



Hypoxia Regulates MicroRNA Expression in the Human Carotid Body

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Abstract

How hypoxia regulates gene expression in the human carotid body (CB) remains poorly understood. While limited information on transcriptional regulation in animal CBs is available, the impact of important post-transcriptional regulators, such as non-coding RNAs, and in particular miRNAs is not known. Here we show using *ex vivo* experiments that indeed a number of miRNAs are

differentially regulated in surgically removed human CB slices when acute hypoxic conditions were applied. Analysis of the hypoxia-regulated miRNAs shows that they target biological pathways with upregulation of functions related to cell proliferation and immune response and downregulation of cell differentiation and cell death functions. Comparative analysis of the human CB miRNAome with the global miRNA expression patterns of a large number of different human tissues showed that the CB miRNAome had a unique profile which reflects its highly specialized functional status. Nevertheless, the human CB miRNAome is most closely related to the miRNA expression pattern of brain tissues indicating that they may have the most similar developmental origins.

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3.1 Introduction

The human carotid body (CB) oxygen sensing and signaling mechanisms are not well understood despite the existence of many and often conflicting hypotheses (Iturriaga and Alcayaga 2004; Lopez-Barneo et al. 2016). One of the contributing factors is the lack of human CB tissues

with notably one study (Ortega-Saenz et al. 2013) describing the morphology and chemosensory response of the organ to hypoxia. However, the contribution and targets of the transcriptional regulation driven by oxygen sensing in the human CB remain largely unexplored. A recently published map of the human CB transcriptome has demonstrated similarities but also significant differences in the expression of many important oxygen sensing CB genes as compared to animal CBs (Mkrtchian et al. 2012).

Among many unanswered questions in CB oxygen sensing, one of the most outstanding is the regulation of the expression of functionally important CB genes. In particular, very little is known about the post-transcriptional regulation by non-coding RNAs, especially microRNAs (miRNAs). These are short (20–22 nt) single-stranded RNA molecules that guide the RNA-induced silencing complex to complementary binding sites in the 3'-UTRs of messenger RNAs (mRNAs) thereby suppressing the translation of the corresponding protein (Wilczynska and Bushell 2015).

MicroRNA-mediated regulation is an important factor for cellular adaptation to a variety of environmental stresses including hypoxia. The hypoxia-inducible factor 1 and 2 alpha subunits HIF-1 α and HIF-2 α are the key transcription factors that are stabilized under hypoxic conditions and mediate the expression of many genes involved in metabolism, angiogenesis, cell cycle, growth and cell death in response to the lack of oxygen (Bristow and Hill 2008). It had also been demonstrated that hypoxia can regulate the expression of a distinct set of miRNAs that were termed hypoxamiRs (Kulshreshtha et al. 2007; Nallamshetty et al. 2013). HIF1- α was reported to activate a subset of these miRNAs and conversely, other hypoxamiRs were shown to regulate HIF-1 α expression (Loscalzo 2010; Bruning et al. 2011; Chen et al. 2016). Therefore, given the prominent role of the HIFs in the regulation of miRNAs, it was possible that miRNAs are also involved in the hypoxic response of the CB.

In the current study using a unique set of primary human CB specimens, we analyzed the CB miRNA expression under acute hypoxia relative

to hyperoxia controls. Many of the hypoxia-regulated miRNAs were predicted to target mRNAs that lead to the upregulation of functions related to cell proliferation and immune response and downregulation of cell differentiation and cell death. Subsequently, we characterized the CB-specific pattern of miRNA expression in the control samples and identified the CB global miRNAome. Comparison of the CB global miRNA profiles with those from other human tissues revealed that while the CB was a highly distinct organ, it was most closely related to brain tissues.

