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Robustness of the Hypoxic Response: Another Job for miRNAs?

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Living organisms are constantly exposed to environmental and genetic perturbations. Biological robustness enables these organisms to maintain their functional stability in the presence of external or internal changes. It has been proposed that microRNAs (miRNAs), small non-coding regulatory RNAs, contribute to robustness of gene regulatory networks. The hypoxic response is a major and well-characterized example of a cellular and systemic response to environmental stress that needs to be robust. miRNAs regulate the response to hypoxia, both at the level of the main transcription factor that mediates this response, the hypoxia-inducible factor (HIF), and at the level of one of the most important systemic outcomes of the response: angiogenesis. In this review, we will take the hypoxic response as a paradigm of miRNAs participating in circuits that provide robustness to biological responses. Developmental Dynamics 241:1842–1848, 2012. © 2012 Wiley Periodicals, Inc.

Key words: robustness; microRNAs; hypoxia; hypoxia-inducible factor; angiogenesis

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miRNAs CONFER ROBUSTNESS TO DEVELOPMENTAL AND PHYSIOLOGICAL PROCESSES

Feedback and Feedforward Loops Provide Biological Robustness

A remarkable feature of developmental and physiological processes is that they are highly reproducible, even under conditions of genetic and environmental variability (Barkai and Shilo, 2007; Herranz and Cohen, 2010). Biological robustness is a property inherent to all living organisms that enables stability. The concept of biological robustness refers to the ability of a biological system to maintain its functions despite endogenous or exogenous perturbations (Kitano, 2004; Silva-Rocha and de Lorenzo, 2010). It should be stressed that robustness not only implies the capacity of a system to buffer perturbations to maintain phenotypic stability, but also contributes to achieve predictable and reproducible responses, allowing phenotypic switches to take place efficiently regardless of perturbations (Freeman, 2000; Kitano, 2004; Ebert and Sharp, 2012). When considering biological systems, noise or variation can have different sources, including stochastic changes in gene expression (i.e., in transcription, translation, and RNA or protein degradation), as well as environmental fluctuations (Raser and O'Shea, 2005).

Among the mechanisms that explain robustness of biological networks is the presence of feedback and feedforward loops (Freeman, 2000; Hartman et al., 2001; Kitano, 2004; Graham et al., 2010; Osella et al., 2011; Pelaez and Carthew, 2012). Bioinformatic analyses indicate that certain loops or circuits that constitute complex gene regulatory networks appear more often than expected by chance; these recurrent circuits have been named "network motifs." Interestingly, feedback and feedforward loops have been found to be network motifs in different organisms, from bacteria to humans (Milo et al., 2002; Shen-Orr et al., 2002; Alon, 2007).

Feedback can be defined as the ability of a system to modify its response

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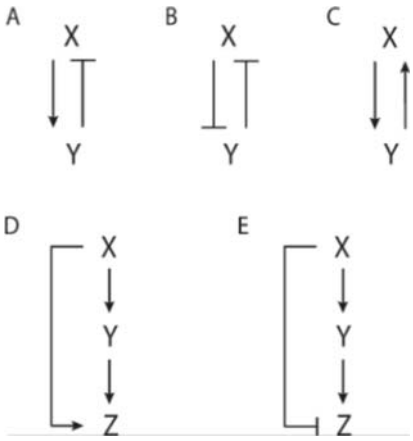

Fig. 1. Feedback and feedforward loops. Only direct interactions are shown, but loops can also be indirect. A: In single-negative feedback loops, the signal (X) induces a factor (Y) that negatively regulates the initial signal. B: In double-negative feedback loops, both factors (X and Y) inhibit each other. C: In positive feedback loops, both factors (X and Y) induce each other. D,E: In feedforward loops, the upstream factor (X) regulates a target gene (Z) both directly and indirectly, via the third component of the loop (Y). In the coherent configuration (D), the signs of the direct and indirect paths are the same. In the case of an incoherent pattern (E), the signs of the direct and indirect paths are opposite. In feedforward loops, the sign of each interaction can be positive or negative, and therefore eight different configurations are possible. Only one example of a coherent and one example of an incoherent pattern are shown.

