**Title:** MIR-30 FOR USE IN THE MODULATION OF ANGIogenesis

**Abstract:** The invention relates to the use of microRNAs in the treatment of angiogenesis related conditions, especially cancer.
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Field of the invention

The invention relates to a microRNA (miRNA) family for use in the treatment of cancer and other conditions, particularly those in which angiogenesis plays a role. Also provided are methods for producing the miRNA, molecules which affect the interaction of the miRNA and its target and the use of such molecules.

Background to the invention

Delta-like 4 (DLL4) is a membrane-bound ligand belonging to the Notch signaling family, which plays a fundamental role in vascular development and angiogenesis (Gridley, 2010; Phng and Gerhardt, 2009). DLL4 haploinsufficiency results in extensive arterial defects and embryonic lethality (Gale et al., 2004) indicating that the developing vasculature is sensitive to minor alterations in DLL4 dosage. Its expression is mainly restricted to the endothelium of nascent vessels, particularly the tip cells, where it maintains stalk cell identity in neighbouring cells, thereby regulating vessel sprouting and branching in response to angiogenic stimuli (Hellstrom et al., 2007). The restraint which DLL4 places on angiogenesis contributes to the switch from the proliferative phase to the maturation and stabilisation phases (Harrington et al., 2008). The importance of optimal DLL4 expression is demonstrated through its regulation of intersegmental vessel (ISV) development in zebrafish. Knock-down of dll4 using morpholinos leads to an increased number of endothelial cells within intersegmental arteries due to aberrant specification of cell identity (Siekmann and Lawson, 2007). When dll4 deficient zebrafish are examined at 2.5 days post-fertilisation (dpf) they have reduced circulation in the dorsal aorta (DA) and post cardinal vein (PCV) and blood flow is almost undetectable in the majority of ISVs (Leslie et al., 2007). Closer examination of these morphant embryos revealed that an aberrant network of vessels had replaced the normal T-junction between each ISV and the dorsal longitudinal anastomotic vessel (DLAV).

The regulatory role fulfilled by DLL4 during physiological angiogenesis is also relevant in pathological settings (Lobov et al., 2007). DLL4 expression is increased in human tumors, often in association with markers of inflammation, hypoxia and angiogenesis (Jubb, 2009; Jubb, 2010; Martinez, 2009; Patel et al., 2005; Patel, 2006), and inhibition of DLL4 in several tumor models blocks tumor growth by promoting non-productive, deregulated angiogenesis.
(Haller et al., 2010; Noguera-Troise et al., 2006; Oishi et al., 2010; Ridgway et al., 2006). The inventors, and others, have shown that DLL4 expression is upregulated in lymphatic endothelial cells (LEC) following infection by Kaposi’s sarcoma herpesvirus (KSHV) (Emuss et al., 2009; Liu et al., 2010). KSHV is an oncogenic γ-herpesvirus that is the etiological agent of Kaposi’s sarcoma (KS), an angioproliferative neoplasm composed of cells of endothelial origin as well as a variable inflammatory component (Douglas et al., 2007; Wang et al., 2004).

MiRNAs are small, non-coding RNAs that influence target gene expression through mRNA degradation and translation inhibition (Carthew and Sontheimer, 2009). Implicated in many key cellular and physiological processes, miRNAs are known to play a role in angiogenesis and cancer (Croce, 2009; Wang and Olson, 2009). The inventors have previously described the miRNA signature in LEC following KSHV infection (Lagos et al., 2010). Further examination of these data indicated significant downregulation of members of the miR-30 miRNA family after infection; we also observed that this family is amongst the most robustly expressed in LEC, suggesting suppression by KSHV could have functional implications. Encoded by six genes and expressed from four different transcripts across the human genome, the members of the miR-30 family share an identical seed sequence and hence have common predicted targets (Grimson et al., 2007). Here we show that miR-30, especially miR-30b and miR-30c, target DLL4 both in vitro and in vivo. Furthermore, overexpression of these miRNAs in the developing zebrafish embryo partially phenocopies dll4 knockdown and the described phenotypes have been shown to occur by way of dll4 targeting. Based on these findings the inventors realised that the miR-30 family can be used to control angiogenesis and to treat conditions in which angiogenesis plays a role.

**Summary of the invention**

The invention provides a polynucleotide comprising a nucleotide sequence having substantial homology to the nucleotide sequence ACAAAUGU for use in the modulation of angiogenesis.

