Abstract:

Title: MODULATION OF MiRNA IN DISEASES WITH ABBERRANT ANGIOGENESIS

The present application relates to the field of microRNAs, and more particular to the role of microRNAs in angiogenesis. It is demonstrated herein that modulation of specific microRNAs, such as miR-144, has therapeutic potential in diseases characterized by aberrant angiogenesis. This is particularly relevant in, for instance, tumour growth and metastasis, which are characterized by excessive angiogenesis and for which inhibition of miR-144 is a novel therapeutic strategy. On the other hand, diseases characterized by insufficient angiogenesis (e.g. associated with ischemia) may benefit from increasing miR-144 activity.
Modulation of miRNA in diseases with aberrant angiogenesis

Field of the invention
The present application relates to the field of microRNAs, and more particularly to the role of microRNAs in angiogenesis. It is demonstrated herein that modulation of specific microRNAs, such as miR-144, has therapeutic potential in diseases characterized by aberrant angiogenesis. This is particularly relevant in, for instance, tumour growth and metastasis, which are characterized by excessive angiogenesis and for which inhibition of miR-144 is a novel therapeutic strategy. On the other hand, diseases characterized by insufficient angiogenesis (e.g. associated with ischemia) may benefit from increasing miR-144 activity.

Background
Cancer remains one of the major causes of mortality and morbidity in the Western society, and constitutes a prime target for drug development. One of the prime leads is the development of inhibitors of blood vessel formation (anti-angiogenic drugs), based on the fact that cancer progression requires angiogenesis to support its growth and metastasis to distant organs (Carmeliet et al., 2009). In addition, recent evidence suggest that the normalization of tumoral blood vessels (as tumors form blood vessels with abnormal structure and function) improves drug delivery and tumor eradication (Mazzone et al., 2009). A milestone in the exploitation of this novel therapeutic paradigm was the clinical development of a monoclonal antibody against a prime regulator of angiogenesis, namely Vascular Endothelial Growth Factor (VEGF). Indeed, adjuvant administration of bevacizumab (trade name Avastin®, Genentech/Roche) to standard chemotherapy proved to prolong the survival of patients with metastatic colon cancer by inhibiting angiogenesis, slowing tumor growth and/or improving the delivery of chemotherapy (Hurwitz et al., 2004; Willett et al., 2004). Currently, Avastin is FDA approved for the treatment of various cancers including metastatic colon cancer and non-small cell lung cancer, and recurring Glioblastoma Multiforme, as well as for the treatment of eye diseases such as age-related macular degeneration (AMD) and diabetic retinopathy. Moreover, the successful development of Avastin catalyzed the further development of a wide spectrum of additional anti-VEGF drugs, of which the class of receptor tyrosine kinase inhibitors (RTKi) such as Sunitinib (Sutent®, Pfizer Inc) and Sorafenib (Nexavar®, Bayer Corporation/Onyx Pharmaceuticals Inc) are most advanced into clinical trials (Carmeliet, 2005).
Despite the successful introduction of Avastin in the clinic, there remain various important limitations of its use in patients. First, to date, its efficacy was only proven in conjunction with standard anti-cancer therapy but not in monotherapy. Second, as VEGF is a prime regulator of endothelial cells in tumors but also in the normal vasculature, treatment with Avastin is endowed with great risk of affecting the non-tumoral endothelium, thereby causing various life-threatening side effects (e.g. hypertension, thrombosis, aggravation of cardiovascular disease, etc), and hence warranting a tight control of its administration and limitation of eligible patients (Carmeliet, 2005). Deleterious side effects are especially present in the clinical experiences so far with RTKi administration in patients, in part amplified by the non-specific targeting of additional tyrosine kinase receptors, expressed in normal endothelium and other cell types. Third, it becomes apparent in preclinical studies that tumors develop resistance against VEGF-inhibitors, by prioritizing alternative pathways to develop new blood vessels, independent of VEGF, including the upregulation of other pro-angiogenic cytokines (Fischer et al., 2007), the recruitment of pro-angiogenic myeloid cells (Shojaei et al., 2007) and the escape of tumor cells from hypoxia (Loges et al., 2009). In summary, anti-angiogenesis constitutes a novel therapeutic paradigm for the treatment of cancer, but the development of novel anti-angiogenic drugs (not targeting the VEGF pathway) with selective efficacy on tumoral blood vessels and an improved safety profile is warranted.

In angiogenesis research, the identification and characterization of endothelial cell subtypes (such as tip and stalk cells) is critical for a better understanding of vascular branching, maintenance and function, with potential clinical translation. Indeed, the cornerstone of vascular sprouting and branching is the initiation of endothelial tip cells, which, after progressive recruitment of stalk cells and a series of other events, leads to a functional branched vessel lined with quiescent endothelial cells (Carmeliet et al., 2009). Mechanistically, various environmental signals such as VEGF and jagged-1 have been identified as initiators of tip cell fate specification (Gerhardt et al., 2003; Benedito et al., 2009), but the cell-intrinsic regulation of tip cell initiation remains unclear. In fact, current dogma states that tip cell initiation is regulated through a "permissive" model in which, amongst several candidate endothelial cells, one single tip cell is selected by negative effects on adjacent endothelial cells via lateral signaling pathways such as Notch (Hellstrom et al., 2007).

Regardless of this, such novel view on angiogenic sprouting might become medically relevant, as targeting tip cells might potentially result in novel antiangiogenic strategies to block angiogenesis in disease states including cancer. The proof-of-principle for such strategies was recently shown in cancer studies in mice targeting established tip cell surface molecules such as Unc5B and DII4 (Noguera-Troise
et al., 2006; Larrivee et al., 2007). Furthermore, targeting tip cells may also allow developing angiogenic strategies in disorders where angiogenesis is impaired, such as limb ischemia.

Angiogenesis equals the formation of a hierarchically branched vascular network. The cellular basis of vessel branching is formed by endothelial heterogeneity, i.e. endothelial subpopulations with specific fates and functions: amongst others the endothelial tip, stalk and quiescent cell (in tumors called phalanx cell) (Mazzone et al., 2009). The cornerstone of vascular branching is the initiation of tip cells, which, after progressive recruitment of stalk cells and a series of other events, leads to a functional branched vessel lined with quiescent endothelial cells (Larrivee et al., 2009). Various environmental signals, such as VEGF and jagged-1, have been identified as initiators of tip cell fate specification (Benedito et al., 2009; Gerhardt et al., 2003), but the cell-intrinsic regulation of tip cell initiation remains unclear. Indeed, current dogma states that tip cell initiation is regulated through a "permissive" model in which, amongst several candidate endothelial cells, one single tip cell is selected by negative effects on adjacent endothelial cells via lateral signaling pathways mediated by, for instance, Notch and Wnt family members (Hellstrom et al., 2007; Phng et al., 2009).

A new class of angiogenesis regulators recently identified are non-coding small RNAs such as microRNAs. MicroRNAs (miRNAs) represent a class of conserved non-coding small RNAs (~22 nt long), which repress gene expression post-transcriptionally by targeting 3'-untranslated regions (3'UTRs) of specific target gene mRNAs, leading to translational repression or mRNA degradation (Bartel, 2009). Via this post-transcriptional regulation (translational repression and/or mRNA degradation) of target genes, miRNAs control cell fate of various cell types, involved in proliferation, differentiation, migration, and so on (Bartel, 2009). Nearly 700 miRNAs have been identified in humans so far, and are predicted to regulate a third of protein coding genes, suggesting that miRNAs are likely to play roles in almost all biological processes (Lewis et al., 2005). A few miRNAs (~2%) have been shown to control blood vessel formation (angiogenesis), both positively (e.g. miR-126, let-7f, the miR-17-92 cluster, miR-31, miR-130a, miR-296, miR-320) (Wang and Olson, 2009) and negatively (e.g. miR-92a, miR-221/222) (Bonauer et al., 2009), thereby affecting the progression of various diseases including malignancy, retinopathy and ischemic cardiovascular disease. A common denominator of these studies was the fact that the selection of these miRNAs was based primarily on expression profiling of cultured endothelial cells (Fish et al., 2008; Kuehbacher et al., 2007; Wang et al., 2008). Relative disadvantages of such screenings are for instance, that (i) such screenings are not performed in a complex microenvironment; and (ii) such screenings do
not implicate a functional role. Hence, when studying the angiogenic potential of the ~98% other miRNAs, one might consider the development of alternative screening platforms.
Most of the miRNAs currently identified as having a role in angiogenesis were predominantly expressed in tumor cells and affected tumor angiogenesis via paracrine mechanisms. The identification of miRNAs primarily expressed by tumor endothelial cells remains an outstanding question.

Although only identified one decade ago, microRNAs are currently envisioned as a major target for personalized medicine and (current) drug development in oncology. Indeed, not only it became clear that specific miRNA expression signatures in various tumors seem to be associated with defined disease states and/or specific cancers, also approaches in which tumor-derived miRNAs in the circulating blood plasma are assayed as a novel biomarker are currently extensively explored. Indeed, recent reports suggest the potential to use circulating miRNAs, analyzed in blood plasma, as a surrogate biomarker of tumor progression (Mitchell et al., 2008).

It would be advantageous to have a novel pro- or anti-angiogenic therapeutic with selective efficacy on blood vessels, particularly one that does not target the VEGF pathway. Most particularly, an anti-angiogenic therapeutic selectively affecting tumoral blood vessels is highly desirable. If a target were identified that is specifically upregulated in these blood vessels, this molecule could also serve as a biomarker in cancer.

Summary

MicroRNAs (miRNAs) critically fine-tune gene expression during blood vessel formation (angiogenesis), but their role in endothelial tip/stalk cell specification remains unknown. By applying a conditional genetic approach in mice, it is shown that endothelial deletion of Dicer limits the development of a branched vascular network in the yolk sac, and miRNA profiling suggested the potential involvement of particularly miR-144 herein. Forced overexpression of miR-144 in endothelial cells stimulated vessel branching both in vitro and in vivo, and was sufficient to initiate an endothelial fate and gene signature, which is reminiscent of branch-inducing tip cells. Conversely, inhibition of miR-144 reduced vessel branching and the formation of endothelial tip cells. By using a combined bio-informatics and biochemical validation approach, we identified e.g. the endothelial Forkhead transcription factor FoxOl as a target gene of miR-144. Consistent herewith, modulation of endothelial FoxOl expression mirrored
the effects after modulation of miR-144 expression. Further studies revealed that endothelial FoxOl represses the expression of the tip cell gene Unc5B. This is consistent with a novel, cell-intrinsic and instructive model of endothelial tip cell initiation, in which increased miR-144 expression in endothelial cells is sufficient to instruct tip cell specification and vessel branching. These findings can be used in diseases where increased angiogenesis is desired, such as ischemia.

Moreover, the fact that it is shown for the first time that e.g. miR-144 promotes (tumor) angiogenesis by instructing endothelial tip cell specification also opens up new therapeutic possibilities, as miRNAs are identified herein that are potential drug targets in cancer. This strategy does not aim to target the tumor cell directly, but rather the tumor endothelial (tip) cell. Indeed, inhibition of miR-144 in in vivo tumor models results in delayed and reduced tumor growth, increased survival and lower incidence of cancer-associated systemic syndrome - see Examples section. Given their well-known apparent safety profile, inhibitors against specific miRNAs (e.g. antagomir-144) represent a new class of angiogenesis inhibitors with a beneficial safety profile and efficacy. As it was found that miR-144 is upregulated in tumours in vivo, most particularly in tumour endothelial cells, the level of miR-144 expression can be used as a biomarker to detect cancer and/or tumour angiogenesis.

According to a first aspect, it is an object of the invention to provide modulators of miR-144 for use as a medicament. According to particular embodiments, the modulators of miR-144 are for use in treatment of a disease characterized by aberrant angiogenesis. Accordingly, methods are provided for treating diseases characterized by aberrant angiogenesis, comprising:

- administering to a subject in need thereof a modulator of miR-144, thereby restoring angiogenesis to a normal level.

In a particular aspect, the modulators of miR-144 are inhibitors of miR-144. These can be used in treatment of diseases characterized by excessive angiogenesis, as inhibition of miR-144 reduces vessel branching and tip cell formation. According to particular embodiments, the diseases characterized by excessive angiogenesis are selected from the group of cancer, diabetic retinopathy and age-related macular degeneration.

In specific embodiments of cancer treatment, the inhibitors of miR-144 are particularly suited to reduce or slow down tumour growth, i.e. when an inhibitor of miR-144 is administered, the tumour may still grow, but will remain smaller than when left untreated.
According to alternative embodiments, the inhibitors of miR-144 reduce tumour metastasis.