3.2 Methods

3.2.1 Patient Carotid Body Collection

Experimental protocols were approved by the ethics committee on human research at the Karolinska Institutet in Stockholm, Sweden. Carotid bodies were collected from five male patients (age 42–80 years) with a BMI <32, scheduled for radical neck dissections not involving the CB. CBs were removed unilaterally under general anesthesia using sevoflurane and intravenous opioids with normoxic and normocarbic mechanical ventilation. Once surgically removed, the CBs were immediately placed in pre-oxygenated hyperoxic (95% O₂, 5% CO₂) ice-cold Krebs-Ringer Solution (KRS, 120 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 21.4 mM NaHCO₃, 1.4 mM NaH₂PO₄, 10 mM glucose) and transported to the laboratory for further experiments within 15 min.

3.2.2 CB Sectioning and Treatments

After removal of connective tissues, CBs were sectioned along their longitudinal axis into 400 μ m slices. Two to four CB slices from each of the 5 patients were used for hypoxia/hyperoxia treatments. Slices were distributed evenly into two 24-well plates containing 300 μ l of pre-oxygenated KRS in each well. Pretreated hypoxic

KRS was added to one plate and moved into an H35 Hypoxia Station (Don Whitley Scientific) set at 10% O₂, 85% nitrogen and 5% CO₂ at 37 °C. The second control plate was fed with hyperoxic KRS and returned to the incubator with settings for hyperoxic conditions (95% O₂ and 5% CO₂ at 37 °C). Both plates were incubated for 1 h prior to harvesting for RNA extraction.

3.2.3 RNA Isolation and miRNA Analysis

Immediately after 1 h incubation, CB slices were immersed into 500 µl of Qiazol (Qiagen) and total RNA was extracted from the lysates using the miRNeasy Micro kit (Qiagen). RNA was reverse transcribed into cDNA using the miRCURY LNA Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon). Each microRNA was assayed by qPCR on the microRNA Ready-to-Use PCR Human panel I + II with the ExiLENT SYBR® Green master mix on a LightCycler® 480 Real-Time PCR System (Roche).

3.2.4 Data Analysis

Differential microRNA expression was identified on the basis of a fold change cutoff of >1.5 for the hypoxia treated carotid slices compared to the hyperoxia controls for each patient and with a t-test p-value <0.05. MicroRNA functional analysis was carried out using Ingenuity Pathway Analysis (IPA, Qiagen) and the mRNA targets of the microRNAs were identified using the microRNA Target Filter in IPA. Gene ontology and pathway analysis of these mRNAs for the determination of their cellular functions was subsequently carried out using IPA.

MicroRNA expression profiles of the human CBs (control hyperoxia samples) were compared with the miRNA profiles of various human tissues from two individuals (Human miRNA tissue atlas, (Ludwig et al. 2016)). For direct comparison, we selected only those miRNAs

from the tissue atlas that have corresponding matches with our identified CB miRNAs. This led to the isolation of 250 miRNAs that are overlapping between the human miRNA tissue atlas and the CB samples. The CB miRNA expression data were represented by the relative expression values $2^{\Delta Cq}$ where $\Delta Cq = \text{individual } Cq - \text{global mean } Cq$ whereas the tissue atlas data were represented by the quantile normalized raw intensity values from the Agilent SurePrint Human miRNA array.