The polynucleotide is preferably a RNA molecule. The polynucleotide could be a shRNA, a siRNA, but is preferably a miRNA. Alternatively the polynucleotide may be a polynucleotide which can processed to produce a miRNA, such as a pri-miRNA or pre-miRNA. The term polynucleotide is also considered, herein, to encompass any molecule which has a base
sequence with a structure similar to that of DNA or RNA so that the base sequence of the molecule can base pair with a complementary base sequence such as an oligodeoxynucleotide or an oligoribonucleotide, a phosphorodiamidate morpholino oligonucleotide (PMO), a 2'-0-methyl (2'OMe) oligonucleotide, a locked nucleic acid (LNA) or a peptide nucleic acid (PNA), oligonucleotides containing phosphorothioate bonds, 2'-fluoro oligonucleotides, hexitol nucleic acid, 2'-0-methoxyethyl oligonucleotide, 2'-0-allyl oligonucleotide, 2'-0-propyl oligonucleotide, 2'-0-pentyl oligonucleotide, or oligonucleotides with multiple modifications, such as those comprising phosphorothioate bonds and fluoro or allyl groups.

In one embodiment, the polynucleotide is preferably between 20 and 30 bases in length and more preferably between 21 and 26 bases in length. It is particularly preferred that the polynucleotide is 21, 22 or 23 bases in length. In this embodiment, the polynucleotide is designed to be structurally similar to a naturally occurring miRNA.

In another embodiment, the polynucleotide may be double stranded and thereby comprise a complementary second base sequence. The two sequences may or may not be connected by a stem-loop like structure in the polynucleotide. If the two sequences are connected by a stem-loop structure the polynucleotide will have a similar structure to pre-miRNA or pri-miRNA. As a result, the polynucleotide can be processed to form a miRNA-like molecule by the Drosha, Pasha (DCGR8) and Dicer proteins. If the polynucleotide has a structure which is similar to pre-miRNA, the polynucleotide will preferably be between about 60 and about 80 bases in length. If the polynucleotide has a structure which is similar to pri-miRNA, the polynucleotide will preferably be between about 500 and about 2 kb in length and more preferably, between about 800 and about 1.2 kb in length. Alternatively, the two sequences may simply be held together by the base pairing between the sequences but are not connected in any way. This structure is similar to a miRNA/miRNA* duplex ("*" denotes the antisense or complementary sequence). If the polynucleotide has a structure which is similar to a miRNA/miRNA* duplex, each strand of the molecule will preferably be between 20 and 30 bases in length.

The polynucleotide comprises a nucleotide sequence having substantial homology to the sequence ACAAAAUGU. In particular, it preferably comprises that nucleotide sequence, or one which differs by only one or two nucleotides. The nucleotide sequence ACAAAAUGU is
preferably found at one end of the polynucleotide molecule, within the first or last 12 nucleotides, more preferably within the first or last 10 nucleotides. Most preferably it provides the first or last 8 nucleotides of the polynucleotide. It may be found at the 5' or 3' terminal, but is preferably at the 5' terminal such that the polynucleotide comprises a U as its final nucleotide at the 5' terminal. The nucleotide sequence ACAAUGU comprises the seed sequence of miR-30 and binds to a region within DLL4 mRNA. The seed sequence of miR-30 is CAAAUG, and it is particularly preferred that the polynucleotide comprises this sequence.

The polynucleotide preferably comprises additional nucleotides found in the sequences of miR-30 family. In particular, the polynucleotide preferably comprises one or more of the nucleotides which flank the ACAAUGU sequence in the miR-30 family sequences. Such nucleotides are preferably found at equivalent positions within the polynucleotide of the invention. The polynucleotide preferably comprises the sequence CCU immediately 3' of the ACAAUGU homologous sequence.

The polynucleotide may comprise additional nucleotides from the miR30 family sequences, again in corresponding locations. For example, the polynucleotide preferably comprises one or both of the nucleotides found at positions 12 and 15 in the miR-30 family sequences, especially of miR-30a, c, d or e, at a position an equivalent distance from the ACAAUGU homologous sequence. At the corresponding position to position 12 in the miR-30 sequence, the nucleotide is preferably U or C. At position 15, the nucleotide is preferably A.

Accordingly, the polynucleotide of the invention preferably comprises one of the following sequences:

ACAAUGU; CCUACAAUGU; UCUCACAAUGU; CCCUACAAUGU; AXXUCCUACAAUGU; AXXCCUACAAUGU; UXXXACAAUGU; CXXXACAAUGU; and AXXXXXACAAUGU, wherein X is any nucleotide.