by monitoring itself (Freeman, 2000). Depending on the overall sign of the interactions, feedback loops can be positive or negative. Single-negative feedback occurs when a signal limits itself by inducing its own inhibitor (Fig. 1A), and is usually related to buffering responses (Becskei and Serrano, 2000; Freeman, 2000). In mutual- or double-negative feedback loops, the signal represses it own repressor (Fig. 1B), conferring robustness by stabilizing gene expression in one state, which can contribute to bistability (Ebert and Sharp, 2012; Pelaez and Carthew, 2012). Positive feedback occurs when a signal amplifies itself, or the cellular response it triggers (Fig. 1C), and confers robustness by ensuring an "all-or-none" outcome, often leading to a bistable system (Kitano, 2004; Graham et al., 2010; Herranz and Cohen, 2010).

Feedforward loops are composed of at least three elements. The upstream factor regulates the target gene through two parallel paths: one is direct and the other one is indirect. In the indirect path, the upstream factor regulates a third component of the loop, which in turn regulates the target gene (Fig. 1D,E). Depending on the sign of the two paths, feedforward loops can be divided in two categories: coherent and incoherent loops. In the coherent pattern, the sign of the direct path is the same as the overall sign of the indirect path (Fig. 1D), while in incoherent structures the signs of the direct and indirect paths are opposite (Fig. 1E). Each of the three interactions in the feedforward loops can be positive or negative, defining eight possible configurations (four coherent and four incoherent) (Mangan and Alon, 2003). Indeed, depending on the configuration, these loops can have different properties and functions, including delaying or accelerating the response and generating pulse-like dynamics (Mangan and Alon, 2003; Alon, 2007). Feedforward loops can confer robustness through different mechanisms. They can buffer the impact of noise on target gene expression, contributing to enhance the robustness of the network (Shen-Orr et al., 2002; Hornstein and Shomron, 2006; Wu et al., 2009a; Osella et al., 2011). In an incoherent feedforward loop, fluctuations of the upstream factor are uncoupled from the final output of the target gene, because the intermediate component of the indirect path of the loop will compensate changes in the input signal (Ebert and Sharp, 2012). Thus, only when the upstream factor reaches certain levels will it induce changes in the expression of the target gene (Pelaez and Carthew, 2012). Coherent feedforward loops can also increase the robustness of the circuit activating, or inhibiting, redundantly the expression of the downstream factor (Ebert and Sharp, 2012; Pelaez and Carthew, 2012).

miRNA-Containing Circuits Contribute to the Robustness of Biological Systems

Genome-scale analyses revealed that feedback and feedforward loops that contain miRNAs are also recurrent network motifs in large gene networks (Tsang et al., 2007; Martinez et al., 2008; Re et al., 2009), so that miRNAs can provide biological robustness by participating in these loops (Hornstein and Shomron, 2006; Wu et al., 2009a; Herranz and Cohen, 2010; Osella et al., 2011; Pelaez and Carthew, 2012). miRNAs are small (approximately 22 nucleotides) non-coding single-stranded RNAs that regulate gene expression post-transcriptionally. They are generally synthesized by the RNA polymerase II, and then processed sequentially by two complexes containing Drosha and Dicer, to be finally incorporated into the RNA-induced silencing complex (RISC). The mature miRNA leads the RISC complex to recognize the target messenger RNA (mRNA) by sequence complementarity, usually found in the 3′-untranslated region (3′UTR) (Kim, 2005; Yeom et al., 2006; Jaubert et al., 2007; Inui et al., 2010). Generally, miRNAs promote degradation and/or inhibit translation of their target mRNAs (Djuranovic et al., 2011).