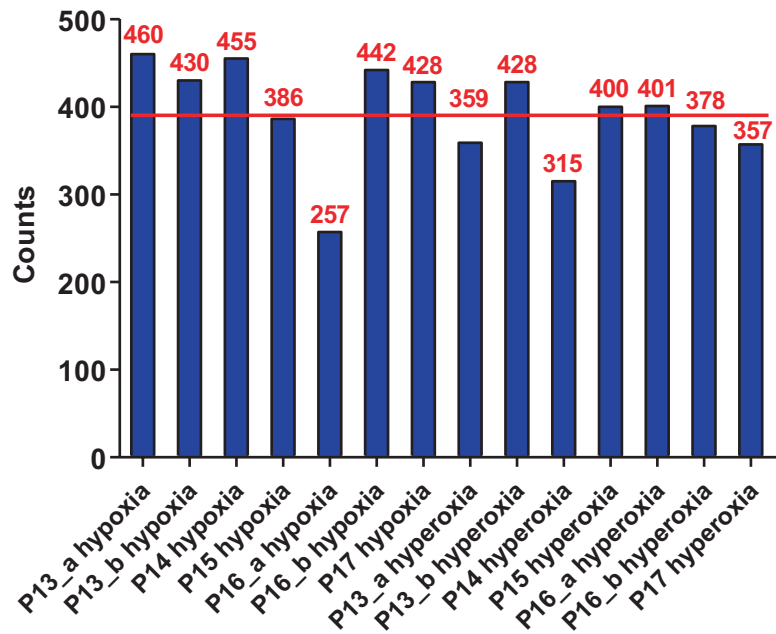
Furthermore, to harmonize the comparisons between these data sets, we used ranked expression values. Heatmap showing mean-centered sigma-normalized ranks for each sample using unsupervised hierarchical clustering and principal component analyses were generated using Qlucore Omics Explorer 3.2 (Qlucore).

3.3 Results

3.3.1 Hypoxia Regulates miRNA Expression in Primary Human CBs

CB slices were divided into two groups, one of which was kept under hypoxic, and another under control hyperoxic conditions for 1 h. The higher oxygen tension treatments at 95% O₂ were used to generate relatively well oxygenated normoxic controls as previous studies have shown that tissue pO₂ levels were less than half that of perfusates (Pepper et al. 1995). For the same reasons, incubations in 10% oxygen for hypoxic treatments are expected to generate oxygen tensions of less than 10% within the carotid slices that more closely reflect oxygenation levels in arterial blood during hypoxic challenge. The treated CBs were further processed for the isolation of RNA and analysis of miRNA expression was carried out using Exiqon qPCR miRNA panels that allow the simultaneous detection of 752 miRNAs. The number of miRNAs detected in each slice varied from 257 to 460 with 392 on average for each slice (Fig. 3.1), and 224 miRNAs were found to be expressed in all of the samples (data not shown).

Fig. 3.1 Number of microRNAs detected in the human carotid body. Bar chart with numbers above each bar shows the number of miRNAs detected in each of 14 CB slices from five patients (P13–P17). Patients 13 and 16 are represented by two slices each, **a** and **b**. The red line shows the average count of 392 miRNAs per sample



Because of the possible contamination of CBs with blood, three miRNAs, miR-144, 142 and 451 were excluded from further analysis due to their known high expression in blood cells (Williams et al. 2013).

Thirty-nine miRNAs were identified to be differentially expressed in the hypoxia treated CB slices with a fold change of at least ± 1.5 and t-test statistical significance of $p \leq 0.05$ (Fig. 3.2). Nevertheless, their physiological effects may be significant as each miRNA is capable of binding up to a few hundred target mRNAs bearing similar miRNA binding sites in their 3'-UTRs (Helwak et al. 2013). Indeed, using the miRNA Target Filter tool in IPA under high stringency conditions where only experimentally validated human targets are considered, this led to the identification of 550 possible mRNA targets (data not shown).

To address what were the biological functions regulated by these microRNA targeted mRNAs, the gene lists were subjected to gene ontology and pathway analysis using IPA. The IPA analysis of the upregulated mRNAs showed that the top most significantly upregulated functions are associated with cellular growth and proliferation,

tissue development functions and cell cycle (Fig. 3.3).

Taken together, all of these analyses indicate that the cell proliferation function was predominantly driven by the mRNAs targeted by the hypoxia-induced microRNAs. In our previous study, we showed that human CB slices release pro-inflammatory cytokines under identical hypoxia conditions. In agreement with this, inflammatory response functions were also found to be among the significantly upregulated cellular processes (Fig. 3.3).

