

## Review Article

# E2F and microRNA regulation of angiogenesis

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**Abstract:** E2F family of transcription factors are best known for regulating genes involved in cell cycle control, cell proliferation, tumorigenesis, and apoptosis. Recent evidences have revealed their critical involvement in modulating cellular response to hypoxia and ischemia in a variety of physiological and pathological processes. Of particular interest are findings that E2Fs act as both regulators and targets of microRNAs that govern hypoxic/ischemic angiogenesis. This review focuses on the crosstalk between E2Fs and microRNAs that have been shown to participate in the regulation of angiogenesis, hypoxia response and ischemic disease.

**Keywords:** E2F, microRNA, angiogenesis, ischemia, hypoxia

### Introduction

Exposure of a cell or an organism to hypoxia, or decrease in the level of oxygen, leads to a massive well studied response characterized by a change of expression of certain genes [1]. Hypoxia may be a necessary part of a physiological process, as in development, or may be a characteristic of some pathological situation such as solid tumor growth or ischemic disease. One of the well characterized organism's responses to hypoxia is an increase of blood supply to tissues through angiogenesis, the process of developing new blood vessels from pre-existing ones.

Recently it became clear that many processes triggered by hypoxia are regulated by microRNAs (miRNAs, or miRs). MiRNAs are short non-coding RNAs ~22 nt long that modulate the stability and/or translation potential of their targets [2]. At present there are more than 1400 miRNAs discovered in humans (miRBase release 17, April 2011) that are predicted to target more than 60% of all mRNAs [3], and this number is constantly growing.

Most mammalian miRNAs are transcribed by RNA polymerase II into primary miRNA transcripts (pri-miRNA) that consists of one or more hairpin structures [4-5]. Many pri-miRNAs are

5'-capped and polyadenylated and often produce more than one functional miRNA. pri-miRNAs are cleaved by the nuclear microprocessor complex containing RNase III enzyme Drosha and DGCR8 protein into ~ 70 nt long hairpin precursor pre-miRNAs which are carried into cytoplasm by Exportin-5. In the cytoplasm pre-miRNAs get further processed and loaded into RISC (RNA-induced silencing complex) by the RISC loading complex (RLC). RLC is a multi-protein complex consisting of RNase III enzyme Dicer and proteins TRBP/PACT and core component Argonaut-2. The pre-miRNA is cleaved into ~ 22 nt miRNA duplex which is separated into guide and passenger strands. The passenger strand typically gets degraded, and the guide strand directs RISC to the complimentary sites within target mRNAs. Usually miRNAs basepair with their target imperfectly and induce translation inhibition. The process of target recognition by miRNAs is not clearly understood yet, but it is generally accepted that Watson-Crick pairing between the "seed region" of miRNA (between nucleotides 2-7) and 3'-UTR of target mRNA is necessary for the inhibition.

According to E2F pathway dogma, E2F transcription factors are tightly linked to cell cycle regulation and cell proliferation, controlling the expression of genes essential for transition from

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G1 to S and initiation of DNA replication [6-7]. E2F transcription factors belong to a large family consisting of eight members (E2F1-8). All family members possess signature winged-helix DNA-binding domain. E2F1-5 factors can interact with retinoblastoma family of proteins, also called pocket proteins. When E2F is bound to a pocket protein, it functions as a transcription repressor. Upon phosphorylation of pocket protein by cyclin-dependent kinases, E2Fs are released and can act as transactivators. Most E2F family members (E2F1-6) bind DNA as heterodimers with one of three dimerization partner proteins (i.e., DP1-3). Traditionally, the E2F family has been divided into activator (E2F1-3) and repressor (E2F4-8) subclasses. Alternative promoters at the E2F3 locus drive the expression of two highly related isoforms, E2F3a and E2F3b. Paradoxically, overexpression of E2F1 can promote both cell proliferation and apoptosis. The apoptotic response is p53-dependent in most cases, through transcriptional activation of p19<sup>Arf</sup> that inhibits p53 degradation and therefore indirectly increases its protein levels. Consistently, mice deficient for E2F1 were shown to have tendency to develop tumors.