In yet further alternative (but non-exclusive) embodiments, the inhibitors of miR-144 reduce the incidence of a disease that is the consequence of the presence of cancer in the body, but is not due to the local presence of cancer cells (typically referred to as a paraneoplastic syndrome). Particularly, they can be used to treat cancer-associated systemic syndrome (CASS) or one or more of its symptoms ( cachexia, anemia, increased chance of thrombosis). Thus, methods are provided for reducing the development or occurrence of CASS, by administering an inhibitor of miR-144. This way, the chances of survival (and thus also the life expectancy) is increased.

According to particular embodiments, the inhibitor of miR-144 is an antagomir (e.g. antagomir-144) or a locked nucleic acid (LNA).

In a further aspect, the modulators of miR-144 are agents that mimic the biological action of miR-144, i.e. miR-144 mimetics or miR-144 itself. These agents can be used in diseases where upregulation of miR-144 is desired, such as diseases characterized by impaired angiogenesis. Indeed, miR-144 (or a mimic thereof) will increase vessel branching, allowing more vessels to be formed and a better perfusion of the tissue. Typical diseases characterized by impaired angiogenesis are those characterized by ischemia, such as ischemic heart disease or, most particularly, ischemic limb disease. According to particular embodiments, the agents that mimic the biological action of miR-144 are administered to endothelial cells. According to very specific embodiments, the agents that mimic the biological action of miR-144 are not administered in cardiomyocytes. According to specific embodiments, the effects of the agents that mimic the biological action of miR-144 are GATA4-independent.

According to yet a further aspect, miR-144 is provided for use as a biomarker. As it was found that miR-144 is specifically upregulated in tumour endothelial cells, miR-144 can be used as a biomarker to detect cancer, i.e. increased levels of miR-144 correlate with tumour progression. As miR-144 is normally absent in many healthy tissues, the presence of miR-144 as such can already be used as a biomarker to indicate the presence of cancer.

According to specific embodiments, the expression levels of miR-144 can be further correlated to the tumor size. Alternatively, the expression levels can be correlated to the nature of the tumour (e.g. breast cancer, pancreatic cancer, etc. This is especially useful in the case of metastases that are not
directly linked to a primary tumour) and/or the expression levels can be correlated to the aggressiveness of the tumour, i.e. the risk of metastasis can be derived from the miR-144 levels. According to further embodiments, when miR-144 expression has been correlated to tumour size, nature and/or aggressiveness, the treatment may be adapted based on this information.

Brief description of the Figures

Figure 1. A, Genotyping of offspring of male Tek^{Cre/lox}:Dicer_{ΔHtmox} and female Dicer_{ΔHtmox} mice. B, Dicer expression in endothelium-selective Dicer-deficient (Tek^{Cre/lox}:Dicer_{ΔHtmox}) mice versus control mice as evaluated by qPCR (top) and microarray (bottom).

Figure 2. qPCR analysis of expression of selected miRNAs in Tek^{Cre/lox}:Dicer_{ΔHtmox} mice relative to control mice.

Figure 3. Relative quantity (RQ) of endothelial nitric oxide synthase (eNOS) in Tie2-GFP positive cells that are CD45 positive or CD45 negative.

Figure 4. miR-144 expression in HUVEC cells is dose-dependently increased upon transfection with miR-144 duplex (left panel) or miR-144 precursor (right panel), both after 24 hours (top) and after 48 hours (bottom). Triangles on Y axis indicate increase in dose; numbers on X axis are fold increase relative to a scrambled control miRNA.

Figure 5. A, miR-144 overexpression increases sprout length, number of sprouts and number of branched sprouts in a spheroid model of sprouting angiogenesis (Bonauer et al., 2009). Left panel: transfection of miR-144 duplex versus scrambled control; right panel: transfection of miR-144 precursor versus scrambled control. B, Microscopic images showing filopodia formation of cells shown in A, left panel.

Figure 6. A, qPCR expression analysis of selected tip cell (Unc5B, VEGFR-1, NP-1, NP-2, PDGF-B, VEGFR-2, Dll4, Notch-1, Notch-4, VEGFR-3) and stalk cell (Narp, Hes1, Robo4) genes in HUVEC cells transfected with miR-144 duplexes, shown as fold increase relative to control. B, co-transfection of control miRNA or miR-144 duplexes with a TP1 Notch signaling reporter construct shows similar Notch activity.
Figure 7. A, Microarray (top) and qPCR (bottom) analysis of predicted target genes of miR-144 that are upregulated in mutant yolk sacs. B, Microarray (left panel) and qPCR (right panel) analysis of FoxOl target genes that are upregulated in mutant yolk sacs. C, FoxOl protein levels in yolk sac extracts of normal or mutant (endothelial-specific Dicer-deficient) mice.

Figure 8. A, Total protein levels of FoxOl in HUVEC cells treated with miR-144 duplex or scrambled control. Tubulin is shown as control. B, FoxOl-dependent luciferase activity in HUVEC cells treated with miR-144 duplex or scrambled control. C, mutating the 3'UTR binding site confirms that FoxOl is a target gene of miR-144. HUVEC cells are treated with miR-144 duplex or scrambled control.

Figure 9. siRNA inhibition of FoxOl increases sprout length, number of sprouts and number of branched sprouts in a spheroid model of sprouting angiogenesis (Bonauer et al., 2009), similar to what is shown in Figure 5.

Figure 10. Expression of miR-144 in syngeneic pancreatic adenocarcinoma tumours of different sizes in C57Bl6 mice, shown relative to expression in the cultured Panc02 cells.

Figure 11. A, Tumor volume time course of C57Bl6 mice subcutaneously inoculated with Panc02 cells and treated with one bolus i.v. injection of antagomir-144 or scrambled control at 8 mg/kg. B, Survival of the mice shown in A. Solid line: control; dashed line: mice injected with antagomir-144.

Figure 12. Weight of tumors of the two groups of mice shown in Figure 11A at the end of the experiment (day 36). Left column: control, right column: mice injected with antagomir-144.

Figure 13. Incidence of paraneoplastic syndrome in the two groups of mice shown in Figure 11A.

Figure 14. A, Tumor volume time course of C57Bl6 mice subcutaneously inoculated with Panc02 cells and treated with one bolus i.v. injection of antagomir-144 or scrambled control at 40 mg/kg. B, Survival of the mice shown in A. Solid line: control; dashed line: mice injected with antagomir-144.
Detailed description

Definitions

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. The drawings described are only schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn on scale for illustrative purposes. Where the term “comprising” is used in the present description and claims, it does not exclude other elements or steps. Where an indefinite or definite article is used when referring to a singular noun e.g. “a” or “an”, “the”, this includes a plural of that noun unless something else is specifically stated.

Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

The following terms or definitions are provided solely to aid in the understanding of the invention. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Plainsview, New York (1989); and Ausubel et al., Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999), for definitions and terms of the art. The definitions provided herein should not be construed to have a scope less than understood by a person of ordinary skill in the art.

The term "microRNA", "mi RNA" or "mi R" is used herein to refer to short (typically 20-24 nt) non-coding RNAs that are involved in post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of mRNAs. miRNAs are transcribed by RNA polymerase II as part of capped and polyadenylated primary transcripts (pri-miRNAs) that can be either protein-coding or non-coding. The primary transcript is cleaved by the Drosha ribonuclease III enzyme to produce an approximately 70-nt stem-loop precursor miRNA (pre-miRNA), which is further cleaved by the cytoplasmic Dicer ribonuclease to generate the mature miRNA and the minor (sometimes referred to as
antisense) miRNA star (miRNA*) products. The mature (single-stranded) miRNA is incorporated into a RNA-induced silencing complex (RISC), which recognizes target mRNAs through imperfect base pairing with the miRNA and most commonly results in translational inhibition or destabilization of the target mRNA (typically, miRNAs bind complementary sequences in the 3-prime UTRs of target mRNAs to induce nucleolytic degradation or inhibit translation).

"miR-144", "MIR144" or "microRNA 144" as used herein refers to the microRNA 144 RNA gene (in humans, also indicated as "hsa-mir-144", characterized by HGNC ID: 31531; Gene ID: 406936; MIMI ID: 612070; miRBase MI0000460; in mice ("mmu-mir-144"): GenelD: 387162; miRBase MI0000168). The primary miRNA transcript (pri-miRNA) that encodes miR-144 also encodes miR-451 (Dore et al., 2008), although recent reports indicate they are differently regulated post-transcriptionally (Cheloufi et al., 2010; Cifuentes et al., 2010).

Up till now, miR144 has been documented to be involved in hematopoiesis, particularly erythropoiesis (Dore et al., 2008; Fu et al., 2009; Rasmussen et al., 2010), but not in angiogenesis. miR-144 has also been implicated in neurodegeneration (An et al., 2010; Persengiev et al., 2010) and bipolar disorder (Dinan, 2010). WO2006/137941 suggests that miR-144 should be administered in cancer, particularly in colon cancer. WO2009/070653 mentions that miR-144 downregulation (amongst others) is associated with lung cancer. Guled et al. report that miR-144*, the minor miR* sequence of hsa-mir-144, is one of the miRNAs severely reduced in malignant mesothelioma (Guled et al., 2009). Similar to these publications on miR-144, downregulation of miR-451 has been reported to be associated with a worse prognosis in gastric cancer (Bandres et al., 2009) and is also associated with pre-B-ALL (Ju et al., 2009). Of note, all these studies in cancer report associations but no causative effects; the administration or inhibition of mir-144 has also not been tested in vivo.

A "modulator of miR-144" as used in the application refers to a compound that modulates the function, activity and/or functional effect of miR-144. Modulators can either inhibit or decrease miR-144 function, in which case they are referred to as "inhibitors of miR-144", or enhance or increase miR-144 function. "Inhibitors of miR-144" are molecules that interfere with the function of miR-144, either at the DNA level (e.g. by interfering with transcription of miR-144) or at the RNA level (e.g. by interfering with the successive cleavage steps, through destabilization of the miRNA so that it is degraded, or typically, by interfering with the miRNA itself, e.g. through hybridization).
Inhibiting at the DNA level can for instance be done by inhibiting functional expression of the miR-144 RNA gene itself. With “functional expression” of the miR-144 gene, it is meant the transcription of functional miR-144 gene product. "Inhibition of functional expression" at the DNA level can e.g. be achieved by removing or disrupting the miR-144 gene, or preventing transcription to take place (in both instances preventing synthesis of the miR-144 gene product).

If inhibition is to be achieved at the DNA level, this may be done using gene therapy to knock-out or disrupt the miR-144 gene. As used herein, a "knock-out" can be a gene knockdown or the gene can be knocked out by a mutation such as, a point mutation, an insertion, a deletion, a frameshift, or a missense mutation by techniques known in the art, including, but not limited to, retroviral gene transfer.

Another way in which genes can be knocked out is by the use of zinc finger nucleases. Zinc-finger nucleases (ZFNs) are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain. Zinc finger domains can be engineered to target desired DNA sequences, which enables zinc-finger nucleases to target unique sequence within a complex genome. By taking advantage of endogenous DNA repair machinery, these reagents can be used to precisely alter the genomes of higher organisms. An alternative genome customization tool that can be used are Transcription Activator-Like Effector Nucleases (TALENs), which are sequence-specific nucleases created by the fusion of transcription activator-like effectors (TALEs) to the catalytic domain of an endonuclease.

Examples of miRNA inhibitors that inhibit by hybridization are miRNA inhibitor molecules that are between 17 and 25 nucleotides in length and comprise a 5’ to 3’ sequence that is at least 90% complementary to the 5’ to 3’ sequence of a mature miRNA (particularly of miR-144).

These antisense oligomers used to inhibit miR function may consist of DNA, RNA or other, synthetic, structures such as phosphorothiates, 2’-0-alkyl ribonucleotide chimeras, locked nucleic acid (LNA) (which will be discussed further), peptide nucleic acid (PNA), or morpholinos. With the exception of RNA oligomers, PNAS and morpholinos, antisense oligomers typically act in eukaryotic cells through the mechanism of RNase H-mediated target cleavage. PNAS and morpholinos bind complementary DNA and RNA targets with high affinity and specificity, and thus act through a simple steric blockade of the RNA translational machinery, and appear to be completely resistant to nuclease attack. An “antisense oligomer” refers to an antisense molecule or anti-gene agent that comprises an oligomer of at least about 10 nucleotides in length. In particular embodiments an antisense oligomer comprises at least 15, 18, 20, 25, 30, 35, 40, or 50 nucleotides. Antisense approaches involve the design of oligonucleotides
(either DNA or RNA, or derivatives thereof) that are complementary to the miRNA of choice, particularly miR-144. Antisense RNA may be introduced into a cell to inhibit translation of a complementary mRNA by base pairing to it and physically obstructing the translation machinery. This effect is therefore stoichiometric. Absolute complementarity, although preferred, is not required. A sequence “complementary” to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double stranded antisense polynucleotide sequences, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense polynucleotide sequence. Generally, the longer the hybridizing polynucleotide sequence, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Recently it has been shown that morpholino antisense oligonucleotides in zebrafish and frogs overcome the limitations of RNase H-competent antisense oligonucleotides, which include numerous non-specific effects due to the non target-specific cleavage of other mRNA molecules caused by the low stringency requirements of RNase H. Morpholino oligomers therefore represent an important new class of antisense molecule. Oligomers of the invention may be synthesized by standard methods known in the art. As examples, phosphorothioate oligomers may be synthesized by the method of Stein et al. (1988) Nucleic Acids Res. 16, 3209 3021), methylphosphonate oligomers can be prepared by use of controlled pore glass polymer supports (Sarin et al. (1988) Proc. Natl. Acad. Sci. USA. 85, 7448-7451). Morpholino oligomers may be synthesized by the method of Summerton and Weller U.S. Patent Nos. 5,217,866 and 5,185,444.

