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Hypoxia response and microRNAs: no longer two separate worlds

Mircea Ivan [a, *], Adrian L. Harris [b], Fabio Martelli [c], Ritu Kulshreshtha [a]

[a] *Molecular Oncology Research Institute, Tufts Medical Center, Boston, MA, USA*
[b] *Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, OX3 9DU, UK*
[c] *Laboratorio di Patologia Vascolare, Istituto Dermopatico dell'Immacolata, IRCCS, Rome, Italy*

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Abstract

MicroRNAs (miRs) are short non-coding transcripts involved in a wide variety of cellular processes. Several recent studies have established a link between hypoxia, a well-documented component of the tumour microenvironment, and specific miRs. One member of this class, miR-210, was identified as hypoxia inducible in all the cell types tested, and is overexpressed in most cancer types. Its hypoxic induction is dependent on a functional hypoxia-inducible factor (HIF), thus extending the transcriptional repertoire of the latter beyond 'classic' genes. From a clinical standpoint, miR-210 overexpression has been associated with adverse prognosis in breast tumours and been detected in serum of lymphoma patients and could serve as a tool to define hypoxic malignancies. We discuss the role of miR-210 and its emerging targets, as well as possible future directions for clinical applications in oncology and ischaemic disorders.

Keywords: miR-210 • hypoxia • cancer • ischemia • microRNAs • HIF

Introduction: miRs, small molecules with wide impact

miRs are a family of short non-coding transcripts involved in the regulation of at least a third of all translated genes [1–3]. They are expressed as primary transcripts, which are subsequently processed by the Drosha RNase, thus generating the hairpin-shaped precursor miRs (pre-miRs). Pre-miRs are then cleaved by another RNase III (Dicer), which leads to the formation of mature duplexes (19–24-nucleotide long). One of the two strands is selectively transferred to the RNA-induced silencing complex (RISC), which interferes with gene expression at post-transcriptional level [4]. The classic view of miR action was that in mammalian cells miRs act predominantly, if not exclusively, by blocking the translation of mRNA targets. However, more recently, it has become apparent that the action is more complex, with mRNA degradation being quite frequent, in a similar fashion to siRNAs [5, 6]. To further increase the intricate nature of miR action, cases of enhanced protein translation have been recently reported [7].

miRs continue to be at the centrestage of the 'non-coding RNA revolution', currently being suspected to regulate virtually all known cellular mechanisms, such as cell differentiation, proliferation, death and metabolism [8–11]. An increasing number of miRs have been associated with the various steps of tumourigenesis [12]. On the one hand, specific miR expression profiles have been associated with human cancers, and in some cases correlated with clinico-pathological features [13–18]. On the other hand, mechanistically individual miRs can function as *bona fide* oncogenes (such as miR-17-92 [19], miR-10b [20], miR-21 [21, 22],) or tumour suppressor genes (such as miR-15-16 clusters [23]).

*Correspondence to: Dr. M. IVAN,
800 Washington Street,
Box 5609, Boston, MA 02111, USA.
Tel.: (617) 636-7514
Fax: (617) 636-6127
E-mail: mivan@tuftsmedicalcenter.org

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A role for hypoxia in the regulation of miR expression

Recent studies have shed significant light into the regulation of miR expression and information is accumulating on the impact of specific stresses on the 'miR-nome'. Arguably, the best-documented cellular stress is oxygen deprivation (hypoxia), which is of relevance for a variety of diseases of major impact [24].

Hypoxia is an essential feature of the neoplastic microenvironment. Tumours with extensive low oxygen tension tend to exhibit poor prognosis and resistance to conventional therapy [25]. Moreover, hypoxia is also a crucial pathogenic component of major cardiovascular diseases, such as myocardial infarction and stroke [26]. The molecular mechanisms of response to oxygen deprivation are extremely complex, a key role being played by hypoxia-inducible factor (HIF), which orchestrates an expression program involving in excess of 100 genes.

While gene induction by low oxygen has arguably dominated hypoxia research, more recently the study of gene repression has received increasing attention [27–30]. One of the interesting features of the latter process is its relative selectivity. Thus, a large percentage of genes continue to be expressed at quasi-normoxic levels, while the translation(transcription of others is significantly suppressed. It is entirely conceivable that specific miRs could be a part of this process.