It is becoming increasingly evident that miRNA-dependent repression of protein translation is usually modest, with a typical down-regulation of no more than 50% (Baek et al., 2008; Selbach et al., 2008). Moreover, in general, individual miRNA knock-out animals don't display dramatic phenotypes under controlled laboratory conditions (Li and Carthew, 2005; Miska et al., 2007; Li et al., 2009; Alvarez-Saavedra and Horvitz, 2010). This is sometimes referred to as the paradox of miRNAs: miRNAs posses a high degree of evolutionary conservation, and yet the majority of them seem to have an apparently non-essential role in cell and organism viability. Interestingly, in spite of these knock-outs being "apparently normal," in many cases they show important phenotypic alterations under different stress conditions (van Rooij et al., 2007; Li et al., 2009; Leung and Sharp, 2010; Mendell and Olson, 2012).

A paradigmatic example of a miRNA granting robustness during development is provided by Drosophila melanogaster miR-7, which participates in interlocked feedback and feedforward loops involved in the differentiation of photoreceptors from their retinal precursor cells (Fig. 2) (O'Neill et al., 1994; Rebay and

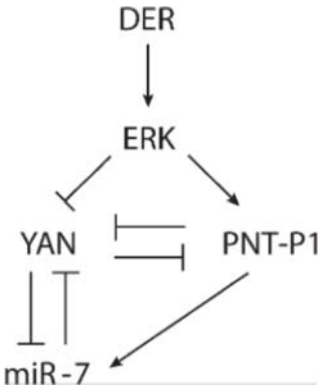

Fig. 2. Regulation of photoreceptor differentiation in Drosophila melanogaster. Yan is a transcriptional repressor that prevents retinal cell differentiation by competing with another transcription factor, Pointed-P1 (Pnt-P1). Epidermal growth factor receptor (DER) signaling activates the extracellular signal-regulated kinase (ERK), leading to Yan degradation and Pnt-P1 accumulation. Yan acts as a repressor of Pnt-P1 function, competing for the binding to specific enhancer regions of target genes, and Pnt-P1 directly represses Yan transcription. One of the Yan and Pnt-P1 target genes is miR-7, whose transcription is activated by Pnt-P1 and inhibited by Yan. In turn, miR-7 directly binds to the 3′UTR of Yan mRNA, inhibiting its translation. This network includes two interlocked double-negative feedback loops, one composed of Yan and miR-7, and the other one of Yan and Pnt-P1. The network also includes three coherent feedforward loops that involve Yan, Pnt-P1 and miR-7, which prevent changes in the network state in response to non-persistent fluctuations in the levels of Pnt-P1 and Yan (Pelaez and Carthew, 2012).

Rubin, 1995; Gabay et al., 1996; Xu et al., 2000; Rohrbaugh et al., 2002; Li and Carthew, 2005; Li et al., 2009). This complex network has been mathematically modeled, predicting that it behaves as a bistable system (Graham et al., 2010). Remarkably, under uniform laboratory conditions, miR-7 mutant flies show normal photoreceptor differentiation, but when these mutant larvae are exposed to fluctuating temperature conditions, abnormalities in photoreceptor differentiation occur (Li et al., 2009; Pelaez and Carthew, 2012). Therefore, both computational and experimental evidence support a role of miR-7 in providing robustness to the network that controls retinal cell differentiation.