Particularly envisaged molecules for inhibition of miRNAs are antagonirs. Antagomirs are chemically engineered oligonucleotides that can be used to silence endogenous microRNA. An antagomir is a small synthetic RNA that is perfectly complementary to the specific miRNA target with either mispairing at the cleavage site of Ago2 or some sort of base modification to inhibit Ago2 cleavage. Usually, antagomirs have some sort of modification to make it more resistant to degradation. Without being bound to a particular mechanism, it is believed that antagomirization (the process by which an antagomir inhibits miRNA activity) operates by irreversibly binding the miRNA.
miRNA may also be inhibited using ribozymes instead of antisense RNA. Ribozymes are catalytic RNA molecules with enzyme-like cleavage properties that can be designed to target specific RNA sequences. Successful target gene inactivation, including temporally and tissue-specific gene inactivation, using ribozymes has been reported in mouse, zebrafish and fruit flies. The feasibility of this approach for miRNA modulation has recently been demonstrated (Suryawanshi H et al., Mol Biosyst. 6(10): 1807-9 (2010)).

Molecules that may be used to enhance or increase miR-144 function include mir-144 mimics or mimetics. miR mimetics typically are molecules consisting of a double-stranded RNA strand. Often, the RNA contains modifications to make them more suitable for administration (e.g. by increasing stability). According to particular embodiments, the miR-144 mimic does not contain modifications and is identical to the native miR-144 or one of its precursor molecules, but in isolated form. Thus, unless otherwise stated, the use of a miR-144 mimic also envisages the use of miR-144.

The term "isolated" means altered or removed from the natural state through human intervention. For example, a miRNA naturally present in a living animal is not "isolated," but a synthetic miRNA, or an miRNA partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated miRNA can exist in substantially purified form, or can exist in a non native environment such as, for example, a cell into which the miRNA has been delivered.

As mentioned already, the miRNA modulators of the invention can comprise partially purified RNA, substantially pure RNA, synthetic RNA, or recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non nucleotide material, such as to the end(s) of the miRNA or to one or more internal nucleotides of the miRNA, including modifications that make the miRNA resistant to nuclease digestion.

The miRNA modulators described herein can be obtained using a number of techniques known to those of skill in the art. According to particular embodiments, the miRNA modulators can be chemically synthesized or recombinantly produced using methods known in the art. For example, the miRNA modulators of the invention are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. Commercial suppliers of synthetic miRNA
modulators (i.e. inhibitors and mimetics) or synthesis reagents include Proligo (Hamburg, Germany), Dhharmacon Research (Lafayette, Colo., USA) (e.g. providing Miridian® miRNA mimics and inhibitor collections of known miRNAs in human, mouse, and rat), Pierce Chemical (part of Perbio Science, Rockford, Ill., USA), Glen Research (Sterling, Va., USA), ChemGenes (Ashland, Mass., USA), Cruachem (Glasgow, UK), Exiqon and Miragen. miRNA modulators have also been described in e.g. WO/2009/029690; and Olejniczak et al., 2010.

According to alternative embodiments, miRNA modulators can also be expressed from recombinant circular or linear DNA plasmids using any suitable promoter. Suitable promoters for expressing miRNA of the invention from a plasmid include, for example, the U6 or HI RNA pol II promoter sequences and the cytomegalovirus promoter. Selection of other suitable promoters is within the skill in the art. The recombinant plasmids of the invention can also comprise inducible or regulatable promoters for expression of the miRNA modulator in a particular tissue or in a particular intracellular environment. The miRNA modulators expressed from recombinant plasmids can either be isolated from culture cell expression systems by standard techniques, or can be expressed intracellularly, e.g. in muscle tissue or in tumours. The miRNA modulators of the invention can also be expressed intracellularly from recombinant viral vectors.

According to a particular aspect, the miR-144 modulators provided herein are used in treatment of diseases characterized by aberrant angiogenesis. The term "aberrant angiogenesis" is used herein to refer to blood vessel growth that is either excessive (i.e. too much formation of new blood vessels) or impaired (not enough new blood vessels are formed).

Diseases characterized or caused by abnormal or excessive angiogenesis in one or more organs include, but are not limited to, cancer, infectious diseases, autoimmune disorders, vascular malformations (e.g. Tie-2 mutation), DiGeorge syndrome, HHT, cavernous hemangioma, atherosclerosis, transplant arteriopathy, obesity, psoriasis, warts, allergic dermatitis, scar keloids, pyogenic granulomas, blistering disease, Kaposi sarcoma in AIDS patients, persistent hyperplastic vitreous syndrome, diabetic retinopathy, retinopathy of prematurity, choroidal neovascularization, primary pulmonary hypertension, asthma, nasal polyps, inflammatory bowel and periodontal disease, ascites, peritoneal adhesions, endometriosis, uterine bleeding, ovarian cysts, ovarian hyperstimulation, arthritis, synovitis, osteomyelitis, and osteophyte formation (see Table 1 of Carmeliet, 2003).
Diseases characterized or caused by insufficient or impaired angiogenesis or vessel regression in one or more organs (and thus typically also accompanied by ischemia) include, but are not limited to, Alzheimer disease, amyotrophic lateral sclerosis (ALS), diabetic neuropathy, stroke, atherosclerosis, hypertension, diabetes, restenosis, gastric or oral ulcerations, Crohn disease, hair loss, skin purpura, telangiectasia and venous lake formation, pre-eclampsia, menorrhagia, neonatal respiratory distress, pulmonary fibrosis, emphysema, nephropathy, osteoporosis, and impaired bone fracture healing (see Table 2 of Carmeliet, 2003). Particularly envisaged ischemic diseases characterized by impaired angiogenesis include, but are not limited to, limb ischemia or critical limb ischemia, chronic obstructive pulmonary disease, ischemia-reperfusion injury, post-operative ischemia, ischemic cardiovascular disease, restenosis, acute myocardial infarction, chronic ischemic heart disease, atherosclerosis, ischemic stroke, ischemic cerebral infarction, or ischemic bowel disease.

In embodiments where diseases are treated characterized by aberrant angiogenesis, it will be particularly envisaged that the modulation of the miR-144 gene function is limited to the tissue where the angiogenesis is aberrant. A typical example hereof is in the case of cancer: inhibition of miR-144 may be restricted to the tissue where the tumour is located, and most particularly, the gene function knock-out is limited to the tumour itself, and miR-144 is not inhibited in the host subject. Also particularly envisaged is that miR-144 function is only modulated in blood vessels and/or endothelial tissue.

Apart from tissue-specific modulation of miR-144 gene product function, the modulation may also be temporary (or temporally regulated). Temporally and tissue-specific gene inactivation may be achieved through targeted administration of miR-144 modulators, or, in case of gene therapy, by using specific expression cassettes including specific promoters and/or enhancers to control expression of the miR-144 modulators.

According to a particular aspect, the disease to be treated is characterized by excessive angiogenesis, particularly cancer. Indeed, because of its pivotal role in vessel branching, miR-144 is an attractive target to specifically affect angiogenesis, most particularly tumour angiogenesis. Inhibitors of miR-144, such as antagomirs against miR-144, have the potential to effectively and specifically inhibit tumor angiogenesis, thereby preventing tumor growth and metastasis.
In these embodiments, an inhibitor of miR-144 gene function can be used to reduce tumour growth (i.e., methods of reducing tumour growth according to these embodiments involve administering an inhibitor of miR-144 to a subject in need thereof).

As used herein, "reducing tumour growth" means that a tumour contacted or treated with the inhibitors described herein will grow less than an untreated tumour in the same time span. Particularly, growth will be 10% less, 20% less, 30% less, 40% less, 50% less, 75% less, 90% less, 95% less or 100% less (i.e., once treatment is started, the tumour does not grow any more). In particular cases, the tumour growth may even be reversed upon treatment, i.e. the tumour starts to shrink (either immediately or after a period of reduced growth). Tumour growth can be assessed by the skilled person using methods known in the art, typically by measuring tumour volume or size. Alternatively, the weight of the tumour can be estimated to assess tumour growth.

According to alternative, but not exclusive, embodiments, the inhibitor of miR-144 can be used to reduce tumour metastasis (or equivalent: methods of reducing tumour metastasis according to these embodiments involve administering an inhibitor of miR-144 to a subject in need thereof). With "reducing tumour metastasis" it is meant that a tumour (or subject) treated with the inhibitors described herein will develop less metastases (i.e. secondary or metastatic tumours) than an untreated tumour in the same time span. Particularly, metastases will be 10% less, 20% less, 30% less, 40% less, 50% less, 75% less, 90% less, 95% less or 100% less (i.e., once treatment is started, the tumour does not metastasise any more).

According to yet further alternative non-exclusive embodiments, the inhibitor of miR-144 can be used to reduce the incidence of paraneoplastic syndrome (herein also referred to as cancer-associated systemic syndrome or CASS) (or equivalent: methods of reducing the incidence of paraneoplastic syndrome according to these embodiments involve administering an inhibitor of miR-144 to a subject in need thereof).

With "reducing the incidence" as used herein, it is meant that the likelihood of developing CASS is reduced upon treatment (i.e., preventing new cases of CASS or, if symptoms of CASS are already present, reducing the severity of at least one symptom of CASS. Reducing the severity may refer to an improvement or may entail the complete disappearance of one or more symptoms (e.g. improving anemia may result in a less anemic state or result in a non-anemic state; improving cachexia may result in gain of lean body mass up to the point that body weight is restored to normal).

The terms "paraneoplastic syndrome", "cancer-associated systemic syndrome" and "CASS" are used herein as equivalents to refer to diseases or symptoms that are the consequence of the presence of
cancer in the body, but is not due to the local presence of cancer cells. These phenomena are typically mediated by humoral factors (by hormones or cytokines) excreted by tumor cells or by an immune response against the tumor. Paraneoplastic syndromes are typical among middle aged to older patients, and they most commonly present with cancers of the lung, breast, ovaries or lymphatic system (a lymphoma). The most common symptoms of CASS include cachexia, anemia and an increased chance of thrombosis.

Cachexia or wasting syndrome is loss of weight, muscle atrophy, fatigue, weakness and significant loss of appetite in someone who is not actively trying to lose weight. The formal definition of cachexia is the loss of body mass that cannot be reversed nutritionally: even if the affected patient eats more calories, lean body mass will be lost, indicating there is a fundamental pathology in place. Cachexia is often seen in end-stage cancer, and in that context is called "cancer cachexia."

According to particular embodiments, the inhibitors of miR-144 can be used in other diseases characterized by excessive angiogenesis, for example diabetic retinopathy.

There are several advantages coupled with the use of miR-144 inhibitors in treating diseases characterized by excessive angiogenesis. First, the standard therapy for these conditions is anti-VEGF therapy. Inhibition of VEGF is poised with deleterious side effects, it seems to effectively reduce tumor growth only when used in association with chemotherapy, and it is sensitive to resistance (i.e. the tumor escapes this therapy by compensatory mechanisms). Here we can show reduced tumour growth without side effects, and without combination with chemotherapy.

Another advantage is that it has recently been shown that a single injection of miRNA inhibitors like antagomirs ensures an efficient reduction of the expression and function of a miRNA for at least 2 months. As the toxicity of antagomirs is little and very transient, these agents are very attractive candidate drugs in the clinic.

According to another particular aspect, modulators of miR-144, more particularly miR-144 mimetics, can be used to treat diseases characterized by impaired angiogenesis, such as ischemic heart disease or ischemic limb disease (which is equivalent to saying that methods of treating diseases characterized by impaired angiogenesis according to these embodiments involve administering a miR-144 mimic to a subject in need thereof). Indeed, miR-144 mimetics can effectively increase revascularization of ischemic hearts and limbs. Thus far, no molecule-based treatment to promote angiogenesis has made it to the
The capacity of miRNAs (such as miR-144) to target various downstream effectors might offer a therapeutic advantage to interfere with the complex modulation of vessel growth, maturation and functional maintenance.