The polynucleotide is indicated as containing the base uracil (U) since the sequences in these tables relate to miRNAs. However, as will be appreciated by one skilled in the art, U can be replaced with the base thymine (T). Whether the base T or U is selected will depend on the type of molecule containing the sequence. For example, if the molecule is a DNA molecule or a PMO, the base may be T whereas if the molecule is a RNA molecule, the base may be U.
Therefore, the molecule of the invention is not limited to a sequence containing U but can also comprise a sequence containing T since the function of the base at these positions is to bind to the base A, a function which both U and T can fulfil.

The polynucleotide of the invention can be used to bind to DLL4 mRNA and to suppress its activity. It binds in a manner similar to the binding of the miR-30 family. The polynucleotide is preferably based on the nucleotide sequence of the miR-30 family. It preferably comprises, and may consist of, a nucleotide sequence having at least 50%, more preferably at least 55%, preferably at least 57%, more preferably at least 60%, even more preferably at least 70%, more preferably at least 80%, more preferably at least 90% homology with one of the miR-30 family sequences, particularly miR-30b or miR-30c, especially miR-30c, which are provided in figure 1C. In particular, the polynucleotide preferably comprises at least 3, more preferably at least 4, 5, 6 or 7 nucleotides that are complementary to the nucleotides in DLL4 mRNA, outside of the seed sequence binding region. The polynucleotide may comprise or consist of the nucleotide sequence of one of miR-30 a, b, c, d or e. The polynucleotide preferably shows similar binding affinity, such as at least 65%, more preferably at least 70%, more preferably at least 75% even more preferably at least 80% of the binding affinity of any of the miR-30 family for DLL4 mRNA. It particularly preferably shows similar binding affinity to that of miR-30b or c for DLL4 mRNA. The polynucleotide preferably comprises the nucleotides required to direct RISC to bind the DLL4 mRNA, as occurs with the miR-30 family.

The term "complementary" means that the majority of the bases in a first sequence are complementary to a second sequence. However, the two sequences will still be able to base pair if there are a small number of mismatched bases or a small "bulge" of non-paired bases in the first sequence. For example, if there are five or fewer mismatched bases or a bulge of five or fewer bases, the two base sequences should still be able to base pair. Preferably, there is no "bulge" of non-paired bases. Preferably, there are four or fewer mismatched bases, more preferably, three or fewer mismatched bases, even more preferably, two or fewer mismatched bases, more preferably still, one or fewer mismatched bases and, most preferably, no mismatched bases. Any mismatched bases etc are preferably found outside the seed region.

Preferably, the polynucleotide is isolated so that it is substantially free from other compounds or contaminants.
The polynucleotide may be conjugated to or complexed with an entity, especially an entity which helps target the polynucleotide to the required site of action.

Also provided by the invention is a vector comprising a polynucleotide as previously described, for use in the modulation of angiogenesis. The vector may comprise components required for expression of the polynucleotide in a mammalian cell. Further provided is a vector comprising a promotor or repressor of miR-30 for use in the modulation of angiogenesis. Any appropriate vector can be used, including, for example, an adenoviral vector, an adeno-associated viral vector, or a lentiviral vector. Also provided is a cell comprising the vector of the invention, especially a mammalian, bacterial or insect cell. The cell is preferably not a human embryonic stem cell.

Further provided is a pharmaceutical composition comprising one or more of the polynucleotides or one or more of the vectors described previously and a pharmaceutically acceptable carrier or excipient. In particular, the composition may comprise a carrier which enables the polynucleotide to be delivered to the relevant site for use. The carrier may target a particular site or otherwise improve delivery to that site. When the pharmaceutical composition comprises a polynucleotide, it may also comprise an excipient which stabilises the polynucleotide. Such stabilisers are well known in the art. Any appropriate stabiliser may be used.

Pharmaceutical compositions of this invention comprise any of the molecules of the present invention, and pharmaceutically acceptable salts thereof, with any pharmaceutically acceptable carrier, adjuvant or vehicle. Pharmaceutically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions include, but are not limited to, ion exchangers, alumina, aluminium stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulphate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene- polyoxypropylene-block polymers, polyethylene glycol and wool fat.
The pharmaceutical compositions of this invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. Preferably, the pharmaceutical compositions are administered orally or by injection. The pharmaceutical compositions may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or vehicles. The term parenteral as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intra-articular, intrasynovial, intrasternal, intrathecal, intralesional and intracranial injection or infusion techniques. Preferably, the route of administration of the composition is transdermal or intrathecal administration.

The pharmaceutical compositions may be in the form of a sterile injectable preparation, for example, as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as, for example, Tween 80) and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant such as Ph. Helv or a similar alcohol.

