Members of the microRNA-17-92 cluster exhibit a cell intrinsic anti-angiogenic function in endothelial cells

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Abstract

MicroRNAs are endogenously expressed small non-coding RNAs that regulate gene expression on the posttranscriptional level. The miR-17-92 cluster (encoding miR-17, -18a, -19a/b, -20a and miR-92a) is highly expressed in tumor cells and is up-regulated by ischemia. Whereas miR-92a was recently identified as negative regulator of angiogenesis, the specific functions of the other members of the cluster are less clear. Here we demonstrate that overexpression of miR-17, -18a, -19a and -20a significantly inhibited 3D spheroid sprouting in vitro, whereas inhibition of miR-17, -18a and -20a augmented endothelial cell (EC) sprout formation. Inhibition of miR-17 and miR-20a in vivo using antagonirs significantly increased the number of perfused vessels in matrigel plugs, whereas antagonirs, that specifically target miR-18a and miR-19a were less effective. However, systemic inhibition of miR-17/20 did not affect tumor angiogenesis. Further mechanistic studies showed that miR-17/20 targets several pro-angiogenic genes. Specifically, Janus kinase 1 (Jak1) was shown to be a direct target of miR-17. In summary, we show that miR-17/20 exhibit a cell intrinsic anti-angiogenic activity in ECs. Inhibition of miR-17/20 specifically augmented neovascularization of matrigel plugs, but did not affect tumor angiogenesis indicating a context-dependent regulation of angiogenesis by miR-17/20 in vivo.
Introduction

Recent studies describe a fundamental role of microRNAs (miRs) in development and diseases.¹⁴ MiRs are small 18-24 nucleotide single-stranded non-coding RNAs that regulate mRNA degradation and translation.⁵ Together with a protein complex known as RNA-induced silencing complex (RISC), miRs bind to sites in the 3'UTR and induce degradation or reduce the translation of the targeted mRNA. About 700 miRs have been identified in humans. Each miR can regulate up to several hundreds of targets and it is considered that translation of about one third of proteins is regulated by miRs.⁶

First evidence that miRs control vessel formation were obtained by depletion of the RNA endonuclease Dicer, which mediates miR maturation. Zebrafish or mice deficient for endothelial Dicer showed an aberrant vessel growth⁷ and silencing of Dicer in endothelial cells reduced in vitro angiogenesis.⁸,⁹ Several specific miRs were shown to control angiogenesis such as miR-126, which is highly expressed in endothelial cells, and is essential for blood vessel growth in zebrafish¹⁰ and mice.¹¹,¹² in addition, miR-221 and miR-222 block angiogenesis,¹³ whereas miR-27b,⁸ miR-130a¹⁴ and members of the let-7 family⁸,¹⁵ increase angiogenesis.

The miR-17-92 cluster was among the first miRs, which were linked to tumor angiogenesis. The miR-17-92 cluster is a typical example of a polycistronic miR cluster encoding the miRs miR-17, miR-18a, miR-19a/b, miR-20a and miR-92a, which are highly expressed in several tumors¹⁶. Particularly, miR-17 and miR-20a were shown to control cellular proliferation and apoptosis by targeting the E2F family of transcription factors¹⁷,¹⁸ and by down-regulating the tumor suppressor p21¹⁹,²⁰ and the pro-apoptotic protein BIM.²⁰ Overexpression of the entire miR-17-92 cluster in myc-induced tumors increased angiogenesis by a paracrine mechanism.²¹ This pro-angiogenic function has been attributed to the down-regulation of the anti-angiogenic molecules thrombospondin-1 (TSP-1) and connective tissue growth factor (CTGF), which are targeted by miR-18 and miR-19²¹. Combined overexpression of miR-17, miR-18a and miR-20a also partially rescued the impaired endothelial network formation induced by silencing of Dicer in vitro.⁷ Injection of miR-17 in combination with let-7b into the ovaries of Dicer-deficient mice partially normalized corpus luteum angiogenesis.¹⁵ In contrast, overexpression of miR-92a suppressed angiogenic sprout formation in vitro and interfered
with intersegmental vessel growth in zebrafish.\textsuperscript{22} Vice versa, inhibition of miR-92a in vivo using antagonirs augmented neovascularization and functional recovery after ischemia.\textsuperscript{22}