In further analysis of the functional significance of the mRNAs targeted by hypoxia-inducible miRNAs in the CB, we examined the upregulated miRNAs that would result in the downregulation of specific mRNA targets. Among various cellular processes inhibited by hypoxia via miRNA suppression, cell differentiation, and apoptosis were among the top significantly dysregulated functions as identified by IPA (Fig. 3.3). This suggested that the human CBs may be undergoing concerted hypoxic upregulation of cell proliferation at the expense of differentiation and the reduction of cell death.

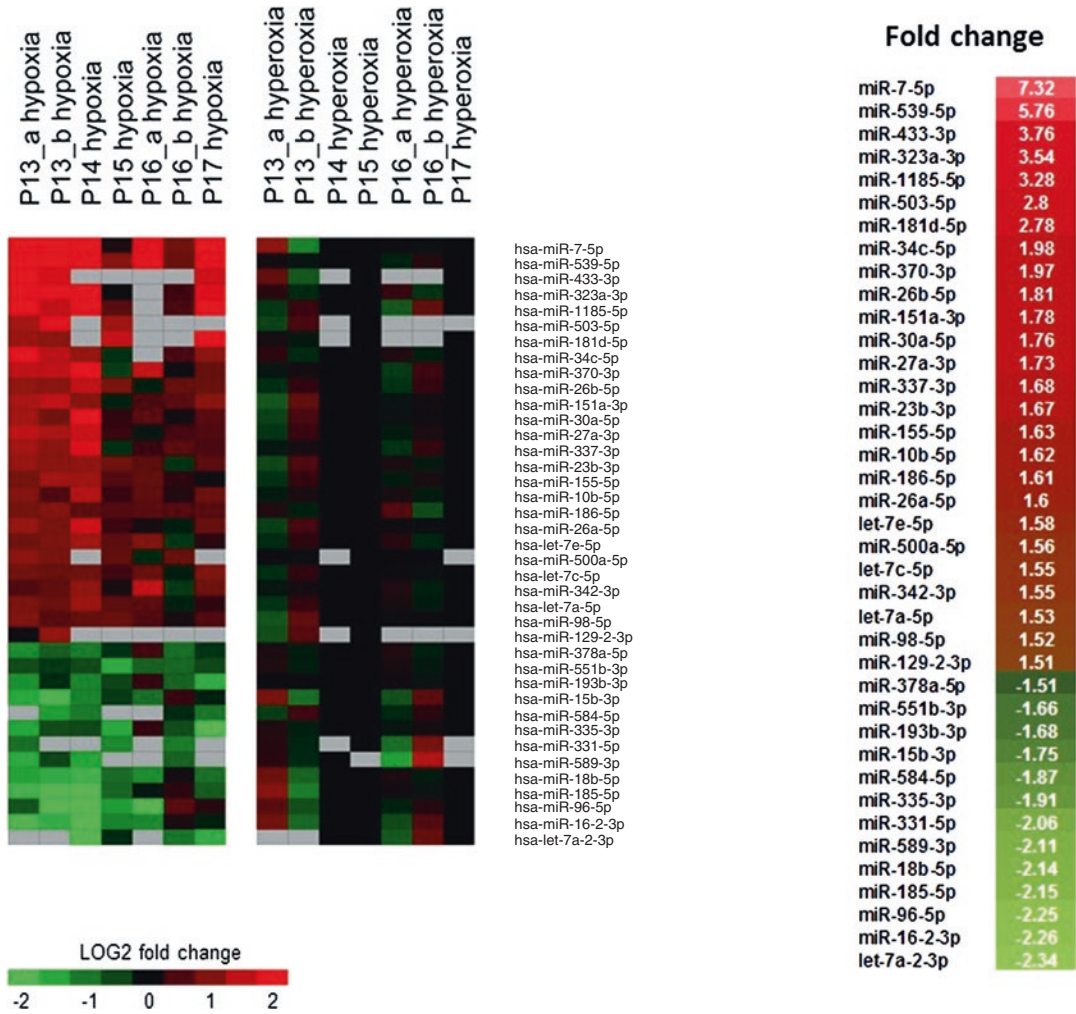


Fig. 3.2 Hypoxia regulates microRNA expression in human carotid bodies. Heatmap shows microRNAs that are differentially expressed by >1.5-fold and with t-test p value <0.05 in hypoxia treated CB slices compared to the average of the hyperoxia treated control slices (left panel). Color bar shows fold change on a Log2 scale in red for upregulation and green for downregulation.