The pharmaceutical compositions of this invention may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, and aqueous suspensions and solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are administered orally, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavouring and/or colouring agents may be added.
The pharmaceutical compositions of this invention may also be administered in the form of suppositories for rectal administration. These compositions can be prepared by mixing a molecule of this invention with a suitable non-irritating excipient which is solid at room temperature but liquid at the rectal temperature and therefore will melt in the rectum to release the active components. Such materials include, but are not limited to, cocoa butter, beeswax and polyethylene glycols.

Topical administration of the pharmaceutical compositions of this invention is especially useful when the desired treatment involves areas or organs readily accessible by topical application. For application topically to the skin, the pharmaceutical composition should be formulated with a suitable ointment containing the active components suspended or dissolved in a carrier. Carriers for topical administration of the molecules of this invention include, but are not limited to, mineral oil, liquid petroleum, white petroleum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, the pharmaceutical composition can be formulated with a suitable lotion or cream containing the active compound suspended or dissolved in a carrier. Suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water. The pharmaceutical compositions of this invention may also be topically applied to the lower intestinal tract by rectal suppository formulation or in a suitable enema formulation. Topically-transdermal patches are also included in this invention.

The pharmaceutical compositions of this invention may be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilising or dispersing agents known in the art.

Also provided is an agent which increases the expression of one or more members of the miR-30 family for use in the modulation of angiogenesis.

A related aspect of the invention provides a method of modulating angiogenesis comprising administering one or more of a polynucleotide, a vector, another agent that alters the
expression of one or more members of the miR-30 family or a pharmaceutical composition as described to a subject.

Also provided is an agent that reduces the expression of one or more members of the miR-30 family or interferes with the interaction between one or more members of the miR-30 family and DLL4 mRNA for use in the modulation of angiogenesis.

Agents that increase or reduce the expression of one or more members of the miR-30 family include agents that increase or reduce transcription activators or inhibitors of miR-30. Also encompassed are agents that increase or reduce the processing of precursors of miR-30 family members into mature miRNA.

Any agent that reduces the expression of one or more members of the miR-30 family may be used, such as a vector as described previously comprising a repressor of one or more members of the miR-30 family.

An agent that interferes with the interaction between one or more members of the miR-30 family and DLL4 mRNA is any agent that reduces the actual interaction between one or more members of the miR-30 family and DLL4 mRNA or any agent that reduces the effectiveness of such interaction. Such agents include agents that compete with the miR-30 family for DLL4 mRNA binding, such as other polynucleotides that bind to DLL4 mRNA without suppressing DLL4 mRNA activity. In particular, there is provided a target protector polynucleotide, which binds to DLL4 mRNA without suppressing its activity. Such a target protector preferably comprises at least 22, more preferably at least 23, more preferably at least 24, more preferably at least 25 nucleotides, including the nucleotide sequence TGTAACA. In particular it preferably comprises the sequence TGTAACA and at least 15, 16 or 17 other nucleotides which specifically bind to DLL4 mRNA. In particular, it preferably comprises 25 contiguous nucleotides found in the following sequence: ACCCATCCAGGATGCAATGTAAACAATGCAGAAGGAAGGTC. For example, the target protector may comprise the sequence: TGTAACAATGCAGAAGGAAGGTC or ACCCATCCAGGATGCAATGTAAACA.

Other agents that interfere with the interaction between one or more members of the miR-30 family and DLL4 mRNA include agents that bind to one or more members of the miR-30
family or otherwise target one or more members of the miR-30 family to prevent binding to DLL4 mRNA. Such agents might include polynucleotides that bind specifically to one or more members of the miR-30 family, such as anti-miRNA oligonucleotides; other binding molecules such as antibodies against one or more members of the miR-30 family; and agents that break down one or more members of the miR-30 family. Alternatively the agent may act as a sponge to mop up one or more members of the miR-30 family and reduce the interaction between one or more members of the miR-30 family and DLL4 mRNA.

A further aspect provides a method of modulating angiogenesis comprising administering an agent that reduces expression of one or more members of the miR-30 family or interferes with the interaction between one or more members of the miR-30 family and DLL4 mRNA to a subject.

The polynucleotides, vectors and agents described herein are useful for the modulation of angiogenesis. This allows them to be used in the treatment of various conditions, including cancer and vascular conditions, such as schema related conditions.

Any cancer can be treated, particularly cancers in which a solid tumour is present or likely to develop. Leukaemias may also be treated.