Here, we investigated the role of individual members of the miR-17-92 cluster for the cell intrinsic angiogenic activity of endothelial cells and determined the effects on neovascularization in vivo. Surprisingly, overexpression of the individual members of the miR-17-92 cluster, namely miR-17, miR-18a, miR-19a and miR-20a reduced endothelial cell sprouting, whereas inhibitors of these miRs augmented angiogenesis in vitro indicating that the miR-17-92 cluster provides a cell intrinsic anti-angiogenic activity in endothelial cells. Combined inhibition of miR-17 and miR-20a by antagonir-treatment in vivo additionally enhanced angiogenic sprouting, but did not affect tumor angiogenesis.
Materials and Methods

Protocols are available online. The animal experiments were conducted according to the “Principle of laboratory animal care” as well as according to the German national laws. The studies have been approved by the local ethical committee (Regierungspräsidium Darmstadt). All microarray data has been deposited into the Gene Express Omnibus (GEO) public database under accession number GSE20745.
Results

Individual members of the miR-17-92 cluster inhibit angiogenesis in vitro

In order to investigate the cell intrinsic function of the miR-17-92 cluster in endothelial cells, precursors of the individual members miR-17, miR-18a, miR-19a and miR-20a were expressed in endothelial cells (Fig. 1A) to study the effect on angiogenesis in vitro. Transfection of human umbilical vein endothelial cells (HUVECs) with precursors of miR-18a, miR-19a and miR-20a specifically increased the expression of the mature miR (Fig. 1B), whereas mature miR-17 and miR-20 were detected when overexpressing the precursor of miR-17 (Fig. 1B). Overexpression of all members of the miR-17-92 cluster significantly inhibited endothelial cell sprouting in a 3D spheroid model (Fig. 1C/D). Likewise, vascular network formation in matrigel models and endothelial cell migration were blocked by overexpressing the individual members of the miR-17-92 cluster (Supplementary Fig. 1A/B). To exclude unspecific effects of the overexpressed precursors, we used a precursor for miR-27b, which did not inhibit angiogenic sprouting (data not shown), and confirmed the anti-angiogenic activity of miR-17 by using miR mimetics (Supplementary Fig. 1C).

To determine whether inhibition of the endogenously expressed members of the miR-17-92 cluster is sufficient to induce angiogenic sprouting, we inhibited the individual miRs using hairpin inhibitors. Inhibition of miR-17, miR-18a and miR-20a increased spheroid sprouting about 1.5-fold, whereas miR-19a only slightly enhanced angiogenesis in vitro (Fig. 1E). As a control, we show that inhibition of miR-27b blocks angiogenic sprouting as previously described. To assess the in vivo relevance of these findings, the individual members of the miR-17-92 cluster were specifically inhibited by antagonim treatment and neovascularization was determined using the matrigel plug assay. First, we confirmed the specific knock-down of the targeted miR in antagonim-treated mice (Fig. 2A/B and supplementary Fig. 2A). Only antagonim-17 exhibited an off-target effect on the expression of the closely related miR-20a as detected by two different probe-based qPCR methods (Fig. 2A/B). These data indicate that the antagonim designed to suppress miR-17 also inhibits miR-20a because of large overlaps in the sequence (differs only in 2 nucleotides), a finding that is consistent with previous studies using LNA-miR-17 inhibitors. Therefore, this antagonim is referred to as antagonim-17/20 from here on. Three injections of antagonim-17/20 (8 mg/kg
body weight each) at days 0, 2 and 4 after subcutaneous implantation of matrigel increased the number of perfused vessels that invaded the matrigel plug in vivo (Fig. 2C/D), whereas specific blockade of miR-18a, miR-19a or mir-20a showed a trend but no significant effect on neovascularization (Fig. 2C). Similar effects were seen when antagonirs were injected only once at day 1 after implantation of the plugs (Supplementary Fig. 2B).

In summary, these data indicate that overexpression of the individual members of the miR-17-92 cluster blocks angiogenesis in vitro, whereas particularly the combined inhibition of miR-17 and miR-20a promotes angiogenesis in vitro and in vivo.