Even thought biological robustness is usually associated with phenotypic stability, developmental and physiological responses to stress also need to be reproducible and robust. In the next two sections, we will discuss the regulation of the hypoxic response by miRNAs. We will argue that miRNAs participate in positive and negative feedback loops, as well as in feedforward loops, which likely provide robustness to the transcriptional response to hypoxia and angiogenesis as an overall process.

miRNAs PARTICIPATE IN NEGATIVE AND POSITIVE FEEDBACK LOOPS THAT REGULATE HIF-a EXPRESSION

The transcriptional response to hypoxia is mainly mediated by a family of transcription factors called hypoxia-inducible factors (HIF) (Maxwell et al., 1993; Wang and Semenza, 1993, 1995; Majmundar et al., 2010). HIF directly regulates the expression of genes involved in the adaptation to hypoxia, decreasing oxygen consumption and improving oxygen delivery to cells, by modifying metabolism, promoting erythropoiesis, vasculogenesis, angiogenesis and vasodilation, among other mechanisms (Semenza, 2007; Lisy and Peet, 2008). HIF is an a/b heterodimer (Wang et al., 1995), in which the b subunit is constitutive and the a subunit is negatively regulated by oxygen. Regulation of HIF-a occurs at different levels, including proteasomal degradation (Huang et al., 1998; Maxwell et al., 1999), recruitment of transcriptional co-activators (Hewitson et al., 2002; Lando et al., 2002) and subcellular localization (Kallio et al., 1998). Regulation of HIF-a protein stability is the most important mechanism controlling HIF activity. In normoxia, two prolyl residues of HIF-a are hydroxylated in a reaction catalyzed by specific prolyl hydroxylases, termed PHD1, PHD2 and PHD3, which use molecular oxygen and 2-oxoglutarate as co-substrates, and Fe(II) as a co-factor. Upon hydroxylation, HIF-a is polyubiquitinated and degraded at the 26S proteasome. Since PHDs utilize dioxygen as a co-substrate in the catalytic reaction, in hypoxia HIF-a is not hydroxylated, stabilizes and accumulates in the nucleus promoting target gene transcription (Jaakkola et al., 2001; Bruick, 2003).

A wide array of miRNAs have been linked to the transcriptional response to hypoxia. There is an extensive and growing list of miRNAs regulated by hypoxia, either positively or negatively, in different experimental settings (Kulshreshtha et al., 2008; Loscalzo, 2010; Bussolati et al., 2012; Du et al., 2012; Fang et al., 2012; Guo et al., 2012; Voellenkle et al., 2012). Other miRNAs are hypoxia-independent, but are capable of negatively regulating HIF; these include miR-107, miR-17-92 and miR-519c, whose expression is induced by p53, c-myc and the hepatocyte growth factor (HGF), respectively (Taguchi et al., 2008; Cha et al., 2010; Yamakuchi et al., 2010). A third group of miRNAs are those regulated by hypoxia that can in turn regulate HIF, participating in either negative or positive feedback loops. Different examples of feedback loops containing miRNAs that regulate HIF expression will be discussed in detail below.

Bruning and coworkers demonstrated in Caco-2 cells exposed to prolonged hypoxia that HIF-1 induces miR-155, which in turn represses HIF-1a expression, through direct binding to the 3′UTR of its mRNA (Fig. 3A). This single-negative feedback loop, where HIF-1a induces its own inhibitor, contributes to set a limit to the activity of HIF-1a in cells exposed to prolonged periods of hypoxia (Bruning et al., 2011).

An example of a miRNA participating in a double-negative feedback loop that regulates HIF-1a expression is provided by miR-20b (Lei et al., 2009) (Fig. 3B). In H22 liver cancer cells exposed to hypoxia, miR-20b levels decrease in a HIF-1-dependent manner, while miR-20b directly targets HIF-1a mRNA, negatively regulating its translation. Thus, whereas in normoxia miR-20b levels are high and repress HIF-1a, in hypoxia HIF-1a accumulation leads to inhibition of miR-20b expression, further enhancing HIF-1a levels.

miR-210 is considered a prototypical hypoxia-induced miRNA, as it has been shown to be consistently upregulated in different cell types under low oxygen tension, in an HIF-dependent manner (Chan and Loscalzo, 2010; Huang et al., 2010; Gorospe et al., 2011). In HEK 293A cells, miR-