The modulators of miR-144, when used to treat diseases characterized by aberrant angiogenesis, will be administered to subjects in need thereof in an effective amount. As used herein, an "effective amount" of the miR-144 modulators is an amount sufficient to obtain the desired effect (e.g. sufficient to hybridize to the target miRNA in case of antagomirs, or an amount sufficient to inhibit tumour growth in a subject; or, in the case of mimetics, an amount sufficient to increase blood flow in ischemic tissue).

One skilled in the art can readily determine an effective amount of the miRNA modulator to be administered to a given subject, by taking into account factors such as the size and weight of the subject; the extent of the disease penetration; the age, health and sex of the subject; the route of administration; and whether the administration is regional or systemic. Generally, an effective amount of the miRNA modulator of the invention comprises an intracellular concentration of from about 1 nanomolar (nM) to about 1 μM, preferably from about 2 nM to about 100 nM, more preferably from about 2.5 nM to about 50 nM or to about 10 nM. It is contemplated that greater or lesser amounts of miRNA modulators can be administered.

According to a further aspect, miR-144 can be used as a biomarker. In this case, endogenous miR-144 is measured. A "biomarker", or biological marker, as used herein is a substance used as an indicator of a biological state. It can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. According to particular embodiments, the expression levels of endogenous miR-144 will be assessed. The way expression levels are measured is not vital to the invention, and the skilled person is well aware of methods to determine expression levels of a given miRNA, e.g. using realtime PCR such as TaqMan® MicroRNA Assay (Ambion), or the microRNA Expression Profiling Assay (Illumina).

According to particular embodiments, miR-144 expression levels can be used as a biomarker to detect diseases with excessive angiogenesis, most particularly cancer. Indeed, in subjects with cancer expression of miR-144 will be increased, so that the increased levels can be used to detect the presence of cancer. To this end, miR-144 expression levels will typically be determined in a sample from said subject, most particularly a sample suspected to contain a tumour. To determine whether miR-144 levels are increased or not, expression will typically be compared to a control sample. Choosing a
relevant control is well within the reach of the skilled person, but typically will be a comparable sample (e.g. from the same tissue and/or in the same form (liquid, solid, ...)) in which it has been verified that no tumour is present.

In some instances, the mere presence of miR-144 may indicate the presence of disease, more particularly cancer (e.g. in tissues/samples where miR-144 is not normally expressed).

According to particular embodiments, the expression levels of miR-144 can not only be correlated with the presence of cancer, but can also be correlated with the nature and/or the aggressiveness of the tumour. In other words, miR-144 is not only a biomarker for the presence of a tumour, but can in these embodiments also be a biomarker to determine malignancy of a tumour and/or to determine the nature of the tumour (e.g. whether it is a breast tumour or not).

According to further specific embodiments, the expression levels of miR-144 can be used to assess pharmacologic responses to a therapeutic intervention (i.e. to check whether a given therapy works) and/or to determine a change in medication or treatment regime (e.g. a switch in therapy if the levels of miR-144 do not decrease after initiation of a given therapy).

According to specific embodiments, a kit is provided comprising material to detect expression levels of miR-144 (e.g. specific probes for miR-144), and/or materials (such as enzymes) needed to facilitate miR-144 detection (e.g. buffers and enzymes to isolate miRNA, reverse transcriptase and primers to convert miR-144 to cDNA, primers and polymerase to perform PCR to quantify miR-144, or (possibly labeled) probes hybridizing to miR-144, optionally fixed to a substrate such as a microarray or chip).

It is to be understood that although particular embodiments, specific configurations as well as materials and/or molecules, have been discussed herein for cells and methods according to the present invention, various changes or modifications in form and detail may be made without departing from the scope and spirit of this invention. The following examples are provided to better illustrate particular embodiments, and they should not be considered limiting the application. The application is limited only by the claims.

Examples

Example 1. Generation of endotheliumselective Dicer deficient mice
To explore the role of miRNAs in angiogenesis, we applied the Cre-LoxP system to target the gene encoding Dicer1 (from hereon termed Dicer), an essential enzyme for miRNA biogenesis (Yi et al., 2006), specifically in endothelial cells. As a model system, we focused on vascular network formation in E13.5 extra-embryonic yolk sacs, as we aimed to study the role of miRNAs once vasculogenesis was completed and vascular network formation was ongoing (Gordon et al., 2008). Female mice homozygous for the floxed Dicer alleles (DicerΔflox/flox) (Yi et al., 2006) were therefore bred with male transgenic mice expressing the Cre recombinase under the control of the endothelium-specific Tek promotor (Koni et al., 2001). Analogous to what is described in this reference (Koni et al., 2001), double-heterozygous male progenies (TekCre+/−:DicerΔflox/flox) were identified and mated with female DicerΔflox/+ mice to obtain endothelium-specific Dicer mutant mice. At E13.5, genotyping of 366 embryos from 49 nests revealed that all possible genotypes were present at the expected Mendelian frequency of ~25% (Figure 1A). By performing lineage-tracing studies via crossings with the R26RLacZ/LacZ indicator mouse (Soriano, 1999), we confirmed that recombination in the yolk sac endothelium was efficient and relatively selective (data not shown), as expected from previous studies (Kisanuki et al., 2001). Quantitative real-time PCR (qPCR) experiments on yolk sac extracts, obtained from TekCre+/−:DicerΔflox/+ mutant embryos (from hereon termed Dicer mutants), showed efficient knock-down of Dicer expression by ~30% compared to control (Figure 1B).

Phenotypic analysis of the mutant yolk sacs revealed an incompletely established vascular network. Indeed, stereomicroscopic analysis of the mutants indicated that, compared to controls, the formation of a hierarchically branched vascular network - with ramifications into large and small branches covering the entire yolk sac - was disturbed (data not shown). These observations suggest impaired vascular branching and networking in the absence of endothelial Dicer and, as a result, large areas of the mutant yolk sacs appeared to lack any blood supply (data not shown). Whole mount staining for CD31 on intact control and mutant yolk sacs confirmed fewer blood vessels in parts of the yolk sacs (data not shown). Similar observations were made with whole mount staining for VE-cadherin (not shown).

Notably, at E13.5, the Dicer mutant embryos exhibited no general developmental delay, but suffered from various organ-specific defects. Taken together, in TekCre+/−:DicerΔflox/+ mutants, we found incomplete and defective branching of the yolk sac vascular network.

To confirm that the Dicer gene was excised in endothelial cells, we crossbred our DicerΔflox/+ mice into the R26RLacZ/LacZ indicator background and subsequently mated them with TekCre+/−:DicerΔflox/+ mice to generate TekCre+/−:DicerΔ/flox,R26RLacZ/+ embryos. A mosaic endothelium-selective LacZ expression was observed (not shown), and the defects of hierarchical vascular branching were practically identical to
the ones in the Dicer mutants (not shown), suggesting that the hierarchical vascular phenotype was indeed caused by a cell-autonomous loss of miRNA production in endothelial cells.

**Example 2. miRNA microarray analysis on yolk sac extracts of Dicer mutants.**

To identify the miRNAs responsible for the defective phenotype in the endothelial Dicer mutants, a microarray profiling for miRNAs (Agilent) was performed on yolk sac extracts of mutant and control embryos (N=4). To enhance fidelity of our screening, 2 different normalization algorithms were applied on the output results, and those miRNAs were selected that showed reduced expression in the mutant yolk sacs in both algorithms. Following this strategy, 20 out of 579 analyzed miRNAs were picked up, of which most were never implicated in angiogenesis before (Table 1 and 2).

Amongst the miRNAs, regulated in the Dicer mutants, was miR-126 (Table 1 and 2), which was recently shown to be an endothelium-selective miRNA (Fish et al., 2008; Wang et al., 2008). By qPCR analysis utilizing miRNA-specific primers, we confirmed in the mutant yolk sacs the reduced expression of miR-126 (Figure 2).

Amongst the other regulated miRNAs, picked up by this platform and practically all validated by qPCR (Figure 2; no specific Taqman probes were available for 3 miRNAs), we noted that both normalization methods ranked as first miR-144 (Table 1 and 2), which was previously shown to be involved in (zebrafish) hematopoiesis but not angiogenesis (Du et al., 2009; Fu et al., 2009). As miR-144 is transcribed in a cluster with miR-451 (Pase et al., 2009), we also analyzed the expression of the latter miRNA. Our microarray results revealed a tendency of miR-451 to be regulated in the Dicer mutants (i.e. not significantly downregulated with Normalization I, whereas significantly downregulated with Normalization II; data not shown). Nevertheless, qPCR analysis showed a significant down regulation of miR-451 expression, consistent with our findings on miR-144. Hence, miR-144 might be an interesting candidate to further investigate for its potential role in angiogenesis.
TABLE 1: Microarray expression results of miRNAs downregulated in endothelial Dicer mutants: Normalization I.

<table>
<thead>
<tr>
<th>Normalization I</th>
<th>control mice</th>
<th>mutant mice</th>
<th>% of control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmu-miR-144</td>
<td>0.47 ± 0.07</td>
<td>0.16 ± 0.06</td>
<td>34</td>
<td>0.016</td>
</tr>
<tr>
<td>mmu-miR-126-3p</td>
<td>3.68 ± 0.24</td>
<td>1.68 ± 0.23</td>
<td>46</td>
<td>0.001</td>
</tr>
<tr>
<td>mmu-let-7b</td>
<td>0.75 ± 0.05</td>
<td>0.37 ± 0.06</td>
<td>50</td>
<td>0.002</td>
</tr>
<tr>
<td>mmu-let-7d</td>
<td>2.10 ± 0.16</td>
<td>1.09 ± 0.12</td>
<td>52</td>
<td>0.002</td>
</tr>
<tr>
<td>mmu-let-7i</td>
<td>3.45 ± 0.22</td>
<td>1.80 ± 0.15</td>
<td>52</td>
<td>0.001</td>
</tr>
<tr>
<td>mmu-miR-143</td>
<td>0.85 ± 0.06</td>
<td>0.45 ± 0.05</td>
<td>52</td>
<td>0.001</td>
</tr>
<tr>
<td>mmu-miR-542-3p</td>
<td>0.88 ± 0.05</td>
<td>0.55 ± 0.05</td>
<td>62</td>
<td>0.003</td>
</tr>
<tr>
<td>mmu-let-7f</td>
<td>2.82 ± 0.24</td>
<td>1.77 ± 0.16</td>
<td>63</td>
<td>0.011</td>
</tr>
<tr>
<td>mmu-miR-199b</td>
<td>3.55 ± 0.20</td>
<td>2.24 ± 0.16</td>
<td>63</td>
<td>0.002</td>
</tr>
<tr>
<td>mmu-let-7c</td>
<td>1.22 ± 0.05</td>
<td>0.77 ± 0.07</td>
<td>63</td>
<td>0.002</td>
</tr>
<tr>
<td>mmu-miR-542-5p</td>
<td>0.47 ± 0.04</td>
<td>0.30 ± 0.03</td>
<td>64</td>
<td>0.013</td>
</tr>
<tr>
<td>mmu-miR-152</td>
<td>1.86 ± 0.08</td>
<td>1.20 ± 0.07</td>
<td>65</td>
<td>0.001</td>
</tr>
<tr>
<td>mmu-miR-214</td>
<td>0.49 ± 0.02</td>
<td>0.32 ± 0.04</td>
<td>66</td>
<td>0.006</td>
</tr>
<tr>
<td>mmu-miR-199a-5p</td>
<td>0.96 ± 0.02</td>
<td>0.64 ± 0.05</td>
<td>66</td>
<td>0.001</td>
</tr>
<tr>
<td>mmu-miR-503</td>
<td>1.03 ± 0.09</td>
<td>0.69 ± 0.02</td>
<td>67</td>
<td>0.009</td>
</tr>
<tr>
<td>mmu-miR-199b*</td>
<td>4.01 ± 0.32</td>
<td>2.70 ± 0.17</td>
<td>67</td>
<td>0.012</td>
</tr>
<tr>
<td>mmu-let-7a</td>
<td>4.37 ± 0.36</td>
<td>2.98 ± 0.22</td>
<td>68</td>
<td>0.017</td>
</tr>
<tr>
<td>mmu-miR-335-5p</td>
<td>0.67 ± 0.05</td>
<td>0.46 ± 0.02</td>
<td>69</td>
<td>0.006</td>
</tr>
<tr>
<td>mmu-miR-223</td>
<td>0.52 ± 0.03</td>
<td>0.40 ± 0.01</td>
<td>77</td>
<td>0.010</td>
</tr>
<tr>
<td>mmu-miR-652</td>
<td>0.43 ± 0.01</td>
<td>0.35 ± 0.02</td>
<td>80</td>
<td>0.009</td>
</tr>
</tbody>
</table>
TABLE 2: Microarray expression results of miRNAs downregulated in endothelial Dicer mutants:
Normalization II.

