt5b-Jup*, and *Eif5a-Mef2d* in hypoxia. Moreover, while the “mountains” of both HRD fabric and CAS-HRD networks are taller in 1 week hypoxia, those of TRA-HRD networks are taller in 1 week normoxia.

Table 2.3 lists the dominant pairs in all conditions. Note the change of dominant pairs both due to development and hypoxia and that the network has the largest scores at 2 weeks normoxia. We found that *Hif1a-Jup*, *Lmna-Pcdh7*, and *Gnao1-Pcdh7* are the most influential at 1, 2, and 4 weeks normoxia, respectively, while *Jup-Slc25a20*, *Cdh16-Vezt*, and *Eif2ak4-Pcdh12* are the leading pairs at 1, 2, and 4 weeks hypoxia.

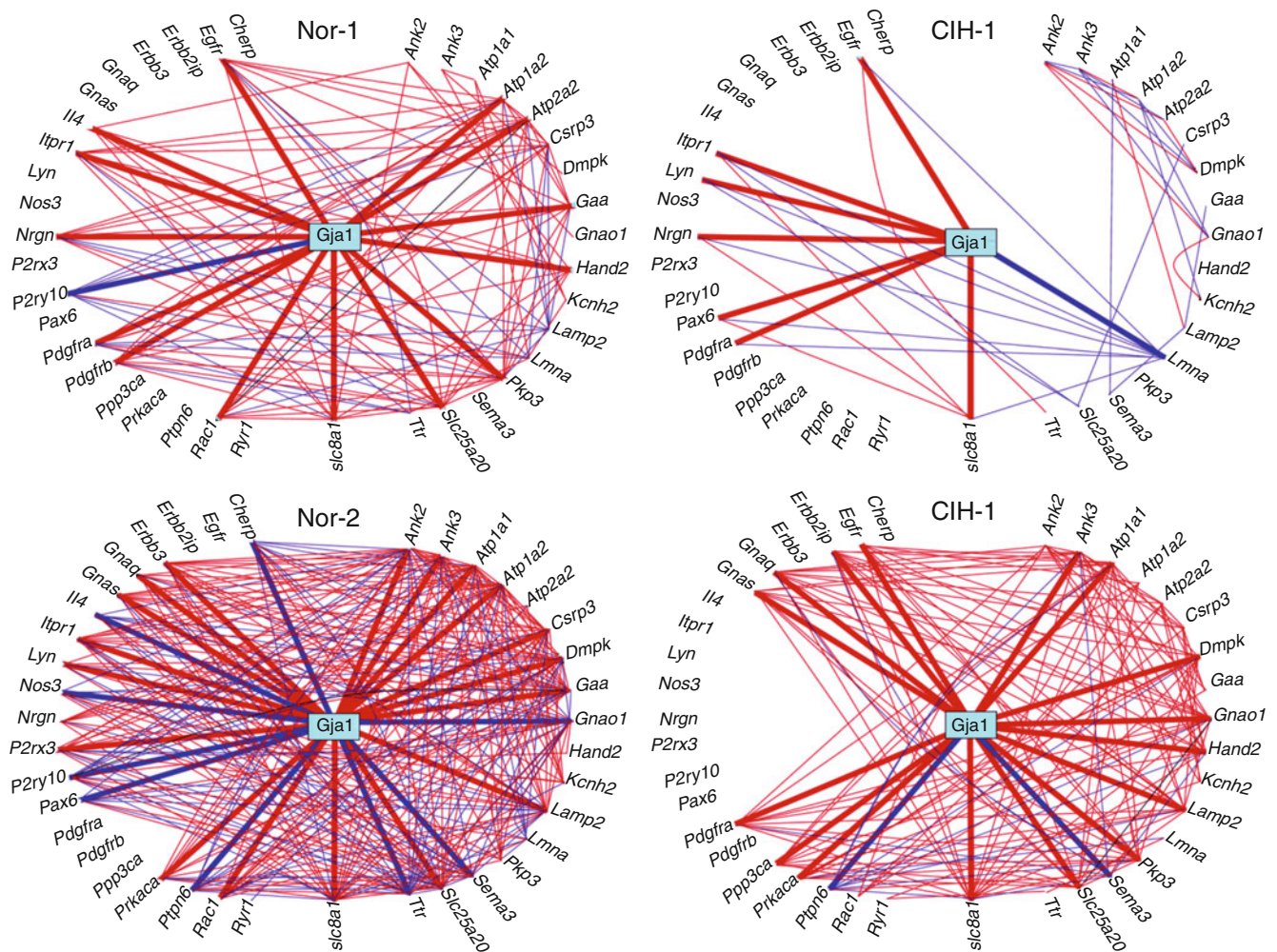


Fig. 2.3 Part of the *Gja1*-dependent network in controlling the heart rhythm determinant and its change during development in normoxic and hypoxic conditions. Red/blue lines (thicker for *Gja1* interlinks) indicate synergistic/antagonistic expression coordination of the linked genes. Note that Cx43 encoded by *Gja1* controls expression of HRD genes both by direct expression coordination and through coordination with its Ca^{2+} -