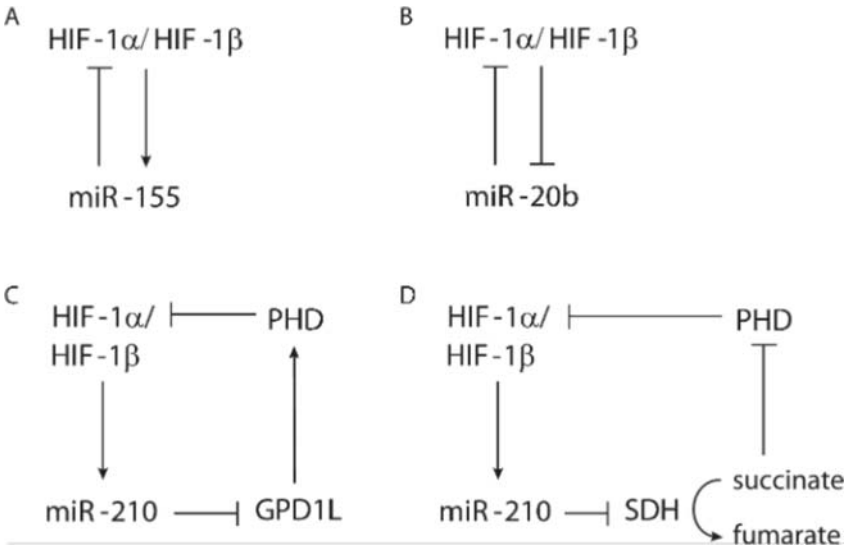

Fig. 3. Feedback loops that contain miRNAs regulate HIF-1a expression. A: HIF-1 and miR-155 are involved in a single-negative feedback loop, where miR-155 is induced by HIF-1 and in turn limits HIF-1a expression. B: HIF-1 and miR-20b participate in a double-negative feedback loop. In hypoxia, HIF-1 represses miR-20b expression, whose down-regulation contributes to further HIF-1a accumulation. C,D: miR-210 participates in two different positive feedback loops regulating HIF-1a expression. In one of the loops (C), HIF-1 induces miR-210 expression in hypoxia, which in turn down-regulates GPD1L translation, leading to decreased PHD activity, ultimately promoting further accumulation of HIF-1a (C). In the other positive feedback loop (D), miR-210 expression is also induced by HIF-1 in hypoxia; miR-210 targets and down-regulates the subunit D of the succinate dehydrogenase (SDH), which catalyzes the oxidation of succinate to fumarate. When SDHD levels decrease, succinate accumulates, leading to PHD activity inhibition, which in turn promotes HIF-1a stabilization (D). These two positive feedback loops (C, D) reinforce the accumulation of HIF-1a in hypoxia, and, therefore, the hypoxic response.

210 binds to the 3′UTR of the glycerol-3-phosphatedehydrogenase1-like (GPD1L) mRNA and down-regulates its translation (Kelly et al., 2011). Independently of its dehydrogenase activity, GPD1L is able to increase PHD activity, leading to augmented proteasomal degradation of HIF-1a (Fig. 3C). Therefore, in normoxia, where HIF-1a activity is suppressed, low levels of miR-210 result in high GPD1L expression, which induces PHD activity, enhancing HIF-1a degradation. In hypoxia, HIF-1a protein accumulates, inducing miR-210 expression, leading to decreased GPD1L levels. This, in turn, potentiates inhibition of PHD activity, increasing HIF-1a protein levels. Thus, miR-210 participates in a positive feedback loop, in which HIF-1 induces miR-210 expression that, in turn, indirectly enhances HIF-1a protein stability.

miR-210 is induced in late stages of lung cancer progression (Puissegur et al., 2011). In lung adenocarcinoma A549 cells, the subunit D of the succinate dehydrogenase complex (SDHD), which is a member of the electron transport chain, is another bona fide miR-210 target mRNA. miR-210 is induced in hypoxia in an HIF-1-dependent manner, leading to inhibition of SDHD expression. As a consequence of SDHD silencing, succinate accumulates in the cell, inhibiting PHD activity, which results in further stabilization of HIF-1a (Fig. 3D). These findings suggest that miR-210 is part of another positive feedback loop, regulating HIF-1a indirectly through SDHD.