<table>
<thead>
<tr>
<th>Normalization II</th>
<th>control mice</th>
<th>mutant mice</th>
<th>% of control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) mmu-miR-144</td>
<td>0.31 ± 0.24</td>
<td>-1.70 ± 0.45</td>
<td>25</td>
<td>0.008</td>
</tr>
<tr>
<td>(14) mmu-miR-199a-5p</td>
<td>1.37 ± 0.06</td>
<td>0.49 ± 0.09</td>
<td>36</td>
<td>0.000</td>
</tr>
<tr>
<td>(2) mmu-miR-126-3p</td>
<td>3.30 ± 0.07</td>
<td>1.86 ± 0.17</td>
<td>37</td>
<td>0.000</td>
</tr>
<tr>
<td>(3) mmu-let-7b</td>
<td>1.00 ± 0.06</td>
<td>-0.31 ± 0.21</td>
<td>40</td>
<td>0.001</td>
</tr>
<tr>
<td>(4) mmu-let-7d</td>
<td>2.49 ± 0.09</td>
<td>1.25 ± 0.14</td>
<td>42</td>
<td>0.000</td>
</tr>
<tr>
<td>(5) mmu-let-7i</td>
<td>3.21 ± 0.07</td>
<td>1.98 ± 0.10</td>
<td>43</td>
<td>0.000</td>
</tr>
<tr>
<td>(6) mmu-miR-143</td>
<td>1.19 ± 0.11</td>
<td>-0.03 ± 0.12</td>
<td>43</td>
<td>0.000</td>
</tr>
<tr>
<td>(7) mmu-miR-542-3p</td>
<td>1.25 ± 0.11</td>
<td>0.28 ± 0.10</td>
<td>51</td>
<td>0.001</td>
</tr>
<tr>
<td>(8) mmu-let-7f</td>
<td>2.91 ± 0.10</td>
<td>1.96 ± 0.12</td>
<td>52</td>
<td>0.001</td>
</tr>
<tr>
<td>(9) mmu-miR-199b</td>
<td>3.25 ± 0.06</td>
<td>2.31 ± 0.07</td>
<td>52</td>
<td>0.000</td>
</tr>
<tr>
<td>(10) mmu-let-7c</td>
<td>1.71 ± 0.03</td>
<td>0.76 ± 0.13</td>
<td>52</td>
<td>0.000</td>
</tr>
<tr>
<td>(11) mmu-miR-542-5p</td>
<td>0.34 ± 0.14</td>
<td>-0.58 ± 0.12</td>
<td>53</td>
<td>0.002</td>
</tr>
<tr>
<td>(12) mmu-miR-152</td>
<td>2.32 ± 0.05</td>
<td>1.41 ± 0.08</td>
<td>53</td>
<td>0.000</td>
</tr>
<tr>
<td>(13) mmu-miR-214</td>
<td>0.40 ± 0.09</td>
<td>-0.51 ± 0.14</td>
<td>53</td>
<td>0.002</td>
</tr>
<tr>
<td>(15) mmu-miR-503</td>
<td>1.47 ± 0.14</td>
<td>0.61 ± 0.05</td>
<td>55</td>
<td>0.001</td>
</tr>
<tr>
<td>(16) mmu-miR-199b*</td>
<td>3.42 ± 0.10</td>
<td>2.58 ± 0.07</td>
<td>56</td>
<td>0.000</td>
</tr>
<tr>
<td>(17) mmu-let-7a</td>
<td>3.54 ± 0.10</td>
<td>2.72 ± 0.10</td>
<td>56</td>
<td>0.001</td>
</tr>
<tr>
<td>(18) mmu-miR-335-5p</td>
<td>0.83 ± 0.07</td>
<td>0.02 ± 0.03</td>
<td>57</td>
<td>0.000</td>
</tr>
<tr>
<td>(19) mmu-miR-223</td>
<td>0.48 ± 0.06</td>
<td>-0.18 ± 0.02</td>
<td>63</td>
<td>0.000</td>
</tr>
<tr>
<td>(20) mmu-miR-652</td>
<td>0.22 ± 0.02</td>
<td>-0.38 ± 0.04</td>
<td>66</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Between brackets were the respective rankings using Normalization I.
Example 3. miR-144 promotes vessel branching in vitro and in vivo

To ensure that miR-144 was expressed in Tek-expressing yolk sac endothelial cells, Tek<sup>C<sup>Cre</sup></sup>- male mice were crossbred with female RA/EGFP stop-flox reporter mice (Constien et al., 2001), and GFP<sup>-CD45</sup>- yolk sac cells were isolated by FACS sorting. These sorted cells indeed represented the endothelial fraction as, compared to GFP<sup>-CD45</sup>+ cells, the expression of the endothelial genes VEGFR-2 (by FACS; not shown) and eNOS, as analyzed by qPCR, was higher in the GFP<sup>-CD45</sup><sup>-</sup> fraction (5.1 ± 1.2 fold; N=4) (Figure 3). Further analysis by qPCR also revealed clear expression of miR-144 in GFP<sup>-CD45</sup>- yolk sac cells (fold increase versus GFP<sup>-CD45</sup>+ yolk-sac-derived blood cells: 14.1 ± 7.1; N=4), suggesting that the vascular phenotype in the yolk sacs of Dicer mutants might indeed be caused by a cell-autonomous loss of miR-144 expression in endothelial cells.

To directly study the role of miR-144 in endothelial cells, we next investigated its angiogenic potential in human umbilical vein endothelial cells (HUVECs), which express miR-144 (Ct values on qPCR are ~33 cycles), and overexpressed miR-144 via transfection with specific duplexes or precursors. Transfection of miR-144 duplex or precursor resulted in a dose-dependent stable increase of the expression of miR-144 for at least 48 hours (Figure 4). Notably, the expression levels of the related miR-451 as well as the unrelated miR-126 were not changed (not shown), indicating that the overexpression of miR-144 was specific, and that transfection with miR-144 did not interfere with miRNA pathways in general. In the 3D in vitro spheroid model of sprouting angiogenesis (Bonauer et al., 2009), forced overexpression of miR-144 by duplexes stimulated the cumulative sprout length (Figure 5A, left panel). Moreover, miR-144 overexpression significantly increased the number of sprouts per spheroid, and especially the number of sprouts with secondary branches (Figure 5A, left panel). Similar results were obtained when miR-144 was overexpressed by transfecting precursors (Figure 5A, right panel). Of note, careful microscopic analysis of the sprouts revealed that, when miR-144 was overexpressed, the tips of the formed sprouts extended much more filopodia compared to control (Figure 5B). These effects were specific as transfection of HUVECs with anti-sense miR-144 duplexes failed to stimulate sprout formation and branching (not shown).

To study the role of miR-144 on vessel branching in vivo, we injected duplexes in the eyes of newborn C57Bl6 mice, and analyzed the retinal vascular development at P5, as described previously (Hellstrom et al., 2007). Compared to scrambled control injected eyes, miR-144 increased the number of branching point (not shown), indicating that miR-144 also promotes vessel branching in vivo. Consistent herewith, intravitreal injection of specific inhibitors (antagomirs (Krutfeldt et al., 2005)) against miR-144 reduced...
retinal vessel branching (not shown), indicating that endogenous miR-144 is required for vessel branching and formation in vivo.

Example 4. miR-144 initiates endothelial tip cell fate

The effects of miR-144 overexpression on vessel branching in the spheroid assays suggest a role for miR-144 in endothelial tip cells (Larrivee et al., 2009). In an initial attempt to study tip cells in more detail, we performed a modified spheroid assay. We stably pre-labelled HUVECs with two different fluorescent tracers by lentiviral transduction to enable tracking of HUVECs, and subsequently transfected each fraction with either scrambled control or miR-144 duplex. We then admixed the two labelled fractions for the preparation of spheroids and analyzed by confocal microscopy the fluorescent label of the tip cells within the formed sprouts. When miR-144-overexpressing HUVECs were admixed in a ratio 1:1 with control-transfected HUVECs, we found that increased levels of miR-144 dose-dependently prioritized these cells to acquire the tip cell position (not shown). Notably, we ruled out experimental biases such as the unequal distribution of the two fractions within the growing sprouts as well as undesired effects of the viral transduction. To exclude the possibility that labelled miR-144-expressing favour the tip cell position because they are more present in growing sprouts, we performed nuclear staining with Dapi and quantified the two fractions. We found an equal distribution of the two labelled fractions within the growing sprouts when admixed spheroids contained the two labelled fractions, when both were transfected with scrambled control (compared to the other labelled fraction: 51 ± 1%), and when one was transfected with miR-144 duplex (compared to the other labelled fraction: 53 ± 1%; 2 independent experiments).

To exclude the possibility that the labelling of HUVECs with two different viral vectors might cause undesired competitive advantage of one fraction over the other to acquire the tip cell position, we transfected both labelled fractions with scrambled control, admixed them 1:1 in a spheroid and quantified the two fractions in the tip cell position. We found equal distribution of the two labelled fractions in the tip cell position (compared to the other labelled fraction: 45 ± 5%; 2 independent experiments).

Even when three-fold more control-transfected HUVECs were admixed with miR-144-overexpressing HUVECs, we found 46 ± 3% of miR-144-overexpressing cells, instead of the predicted ~25%, in the tip cell position (N=18; P<0.05). Thus, miR-144-expressing endothelial cells favour the tip cell position within growing sprouts.
Detailed qualitative analysis of our dual fluorescent sprouts revealed two additional observations of interest. First, we noticed that, compared to control, the tip cells extended more filopodia, when miR-144 was overexpressed (Figure 5B). Secondly, in sprouts with branching ends, we observed that, in ~100% of cases, both branching tip cells were expressing the same label (not shown). The latter finding suggests that, at the sprouting forefront, symmetrical tip cell division yielding a second tip cell precedes further branching of the sprout (see further).

If miR-144 promotes an endothelial tip cell fate, also the genetic program of these cells should change, as endothelial tip cells have a distinct gene signature (Larrivee et al., 2009). We therefore transfected HUVECs with miR-144 duplexes and analyzed the expression of several putative tip cell and stalk cell genes by qPCR. Consistent with our hypothesis, miR-144 increased the expression of various tip cell genes including Unc5B (Figure 6A). By contrast, miR-144 did not or only minorly alter the expression of VEGFR-2, Dll4 and a panel of stalk cell genes, including effector genes of the Notch pathway (Figure 6A). Accordingly, co-transfection with a Notch reporter construct showed that miR-144 duplexes failed to affect Notch signalling (% Firefly/Renilla activity of control: 87 ± 9; N=3; P=NS; Figure 6B). Hence, increased miR-144 expression in endothelial cells suffices to initiate a gene program reminiscent of tip cells.

Quantification of the number of tip cells at the vascular front of P5 retinas confirmed that, compared to control, miR-144 stimulated the formation of endothelial tip cells and filopodia (not shown). Conversely, antagomirs against miR-144 reduced the number of endothelial tip cells and filopodia (not shown), indicating that miR-144 can instruct tip cell initiation and activation in vivo.

Example 5. Target gene analysis on yolk sac extracts of Dicer mutants
miRNAs regulate gene expression by binding to target mRNAs, resulting in translational repression or degradation. As bio-informatical analysis using prediction software (TargetScan, miRanda) revealed that none of the tip or stalk cell genes were predicted targets of miR-144, we took a step back and first studied the transcriptome of the mutant yolk sacs by comparative genome-wide expression analysis. Subsequently, we focussed on genes whose expression were upregulated, as they could constitute potential miRNA target genes. To this end, we hybridized control and mutant yolk sac extracts onto Affymetrix microarray chips in two independent experiments (N=4 per experiment) and potential target genes were considered when, in both experiments, their expressions in the mutants showed a greater
than 1.05-fold increase with P<0.05. Of note, consistent with our qPCR data, the expression of Dicer was significantly reduced by ~30% (not shown).

This approach resulted in the identification of 697 upregulated genes. We first validated our microarray results by looking for target genes, predicted for miR-126 and the let-7 family. When cross-analyzing the list of 697 upregulated genes for predicted target genes of miR-126-3p, we found the expression of various genes, including Vegfa and Adam33, to be upregulated in the mutant yolk sac (not shown). Further qPCR analysis confirmed that their expression was significantly upregulated (not shown). In the microarray, the expression of the previously identified miR-126 target gene Spred-1 (Fish et al., 2008; Wang et al., 2008) was upregulated by 1.31-fold, but only in one experiment, and was only increased 1.1-fold as analyzed by qPCR (not shown). When searching potential target genes of the let-7 family, we found that the expression of the predicted targets VLDLR and Lin28 was upregulated in the microarray, and we confirmed this increase by qPCR (not shown).