Vascular conditions include conditions in which the vasculature is disordered, whether because of the presence of abnormal vessels or an excessive number of vessels or due to the loss of normal vessels or their function. Vascular conditions and ischemia related conditions can occur in various parts of the body. It is particularly relevant to treat eye disorders such as retinopathy of prematurity, ischemic retinopathy, retinal vein or artery occlusion, diabetic retinopathy, choroidal neovascularization, age related macular degeneration, corneal neovascularization, neovascular glaucoma or corneal transplantation. More broadly, though, treatment of any condition caused by or related to disordered vasculature or ischemia is envisaged. Accordingly, the treatment of any ischemic disease or condition caused by insufficient blood supply due to blood vessel loss and/or poor perfusion, for example ischemic injury, cerebral ischemia, cardiac ischemia, ischemic conditions affecting the limbs and other organs or tissues, arteriovenous malformations, wound healing, organ or tissue transplantation, placental insufficiency, arterial narrowing and occlusion, atherosclerosis, and systemic or pulmonary hypertension is provided by the invention.
It is preferable to treat cancer and ischemic retinopathy by administering one of the polynucleotides of the invention, one of the vectors of the invention which increases expression of one of the polynucleotides of the invention or by increasing the expression of miR-30.

It is also generally preferable to treat other ischemic conditions in the same way, by administering one of the polynucleotides of the invention, one of the vectors of the invention which increases expression of one of the polynucleotides of the invention or by increasing the expression of miR-30, thereby encouraging the sprouting and growth of new vessels and revascularisation of the ischemic areas. However, angiogenesis may be modulated as appropriate for the condition in question.

The polynucleotides, vectors and agents described herein may be for administration with other agents that complement or enhance their activity. For example, agents and polynucleotides that are for use in suppressing DLL4 activity can be administered with DLL4 antibodies or other antagonists. Alternatively they may be administered with agents that otherwise modulate angiogenesis, such as agents that modulate VEGF.

When the term miR-30 is used herein, it may refer to any member of the miR-30 family, miR-30a, b, c, d or e. It can preferably mean miR-30a, c, d or e, especially miR-30c.

The present invention will now be described, by way of example only, with reference to the figures.

**Brief Description of the Drawings**

**Figure 1. KSHV regulates expression of the miR-30 family, which is predicted to target DLL4.** (A) Heatmap representing relative changes in expression of hsa-miR-30 family members in LEC following KSHV infection. miRNAs are ordered alphabetically. Original GEM data from (Lagos et al., 2010) **, £<0.01; ***, $<0.001. Black and white denote low and high expression respectively. (B) Downregulation of miR-30b and miR-30c in KLEC, confirmed by qRT-PCR. Columns are the average of three independent experiments, expression is relative to LEC. Values are shown as mean ± standard error of the mean (SEM).
**, P<0.01. (C) Complementarity between miR-30 family members and the DLL4 3'UTR. Solid lines indicate canonical Watson and Crick base-pairing, dashed lines indicate G:U wobbles. The predicted target site within the DLL4 3'UTR, positions 59-66, is shown in bold, the miR-30 seed region is shown in bold italics. (D) Alignment demonstrating the conservation of the miR-30 target site within the DLL4 3'UTR across 24 species, including all the species utilized by TargetScan. Positions 53-66 of the 3'UTR are shown and the predicted miR-30 target site is highlighted in bold.

**Figure 2. miR-30b and miR-30c target DLL4.** (A) Mean fold-change in DLL4 mRNA in LEC transfected with hsa-miR-30b or hsa-miR-30c mimics. The unrelated mRNA, E-selectin, is used as a control for global mRNA suppression. Columns are the average of four independent experiments. Expression measured by qRT-PCR relative to non-targeting control (NTC) mimic. ***, P<0.001. (B) Upper panel, representative western blot showing protein levels in LEC transfected with hsa-miR-30b or hsa-miR-30c mimics. Lower panel, intensity of antibody ECL signal in upper panel relative to NTC mimic. Columns are the average of two independent experiments **, P<0.01. (C) Left-hand panel, DLL4 mRNA downregulation in LEC following transduction with hsa-miR-30b- and hsa-miR-30c-expressing lentiviruses. Columns are the average of three independent experiments. Expression is relative to empty vector, pSIN. **, P<0.01, Right-hand panel, western blot showing protein levels in LEC transduced with hsa-miR-30b- and hsa-miR-30c-expressing lentiviruses. Values indicate intensity of antibody ECL signal relative to empty vector, pSIN. (D) Upper panel, representative western blot showing protein levels in LEC transfected with has-miR-30b or has-miR-30c inhibitors. Lower panel, intensity of antibody ECL signal in upper panel relative to NTC inhibitor. (E) Reporter assay indicating the response of wildtype (wt) or mutant (mut) DLL4 3'UTR to exogenous hsa-miR-30b and hsa-miR-30c. Columns are the average of three independent experiments. Change in relative light units (RLU) is relative to NTC mimic. MTO1 is a control reporter, lacking a 3'UTR sequence but containing the Firefly and Renilla luciferase genes. *, P<0.05; **, P<0.01; ***, P<0.001 and related statistically significant values are indicated by horizontal bars. Error bars indicate column values ± SEM.