**Regulation of tumor angiogenesis**

Previous studies suggested that overexpression of the miR-17-92 cluster in tumor cells enhances tumor angiogenesis. The pro-angiogenic activity in this model has been attributed to the suppression of anti-angiogenic factors by tumor cells, which might act in a paracrine manner on endothelial cells. Therefore, we compared the angiogenic activity of conditioned medium of tumor cells, which had been transfected with the precursor molecules for the individual members of the miR-17-92 cluster, with that of likewise transfected endothelial cells (Fig. 3A/B). Indeed, conditioned medium of LLC-1 tumor cells overexpressing miR-17, miR-19a and miR-20a (Supplementary Fig. 3) slightly enhanced angiogenic sprouting of endothelial cells (Fig. 3B). In contrast, conditioned medium derived from endothelial cells transfected with miR-17, miR-18a or miR-19a showed a trend towards reduction of angiogenic activity of endothelial cells (Fig. 3B). To determine the net effect on tumor growth in vivo, we tested the effect of antagonir-17/20 in a Lewis lung carcinoma tumor model. Injection of antagonir-17/20 induced a minor non-significant increase in tumor size, volume and weight (Fig. 3C/D), however, tumor vascularization as measured by counting endomucin-stained capillaries was not increased (Fig. 3E). These data were confirmed by detecting in vivo perfused lectin-positive vessels in the tumor sections showing that antagonir-17/20 treatment did not increase the perfusion of the implanted tumors (Fig. 3F). Moreover, increasing the number of antagonir-17/20 injections did not affect tumor growth despite a sufficient inhibition of miR-17 expression in explanted tumors (Supplementary Fig. S4). Overall, these data suggest that antagonir-17/20 selectively enhances neovascularization of matrigel plugs but does not affect tumor angiogenesis.
Targets of the members of the miR-17-92 cluster

The miR-17-92 cluster was shown to target several proteins involved in cell cycle progression and apoptosis in hematopoietic and tumor cells. Among others, the miR-17-92 cluster was shown to target the protein Bim, a pro-apoptotic protein, which induces apoptosis of hematopoietic cells.\textsuperscript{29,30} Therefore, we first determined the effect of the individual members of the cluster on Bim expression in endothelial cells. Overexpression of individual miR-17-92 cluster members caused only a minor reduction of Bim expression (Fig. 4A) and most of the members of the miR-17-92 cluster did not exert an anti-apoptotic effect on endothelial cells under basal conditions or H\textsubscript{2}O\textsubscript{2} stimulation (Fig. 4B). Only miR-18a significantly enhanced apoptosis under basal conditions, but not after treatment with low and high concentrations of H\textsubscript{2}O\textsubscript{2} (Fig. 4B). Overall, these data indicate that Bim is not a functionally relevant target of the miR-17-92 cluster in endothelial cells. Moreover, we observed a minor down-regulation of the anti-angiogenic proteins CTGF and Tsp1 by overexpression of miR-18a and miR-19a but not by miR-17/20 in endothelial cells (Supplementary Fig. 5).

Having shown that miR-17 is the miR-17-92 member most significantly affecting angiogenesis in vitro and in vivo, we intended to identify relevant targets mediating the anti-angiogenic activity of miR-17 in endothelial cells. In a first approach, we performed a microarray mRNA profile. Overexpression of miR-17 significantly reduced several predicted targets, including the cell cycle inhibitor p21, the S1P receptor EDG1, and the protein kinase Jak1 (Fig. 5A/Supplementary Table 1/2). The reduction of p21 mediated by miR-17 was associated with an increased proliferation of pre-miR-17 transfected endothelial cells (Supplementary Fig. 6). In contrast, the reduction of EDG-1 expression did not affect the chemotactic response of the ECs to the EDG-1-ligand sphingosine-1-phosphate (Supplementary Fig. 7). Since Jak1 was efficiently down-regulated on mRNA as well as on protein level by miR-17 and the closely related miR-20a (Fig. 5A/B/C) and we additionally demonstrated that inhibition of miR-17 increased Jak1 expression (data not shown), we further tested the function of Jak1 in endothelial cells. SiRNA-mediated silencing of Jak1 reduced angiogenesis in vitro (Fig. 5D) and abrogated cytokine and growth factor-induced phosphorylation of Stat3 (Supplementary Fig. 8). Moreover, silencing of Jak1 partially reduced the pro-angiogenic effect mediated by miR-17 inhibitors (Fig. 5E) indicating that Jak1 is one of the miR-17 downstream targets. To confirm a direct regulation of Jak1 by miR-17, we performed luciferase assays in which the miR-17 target sequence in the Jak1 3’UTR
was cloned in the luciferase 3′UTR. Overexpression of miR-17 reduced luciferase activity, but exhibited no effect on a mutated construct (Fig. 5F) showing that miR-17 directly targets the Jak1 3′UTR.
Discussion