signaling gene partners. Genes: *Atp1a1/2* ATPase, Na^+/K^+ -transporting, alpha 1/2 polypeptide, *Atp2a2* ATPase, Ca^{2+} -transporting, cardiac muscle, slow twitch 2, *Csrp3* cysteine and glycine-rich protein 3, *Dmpk* dystrophin myotonic-protein kinase, *Gaa* glucosidase, alpha, acid, *Hand2* heart and neural crest derivatives expressed transcript 2, *Lamp2* lysosomal membrane glycoprotein 2, *Lmna* lamin A

2.3.5 Overall Transcriptomic Changes

Figure 2.5 presents the average transcriptomic alterations induced by chronic hypoxia in expression level, control, and coordination of the selected 127 genes as well as the differences between successive time points in the same treatment. Interestingly, coordination alteration is the largest at each treatment duration, pointing out that network remodeling is the most important arrhythmogenic transcriptomic factor and not the regulation of the expression levels of HRD genes. An important observation is that the overall alteration diminishes continuously from 1 to 4 weeks hypoxia indicating activation of transcriptomic adaptation mechanisms to the hypoxia stress. Although chronic hypoxia is a major stress, the developmental changes appear of similar

magnitude if not even larger than those induced by hypoxia (compare values of different treatments for the same duration with different durations for the same treatment).

2.4 Discussion

2.4.1 Advantages and Limitations of the Transcriptomic Studies on Ischemic Animal Models

Numerous microarray studies have compared the heart transcriptomes of arrhythmic and non-arrhythmic humans and animal models (e.g., [44–48]), identifying significantly