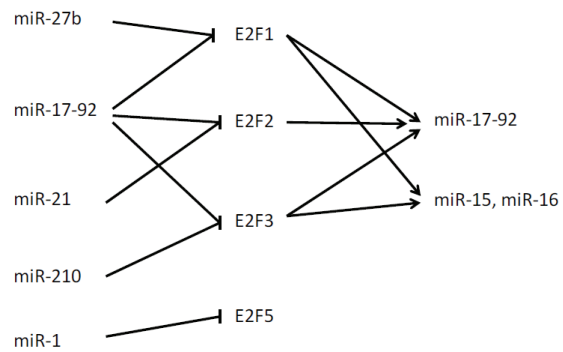
We had previously shown that E2F1 can regulate ischemic angiogenesis *in-vivo* [8]. Mice deficient for E2F1 demonstrated enhanced angiogenesis in hind-limb ischemia and tumor graft models, and we showed that the E2F1-dependent regulation of VEGF expression was responsible for this angiogenic response. Another E2F family member, E2F2 regulates endothelial function, arterial contractility, and blood pressure [9].

In recent years E2F transcription factors were identified as both targets of miRNAs and regulators of their biogenesis. In this review we describe some of well-known hypoxia-, angiogenesis-, and ischemia-related miRNAs that can regulate or are regulated by E2Fs (**Figure 1**).

### Hypoxia-induced miRNA

#### *miR-210*

Hypoxia inducible factor (HIF) is a key mediator of gene expression following hypoxia [1]. HIF is a heterodimeric complex consisting of an  $\alpha$  and a  $\beta$  subunits. HIF- $\beta$  is constitutively expressed in all cell types, but  $\alpha$ -subunit levels are tightly controlled by oxygen. Under normoxia, prolyl



**Figure 1.** Schematic representation of E2F family members and microRNA regulatory network.

hydroxylases (PHDs) catalyze hydroxylation of HIF- $\alpha$ . The hydroxylated HIF- $\alpha$  subunits are then polyubiquitinated and targeted for proteasome mediated degradation. If the level of oxygen drops, the  $\alpha$  subunit does not undergo prolyl hydroxylation and, as a result, forms a stable complex with the  $\beta$  subunit. This complex binds as a heterodimer to hypoxia response elements (HREs) in the promoter regions of hypoxia-sensitive genes to induce gene transcription. Significant number of HIF target genes have been identified and validated in humans. HIF-dependent transcriptional changes regulate a broad spectrum of cellular functions, including metabolism, proliferation, apoptosis, and angiogenesis.

A number of microRNAs have been shown to be induced by hypoxia, and the term “hypoxamiRs” have been coined to describe them [10]. One of the most consistently and robustly induced miRNA is miR-210 [11]. Its regulation has been shown in a variety of aspects of hypoxia biology: angiogenesis, apoptosis, DNA damage repair, tumor biology, cell cycle regulation and stem cell biology (reviewed in [12]). The hypoxic regulation of miR-210 was first identified in cancer cell lines and HIF-1 $\alpha$  was shown to directly bind the HRE on the proximal miR-210 promoter [13]. miR-210 has been shown to play an important role in endothelial cell response to hypoxia. It is strongly upregulated by hypoxia in HUVECs and shown to regulate VEGF-induced chemotaxis and ability of endothelial cells to form capillary-like structures [14]. The importance of miR-210 regulation was confirmed in *in-vivo* studies. miR-210 had been shown to be upregulated in mouse hind limb ischemia and rat brain tran-

sient focal ischemia [15-16]. Given the universal regulation of miR-210 by hypoxia, not surprisingly, a variety of miR-210 targets have been identified. Among them are Ephrin A3 [14, 16], RAD52 [17], ACVR1B [18], MNT [19] and FGFRL2 [20].

Involvement of E2F family in the miR-210 signaling pathway was demonstrated by Giannakakis et al. [21]. In luciferase assays miR-210 directly targets transcription factor E2F3. This regulation was confirmed at the protein level in Western blot experiments where authors showed the suppression of E2F3 expression by miR-210. Using antibody against the C-terminal region of the E2F3 protein that recognizes both E2F3a and E2F3b, the authors showed that overexpression of miR-210 in HeLa cells caused significant decrease of the protein levels of both isoforms of E2F3.

### Angiogenesis-related miRNAs

The crucial role played by microRNAs in the regulation of angiogenesis became obvious after several studies demonstrated that deletion of Dicer, enzyme required for miRNA biogenesis, resulted in severe *in-vivo* and *in-vitro* angiogenic defects [22-25]. Since then miRNAs were shown to play important roles in regulation of angiogenesis during development and normal physiological processes, as well as pathological angiogenesis (for review, see [26-28]).