**Figure 3. Regulation of DLL4 by miR-30b and miR-30c has relevance in pathophysiological settings.** (A) Mean fold-change in DLL4 mRNA in LEC or KLEC transfected with hsa-miR-30b- and hsa-miR-30c-expressing lentiviruses. Columns are the average of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001. (B) Left-hand panels, representative photograph at indicated time points of HUVEC spheroids
embedded in Matrigel, taken at 5x magnification in phase contrast. HUVEC were transfected with mimic before being induced to form spheroids through the hanging drop method. Right-hand panels, quantification of total sprouts per spheroid. Box plot indicates inter-quartile range, whiskers indicate total range, black line denotes median, n=60. ***. Error bars indicate column values + SEM.

Figure 4. miR-30b and miR-30c overexpression induce aberrant endothelial cell behaviour in vivo. (A) dll4 and dre-miR-30b and dre-miR-30c expression correlate during the period immediately prior to, and during intersegmental vessel formation in the developing zebrafish embryo. Time points are shown as hours post fertilisation (hpf). Expression is relative to the shield stage of development (6hpf). RNA was obtained from whole embryos at each time point. (B) Photograph of developing vessels in uninjected Tg(kdrl:GFP) zebrafish embryos or embryos injected with dll4 MO or indicated miRNA mimic. Left panels: aberrant endothelial cell migration is indicated by white arrowheads at 25hpf. Right panels: increased branching of the intersegmental vessels is indicated by white arrows at 72hpf. (C) Graph describing the relative size of the dorsal aorta (DA) in zebrafish embryos injected with dll4 MO or miR-30 mimic compared to wt embryos. Column values are the average of three embryos per condition. Six DA measurements were made for each embryo. ***. Error bars indicate column values + SEM. (D) Photograph of developing vessels in Tg(kdrl:GFP) zebrafish embryos under the same conditions as 4B, with or without co-injection of dll4-TPm30. Left panels: aberrant endothelial cell migration is indicated by white arrowheads at 25hpf. Right panels: increased branching of the intersegmental vessels is indicated by white arrows at 72hpf.

Supplementary Figure 1.
(A) Downregulation of pre-miR-30c-1 and pre-miR-30c-2 in KLEC. Columns are the average of four independent experiments. Expression is relative to LEC. *, P < 0.05; **, P < 0.01. Error bars indicate column values ± SEM.

Supplementary Figure 2.
(A) Downregulation of DLL4 mRNA by miR-30 mimics can be titrated. Triangles indicate increasing concentrations of mimics: 10nM, 20nm, 40nM, 80nM and 100nM. Columns are the average of three independent experiments. Expression is relative to NTC mimic at the same concentration. **, P<0.01, ***, P<0.001. (B) Expression of miR-30 and corresponding downregulation of DLL4 mRNA by miR-30 transduction can be titrated with increasing
copies per cell (c/c) of lentivirus. Expression is relative to empty vector, pSIN. (C) Schematic of positions 44-66 of the DLL4 3’UTR. The predicted target site for the miR-30 family, positions 59-66, is shown in bold. The mutated nucleotides are crossed out and their replacements are shown underneath underlined. Error bars indicate column values ± SEM.

5 Supplementary Figure 3.

(A) miR-30b and miR-30c levels in LEC transduced with vGPCR at the indicated timepoints. Columns are the average of two independent experiments. Expression is relative to empty vector, pSIN. (B) miR-30b and miR-30c levels in LEC grown at 1% oxygen for 24h. Columns are the average of two independent experiments. Expression is relative to LEC grown at 20% oxygen for the same time period. (C) Mean fold-change in DLL4 mRNA in HUVEC transfected with hsa-miR-30b or hsa-miR-30c mimics. Columns are the average of two independent experiments. Expression measured by qRT-PCR relative to non-targeting control (NTC) mimic. *, P<0.05, **, P<0.01

10 Supplementary Figure 4.

15 (A) Expression levels of dll4 and the miR-30b and miR-30c during the course of zebrafish development. Time points are shown as hours post fertilisation (hpf). Expression is relative to 6hpf. (B) Overexpression of miR-30 in whole zebrafish embryos following microinjection of dre-miR-30b and hsa-miR-30c at the one- to four-cell stage. Values indicate amount of injected mimic as ng per embryo. Expression is relative to uninjected control embryos. (C) Downregulation of dll4 mRNA in whole zebrafish embryos following microinjection with dre-miR-30b and hsa-miR-30c. Values indicate amount of injected mimic as ng per embryo. Expression is relative to uninjected control embryos.