The data of the present study demonstrate that all individual members of the miR-17-92 cluster tested block angiogenic sprouting of endothelial cells in vitro. The findings that members of the miR-17-92 cluster inhibit angiogenesis and neovascularization are surprising and are in contrast to previous reports showing that overexpression of the miR-17-92 cluster promotes tumor angiogenesis.\(^{21}\) The pro-angiogenic activity was attributed to the block of the endogenous angiogenesis inhibitors Tsp-1 and CTGF by miR-18 and miR-19 in tumor cells. The reduction of these inhibitors was suggested to tip the delicate balance of angiogenesis activators and inhibitors towards promotion of angiogenesis. Although we confirmed a slight regulation of Tsp-1 and CTGF by overexpressing miR-18a and miR-19a in ECs, a pro-angiogenic activity of these miRs was not detected in any of the assays used. Thus, 3D spheroid sprouting was significantly inhibited by overexpression of both miR-18a and mir-19a and inhibition of miR-18a and miR-19a slightly increased sprouting in vitro and matrigel plug neovascularization in vivo. One may speculate that targeting of other endothelial cell intrinsic genes by miR-18a and miR-19a mediates these effects. In addition, one should note that Tsp-1, which has been described to be the major mediator of the pro-angiogenic activity of miR-18a and miR-19a in tumor cells, can act in a context dependent manner to either promote or block angiogenesis.\(^{31,32}\)

Among the members of the miR-17-92 cluster tested in the present study, miR-17/20 exhibited the most profound effects in endothelial cells in vitro and systemic blockade by antagonomirs promoted neovascularization in vivo. Interestingly, the identical concentration of antagonomir-17/20, which induced sprouting in implanted matrigel plugs, did not enhance tumor angiogenesis in a LLC1 tumor model suggesting that inhibition of miR-17/20 differentially affects neovascularization of matrigel plugs compared to tumor angiogenesis. This observation might be explained by direct effects of miR-17/20 inhibition on tumor cells, thereby altering the secretome of tumor cells, which may antagonize the cell intrinsic pro-angiogenic effects of the antagonomirs on endothelial cells. This hypothesis is supported by our findings that overexpression of miR-17 and miR-20a slightly enhanced the paracrine pro-angiogenic activity of tumor cells. Thus, a reduction of miR-17/20 in tumor cells might cause a reduced secretion of factors contributing to a pro-angiogenic environment surrounding the tumor cells.
In addition, we cannot exclude a direct effect on tumor cell survival or proliferation mediated by the systemic effects of antagonmir-17/20 injection, although Antagonmir-17/20 treatment of LLC1 cells in vitro had no profound effect on cell cycle distribution (Supplementary Fig. 9). Other groups reported that a direct and repeated injection of antagonmir-17 in the implanted tumors reduced tumor growth. However, in our study, the systemic i.v. injection of one or two doses of the rather low concentration of 8 mg/kg antagonmir-17/20 did not reduce tumor growth suggesting that these rather low systemically supplied concentrations of antagonmir-17/20 are not sufficient to mediate such direct anti-proliferative effects on tumors. One may envision that the slight increase in tumor weight and volume seen in our study might have been caused by an effect on the cell cycle promoter Cyclin D1, which has recently been shown to be targeted by miR-17 in tumor cells and might be increased under conditions of miR-17 inhibition.

In endothelial cells, the combined overexpression of miR-17, miR-18a and miR-20a was shown to rescue impaired endothelial cell functions induced by depletion of Dicer. However, in our experiments, none of the members of the miR-17-92 cluster exhibited a pro-angiogenic effect with respect to sprouting and particularly miR-17 suppressed the angiogenic activity of endothelial cells in vitro and in vivo. Since our results are consistent with recent data in immortalized endothelial cells showing that miR-17 blocks cell adhesion and migration, it is likely that the miR-17-92 cluster might have had specific effects under the stress imposed by the depletion of Dicer.