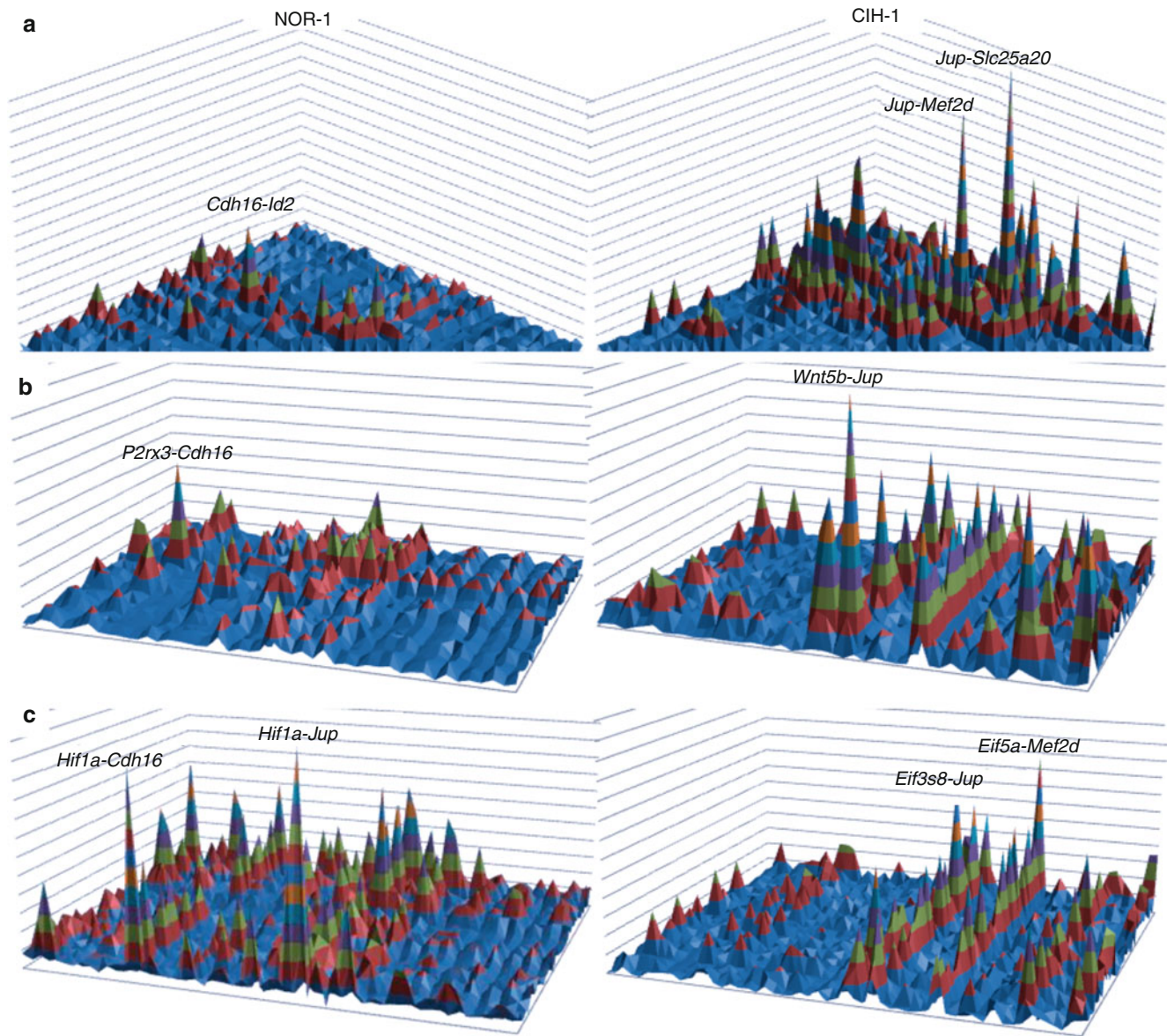


Fig. 2.4 Gene network landscapes at 1 week. (a) HRD fabric. Since the same set of HRD genes was considered on both horizontal axes, the landscape is mirrored with respect to the diagonal and therefore only half of it was represented. (b) SIG-HRD network. (c) TRA-HRD net-

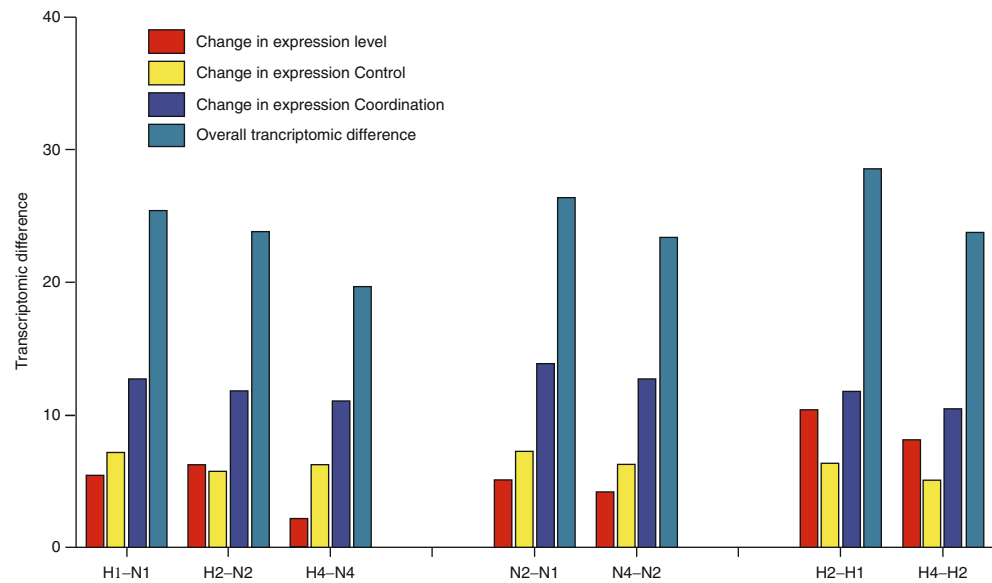
work. Note the profound remodeling of the landscapes, the differences in “mountain heights,” and the change of dominant gene pairs. Genes: *Cdh16* cadherin 16, *Eif3s8* eukaryotic translation initiation factor 3, subunit 8

Table 2.3 The dominant gene pairs of each network in each condition

	HRD–HRD	SIG–HRD	TRA–HRD
NOR-1	<i>Cdh16–Id2</i> (12.38)	<i>P2rx3–Cdh16</i> (12.08)	<i>Hif1a–Jup</i> (25.56)
CIH-1	<i>Slc25a20–Jup</i> (34.71)	<i>Wnt5b–Jup</i> (23.68)	<i>Eif5a–Mef2d</i> (17.62)
NOR-2	<i>Lmna–Pcdh7</i> (61.56)	<i>Cacnb1–Pcdh7</i> (48.82)	<i>Eif1b–Pcdh7</i> (50.16)
CIH-2	<i>Cdh16–Vezt</i> (20.27)	<i>Pax–Vezt</i> (17.46)	<i>Eif3s12–Pcdh12</i> (11.12)
NOR-4	<i>Gnao1–Pcdh7</i> (20.35)	<i>Cacnb1–Pcdh7</i> (11.20)	<i>Eef1a2–Gnao1</i> (14.88)
CIH-4	<i>Pcdh12–Sema3a</i> (9.21)	<i>P2ry10–Pcdh12</i> (14.94)	<i>Eif2ak4–Pcdh12</i> (16.56)