Detailed Description of the Invention

EXAMPLES

Experimental Procedures

Cell culture

LEC were purchased from PromoCell and cultured as previously described (Lagos et al., 2007). HUVEC were purchased from PromoCell and cultured in MV2 (PromoCell). For both LEC and HUVEC, experiments were performed before passage 8. BCBL-1 cells, latently
infected with recombinant GFP-KSHV (Vieira et al., 2001) were cultured as previously described (Vart et al., 2007). 293T and human fibroblast cells were grown in Dulbecco modified Eagle medium (Invitrogen), supplemented with 10% FBS.

**KSHV production and infection of LEC**

KSHV was produced and used to infect LEC as previously described (Lagos et al., 2007). This procedure reproducibly resulted in 30% to 50% LEC expressing GFP 3 days after infection.

**microRNA mimics and inhibitors and RNA interference**

LEC, HUVEC or 293T were seeded in 6- or 12-well plates respectively, 16 h prior to transfection using Oligofectamine (Invitrogen), as per the manufacturer's instructions. miRIDIAN miRNA Mics for hsa-miR-30b, hsa-miR-30c and the Negative Control #1 (non-targeting control) (Thermo Fisher Scientific) were transfected at 100nM, unless otherwise specified. Cells were harvested, utilised for the hanging drop assay or transfected with luciferase reporter plasmids 48h post-mimic transfection. miRIDIAN miRNA Inhibitors (ThermoScientific) were transfected into cells (100nM) following the same protocol as mimic transfection.

**Western blotting**

Forty-eight hours post-transfection with mimics or inhibitors, or 48h post-lentiviral or KSHV infection, LEC or HUVEC were lysed in Pierce M-PER buffer (ThermoScientific). Protein was quantified using Pierce BCA Protein Assay (ThermoScientific) and equal concentrations of protein were resolved on a 10% polyacrylamide gel. Antibodies against DLL4 (#2589, Cell Signaling Technology) and GAPDH (Monoclonal 6C5, Advacned Immunochemical Inc) were detected with HRP-conjugated secondary antibodies and were quantified using ECL or ECL Plus (GE Healthcare).

**Lentivirus production and infection of LEC**

Genomic fragments containing pre-miR-30b and pre-miR-30c-1 were cloned from LEC and were expressed using a modified pSIN-MCS lentiviral vector as described (Vart et al., 2007). The number of lentiviral copies per cell was determined by qPCR and miRNA expression was confirmed by RT-PCR. Experiments were performed 2-3 days post-lentivirus infection.
qPCR and qRT-PCR

Genomic DNA for qPCR was extracted using the QIAamp DNA mini-kit (Qiagen). The number of lentiviral copies per cell (c/c) was determined as described previously (Vart et al., 2007). Total RNA was extracted using miRNeasy mini-kit (Qiagen) and subjected to DNase I treatment (Qiagen). About 50 to 1,000 ng of total RNA was used for cDNA synthesis using the Superscript II reverse transcriptase (Invitrogen). GAPDH (housekeeping reference gene) and E-selectin mRNA levels were quantified by qRT-PCR using optimized forward and reverse primers and SYBR Green PCR Master Mix (Applied Biosystems). qRT-PCR quantification of DLL4, dll4, bactinl, hsa-miR-30b and hsa-miR-30c was performed using Taqman Gene Expression or Taqman MicroRNA assays (Applied Biosystems). Quantification of pre-miR-30c-1 and pre-miR-30c-2 was performed using miRNA qRT-PCR Kit and Primer Set (GenoExplorer).

Luciferase reporter assays

The reporter plasmids (50ng), either empty vector (pEZX-MTOI) or the DLL4 3'UTR containing plasmid (pEZX-DLL4), were transfected into 293T cells, 48h post-transfection with miRNA mimic. Cells were harvested 24h post-transfection according to the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured using a Fluoroskan Ascent FL luminometer (ThermoScientific). Firefly activity was normalised to internal renilla luciferase levels.