We have discussed examples of miRNAs that participate in feedback loops that regulate the hypoxic response. As we have seen, depending upon the type of feedback loop they are involved in, miRNAs can either contribute to restore the original gene expression pattern, or to reinforce a new expression program (Leung and Sharp, 2010; Mendell and Olson, 2012). If the miRNA participates in a single-negative feedback loop (e.g., Fig. 3A), it will tend to restore the original state of the system, provoking desensitization of the response (Pelaez and Carthew, 2012). On the other hand, double-negative (e.g., Fig. 3B) or positive (e.g., Fig. 3C and D) feedback loops reinforce the activation of the response to hypoxia. Thus, miRNAs participating in single-negative, double-negative or positive feedback loops can provide robustness to the hypoxic response through different molecular mechanisms.

miRNAs PARTICIPATE IN THE REGULATION OF HIF-DEPENDENT ANGIOGENESIS

Large organisms, with sizes beyond the oxygen diffusion limit, have developed delivery systems to transport oxygen to different tissues. In insects, such as Drosophila, oxygen transport occurs through the tracheal system, a complex network of ramified tubules that reaches every tissue of the body. Similarly, in vertebrates, oxygen is transported by the bloodstream through vessels that reach organs and tissues (Dunwoodie, 2009; Fraisl et al., 2009). Vascular formation and remodeling occur by means of different mechanisms: Vasculogenesis is the process of de novo formation of blood vessels, from vascular progenitor cells; angiogenesis is defined as the generation of new capillaries from pre-existing vessels (Rey and Semenza, 2010). Hypoxia is an essential stimulus for both vasculogenesis and angiogenesis (Simon and Keith, 2008), and HIF directly induces the transcription of the vascular endothelial growth factor (VEGF), a central player in these two processes (Forsythe et al., 1996). Remarkably, the role of HIF in angiogenesis is far more general, as it controls hypoxia-dependent expression of most critical angiogenic factors, including stromal-derived factor 1 (SDF1), angiopoietin 1 and 2 (ANGPT1 and ANGPT2), placental growth factor (PGF), and platelet-derived growth factor B (PDGFB) (Kelly et al., 2003; Ceradini et al., 2004; Rey and Semenza, 2010). Importantly, adult angiogenesis can be in some cases physiological (i.e., in skeletal growth, menstrual cycle, and pregnancy), or in others pathological, such as in tumorigenesis and

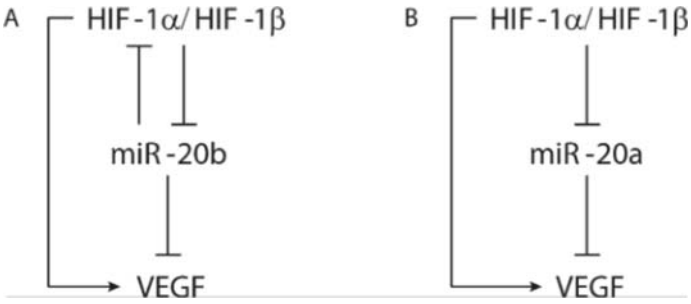

Fig. 4. Feedforward loops that include miRNAs regulate angiogenesis. A: HIF-1 induces VEGF expression both directly, at a transcriptional level, and indirectly, by down-regulating miR-20b expression, which in turn inhibits VEGF translation. This coherent feedforward loop is interlocked with a double-negative feedback loop between HIF-1 and miR-20b (also shown in Fig. 3B). B: Hypoxia induces VEGF expression by a HIF-1 direct transcriptional activation of VEGF, and also through an indirect path, down-regulating miR-20a, presumably in a HIF-1-dependent manner, and releasing the repression exerted by this miRNA on VEGF. These coherent feedforward loops regulate VEGF protein levels and, ultimately, angiogenesis.

ischemic diseases (Shi, 2009; Lu and Kang, 2010; Chung and Ferrara, 2011).