Example 6. miR-144 reduces FoxOl expression

We then aimed at identifying potential target genes for miR-144 in the mutant yolk sac. Cross-analysis of the list of 697 upregulated genes with predicted target genes for miR-144 revealed 26 genes, including FoxOl, Bcl2lll (Bim), GdflO, Ptgs2 (Cox2), Sgppl, JmjdIA, and Ypel2. Validation by qPCR analysis confirmed the upregulated expression of FoxOl, Bcl2lll, GdflO, Ptgs2, Sgppl and JmjdIA (Figure 7A). Further bioinformatics using Gene Ontology (GO) Analysis and the DIANA target prediction algorithm (Maragkakis et al., 2009) specified our attention to the Forkhead transcription factor FoxOl, a conserved target gene of miR-144 (not shown), and an established negative regulator of (developmental) angiogenesis (Potente et al., 2005).

We first analyzed whether the expression of FoxOl target genes and co-factors were also upregulated in our microarray. The expression of FoxOl target genes such as Igfbpl and PpargClA, as well as its co-factor Fox03a were indeed upregulated in our microarray as well as by qPCR validation (Figure 7B). Moreover, immunoblotting on yolk sac extracts revealed higher levels of FoxOl protein in the endothelial Dicer mutants (Figure 7C). Hence, these results suggest that endothelial deletion of Dicer affected FoxOl bioactivity.

We therefore performed further biochemical validation of the miR-144/FoxOl axis in HUVECs. When miR-144 was overexpressed in HUVECs, Affymetrix microarray profiling revealed reduced levels of FoxOl, and qPCR validation experiments confirmed the reduced expression levels of FoxOl (not shown). Consistent herewith, the total protein levels of FoxOl were reduced (Figure 8A). Forced overexpression
of miR-144 also reduced the nuclear protein levels of FoxOl (not shown). Additional functional assays corroborated the notion that miR-144 is truly a negative regulator of FoxOl protein levels and bioactivity. Indeed, when utilizing HUVECs transfected with a FoxOl-responsive luciferase reporter construct (Potente et al., 2005), miR-144 overexpression resulted in lower luciferase activity (Figure 8B).

Moreover, luciferase assays with wild-type and mutated 3'UTR binding sites (1x binding site), transfected in HUVECs, confirmed that FoxOl is a target gene of miR-144 (Figure 8C). Currently some additional miR-144 target genes are being validated.

Example 7. FoxOl knock-down phenocopies miR-144 overexpression

Previously, it was shown that genetic knock-down of FoxOl expression in HUVECs increases cumulative sprout length in the spheroid assay (Potente et al., 2005), but the effect on vessel branching was not studied. Vessel branching was now analyzed, and it was found that, analogous to miR-144 overexpression, lower FoxOl expression indeed also increased the number of sprouts per spheroid, and especially the number of sprouts with secondary branches (Figure 9). When repeating these experiments with a lower siRNA concentration (to achieve a ~50% reduction in FoxOl expression (not shown) which more closely mimics the conditions when miR-144 is overexpressed), we found less increased cumulative sprout length but still clear effects on sprout branching (not shown). Thus, analogous to overexpression of miR-144, vessel branching is promoted by modest reduction of the FoxOl levels in endothelial cells via siRNA.

In conclusion of the first set of experiments, to explore the role of miRNAs in endothelial tip cell specification, a screening platform was developed using endothelial-selective dicer mutant mice (Tek-dicer^-/-; no production of miRNAs in endothelial cells) as well as some HUVEC-based in vitro functional validation assays (gain-of-function (GOF) and loss-of-function (LOF)), to identify miRNAs with a cell-intrinsic role in the specification of endothelial cell fate. Compared to control mice, the yolk sac vasculature of Tek-dicer^-/- mice was less branched. Profiling experiments showed strongly reduced expression of at least 20 miRNAs, suggesting that these miRNAs might be involved in angiogenesis. Amongst them, miR-126 was already recognized as a pro-angiogenic miRNA (Fish et al., 2008), but the strongest regulated miRNA, miR-144, was only known for its hematopoietic involvement (Fu et al., 2009). miR-144 was (lowly) expressed in HUVECs, and GOF studies with 3D spheroid assays, modified to allow fluorescent-based tracing in angiogenic sprouts (not shown), revealed that miR-144 prioritized endothelial cells to acquire the tip cell position, thereby promoting sprout branching in vitro. This
phenotype was not found with its cluster member miR-451. Mechanistic pilot studies using bioinformatical, biochemical and molecular-biological assays suggested that miR-144 might instruct endothelial tip cell fate at least in part by repressing the expression of the endothelial Forkhead transcription factor FoxOl (known to be a negative regulator of angiogenesis (Potente et al., 2005)) but, notably, not by affecting Notch signaling. In sprouting assays, FoxOl LOF phenocopied the effects of miR-144 GOF. GOF studies in vivo confirmed that miR-144 indeed stimulates branching of the retinal vasculature.

Example 8. miR-144 as anti-angiogenic target

miR-144 is primarily expressed in tumor endothelial cells in vivo

The abovementioned findings strongly suggested that miR-144 might be an interesting anti-angiogenic target during tumor growth. We therefore quantified by qPCR the expression of miR-144 in C57Bl6 mice, which were inoculated subcutaneously with the syngeneic pancreatic adenocarcinoma tumor cell line Panc02. Interestingly, while cultured tumor cells expressed little miR-144, the expression of miR-144 progressively increased ~35-fold in Panc02 tumors in vivo, reaching a maximum around ~900-1000 mm³ (see Figure 10). Pilot FACS sorting and in situ hybridization experiments on these tumors showed that miR-144 was primarily expressed in tumor endothelial cells (not shown). These data clearly suggested that, in growing tumors, the expression of miR-144 increased in tumor endothelial cells.

Inhibition of miR-144 reduces tumor growth in vivo

Next, we performed a LOF study with tumors in vivo, with the use of specific "antagomirs", i.e. chemically engineered oligonucleotides which silence specific miRNAs in vivo with very little toxicity (Krutzelfdt et al., 2005). To this end, groups of C57Bl6 mice were inoculated subcutaneously with Panc02 cells. When the tumors reached a size of ~100 mm³, mice were randomized to receive one bolus i.v. injection of specific antagomirs against miR-144 (antagomir-144) or a control scrambled version (antagomir-co) at 8 mg/kg (i.e. a low dose (10-fold lower than the previously reported dose of 80 mg/kg for antagomir administration (Krutzelfdt et al., 2005)), which primarily targets vascular endothelial cells while penetrating little into tissues (Bonauer et al., 2009)). Further follow-up of these mice showed delayed and reduced tumor growth (see Figure 11A). At the end of the experiment (i.e. day 36 after inoculation), the tumor size and weight were reduced by ~20% and ~30%, respectively, in tumor-bearing mice treated with antagomir-144 versus antagomir-co (Figure 11A and 12). Thus, a bolus injection of antagomir-144 at 8 mg/kg delayed and reduced tumor growth in mice.
Interestingly, we also found differences in the survival of tumor-bearing mice. Indeed, while >50% of tumor-bearing mice treated with antagomir-co succumbed before day 36, all tumor-bearing mice treated with antagomir-144 survived (Figure 11B). Associated herewith, we found that the tumor-bearing mice treated with antagomir-co displayed an increased incidence of a cancer-associated systemic syndrome (CASS or paraneoplastic syndrome), consisting of cachexia, anemia, and a relative pro-thrombotic status: 9/13 mice developed paraneoplastic syndrome in the control group, compared to only 3/13 mice in the antagomir-144 treated group (Figure 13). The anemia in these mice was macrocytic of nature (i.e. increased MCV), with normal serum iron levels, and not associated with abnormal circulating levels of Epo or VEGF (previously shown to cause CASS (Tarn et al., 2008; Xue et al., 2008)). The pro-thrombotic status was characterized by increased platelet activation, decreased aPTT, lower circulating levels of fibrinogen, and elevated circulating levels of PAI-1 (known to be primarily derived from the endothelium (Fischer et al., 2007)), but with absence of systemic coagulopathy (e.g. normal FVII levels) or acute phase response (e.g. normal hepatic synthesis of fibrinogen). Thus, antagomir-144 treatment protected mice from death and the development of CASS.

To evidence a dose effect, we also performed an additional experiment in which tumor-bearing mice were injected once with 40 mg/kg antagomir. Although a small experiment (n=6 per group), we found that primary tumor growth was now inhibited by ~50% (Figure 14A), thereby evidencing a dose effect. In addition, we also found again survival benefits after treating mice with antagomir-144 (Figure 14B), which was associated with absence of CASS. Histological analysis of these tumors revealed various additional insights: (1) compared to control treatment, administration of antagomir-144 resulted in less tumor necrosis; (2) compared to control treatment, administration of antagomir-144 did not affect the vascular density per se, but rather the overall perfusion of the tumor: whereas tumors of lectin-FITC-infused control-treated mice consisted of a dispersed presence of hyperdense vascular hot-spots surrounded by large hypoperfused areas, the perfused vessels in the tumors of lectin-FITC-infused antagomir-144-treated mice were homogenously distributed over the whole tumor (not shown). These data suggest that antagomir-144 might reduce primary tumor growth and prevent the onset of CASS by normalizing tumor vasculature.

Example 9. Further validation of miR-144 as anti-tumor target
The therapeutic potential of antagomir-144 will be validated in various mouse tumor models. First, we will optimize the mode of administration of antagomir-144 in C57Bl6 mice subcutaneously inoculated with syngeneic Panc02 pancreatic adenocarcinoma tumor cells (as this model was used to generate our preliminary promising data). In a second phase, we will use various other syngeneic and xenograft mouse tumor models to test the therapeutic potential of antagomir-144.

Optimization of administration
Antagomirs have medically seen enormous potential as it has been reported that a single bolus i.v. injection of antagomirs (80 mg/kg) efficiently silences the expression of a specific endogenous miRNA up to 8 weeks after administration (Krutzfeldt et al., 2005). In addition, toxicity is very little, with only documented findings on transiently (non-specific) elevated liver enzymes and disturbed levels of cholesterol, and a slight inflammatory response (Krutzfeldt et al., 2005). Moreover, it was reported that a 10-fold reduced dose of antagomirs compared to the standard dose (8 mg/kg), injected in bolus i.v., still effectively silences endogenous miRNA expression for up to 6 weeks in vascular endothelial cells with little tissue penetration (Bonauer et al., 2009). In the experiments using C57Bl6 mice subcutaneously inoculated with Panc02 cells (Example 9), we injected a single bolus i.v. 8 mg/kg, with promising results. We will therefore use this model to optimize the mode of administration, and focus on number of injections and antagomir dose versus efficiency to knock-down the expression of miR-144 in tumors in vivo, and the biological response.

To this end, we will inoculate 1 x 10^6 Panc02 cells in the dorsal flank of age- and gender-matched C57Bl6 mice. When the tumors have reached a size of ~100 mm^3, we will randomize the mice into groups which receive i.v. injections of antagomir-144 or control scrambled antagomir (both dissolved in saline):
- Different doses of antagomirs (e.g. 8 mg/kg, 20 mg/kg, 40 mg/kg) will be further evaluated in a single bolus injection i.v. when the tumors reached a size of ~100 mm^3;
- We will test an additional bolus i.v. injection of antagomirs when the tumor reached a size of ~800-900 mm^3 (i.e. when miR-144 expression is upregulated the most - see Figure 10).

Mouse health and survival will be followed up daily, and tumor growth will be followed up daily by measurements of the length (L) and width (W) of tumors via an accurate electronic caliper, and by an investigator blinded to the treatment. Mouse tumor volumetric size is calculated by the following formula : (L x (W^2) x \Pi)/6 (Loges et al., 2010). Mouse total body weights will be documented regularly.

For analyzing knock-down efficiency, tumors will be taken out at sizes around ~1000 mm^3, or at the end of the experiment (day 36 after inoculation, or when the tumor size has reached the ethical limit of ~2500 mm^3). Tumor tissue will be subjected to RNA extraction using previously optimized protocols.
(miRNAEasy, Qiagen), and the expression of miR-144 and the housekeeping small RNA U6 will be measured by Taqman qPCR (commercially available via Applied Biosystems). For specificity reasons, we will also measure the expression of related (miR-451) and unrelated (miR-126) miRNAs.