MicroRNAs with no data on their expression are indicated in gray. Average fold-change in hypoxia treated CB slices compared to the average of the hyperoxia treated control slices are shown in the right panel. The differentially expressed miRNAs are ranked by fold change in descending order. P13-P17, individual CB slices from five different patients

3.3.2 The miRNAome of the Human Carotid Body Is Highly Distinct from Other Human Tissues

The miRNA expression profile of human CBs remains poorly understood, yet this is important for the understanding of the underlying functions of the CB itself. To address what the CB expressed microRNAs may be involved with in terms of CB

biology, we used the control CB slices incubated under hyperoxia that approximate for well oxygenated normoxic conditions and compared their miRNA profiles with those of different tissues. For this purpose, we used the human miRNA tissue atlas that consists of microarray-based miRNA expression from 61 tissue biopsies of two individuals (Ludwig et al. 2016).

We have examined the relation between the miRNA signature of CBs and diverse body tissues

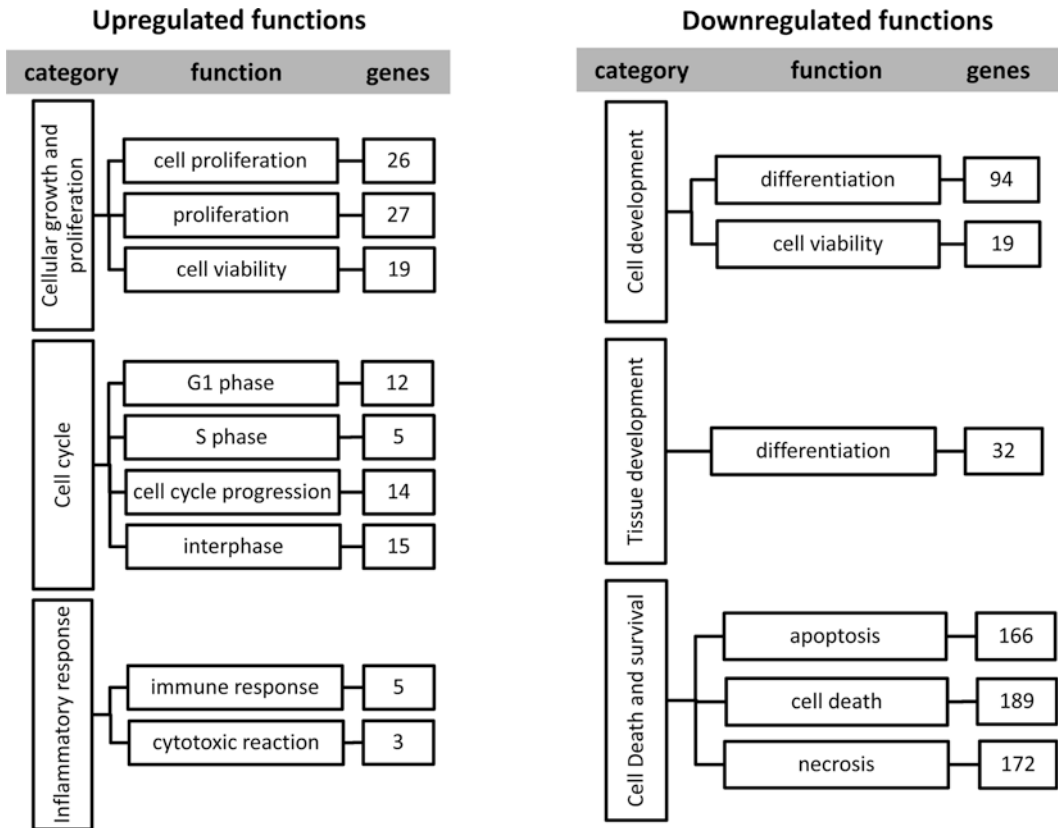


Fig. 3.3 Hypoxia regulated microRNAs target mRNAs that are involved in diverse cellular functions. Messenger RNA targets of the hypoxia regulated microRNAs were identified using the Ingenuity pathway (IPA) MicroRNA Target Filter and further subjected to the IPA core analysis for identification of gene ontology and bio-