Studies from several groups identified a variety of hypoxia-regulated miRs, providing a link between a tumour-specific stress factor and gene expression control [31–39]. The one miR that all the studies had in common was miR-210. For example, one group [31] identified a wide set of hypoxia-induced miRs in breast- and colon-cancer cells, which in addition to miR-210 included miR-21, 23a, 23b, 24, 26a, 26b, 27a, 30b, 93, 103, 106a, 107, 125b, 181a, 181b, 181c, 192, 195 and 213. Of these, only miR-30b, 93 and 181b were independently confirmed by a separate study [38]. A more recent set of data identified only three miRs (miR-210, ambi-miR-7105 and mmu-miR-322–3p), which showed at least 2-fold induction in response to hypoxia [33]. In non-cancer cells, miR-210 was identified as a key player of endothelial cells response to low oxygen tension [34], and therefore this miR is emerging as a universal responder to hypoxia, with likely deep biological impact in the response to this type of stress.

Finally, according to two studies, hypoxia can also lead to miR down-regulation, including: miR-15b, 16, 19a, 20a, 20b, 29b and 197 [38, 39]. Whether these represent specific targets of HIF, or this process is simply the result of cell cycle arrest, is not known at this point.

As results from above, beyond general agreement with regards to miR-210, there has been rather limited overlap in miRs regulated by hypoxia. These discrepancies are not necessarily surprising, and could be explained by the differences in cells examined, technology employed, differences in the thresholds and time investigated, as well as oxygen concentrations [35, 36].

In most tumours, the expression of miR-210 is significantly up-regulated compared to the corresponding non-malignant tissue [14, 16, 18, 33]. The exception, according to one study, is represented by the ovarian carcinomas that tend to lose the locus encoding for miR-210, and as consequence exhibit decreased expression of this miR [32]. More significantly than altered expression in cancers, miR-210 has been shown to correlate with a hypoxia signature score in human breast cancers and strongly associate with an adverse clinical outcome [33].

Role of HIF in the regulation of miR-210

A variety of transcription factors involved in the regulation of 'classic' genes are now known to regulate the expression of specific miRs, therefore miRs may be rather common targets of transcription factors. From a historic perspective, the first transcription factors shown to involve miRs are c-MYC and E2F, which activate the miR-17-92 oncogenic cluster [40–42]. Recently, miR-34a joined the targets of the transcription factor (and tumour suppressor gene product) p53, and has been shown to contribute to its function [43–45].

It is not surprising that the HIF was interrogated first with regards to a role in miR regulation. At least for miR-210, HIF is clearly a critical factor for the hypoxic induction, as determined in several studies [31, 33, 34] by multiple strategies: transfection of active HIFs, chromatin immunoprecipitation and luciferase-based reporters driven by fragments of select HRM promoters. Additionally, miR-210 hypoxic induction was shown to be dependent on the von Hippel-Lindau (VHL) tumour suppressor gene in RCC4 renal carcinoma cells. Inactivating mutations of the VHL gene block proteolytic degradation of HIF, leading to constitutive activation of hypoxia pathways, therefore further confirming the central role of HIF in miR-210 induction (Fig. 1). Finally, siRNA-mediated suppression of HIF-1 or HIF-2 in MCF7 cells led to abrogation of miR-210 induction under hypoxia. In primary endothelial cells, the situation seems more complex: the knock-down of HIF2, the main HIF species in this cell type, does not affect miR-210, while siRNAs to HIF1 effectively prevents miR-210 induction, suggesting a potential HIF isoform specificity, at least in certain cell systems [34]. Moreover, the dynamic of miR-210 induction exhibits a notable difference compared to HIF, as it continues to increase beyond the peak of HIF protein level [31, 34].

Several issues remain to be elucidated with respect to miR-210 regulation, for example the existence of other possible mechanisms. Growth factor deprivation, osmotic stress, acidosis and oxidative stress did not elicit miR-210 increase, but additional conserved candidate transcription factor sites are present in the proximity of miR-210: Oct-C, AP2, PPAR γ and E2F [35, 36]. These could potentially regulate its expression as part of the hypoxia response - in the case of Oct-4, itself a hypoxia regulate gene [46] - or as part of unrelated pathways.