The numbers are the “heights” of the pairs. Note that the “tallest” pairs are at 2 weeks normoxia
Genes: *Cacnb1* calcium channel, voltage-dependent, beta 1 subunit, *Pcdh12* protocadherin 12

Fig. 2.5 Transcriptomic changes induced by hypoxia (H1-N1, H2-N2, H4-N4) or by development under normoxic (N2-N1, N4-N2) or hypoxic (H2-H1, H4-H2) conditions. Letters *N* and *H* indicate normoxia and hypoxia, while numbers indicate the duration of the treatment (in weeks). Note that change in expression coordination has the largest contribution in each comparison and that changes induced by hypoxia are of similar magnitude as those caused by development either in normoxic or hypoxic conditions



regulated genes and clustering them according to gene ontology (GO) terms and fold expression changes. These studies have had a high impact in delineating essential genomic alterations induced by or predisposing to the disease and in describing transcriptomic differences between normal and pathological conditions. However, although use of lab animals offers unlimited possibilities to investigate consequences of oxygen deprivation, no animal model reproduces exactly what happens in the human body. Even so, animal experiments are critical for narrowing the spectrum of possibilities since the hard cores of a great number of functional pathways were preserved during evolution.

2.4.2 The Endless Story of Gene Expression Analysis

For now, the most powerful and cost-effective tool to investigate complex molecular phenomena is the gene expression array. By quantifying thousands of genes at a time, practically the whole genome, gene expression studies allow endless possibilities to analyze data. The unlimited number of perspectives comes from the fact that genes are not lonely but team players, forming partially overlapping webs whose composition and topology adapt continuously to the variable local conditions. If only gene composition is considered, the complex gene network can be formally deconvoluted into 20,000 or so individual genes, ~200 millions distinct gene pairs, >1.3 trillion distinct triplets, $>6.6 \times 10^{15}$ quadruplets, and so on. This means as many distinct pathways in which genes may be involved as individuals, doublets, triplets, quadruplets, etc. Moreover, the same pathway may perform differently when conditions change. Because of this, interpretation of microarray data is a never-ending story.

This is our fourth paper analyzing from new perspectives the transcriptomic alterations in hearts of mice subjected in their early life to chronic intermittent hypoxia based on a gene expression experiment. In the first paper [13], we have identified the genes that were up- or downregulated in hypoxia when compared to normoxic counterparts and grouped them according to their GO categories as biological process, molecular function, or cellular localization. Expression regulation of selected genes was confirmed by qRT-PCR, Western blotting, and morphological and physiological observations. Although this was a huge amount of information by itself, it represents only the very tip of what we could learn from such studies.

Table 2.1 shows that in the first and second week of hypoxia, numerous translation regulators and signaling genes were downregulated, while after 4 weeks, only *Eif5a* was downregulated, *Eef1b2* and *Ve2t* being upregulated. Interestingly, *Eif5a* was downregulated at all 3 time points that explain why apoptosis was enhanced in chronic constant hypoxia (where it was upregulated at 4 weeks) but not in CIH [13], confirming the role of *Eif5a* in the apoptosis induction [49].

2.4.3 Expression Variability

An important addition was to determine whether the new condition changed the strength of the control of transcript abundance by determining the change of the relative expression variability (REV score) and gene expression stability (GES percentile) [16]. Although the transcriptomic machinery is similar in (here) hearts of mice from the same litter, subjected to the same treatment, the local conditions in which genes are transcribed are never identical, making the genome a noisy system. Therefore, genes exhibit different expression levels in biological replicas, expression variability resulting

from the transcriptomic tuning to the local conditions. Moreover, as illustrated in Fig. 2.1b, the variability is not uniform among the genes, our study revealing from very stably expressed (standard deviation negligible when compared to the average expression level) to very unstably expressed (standard deviation exceeding the average value). Since gene expression is regulated by numerous controlling mechanisms, a lower variability indicates a stricter control of the transcript abundance. Expression variability is expected to increase with relaxation of the control of transcript abundance by the homeostatic mechanisms. Very stably expressed genes are likely to be essential for cell survival, phenotype expression, and/or integration in superior, multicellular structures. Correspondingly, certain very unstably expressed genes may be involved in the adaptation to environmental changes. Thus, the analysis of expression variability may provide valuable indication with regard to the role of various genes in maintaining the function or ensuring adaptation of the system to the environment changes. Remarkably, genes such as *Smyd1*, *Eef2*, and *Lyn* were switched between stably and very unstably expressed, indicating profound reconfiguration of transcription control priorities. The relaxed control of *Eef2* at 1 and 4 weeks hypoxia may help its control by *Ampk* whose α_1 and α_2 subunits of AMPK are activated during myocardial ischemia [50].