logical pathways. Top ranking biological and cellular functions with Fisher's Exact Test $p < 0.05$ are shown in the dendrograms with the number of microRNA target genes predicted to be upregulated (left panel) and downregulated (right panel) in each functional category indicated

using principal component analysis (Fig. 3.4, right panel). We found all CB samples grouping closely together suggesting their miRNA profiles were consistent and similar to each other and there was comparatively little variation in the CB samples from different patients themselves. However, the grouping of CB samples was highly distinct with clear separation from other tissues. To further examine the relationship of the CBs with respect to the other body tissues, we next carried out hierarchical clustering analysis. The heatmap showing the clustering and miRNA expression patterns confirm the distinctiveness of the CBs from the other tissues (Fig. 3.4, left panel). Interestingly, despite the large differences between the CBs and other tissues, the CB

microRNA signature appears to be most closely linked to the brain tissues supporting that they may have the most similar developmental origin (Hempleman and Warburton 2013).

3.4 Discussion

Cellular adaptation to hypoxia involves a complex interplay of transcriptional, post-transcriptional and post-translational events aimed at maintaining cellular homeostasis. In addition to the known regulatory mechanisms, another layer of regulation represented by non-coding RNAs including miRNAs, appears to play an important role in the hypoxic response. This

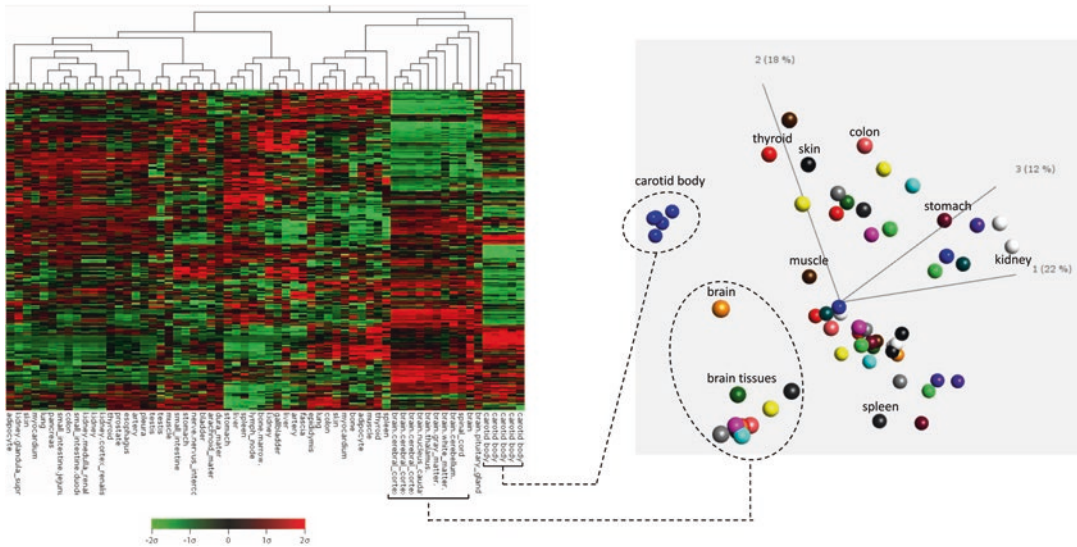


Fig. 3.4 The human carotid body miRNAome is most closely related to those of brain tissues. Heatmap (left panel) shows mean-centered, sigma-normalized ranks of miRNA expression data of individual patient CB samples under hyperoxia and human tissue expression data from the Human miRNA tissue atlas. Normalized gene expres-

sion data is shown in red for upregulation and green for downregulation over the mean. The right panel shows principle component analysis of the human carotid bodies and human tissue miRNA expression data (see Materials and Methods for details)

study is the first attempt to decipher the miRNA response under hypoxic conditions and also to map the miRNAome of primary human CBs.