While miR-210 was initially thought to be intergenic, a more recent study showed that it is in fact contained within the

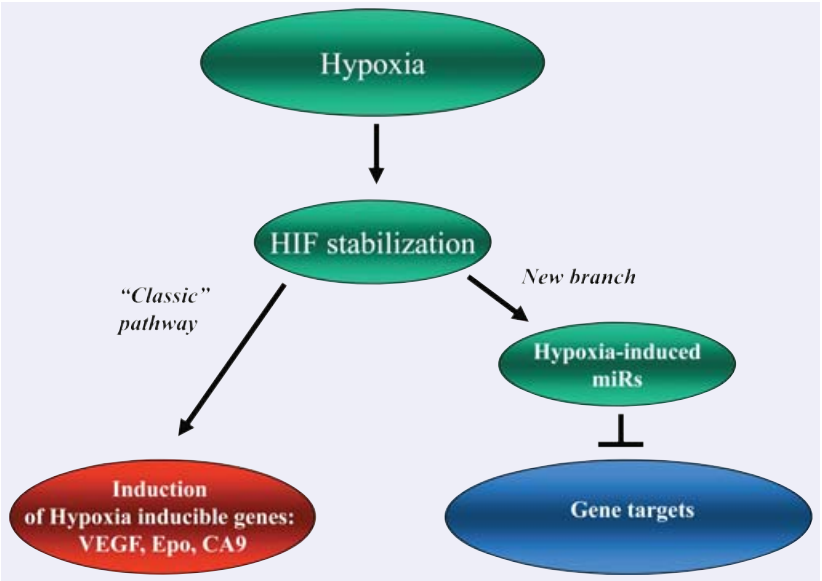

Fig. 1 The dual aspect of the hypoxic response. In addition to the well-documented hypoxia-inducible genes that are direct transcriptional targets of HIF, the response to low oxygen triggers expression of select miRs, which in turn down-regulate select genes.

sequence of a transcript with virtually unknown function (AK123483) [33]. This transcript is also hypoxia inducible, consistent with published observations of coordinated expression of miRs and the corresponding host genes. Whether this transcript encodes for a protein or plays any biological role in the hypoxia response is not known as yet.

The ongoing search for miR-210 targets

Identification of miRs targets remains without a doubt a highly complex endeavour. An increasing number of prediction programs for target identification are currently available, such as PicTar, TargetScan and Miranda [47–49]. When more than one search program is used, the *in silico* predictions often exceed one hundred genes, thus posing significant challenges to the effort to identify the biologically relevant targets.

The first clues about genes(pathways that may be targeted by miR-210 were provided by a computational analysis [50]. Over 70 targets of miR-210 were predicted in Drosophila, with a significant overrepresentation of genes involved in female gamete generation (according to Gene Ontology), including: *cut, egghead, germ cell-less, gurken, lozenge, par-1, rhomboid-4, RNA-binding protein 9, singed* and *slalom*. Biochemically, these genes belong to receptor tyrosine kinases, Notch, wingless, or hedgehog signalling, and also function at more advanced stages during embryogenesis. Based on the available prediction programs, most of these targets are not conserved in more complex organisms, such as mammals.

For human miR-210, *in silico* searches reveal a highly complex spectrum of candidate targets, including genes involved in proliferation, DNA repair, chromatin remodelling, metabolism and cell migration [31, 33–36]. This diversity, combined with the salient discrepancies between prediction programs, raises experimental challenges, one prediction being that manipulation of any individual target will fail to fully capture the phenotypic impact of the corresponding miR in low oxygen.

Despite the above challenges, biologically relevant miR-210 targets have started to emerge [32, 34] (Fig. 2). One study focusing on the response of endothelial cells to hypoxia identified EphrinA3 as target of miR-210 in hypoxic conditions [34]. miR-210 was shown to play an important role in this system, as its inactivation decreases the ability of HUVEC cells to form capillary-like structures and migrate in response to VEGF. Ephrin ligands and their corresponding receptors are known to be involved in the development of the cardiovascular system, and at least for the EFNA1/EphA2 system there is evidence for involvement in VEGF signalling/angiogenesis [51–53]. The finding that EFNA3 inhibition is necessary for miR-210-mediated stimulation of tubulogenesis suggests that this particular ephrin may also be part of angiogenic regulation. Whether this mechanism functions only in the cardiovascular system, or its significance can be extended to neoplastic angiogenesis, remains to beelucidated.