#### *miR-17~92 cluster*

The miR-17~92 cluster is a polycistronic miRNA gene. In the human genome, the miR-17~92 cluster encodes six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1). In vertebrates the sequences of these mature miRNAs are highly conserved. The human miR-17~92 cluster is located in the third intron of an approximately 7 kb primary transcript known as C13orf25 [29]. The miR-17~92 cluster first attracted attention following a series of observations linking these miRNAs to cancer pathogenesis [30]. Mice deficient for miR-17~92 die after birth with lung hypoplasia and a ventricular septal defect [31].

The roles of the cluster members appear to be different in physiological angiogenesis and tumor angiogenesis. Dews et al. [32] showed that overexpression of miR-17~92 in murine carci-

noma cells resulted in enhanced tumor angiogenesis. The mechanism involved downregulation of the potent endogenous inhibitor of angiogenesis thrombospondin-1 together with several proteins containing thrombospondin type 1 repeats. miR-17~92 downregulate thrombospondin type 1 repeat protein clusterin indirectly through transforming growth factor- $\beta$  (TGF $\beta$ ) pathway. The expression of type II TGF $\beta$  receptor was suppressed by miR-17-5p and miR-20a, and miR-18a reduced Smad4 level [33]. Similarly, injection of miR-17-5p in combination with let-7b into the ovaries of Dicer-deficient mice partially normalized corpus luteum angiogenesis [34]. However, Bonauer et al [35] demonstrated that overexpression of miR-92a in endothelial cells blocked angiogenesis *in vitro* and *in vivo*. In mouse models of limb ischemia and myocardial infarction, systemic administration of miR-92a antagomiR led to enhanced blood vessel growth and functional recovery of the injured tissues. Besides, same group [36] showed that overexpression of miR-17, -18a, -19a, and -20a significantly inhibited HUVEC sprouting in a 3-dimensional spheroid model and inhibition of miR-17, -18a, and -20a increased sprouting. *In-vivo* inhibition of miR-17 and miR-20a increased the number of blood vessels in Matrigel plugs, but antagomiRs that target miR-18a and miR-19a were less effective. Comparing the angiogenic properties of conditioned media obtained from tumor cells overexpressing miR-17, miR-19a, and miR-20a with that of likewise transfected ECs the authors found that conditioned media of LLC1 tumor cells slightly enhanced angiogenic sprouting of ECs, but media derived from transfected endothelial cells showed a trend toward reduction of angiogenic activity of ECs. In Lewis lung carcinoma tumor model inhibition of miR-17 and miR-20 slightly increased tumor size but did not increase angiogenesis.

Several E2F family members were shown to be both targets and regulators of miR-17~92 cluster. O'Donnell et al. [37] demonstrated that members of miR-17~92 cluster directly regulate expression of E2F1. Inhibition of miR-17-5p and miR-20a led to approximately 4-fold increase in E2F1 protein level, and overexpression of miR-17 cluster resulted in decrease of E2F1 protein. Another group [38] also showed that miR-20a can bind to 3'-UTR region of E2F1, E2F2 and E2F3 factors and inhibition of miR-17 and miR-20a led to increase in protein levels of E2F1

and E2F2. Interestingly, the authors demonstrated that E2F1-3 themselves directly regulate the expression of miR-17~92 cluster and over-expression of E2F1-3 in HeLa cells led to increase of miR-20a. These results suggest a self-regulatory mechanism, where miR-20a controls the translation of E2F1-3 which, in turn, regulate miR-20a transcription. The binding of E2F3 to miR-17~92 cluster promoter region was reported by another group [39]. Interestingly, Aurora kinase A upregulates miR-17~92 cluster by increasing E2F1 binding to its promoter region [40]. Pickering et al [41] demonstrated that in normal human fibroblasts inhibition of miR-17 and miR-20a led to earlier peak accumulation of E2F1 after serum starvation (9-12 hrs vs. 15-18 hrs in control cells), and this premature accumulation resulted in a DNA damage induced cell cycle arrest. Interestingly, according to their data, it was the miRNA-regulated timing of E2F1 accumulation but not the gross level of protein, required for initiation of DNA damage response.