Because miR-17 exhibited the most profound effects on sprouting angiogenesis, we focused on the identification of the targets of this member of the miR-17-92 cluster. First, we confirmed previously identified targets of miR-17 and miR-20a and showed that the pro-apoptotic protein Bim and the cell cycle inhibitor p21 were suppressed by overexpression of miR-17. However, apoptosis of endothelial cells was only slightly reduced by miR-17 when using very high concentration of hydrogen peroxide. The lack of a profound anti-apoptotic effect of miR-17/20 mediated regulation of Bim is likely explained by the finding that Bim is not a major regulator of apoptosis in endothelial cells, but was shown to selectively affect hematopoietic progenitor cells. In contrast to the modest effects on apoptosis, miR-17/20 significantly increased cell cycle progression. Proliferation and sprouting are tightly regulated
during vessel growth, as nicely illustrated in model organisms like the zebrafish embryo, where the formation of intersegmental vessels is associated with proliferation of stalk but not tip cells.

Moreover, cell cycle regulators such as p21 do not only function as cell cycle inhibitors, but also are required for migration of cells. Thus, a reduction of p21 as seen by overexpression of miR-17 may disturb the balance of coordinated vessel growth by activating proliferation but reducing migration. This may also explain why the effects of miR-17/20 are much more profound in spheroid sprouting models (Fig. 1C), which mimic tip-stalk cell mediated angiogenic growth, compared to the network forming activity detected by in vitro matrigel assays (Supplementary Fig. 1A). However, the complex and dose-dependent activity of p21 on cell cycle progression, apoptosis and migration of endothelial cells challenges to pinpoint p21 effects on vessel growth.

Screening for additional targets revealed several new targets of miR-17, namely the tyrosine kinase Jak1 that was one of the most efficiently down-regulated targets. The Jak/Stat signaling pathways play a crucial role in vascular homeostasis and disease. Jak1, Jak2 and Tyk2 are expressed in endothelial cells and cardiomyocytes. In contrast, highest levels of Jak3 are found in the thymus. Unspecific inhibition of Jak/Stat signaling by AG-490 blocked angiogenesis. Moreover, mice with a cardiomyocyte-restricted STAT3 deletion showed a profoundly inhibited neovascularization capacity. The specific functions of Jak1 versus Jak2 are largely unknown, but selective disruption of the Jak1 gene demonstrates non-redundant roles of Jak1 in cytokine-responses of hematopoietic cells. In endothelial cells, Jak1 siRNA was shown to reduce HIF protein expression. Our data further confirmed that Jak1 is indeed required for sprouting angiogenesis and growth factor mediated activation of STAT3. The causal involvement of Jak1 was further evidenced by the findings that silencing Jak1 by siRNA partially prevented the pro-angiogenic activity of miR-17 inhibition. However, our data do not exclude a role of various additional targets such as the EDG1 receptor to the observed phenotype. Our findings that miR-17 mediated downregulation of EDG-1 did not affect sphingosin-1-phosphate induced migration is likely explained by compensation via the EDG-3 receptor that is highly expressed in endothelial cells. However, EDG-1-deficient mice exhibit a profound defect in vascular development and
die during embryonic development suggesting that under certain conditions, EDG-1 plays a unique role in the vasculature.

In summary, the findings of the present study provide novel insights into the complex regulation of angiogenesis and vascular growth by members of the miR-17-92 cluster and specifically miR-17/20. The differential effect of systemic inhibition of miR-17/20 on neovascularization of matrigel plugs compared to LLC-1 tumors suggest that the miR-17-92 cluster is differentially involved in regulation physiological versus tumor angiogenesis. Conceptually, targeting specific members of the miR-17-92 family may provide an interesting therapeutic perspective to specifically enhance therapeutic angiogenesis.