2.4.4 Remodeling of the HRD Fabric

Figure 2.3 illustrates the plasticity of the HRD fabric whose topology was profoundly remodeled during development and altered by oxygen deprivation. However, when a particular relationship is fundamental for the cardiac function, it tends to be preserved, as the antagonistic expression of *Gjal* and *Sema3* at 2 weeks. *Sema3a* is important in maintaining normal heart rhythm through sympathetic innervation patterning, both disruption and overexpression of *Sema3a* being associated with reduced sympathetic innervations and attenuation of the epicardial-to-endocardial innervation gradient [51]. On the other hand, Cx43 encoded by *Gjal* influences the heart rhythm by modulating the innervation of the cardiac tissue as we have shown by both transcriptomic and immunostaining comparisons of the left ventricles of wild-type and Cx43 null neonatal mice [52].

2.4.5 Why Have We Focused on Functional Genomic Fabric?

All of our transcriptomic studies indicate that genes are not independently but rather coordinately expressed in transcellular transcription networks (e.g., [36–38]). Therefore, manipulation of a single gene has ripple effects on numerous

others, located on all chromosomes and whose protein products are involved in a wide diversity of processes [52]. Because of expression coordination, practically, all genes are directly or indirectly involved in every functional pathway. However, genes are not of equal importance in controlling the pathway and an arbitrary cutoff (either as influence of a gene or as number of genes considered) should be established. Therefore, we have developed and tested the prominent gene analysis (PGA) [17, 39, 40] to identify the genes forming the most interconnected and stably expressed functional web. Expression coordination of web genes forces their expression levels to oscillate in synchrony. PGA is an alternative to the principal component analysis (PCA), both methods aiming to reduce the huge number of variables to the most informative ones. The difference is that while PCA considers “centroids” as the most alterable genes in a set of distinct conditions, PGA selects the most interconnected and stably expressed ones in the same condition. Genomic fabrics exhibit characteristic gene composition and topology depending on region, sex [39], and age [14, 16, 17]; responds by transcriptomic tuning to variable local conditions and environmental constraints [14, 16, 17]; and remodels in disease.

2.4.6 Network Landscape

The novel analysis of network landscape quantifies the role of each gene pair in interconnecting and stabilizing the web. Remarkably, genes encoding the protocadherins *Pcdh7* and *Pcdh12* and cadherin *Cdh16* are components of the most influential pairs within HRD fabric, underlying the role of the intercalated discs in synchronizing contraction of cardiomyocytes. The cadherins are a superfamily of calcium-dependent adhesion molecules with auxiliary functions in embryogenesis, stem cell fate and maintenance, cell polarity, and signal transduction [53]. However, genes encoding signaling proteins such as the purinergic receptors *P2rx3* and *P2ry10* and *Wnt5b*, as well as the translation regulators *Eef1a2*, *Eif1b*, *Eif2ak4*, *Eif3s12*, and *Eif5a* are forming also very influential pairs with HRD genes.

2.4.7 Overall Transcriptomic Changes

This new measure provides the most comprehensive way to evaluate the transcriptomic differences between two conditions. It includes the “transcriptomic recovery” that we have recently used to determine the reversion of gene expression alterations in hearts of mice with chronic chagasic cardiomyopathy after transplantation of bone marrow cells [54]. The analysis revealed two very interesting aspects: (1) that remodeling of the coordination network has the major

contribution when comparing any two conditions and (2) that development and chronic hypoxia induce similar overall transcriptomic changes. The first aspect tells us that analysis of expression regulation is not enough to characterize the transcriptomic alterations induced by pathological conditions/development. Therefore, the list of biomarkers should be extended to include also the genes exhibiting large changes in expression control and coordination.

In summary, from this study, we have learned that chronic intermittent hypoxia is a major stress that profoundly remodels the heart rhythm genomic fabric and signaling pathways controlling this fabric, the overall transcriptomic alterations being comparable to the substantial changes during the development.

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