Tests in various syngeneic and xenograft mouse tumor models

The efficacy of antagomir-144 will also be explored in various subcutaneous syngeneic and xenograft mouse tumor models, including the use of tumor models previously shown to be relatively resistant to anti-VEGF treatment (Loges et al., 2010):

- Syngeneic anti-VEGF-sensitive models: Panc02 (pancreas adenocarcinoma, C57Bl6), and 4T1 (breast adenocarcinoma, BalbC);
- Syngeneic anti-VEGF-resistant models: CT26 (colon adenocarcinoma, BalbC), and EL-4 (T-cell lymphoma, C57Bl6);
- Xenograft models (using nu/nu nmri mice as recipients): MDA-MB-231 (human breast adenocarcinoma), and DanG (human pancreas adenocarcinoma).

To this end, age- and gender-matched recipient mice will be inoculated in the dorsal flank with $1 \times 10^6$ cells, and followed up for survival and tumor growth as described above (the duration of follow-up depends on tumor cell type). We will first do an expression analysis for miR-144 by qPCR at different tumor sizes and in the different tumor cell lines, as previously performed in the subcutaneous Panc02 model (Figure 10). Then, we will apply the optimal mode of administration of antagomir-144, and analyze, compared to control treated mice, the following parameters:

- Dissection: at the end of each experiment, we will carefully dissect the tumors from the mice, paying macroscopic attention to tumor invasiveness, signs of metastasis, etc. Parts of the tumors will be processed for histological analysis (see below), and parts will be stored at -80 °C for RNA or protein analysis (see mechanistic studies).

- Vascular density: ex vivo analysis of vascular densities includes staining for endothelial markers (CD31, lectin) on paraffin sections. In separate groups, we will also analyze the number of in vivo perfused vessels by injecting i.v. 200 μl lectin-FITC (1 mg/ml; Sigma) at 30 min before dissection in tumor-beating mice (Loges et al., 2010). Imaging will be performed on a Zeiss fluorescence microscope, and quantitative (morphometric) analysis will be performed using predesigned AxioVision software and macros.

- Further histological analysis of tumors: the area of tumor cell proliferation will be determined by staining for proliferation markers (Ki-67, PCNA) or by pre-injection of BrdU 24. The necrotic area will be
determined by measuring the area of autofluorescence (Loges et al., 2010). Differences in the recruitment of inflammatory cells will be analyzed by staining for leukocyte (CD45) and macrophage (Mac3) markers (Loges et al., 2010). Differences in the cancer-associated stromal fibroblast compartment will be analyzed by staining for vimentin (Loges et al., 2010). The presence of thrombi will be analyzed by staining for fibrinogen and VWF (Loges et al., 2010).

- Hypoxia analysis: tumor hypoxia will be detected at 120 min after injection of 60 mg/kg pimonidazole hydrochloride into tumor-bearing mice (Loges et al., 2010). To detect pimonidazole, tumor sections will be immunostained with hypoxyprobe-I-Mab (Chemicon) following the manufacturer’s instructions.

- CASS (paraneoplastic syndrome) analysis: blood will be repetitively sampled from tumor-bearing mice by puncturing the retro-orbital plexus under isoflurane anesthesia. Specific blood samples (plasma, serum) will be taken for mechanistic studies (see below). Full blood counts will be obtained via an automated blood counter (Abbott Cell-Dyn 3500), including mean platelet volume (MPV) as a surrogate marker of platelet activation. At the end of the experiment, we will analyze extramedullar hematopoiesis (e.g. spleen weights, histological analysis of liver and spleen), the bone marrow (e.g. histological analysis of femurs), and Epo synthesis in liver and kidney (see mechanistic studies).

- Anti-VEGF treatment: in the syngeneic anti-VEGF-resistant models (CT26, EL-4), we will compare the effect of antagonim-144 with treatment with neutralizing anti-VEGFR-2 antibodies (DC101), available through ThromboGenics. To this end, tumor-bearing mice will be injected i.p. 3x per week with 50 mg/kg DC101 or IgG control antibodies (Sigma), as done previously (Fischer et al., 2007).

- Endothelial tip cells: To visualize endothelial tip cells in tumors, we will use tumor-bearing Tek-YFP mice (C57Bl6 background; available) treated with antagonim-144, and confocal imaging of whole tumor sections and tip cells in 70 μm vibratome tumor slices (embedded in agar). Pilot experiments showed its feasibility.

**Mechanistic studies**

To gain further mechanistic insights into the potential effects of antagonim-144 in mouse tumor models, we will investigate the potential mechanism by which antagonim-144 might affect tumor angiogenesis. To this end, based on the in vitro data (e.g. Example 6), we will analyze the effects of antagonim-144 on the expression of FoxO1 in tumor endothelial cells. Also, the underlying molecular mechanisms by which antagonim-144 might protect against (cancer-associated) platelet activation and thrombosis as well as anemia will be studied. Further, we will study how miR-144 expression in (tumor) endothelial cells is regulated. We have also generated miR-144**+/−** mice which, by intercrossing with endothelial-selective
Cre deleter mice, will allow to generate cell-type specific genetic data to corroborate the role and potential of miR-144 as a novel anti-angiogenic target in cancer.

**Effects of antagomir-144 on tumor endothelial cells**

One of the goals is to identify the molecular mechanism by which antagomir-144 is capable of affecting tumor angiogenesis. Based on the data showing that miR-144 targets the endothelial forkhead transcription factor FoxO1, we will therefore FACS sort tumor endothelial cells from tumor-bearing ring mice and analyze the expression levels of FoxO1. We have already established the experimental approach for this by using Panc02 tumor-bearing Tek-YFP mice (C57Bl6 background; available) treated with antagomir-144 as recipients. Tumor endothelial cells will be isolated from tumor cell suspensions with a FACS Aria Cell Sorter by positive staining for YFP and CD31, and negative staining for Mac1/CD11b (operational). RNA extraction of sorted cells using miRNAEasy (Qiagen) will be performed to analyze the expression of miR-144, FoxO1 and the FoxO1 target gene eNOS (Taqman probes are commercially available with Applied Biosystems). Protein extracts of sorted cells will be used for western blotting of FoxO1 and eNOS, using commercially available antibodies.

We will also perform experiments on tumors from C57Bl6 mice, and analyze the expression of miR-144, VEGF, other angiogenic cytokines, etc. by qPCR and ELISA.

**Effects of antagomir-144 on cancer-associated platelet activation and anemia**

Our findings suggest that antagomir-144 protects against cancer-associated platelet activation, thereby affecting hemostasis. Preliminary (bioinformatical and experimental) evidence suggests the involvement of the platelet-activating lipid SIP as a potential explanation: miR-144 seems to repress the SIP degrading enzyme sgppl, thereby increasing the bioavailability of SIP. We will therefore measure the SIP levels in the circulation and tumor extracts of tumor-bearing mice.

Sorted tumor endothelial cells will be used for the measurement of sgppl by qPCR (Taqman probe available via Applied Biosystems) and western blot using commercially available antibodies.

We will also further characterize the pro-thrombotic status in tumor-bearing mice. To this end, the levels of aPTT, coagulation factors and circulating fibrinogen will be determined on an automated machine (Siemens), previously validated for mouse samples. Analysis of circulating PAI-1 levels (as a parameter of endothelial pro-thrombosis) will be performed with an in-house developed ELISA. Levels of circulating sP-selectin as a surrogate marker of platelet activation will be measured by commercially available ELISA (R&D Systems).
Further mechanistic studies will be performed in HUVECs. We will transfect HUVECs with miR-144 duplexes or sgppl siRNA (Qiagen) and analyze the levels of secreted SIP. In addition, freshly isolated calcein-labeled mouse platelets will be added, and the number of platelet aggregates will be analyzed by fluorescence microscopy.

We strongly believe that miR-144 targets FoxO1 and sgppl with different biological read-out: miR-144 represses FoxO1 expression to instruct endothelial tip cell fate, whereas it represses sgppl expression to increase its thrombogenicity. To exclude that miR-144-mediated repression of sgppl affects endothelial tip cell fate, we will use our previously established dual-labeled spheroid assay and analyze the role of sgppl in endothelial tip cell specification, using HUVECs transfected with sgppl siRNA. Vice versa, we will use HUVECs transfected with FoxO1 siRNA to analyze aggregation of co-cultured platelets. To further establish sgppl as a target gene of miR-144, we will use state-of-the-art 3'UTR luciferase reporter assays: i.e. HUVECs are transfected with luciferase constructs in which the binding sequence of miR-144 to sgppl is cloned (operational).

To analyze the specificity of antagonim-144 to affect platelet activation in tumor-bearing mice, we will also study the effect of antagonim-144 in non-tumor mouse thrombosis models. To this end, we will use a mouse model of pulmonary thrombo-embolism to test the effect of antagonim-144. C57Bl6 mice are injected with antagonim-144 or control scrambled antagonim, and 3 days later with a mixture of collagen (0.5 mg/kg) and epinephrine (60 mg/kg) (Angelillo-Scherrer et al., 2005). Thrombo-emboli will be formed and the time of survival will be followed.

Our findings also show that antagonim-144 protects against cancer-associated anemia. The anemia in these mice was macrocyclic of nature (i.e. increased MCV), and with normal serum iron levels. Previous reports showed that CASS could be caused by abnormal circulating levels VEGF thereby causing regression of hepatic blood vessels and reduced levels of circulating Epo (Tarn et al., 2008; Xue et al., 2008), this appears not to be the case in our experiments (i.e. no differences in plasma levels of VEGF and Epo, absence of hepatic vessel regression). We believe that antagonim-144 protects against anemia by preventing repression of erythropoiesis. Notably, as potential target gene of miR-144, bioinformatical analysis predicted the cKit-liga nd SCF, and it is known that SCF deficient mice display macrocyclic anemia (McCulloch et al., 1964). Therefore, we believe that, in tumor-bearing mice, miR-144 represses the expression of SCF, thereby causing macrocyclic anemia, and that this is consequently prevented in mice treated with antagonim-144. We will therefore measure the SCF levels in the circulation and tumor extracts of tumor-bearing mice by commercially available ELISA (R&D Systems). Sorted tumor endothelial cells will be used for the measurement of SCF by qPCR (Taqman probe available via Applied
Biosystems). Further mechanistic studies will be performed in HUVECs. We will transfect HUVECs with miR-144 duplexes and analyze the levels of (secreted) SCF by qPCR and ELISA. As we strongly believe that miR-144 targets FoxO1, sgpp1 and SCF with different biological readout (see above), we will use our previously established dual-labeled spheroid assay and analyze the role of SCF in endothelial tip cell specification, using HUVECs transfected with SCF siRNA (Qiagen). To further establish sgpp1 as a target gene of miR-144, we will use state-of-the-art 3’UTR luciferase reporter assays: i.e. HUVECs are transfected with luciferase constructs in which the binding sequence of miR-144 to SCF is cloned (operational).

10 Regulation of miR-144 expression

Preliminary (bioinformatical and experimental) evidence suggests the involvement of hypoxia and the endothelial transcription factor Gata2. We will therefore use previously established genetic (Gata2 GOF and LOF: Gata2 overexpression construct available), molecular- and (HUVEC-based) cell-biological approaches to further investigate this. We will also attempt to identify the upstream cytokine regulating Gata2 activation and miR-144 upregulation, by in vivo cytokine profiling (R&D Systems) in plasma samples of tumor-bearing mice treated with antagonmir-144, and further in biochemical and molecular biological validation studies using HUVECs in vitro.

Genetic studies using newly generated conditional miR-144 deficient mice

A key research tool will be the availability of miR-144fl/fl mice. We developed this unique mouse model via outsourcing to GenOway, and these mice are currently available. They can be used for cross-breeding. We will generate and phenotype mice lacking miR-144 expression in a conditional cell-type specific fashion (i.e. VE-Cad-CreERT), to further explore its role in tumor angiogenesis using the syngeneic models as described above (Tamoxifen will be administered to induce genetic recombination in VE-Cad-expressing host endothelial cells). These mice will also be instrumental for our mechanistic studies as described above.

These mice will also be instrumental to perform expression analysis and genetic lineage tracing. Therefore, we plan to cross-breed the miR-144fl/fl mice with Rosa26LacZ, Rosa26YFP and/or Rosa26mFP reporter mice (all available). Further intercrossing with VE-Cad-CreERT will allow to genetically trace down miR-144 expressing endothelial tip cells by confocal microscopy (see above), and to isolate them by FACS sorting for further mechanistic studies.
Extension of clinical data

The effects of antagomir-144 will be studied in some clinically relevant settings, including safety/toxicity studies, studies on co-administration of antagomir-144 together with chemotherapy, studies on potential effects on metastasis and lymphangiogenesis, and studies in a mouse model of choroidal neovascularization (CNV).