Another miR-210 target derived from computational analysis and subsequently backed by experimental studies is the E2F transcription factor 3 (E2F3). This protein plays a pivotal role in the control of the cell cycle [54], therefore the study potentially reveals a novel link between hypoxia and cell proliferation. However, there are limited data about a possible biological impact of this connection.

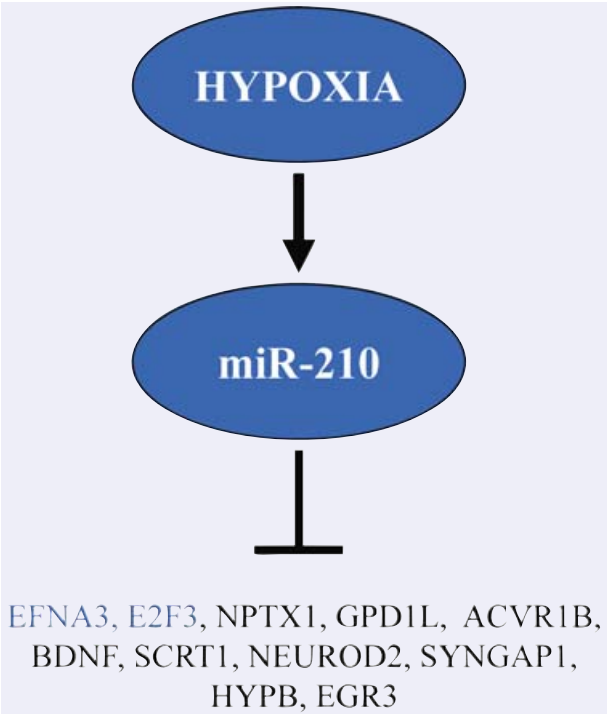

Fig. 2 Predicted wide impact of miR-210 in the hypoxic response. A selection of potential targets is shown (identified using TargetScan 4.1 and PicTar programs). Standard Gene ID nomenclature was used and experimentally confirmed targets are shown in blue.

Perspectives: towards clinical applications of miR-210 manipulation

The recent study by Camps *et al.* [33] provided the first evidence that miR-210 represents not only a marker of tumour hypoxia *in vivo*, but is also a prognostic indicator of adverse prognosis in breast cancer, and may represent a valuable drug target. Although proteins represent the overwhelming majority of therapeutic targets, recent developments of miR derivatives such as anti-miR oligonucleotides (AMOs) and locked nucleic acids (LNAs) are regarded as important steps toward clinical applications [55–59]. While classic gene therapy strategies have failed to fulfil expectations in most cases, the small size of miRs and their high rate of transduction in eukaryotic cells represent distinctive advantages. Indeed, the feasibility of oligonucleotide-based therapeutics such as microRNAs and siRNAs is already supported by ongoing clinical trials. To list only a few, Sirna, Inc. developed siRNA-027 [60], which targets VEGFR-1 and is now in a Phase II clinical trial for age-related macular degeneration. Additionally, Alnylam has initiated a Phase I clinical trial of an inhaled siRNA-based drug for the treatment of Respiratory Syncytial Virus (RSV) infections [61], and Santaris Pharma has developed SPC3649 [62], which specifically targets miR-122, which is important for hepatitis C virus replication.

In cancer, one can hypothesize that inactivation of an miR critically important for the response to lox oxygen could have a beneficial effect against a tumour compartment notoriously resistant to therapy [24–26]. miR-210 analysis also serves as proof of principle for a novel class of non-invasive cancer diagnostic tools, as it is readily detectable in the serum from patients with diffuse large B-cell lymphoma (DLBCL) *versus* healthy controls [63].

The impact of miR-210 (and by extension, of other less thoroughly validated hypoxia-regulated miRs) may not be limited to cancer. Hypoxia represents a central component of other clinical conditions with major impact on morbidity and mortality, such as cardiac ischaemia and cerebrovascular diseases. Although to date there are no formal data to substantiate a mechanistic role in such disorders, miR-210 was recently found up-regulated in a mouse model of cardiac hypertrophy/cardiac failure and in response to brain transient focal ischaemia in rats [64–66].

Another example of disease with a hypoxic component is preeclampsia, which is associated with accumulation of HIF-alpha proteins at placental level [67]. Consistent with its status of HIF target, miR-210 was found up-regulated in placentas from patients with preeclampsia, compared to normal pregnant women. Again, whether miR-210 contributes to the clinical manifestations of this condition is yet to be determined.

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