Comparison of the differentially expressed CB miRNAs under hypoxic conditions with hypoxamiRs identified in other studies shows only small overlaps. The upregulation of miR-27a-3p, 26a, 7, 98, 155, let-7e and downregulation of let-7a-2-3p, miR-15b, 18a are often included in the miRNA signature of hypoxia (Kulshreshtha et al. 2007; Nallamshetty et al. 2013), and we found this was also the case for human CBs. However, other prominent constituents of the hypoxia miRNA signature such as miR-210 that is targeted by HIF-1 α (Chan and Loscalzo 2010) was not affected in our CB model of hypoxia.

HIF-1 α is one of the key downstream effectors of the oxygen response in cells that mediates their adaptation to hypoxic stress (Poellinger and Johnson 2004). Its expression is regulated by miRNAs amongst other factors. In particular, miR-18b is predicted to bind to the 3'-UTR of HIF-1 α and thus inhibit *HIF1A* mRNA translation (Chen et al. 2016). Incidentally, under our

experimental conditions miR-18b is downregulated by hypoxia, which may hypothetically lead to an increase in HIF-1 α levels, thereby completing a positive feedback loop. It remains to be investigated whether regulation of HIF-1 α level and activity does indeed occur in the human carotid body under hypoxia.

Along with the downregulation of miR-18b, hypoxia also induces the expression of another HIF-1 α negative regulator miR-155. As HIF-1 α itself is known to control the expression of miR-155, this mechanism may in principle represent a negative feedback loop to dampen and prevent runaway activation of hypoxia signaling (Bruning et al. 2011). Interestingly, miR-155 is also implicated in the activation of pro-inflammatory agents such as TNF α and interleukin 6 (IL-6) and in general the activation of innate immunity as well as positive regulation of antigen presentation (Rodriguez et al. 2007; Tili et al. 2007). Therefore, the upregulation of miR-155 may be one of the factors involved in the hypoxia-mediated release of pro-inflammatory cytokines including TNF α and IL-6 from the identical set of human CB

slices characterized in our previous report (Kahlin et al. 2014).

It has been shown that enhanced HIF-1 α signaling in the CB promotes hyperplasia (Bishop et al. 2013; Hodson et al. 2016). The analysis of biological processes potentially targeted by the differentially expressed miRNAs under hypoxia indicates upregulation of proliferative pathways with concomitant downregulation of differentiation and cell death related processes (Fig. 3.3). It is, therefore, possible that the phenomenon of hypoxic CB hyperplasia (Saldana et al. 1973; Kay and Laidler 1977) may be in part attributed to hypoxia-driven miRNA regulation.

This is the first study to demonstrate the global expression of miRNAs in the human carotid body where no data is presently available including for animal CBs. Similar to the human CB transcriptome, the CB miRNAome appears to have a unique profile as compared to other body tissues, which reflects its highly specialized functional status. Similar to the CB transcriptome (Mkrtchian et al. 2012), the human CB miRNAome shares to a certain extent the miRNA expression patterns of brain tissues. One of the highly expressed miRNAs in both the CB and the brain is miR-29b, which has been shown to be important for neuronal maturation (Kole et al. 2011). A group of brain-specific let-7 miRNAs and also miR-7 that are important for neuronal differentiation are also highly expressed in CBs (Nowak and Michlewski 2013; Zhu et al. 2016). Therefore, not only do these microRNAs demarcate the neural origin of the CBs, they may also direct the primary function of the CB in neural signaling.

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