Safety/toxicity studies of antagomir-144

Imperative for potential further development of antagomir-144 as a novel anti-angiogenic drug will be the lack of toxicity in vivo. Antagomirs have medically seen enormous potential as it has been reported that a single bolus i.v. injection of antagomirs (80 mg/kg) efficiently silences the expression of a specific endogenous miRNA for many weeks (Krutzfeldt et al., 2005). In addition, toxicity is very little, with only documented findings on transiently (non-specific) elevated liver enzymes and disturbed levels of cholesterol, and a slight inflammatory response (Krutzfeldt et al., 2005). We will therefore carefully monitor the mice treated with antagomir-144, and analyze regularly the full blood counts, as well as various parameters of systemic toxicity, including liver and kidney panels (in collaboration with Clinical Biochemistry, University Hospital Gasthuisberg).

Importantly, like anti-PIGF (Fischer et al., 2007), we believe that antagomir-144 does not affect the quiescent normal vasculature and only targets the tumor vasculature, as normal endothelial cells have only low expression of miR-144. We will therefore, in tumor-bearing mice, analyze microvascular pruning in healthy organs such as the trachea, as done previously (Fischer et al., 2007). Age- and gender-matched C57Bl6 mice, inoculated subcutaneously with Panc02 cells and treated with antagomir-144, will be injected with lectin-FITC, and the density of capillary branching points in the trachea will be determined by fluorescence microscopy. As control, we will administer to tumor-bearing mice neutralizing antibodies against VEGFR-2 (DC101, available through ThromboGenics).

Co-administration of antagomir-144 with chemotherapy

Analogous to anti-VEGF, antagomir-144 might synergistically amplify the efficacy of chemotherapy. We will therefore test the efficacy of combined administration of antagomir-144 and chemotherapy in mice, subcutaneously inoculated with Panc02 cells, as done previously (Fischer et al., 2007). We will administer antagomir-144 or scrambled control antagomir and/or the chemotherapeutic agent gemcitabine (125 mg/kg, 2x per week), and mouse survival and tumor growth will be followed as described above.
Effects of antagomir-144 on metastasis

As antagomir-144 might block tumor angiogenesis, it might also reduce the hematogenic metastasis of tumor cells to distant organs. To this end, we will use the previously established model of orthotopic inoculation of 4T1 tumor cells in the mammary fat pad, which allows the analysis of hematogenic dissemination of tumor cells to the lung (Loges et al., 2010). Age-matched female BalbC mice will be injected with $1 \times 10^6$ 4T1 cells in the left mammary fat pad, and mouse survival and tumor growth will be followed as described above. First, we will perform a time course study on the expression of miR-144 by qPCR in different primary tumor sizes. Second, antagomirs will be administered in bolus i.v., when the primary tumor reached a size of $\sim 100\text{mm}^3$. At day 28-30 after inoculation, primary tumors will be removed, and tumor weight and volume will be determined. The incidence of tumor invasion into the lungs will be analyzed by tracheal instillation with Indian Ink, and macroscopic quantification of metastatic nodules on the lung surfaces (white after destaining), and histologically confirmed. We will carefully analyze that potential differences are not due to differences in primary tumor size.

Effects of antagomir-144 on lymphangiogenesis

Analysis of effects of antagomir-144 on lymphangiogenesis is clinically relevant, as the formation of lymphatic vessels promotes lymphatic metastasis (occurring frequently in pancreatic and breast cancer). To this end, we will use the previously established model of orthotopic inoculation of Panc02 tumor cells in the mouse pancreas, which allows the analysis of lymphangiogenesis and lymphatic metastasis (Fischer et al., 2007; Loges et al., 2010). Age- and gender-matched C57Bl6 mice, anesthetized with isoflurane, will be injected with $1 \times 10^6$ Panc02 cells in the pancreas head via a small abdominal midline incision, and mouse survival and tumor growth will be followed as described above. First, we will perform a time course study on the expression of miR-144 by qPCR in different primary tumor sizes. Second, antagomirs will be administered in bolus i.v. at 2 days after inoculation (when the primary tumor reached a size of $\sim 30\text{mm}^3$). At day 11 after inoculation, primary tumors will be removed, and tumor weight and volume will be determined. The incidence of tumor invasion into adjacent organs, hemorrhagic ascites, and regional celiac and mesenteric lymph node metastasis will be recorded, and histologically confirmed. We will carefully analyze that potential differences are not due to differences in primary tumor size. Apart from analyzing the density of blood vessels using techniques described above, we will also determine the peritumoral lymphatic vessel density in primary tumors, by staining with Lyve-1. We will also determine the effect of antagomir-144 treatment on the expression of lymphangiogenic genes (e.g. VEG-F-C) by qPCR in tumor extracts.
Effects of antagomir-144 on CNV

Analogous to anti-VEGF, antagomir-144 might hold the potential to become a novel anti-angiogenic drug in diseases other than cancer, such as eye diseases. We will therefore explore its efficacy in a previously established mouse model of choroidal neovascularization (CNV), which represents a surrogate model of age-related macular degeneration or AMD (accessible through collaboration with Dr I Stalmans (KUL) and ThromboGenics). In brief, 3 laser burns will be placed with a 532 nm green laser at 9, 12, and 3 o'clock positions around the optic disk using a slit lamp delivery system in age- and gender-matched C57Bl6 mice, anesthetized with Nembutal and pretreated with the pupil dilator Tropicamide. First, we will perform a time course study on the expression of miR-144 by qPCR in the lasered mouse eye. Second, we will test intravitreal and systemic injection of antagomir-144, administered a few hours after laser-burning. On day 14 after the laser treatment, the eyes will be enucleated after i.v. perfusion with FITC-conjugated dextran (50 mg/ml, Mr 2 x 106 Da; Sigma) and fixed in 1% paraformaldehyde, or immediately processed for RNA extraction and analysis of miR-144 expression by qPCR. The FITC-vascular area will be quantified on flat mount preparations using a Zeiss fluorescence microscope with automated macros. We will also be able to perform genetic studies using our newly developed miR-144fl/fl mice intercrossed with PDGFB-CreERTdeleter mice.

Conclusion

1. We have shown that antagomir-144 is an innovative angiogenesis inhibitor with high therapeutic potential, which will be further explored. Given the high efficacy of antagomirs, as well as their low toxicity with a single bolus injection, the benefits of this approach are clear. As far as we know, this is the first suggestion to use antagomirs as anti-angiogenic drug in cancer.

2. In angiogenesis research, the identification and characterization of endothelial tip cells is critical for a better understanding of vascular branching and sprouting in health and disease, with potential clinical translation. Indeed, drug development focuses on specifically targeting these endothelial tip cells as anti-angiogenic strategy, as exemplified by the recent findings to target tip cell molecules such as Dll4 and Unc5B. The present approach allows specific targeting of endothelial tip cells in a hereto unrecognized manner.

3. We have generated in vivo data in a mouse model with both miR-144 and antagomir-144, and several unique mouse models (e.g. miR-144fl/fl mice) are currently being used for further validation, particularly for the development of antagomir-144 as a novel anti-angiogenic drug in cancer.
The current dogma for endothelial tip cell specification states a "permissive" model, i.e. negative selection. Our results indicate an unprecedented "instructive" model, i.e. positive selection of tip cells by cell-intrinsic upregulation of specific miRNAs. Moreover, beyond the established role of endothelial tip cells as migrating cells at the vascular forefront enabling vessel sprouting and branching, we have preliminary evidence for novel and unexpected biological functions of tip cells, i.e. regulating hemostasis and RBC homeostasis (see above).

References


Claims

1. A modulator of miR-144 for use as a medicament.

2. A modulator of miR-144 for use in treatment of a disease characterized by aberrant angiogenesis.

3. The modulator of claim 2, which is an inhibitor of miR-144, for use in treatment of a disease characterized by excessive angiogenesis.

4. The inhibitor of claim 3, wherein the disease characterized by excessive angiogenesis is selected from the group of cancer and diabetic retinopathy.

5. The inhibitor of claim 4 for use in treatment of cancer, wherein the inhibitor of miR-144 reduces tumour growth.

6. The inhibitor of claim 4 or 5, wherein the inhibitor of miR-144 reduces tumour metastasis.

7. The inhibitor of any one of claims 4 to 6, wherein the inhibitor of miR-144 reduces the incidence of paraneoplastic syndrome. CASS

8. The modulator of any one of claims 1 to 7, which is an antagomir or LNA.

9. The modulator of claim 2, which is a miR-144 mimetic, for use in treatment of a disease characterized by impaired angiogenesis.

10. The mimetic of claim 9, the disease characterized by impaired angiogenesis is selected from ischemic heart disease or ischemic limb disease.

11. A method for treating diseases characterized by aberrant angiogenesis, comprising:

- administering to a subject in need thereof a modulator of miR-144, thereby restoring angiogenesis to a normal level.

12. miR-144 for use as a biomarker.

13. miR-144 for use as a biomarker to detect cancer.

14. A method to detect cancer in a subject, comprising:
- determining the expression levels of miR-144 in a sample from said subject;
- correlating the expression levels of miR-144 to the presence of cancer.

5 15. A method to characterize cancer in a subject, comprising:
- determining the expression levels of miR-144 in a tumour sample from said subject;
- correlating the expression levels of miR-144 to the nature and/or aggressiveness of the tumour.
**Figure 1**

### A. Genotype

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<th>Dicer$^{\text{flox/flox}}$</th>
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<td>(28%)</td>
<td>(22%)</td>
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### B. Dicer expression

**qPCR**

- **Control**: [Bar Graph]
- **Mutant**: [Bar Graph] * (Significant Difference)

**Microarray**

- **Control**: [Bar Graph]
- **Mutant**: [Bar Graph] * (Significant Difference)
Figure 2

qPCR validation

- mmu-miR-144
- mmu-miR-126-3p
- mmu-let-7b
- mmu-let-7d
- mmu-let-7i
- mmu-miR-143
- mmu-miR-542-3p
- mmu-let-7f
- mmu-miR-199b
- mmu-miR-152
- mmu-miR-214
- mmu-miR-199a-5p
- mmu-miR-503
- mmu-miR-335-5p
- mmu-miR-223
- mmu-miR-652

Expression in mutants relative to control (%)

Figure 3

RQ of eNOS

- Tie2-GFPpos CD45pos
- Tie2-GFPpos CD45neg

Expression levels ranging from 0 to 1.2
Figure 4

miR-144 duplex

miR-144 precursor

Figure 5

A

Cumulative sprout length per spheroid
Number of sprouts per spheroid
Number of branched sprouts per spheroid

Expression in mutants relative to scrambled control (%)

scrambled control
miR-144 duplex

B

scrambled control
miR-144
miR-144 duplex
Figure 6

A

miR-144-initiated gene signature

Expression relative to control (fold increase)

Tip cell genes  Stalk cell genes

B

TP1 Notch signaling reporter

Relative activity versus control (%)
Figure 7

A

miR-144 target genes

Microarray

FoxO1
Bcl2l11
Gdf10
Ptgs2
Sgpp1
Jmjd1A
Ypel2

Expression relative to control
(fold increase)

qPCR

Expression relative to control
(fold increase)

B

FoxO1 target genes

Microarray

Igfbp1
Ppargc1A
FoxO3a

Expression relative to control
(fold increase)

qPCR

Expression relative to control
(fold increase)

C

Yolk sac

FoxO1

FoxO1 levels (% of control)

control

mutant
Figure 9

HUVEC spheroid assay

- Cumulative sprout length per spheroid
- Number of sprouts per spheroid
- Number of branched sprouts per spheroid

Expression in mutants relative to scrambled control (%)

- Scrambled control
- FoxO1 siRNA

Figure 10

Expression of miR-144 in tumors relative to tumor cells

- Tumor cells
- \approx 400 \text{ mm}^3
- \approx 700 \text{ mm}^3
- \approx 1000 \text{ mm}^3
- \approx 1200 \text{ mm}^3
- \approx 1900 \text{ mm}^3
- \approx 2500 \text{ mm}^3

Fold increase versus tumor cells
Figure 11

A

***Tumor Volume Time course 8 mg/kg

![Graph showing tumor volume over time for control and Antago miR-144 groups.]

Days post tumor cell implantation

B

Survival of Exp02 and Exp03

![Graph showing survival over time for different groups.]

Logrank test: P=0.0012
Figure 14

A

Tumor Volume time course Exp05 35d

- Control
- Antago mTR-144

Days post tumor cell implantation

B

Survival of tumor-bearing mice (40 mg/kg)

ctr (n=12)
144 (n=6)

Percent survival

Logrank test: P=0.1741
A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113 A61K31/713
ADD. A61P35/00

According to International Patent Classification (IPC) and/or both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal , WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Relevant to claim No.</th>
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[ ] Further documents are listed in the continuation of Box C.  
[ ] See patent family annex.

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"Z" document member of the same patent family

Date of the actual completion of the international search: 6 June 2012

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