Growing evidence suggests that specific miRNAs participate in different aspects of the angiogenic response, from proliferation and migration of endothelial cells to morphogenesis of the angiogenic sprouts (Wu et al., 2009b). Importantly, some miRNAs are pro-angiogenic, whereas others have anti-angiogenic effects. The complete analysis of the miRNAs that regulate angiogenesis is out of the scope of this article, as this topic has been excellently reviewed by others (Wang and Olson, 2009; Staszel et al., 2011). Nevertheless, it is important to point out that it has not been established whether the regulation of angiogenesis by most of these miRNAs requires and/or depends on HIF. We will, therefore, discuss examples in which miRNAs regulate angiogenesis as part of feedforward loops that also include HIF.

As mentioned in the previous section, miR-20b and HIF-1 are engaged in a double-negative feedback loop in which both factors inhibit each other's expression. Noteworthy, miR-20b does not only down-regulate HIF-1a translation, but also directly targets VEGF (Lei et al., 2009). Thus, in hypoxia, HIF-1 increases VEGF expression through both a direct and an indirect path. On one hand, HIF-1 directly induces VEGF transcription (Forsythe et al., 1996), and on the other hand, it inhibits miR-20b expression, releasing the repression exerted by this miRNA on VEGF. This second circuit involving HIF-1, miR-20b and VEGF is defined as a coherent feedforward loop (Fig. 4A). Hence, by looking at the whole picture, there is a network composed of two interlocked loops: a coherent feedforward loop and a double-negative feedback loop that interplay, likely conferring robustness to the angiogenic process.

In CNE cells, the expression of miR-20a is down-regulated in hypoxia, presumably in an HIF-1-dependent manner; this miRNA in turn directly inhibits its VEGF translation (Hua et al., 2006) (Fig. 4B). Thus, a coherent feedforward loop apparently occurs, in which HIF-1 directly induces VEGF transcription (Forsythe et al., 1996) and simultaneously reduces the repression exerted by miR-20a, enhancing VEGF expression indirectly.

Thus, miRNA-mediated feedforward loops involved in angiogenesis regulation might be an additional way to provide robustness to the hypoxic response. Occurrence of robust mechanisms controlling angiogenesis is crucial to prevent an onset of the process under weak or non-persistent hypoxic stimuli, and, conversely, to ensure a timely and reproducible response under sustained hypoxic conditions.

PERSPECTIVES

In the present article, we have proposed that miRNA-containing feedback and feedforward loops regulating HIF-a expression and angiogenesis can enhance robustness of the hypoxic response. Even though bioinformatic analyses have demonstrated that these loops can provide robustness, it is important to take into account that properties of the network motifs are not exclusively dependent on qualitative interactions. Quantitative parameters, such as stoichiometry of the components and kinetics of the interactions in the loop, also determine the functionality of these motifs (Graham et al., 2010; Pelaez and Carthew, 2012). The context in which the network operates can also be critical, since circuits do not act in isolation (Ebert and Sharp, 2012). Therefore, experimental and computational evidence will be extremely relevant to confirm an actual role of miRNAs in conferring robustness to the hypoxic response.

The regulatory role of miRNAs in complex networks is only beginning to be understood. These small RNAs have unique properties as gene expression regulators, and studying their involvement in interlocked circuits and complex networks will help us to further understand their biological functions. One of the most important roles of miRNAs may be to provide robustness to the networks they are involved in, regulating many different biological processes, the hypoxic response being one of them.

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