### *miR-27b*

E2F1 levels has also been shown to be regulated by another angiogenesis-related microRNA, miR-27b.

miR-27b belongs to the intronic miR-23b~miR-27b~miR-24-1 cluster and have been shown as an angiogenesis-related microRNA in experiments using Dicer depletion to identify endothelial miRNAs [24]. Its inhibition was shown to significantly reduce endothelial sprouting. Recent data show that inhibition of miR-27b impairs angiogenesis *in vitro* and postnatal vascular retinal development *in-vivo* [42]. In developing mouse heart miR-27b was highly expressed in myocardium at day E10.5 and its expression remained high at later developmental stages [43]. Cheng et al [44] also observed high levels of miR-27b expression in normal mouse heart. miR-27b was upregulated in a rat model of early heart hypertrophy [45].

In addition, miR-27b expression was regulated by shear stress [46]. In this paper the authors found that the upregulation of miR-23b and miR-27b was correlated with the shear flow-induced growth arrest of endothelial cells. Pulsatile shear flow led to a reduction of E2F1 protein level and Rb hypophosphorylation. The Rb hypophosphorylation was reversed by inhibition of

miR-23b, but not miR-27b, and inhibition of both microRNAs could reverse shear flow-induced decrease of E2F1 levels.

### *miR-15, miR-16*

Though miR-15 and miR-16 are better known for their involvement in cancer [47-48], several studies profiling miRNA expression in endothelial cells demonstrated that these microRNAs are expressed at high levels, indicating their potential importance in angiogenesis regulation [23, 27, 49]. miR-15 and miR-16 were first reported to inhibit VEGF expression in human carcinoma cell line [50]. Hypoxia-induced VEGF expression was significantly decreased by transfection of miR-15,16 and inhibition of these microRNAs under normoxia led to increase of VEGF level. Later it was demonstrated that miR-16 negatively regulates VEGF translation by targeting VEGF 3'-UTR [51]. miR-15, miR-16 were shown to be involved in various cancers. In chronic lymphocytic leukemia both miR-15a and miR-16-1 negatively regulate antiapoptotic B cell lymphoma 2 (Bcl2) protein at a posttranscriptional level [52], and Bcl2 repression by these microRNAs induces apoptosis in a leukemic cell line model. Growing experimental evidence suggest that miR-15a/miR-16 constitute key tumor suppressors whose deletion contributes to cancer. mRNAs targeted by miR-15 and miR-16 include CCND3, CCNE1, CDK6, CAPRN1, and HMGA1 (reviewed in [53]). During *Xenopus* embryonic development, miR-15, miR-16 were shown to interfere with TGF $\beta$  signaling by targeting *Acvr2a*, receptor for Nodal, ligand belonging to TGF $\beta$  superfamily. At the same time, miR-15 and miR-16 are negatively regulated by Wnt/beta-catenin pathway, linking these two crucial developmental pathways [54].

MiR-15 and miR-16 were shown to participate in the regulation of cell-cycle. Thus, Linsley et al. [55] demonstrated that miR-16 family negatively regulates cell cycle progression. Recently Ofir et al. [56] demonstrated that miR-15a, miR-15b and miR-16 are significantly induced by E2F1 in human lung carcinoma cells. In chromatin immunoprecipitation experiments E2F1 was found binding to human *Dleu2* and *SMC4* gene promoters, therefore regulating miR-15b, miR-16-2 cluster and miR-15a, miR-16-1 cluster. Interestingly, ectopic expression of miR-15 resulted in reduced levels of cyclin E, a key direct transcriptional target of E2F pivotal for the G1/S

transition [57]. This observation suggests that E2F1, miR-15, and cyclin E form a self-regulating loop that modulates E2F activity. Bueno et al. [58], analyzing the transcription profiles of miRNAs in response to mitogenic stimulation in primary fibroblasts, demonstrated that miR-15b and miR-16-2 miRNAs are direct targets of E2F1 and E2F3. These miRNAs were specifically induced by E2F1 or E2F3 during the G1/S transition and repressed in E2F1-, and E2F3-knockout cells.

### Ischemia-related miRNAs

Another microRNAs involved in E2F signaling are miR-1 and miR-21, known for their important role in cardiac ischemia.