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Authors contribution

C.D., A.B., and A.F. performed research and analyzed data; A.S. and Y.R. provided support with the tumor model; C.U. and W.H. provided support with the microarray; A.M.Z. and S.D. designed the study. C.D., A.B. and S.D. wrote the manuscript. The authors have no conflict of interest.
References


Figure legends

Figure 1: Effect of the members of the miR-17-92 cluster on angiogenesis in human ECs. (A) Schematic illustration of the miR-17-92 cluster and sequences of the individual members (B) HUVECs were transfected with miR-precurors or a control precursor (Pre-Co) as indicated and expression of mature miRs was detected by stem loop PCR after 24 hours. Data were normalized to RNU48. n=3. (C/D) Inhibition of sprout formation in the spheroid assay (n=10 spheroids/experiment, n=5-11 experiments). *p<0.05 versus Pre-Co. Representative images are shown in D. Scale bar: 100 µm (E) HUVECs were transfected with miR inhibitors as indicated and sprout length of spheroids was quantified (n=10 spheroids/experiment, n=6 experiments). *p<0.05 versus MiR-Inhib-Co2.

Figure 2: Inhibition of miR-17 enhances angiogenesis in mice. (A/B) Effect of systemic infusion of three i.v. injections of antagomirs targeting miR-17, miR-18a, miR-19a, and miR-20a (8 mg/kg bw, n=3-5 mice per group, 2 plugs/mouse) or a control antagomir (antagomir-Co, n=6) on miR expression in hearts harvested 7 days after the first injection. MiR expression was detected by a real-time PCR method using a universal TaqMan probe in combination with a miR specific forward primer (A) and a TaqMan MicroRNA Assay with higher specificity (B). (C/D) Effect of antagomir i.v. infusion at day 0, 2, 4 on the number of lectin-perfused vessels in matrigel plugs in vivo after 7 days. n=4-10 per group. *p<0.05 versus Antagomir-Co. Representative images are shown in D. Scale bars: 20 µm.

Figure 3: Effect of members of the miR-17-92 cluster on paracrine activity of tumor cells in vitro and tumor angiogenesis in vivo.
(A/B) HUVEC or LLC1 tumor cells were transfected with the respective precursors. Medium was changed to DMEM with 0.05% BSA after 1 day. Conditioned medium was collected at day 2 and 10x concentrates were transferred to collagen embedded spheroids with wildtype HUVECs as illustrated in A. Quantification of spheroid sprout length was performed after incubation of the spheroids with the conditioned medium for 24 hours. n=4. (C/D) LLC1 tumor cells were subcutaneously injected in mice. Antagomir-17/20 (8mg/kg bw) was i.v. injected once as indicated. Tumor size was measured daily (C) and tumor volume and weight...
were detected in explanted tumors (D) at day 13. n=7 for Antagomir-Co and n=6 for Antagomir-17. (E) Tumor angiogenesis was detected in sections stained with the endothelial marker endomucin. A secondary antibody conjugated to Alexa Fluor 555 was used. The number of vessels was counted manually. n=7 for Antagomir-Co and n=6 for Antagomir-17. (F) Perfused vessels were detected by i.v. infusion of FITC-conjugated lectin and were quantified by automatical measurement of the pixel region in each section. n=7 for Antagomir-Co and n=6 for Antagomir-17. Scale bars: 20 µm.

**Figure 4: Effect of members of the miR-17-92 cluster on apoptosis**  (A) Expression of the pro-apoptotic protein Bim in HUVECs after transfection with precursors for the indicated miRs. A representative Western blot is shown. n=3 experiments. (B) Detection of Annexin positive HUVECs after transfection with the miR precursors in the presence or absence of H2O2 for 14 hours. n=3-11 experiments. *p<0.05.

**Figure 5: Identification of miR-17 targets.**  (A) mRNA expression of the putative targets after overexpression of pre-17 in HUVECs for 24 hours. n=3. *p≤0.05. (B/C) Expression of Jak1 in HUVECs overexpressing the indicated miR precursors. A representative Western blot is shown in B. Quantification is provided in C. n=4. (D) Effect of Jak1 silencing using 40 nM siRNA on spheroid sprouting. A siRNA directed against firefly luciferase or cells treated with the transfection reagent were used as controls. n=5-6. Down-regulation of Jak1 protein 48h after siRNA transfection is shown in the representative Western blot. (E) Jak1 siRNA reduces the pro-angiogenic effect of miR-17 inhibition. HUVECs were transfected with Jak1 siRNA (40 nM), miR-17 inhibitor (50 nM) and the respective controls as indicated. Spheroids were generated and sprouting was quantified. n=3. *p<0.05. (F) Firefly luciferase activity normalized to Renilla luciferase activity measured in homogenates of HEK cells transfected with the wild type (wt) or mutated luciferase constructs and Pre-17 or control Pre-miR. Measurements were done 48 hours after transfection. n=4.
Figure 1: Doebele et al