#### *miR-1*

miR-1 involvement in cardiac disorders is well documented [59]. Downregulation of miR-1 after pressure overload in mouse heart was reported by Sayed et al [60]. Ikeda et al [61] showed miR-1 decrease in cardiac hypertrophy, where it targets calmodulin and Mef2a. Reduction of miR-1 was shown to be required for an increase of cell mass [62-63]. miR-1 was demonstrated to lead to pro-apoptotic signaling in cardiomyocytes [64], and in cardiac ischemia miR-1 was shown to regulate apoptotic pathways through regulation of Bcl2 [65]. In the failing human heart the reported levels of miR-1 have been inconsistent [59], but overall miR-1 regulates the genes involved in cardiac hypertrophy. Targeted deletion of miR-1-2 was demonstrated to result in disrupted cardiac morphogenesis and, surprisingly, cell cycle abnormalities [66]. Adult miR-1-2 mutants had significant increase in heart/body weight ratio due to hyperplasia. Analysis showed 20% increase in the myocytes number in the mutants, and many adult cardiomyocytes were undergoing division. Authors observed significant increase in the number of mitotic marker-positive myocytes at the postnatal day P10 compared to wild type animals, and even at the age of 2-3 months there were mitotic myocytes in the hearts of miR-1-2<sup>-/-</sup> mutants.

It turned out that E2F factors are among miR-1 targets. Thus, Zhang et al [67] have shown that in hepatoma cells overexpression of miR-1 led to the cell cycle arrest at the G1 phase. E2F5 was identified as a direct target of miR-1, and

overexpression of miR-1 resulted in marked dose-dependent decrease of E2F5 protein. pRb levels were also decreased by miR-1 overexpression.

#### *miR-21*

miR-21 is among most significantly upregulated microRNAs in hypertrophic mouse heart [44, 60]. Following aortic banding, its expression increased more than 4-fold at day 7. These results were confirmed in *in-vitro* experiments with rat neonatal cardiac myocytes with stimulated hypertrophy [44, 68]. Upregulation of miR-21 was shown in the stimulated cells, and inhibition of miR-21 led to a decrease in hypertrophy. miR-21 was shown to target pro-apoptotic PDCD4 [69], tumor suppressor PTEN and anti-angiogenic Spry-2 [70-71].

Comparing microRNAs expression of left ventricular tissue in failing, healthy and fetal human heart Thum et al found several fold increase in miR-21 expression levels in fetal and failing sample versus healthy ones [72]. In human infarcted hearts there was a striking increase of miR-21 expression in border zone [73]. Following experimental data showing marked increase of miR-21 in mouse heart failure models as well as in human heart, Thum et al [74] showed that this increase happens mostly in cardiac fibroblasts, and not in myocytes. miR-21 was shown to have an anti-apoptotic role that was dependent on ERK activity. Spry1, an inhibitor of RAS/MEK/ERK pathway was identified as a direct target of miR-1 that was responsible for ERK activation. Treatment of mice with chemically modified antisense oligonucleotide (antagomiR) specific for miR-21 following pressure overload of left ventricle by transverse aortic constriction had reversed the negative effects of procedure such as myocyte size, heart weight and interstitial fibrosis. Moreover, the authors reported that treatment with miR-21 antagomiR had curative effect in pressure overload model. Surprisingly, these results were not confirmed by genetic deletion of miR-21 [75]. miR-21-null mice had no abnormalities in heart size, structure or cardiac contractility, and demonstrated no significant differences in pathologic remodeling from wild type control after being subjected to 4 different cardiac stresses: acute pressure overload, chronic calcineurin activation, infusion of Ang II and myocardial infarction. Similarly, inhibition of miR-21 using LNA-antimiR resulted in

comparable cardiac hypertrophy between control and treated groups. These discrepancies might be explained in part by the possible compensatory mechanisms activated in mice due to the deletion of miR-21 and difference between cholesterol-conjugated antagomiR used by Thum et al. and short 8-nt LNA-modified antimiR used by Patrick et al. Another explanation might be a redundancy of microRNAs, resulting in inhibition of multiple microRNAs sharing similar seed sequence [76].

Involvement of miR-21 in E2F regulation was demonstrated by Bhat-Nakshatri et al [77]. In experiments with MCF-7 breast cancer cells authors showed that inhibition of miR-21 increased E2F2 basal level, but had no effect on E2F1 protein. Estradiol-stimulated increase of E2F1 and E2F2 proteins was not significantly affected by miR-21 inhibition.

### Outlook

E2F family of transcription factors have long been a focus of intensive research, and its role in the cell cycle regulation and cell survival is well established. It is known that members of the family are involved in the signaling cascades induced by hypoxia. Rapidly accumulating data show that miRNAs actively participate in the modulation of E2F activity as both regulators of E2F transcription and their targets, forming intricate signaling network. Studying these complex interactions gives a new dimension to our understanding of the role of E2F transcription factors in hypoxic conditions and their mode of action, and can potentially lead to the development of new therapeutic targets in cardiovascular and cancer medicine.

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