A

B

C

D

E

Relative expression 2^△Ct (% Pre-Co)

** Relative expression 2^△Ct (% Pre-Co)

- miR-17: CAAAGUGCUUACAGUGCAGGUAG
- miR-18a: UAAAGUGCUUAAUGUGCAGGUAG
- miR-19a: UGUGCAAUUCUGCAAAACUGA
- miR-19b: UGUGCAAAUCUGCAAAACUGA
- miR-92a: UAUUGCACUGGCCGCGCGU

** Relative expression 2^△Ct (% Pre-Co)

- miR-17: UUAAGGUGCAUCUAGUGCAGAUAG
- miR-20a: UAAAAGUGCUUAAGUGCAGGUAG
- miR-19a: UGUGCAAAUCUGCAAAACUGA
- miR-19b: UGUGCAAAUCUGCAAAACUGA
- miR-92a: UAUUGCACUGGCCGCGGCB

** Relative expression 2^△Ct (% Pre-Co)

- Pre-Co
- Pre-17
- Pre-18a
- Pre-19a
- Pre-20a

** Relative expression 2^△Ct (% Pre-Co)

- Pre-Co
- Pre-17
- Pre-18a
- Pre-19a
- Pre-20a

** Relative expression 2^△Ct (% Pre-Co)

- Pre-Co
- Pre-17
- Pre-18a
- Pre-19a
- Pre-20a

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- Pre-Co
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- Pre-18a
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- Pre-20a

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- Pre-Co
- Pre-17
- Pre-18a
- Pre-19a
- Pre-20a

** Relative expression 2^△Ct (% Pre-Co)

- Pre-Co
- Pre-17
- Pre-18a
- Pre-19a
- Pre-20a

** Relative expression 2^△Ct (% Pre-Co)

- Pre-Co
- Pre-17
- Pre-18a
- Pre-19a
- Pre-20a
Figure 2: Doebele et al

A

Relative expression $2^{-\Delta\text{Ct}}$ (% Antagomir-Co)

Antagomir-Co  Antagomir-17  Antagomir-18a  Antagomir-19a  Antagomir-20a

B

Relative expression $2^{-\Delta\text{Ct}}$ (% Antagomir-Co)

Antagomir-Co  Antagomir-17  Antagomir-20a

C

Lectin positive vessels (% Antagomir-Co)

Antagomir-Co  Antagomir-17/20  Antagomir-18a  Antagomir-19a  Antagomir-20a

D

Matrigel Plug  i.v. Antagomir  i.v. Lectin  Harvest

Antagomir-Co  Antagomir-17/20

FITC-Lectin  DAPI

FITC-Lectin  DAPI
Figure 3: Doebele et al

A

B

C

D

E

F

Endomucin positive vessels (% Antagomir-Co)

Lectin positive vessels (% Antagomir-Co)

FITC-Lectin
Figure 4: Doebele et al

A

BIM

Tubulin

B

Total apoptotic cell death (%)

Pre-Co
Pre-17
Pre-18a
Pre-19a
Pre-20a

Standard Culture
200 µM
1 mM

H2O2

* p = 0.07
Figure 5: Doebele et al

A

Control
Cyclin D1
E2F1
p21
EDG1
JAK1

mRNA level (% Control)

B

Mean cumulative sprout length per spheroid (% Control siRNA + miR-Inhib-Co)

C

JAK1 Expression (% Pre-Co)

D

Mean cumulative sprout length per spheroid (% Control siRNA)

E

Mean cumulative sprout length per spheroid (% Co siRNA + miR-Inhib-Co)

F

Firefly/Renilla Luciferase activity (% Pre-Co)

Control siRNA
Jak1 siRNA
MiR-Inhib-Co
MiR-Inhib-17

JAK1 binding sequence wt
JAK1 binding sequence mut
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