Spheroid-based sprouting angiogenesis assay

HUVEC were transfected with miRNA mimic (ThermoScientific) and after 24h spheroids were generated as previously described {Alajati, 2008 77 /id}. One hundred spheroids were generated per condition, collected after 24h and embedded in Matrigel Basement Membrane Matrix (BD Biosciences). Spheroids were monitored for 120h. To analyse average sprouts per spheroid, sprouts were counted using Adobe Photoshop CS2 (n=60). Average sprout length was measured using the segmented lines tool in Image J (NIH). Five sprouts were measured per spheroid (n=20).

Embryo Manipulation and In Situ Hybridization Procedures

Zebrafish embryos were obtained by natural spawning of adult zebrafish. Embryos were raised and maintained at 28.5°C in system water and staged as described {Westerfield, 1993
Tg(kdrkGFP) zebrafish were used to examine the developing vasculature. Antisense morpholinos (MOs) (GeneTools) and miRNA mimics (ThermoScientific) were injected into 1- to 4- cell stage embryos. The MOs used in this work were dll4-M01 (Siekmann, 2007) (5ng) and dll4-TPmiR-3 (Figure S4D) (10ng). miRNA mimics were injected in the quantities stated. In situ hybridization was performed as described (Gering, 2005). RNA probes were labelled with digoxigenin (Roche) and detected using BM Purple (Roche). Endothelial cells were visualised in Tg(kdrl:GFP) embryos using UV light.

Statistical analysis

All experiments were performed in independent replicates and error bars correspond to standard deviation from the mean. Statistical significance (p values) was calculated with a two-sided unpaired Student's t test. Statistical analysis of the KLEC GEM was performed as described using a moderated t statistic and a false discovery rate correction (Lagos, 2010).

Results

KSHV regulates expression of a miRNA family predicted to target DLL4

During tumorigenesis, cellular miRNA expression profiles are subject to specific changes (Volinia et al., 2010). Primary human LEC are the putative cellular targets of KSHV during the development of KS (Mesri et al., 2010) and the inventors recently described the miRNA signature of these cells during viral infection; focussing on upregulated microRNAs (Lagos et al., 2010). The inventors analyzed these data with respect to downregulated microRNAs and observed significantly decreased expression of members of the miR-30 family in KSHV-infected LEC (KLEC) 72hrs p.i. (Figure 1A). The most significant reduction in signal was recorded for probes corresponding to miR-30b and miR-30c and these microarray data were validated by qRT-PCR (Figure 1B). The mature microRNA of miR-30c detected by the microarray can be produced from two distinct precursor hairpins (miR-30c-1 and miR-30c-2) and the inventors confirmed that both sources of the mature miRNA are downregulated in KLEC (Figure S1A). Downregulation of the miR-30 family has been associated with enhanced tumorigenesis in breast cancer and anaplastic thyroid carcinoma (Braun et al., 2010; Yu et al., 2010). When the inventors examined the miRNA expression profile of
uninfected LEC, they discovered that this family is amongst the most robustly expressed, suggesting that the suppression of these miRNAs by KSHV could be functionally relevant.

The human miR-30 family members are encoded by six genes and expressed from four transcripts, but share an identical seed sequence and therefore have common predicted targets (Grimson et al., 2007). To establish a role for miR-30 in LEC the inventors used TargetScan software to identify these targets and ranked them according to total context score (Grimson et al., 2007). They analyzed this list with respect to genes significantly altered in KLEC and discovered that the 3'UTR of the Notch ligand DLL4, one of the most significantly upregulated genes in KLEC (Emuss et al., 2009), scored favorably (total Context Score = 0.39). The inventors repeated the TargetScan analysis using the DLL4 3'UTR sequence and discovered that the miR-30 family are the best scoring miRNAs for this 3'UTR. The family members demonstrate a full 8mer target sequence with additional 3' pairing that is located close to the start of the 3'UTR (Grimson et al., 2007) (Figure 1C). This miR-30 target site is absolutely conserved within the DLL4 3'UTR from 24 species and the inventors' alignment indicates that the surrounding sequence is also highly conserved (Figure 1D). miRNAs have been shown to target Notch components during tumor development (Zhiwei et al., 2010), but a function for this cross-talk during pathogenesis is unclear and no miRNAs targeting DLL4 have been identified. DLL4 is one of the most significantly upregulated genes in KLEC (Emuss et al., 2009) and the corresponding suppression of a regulatory miRNA in response to KSHV infection suggests a functional relationship between DLL4 and miR-30 in these cells.

**miR-30b and miR-30c target DLL4**

The inventors' GEM experiments show that miR-30b and miR-30c are amongst the most significantly suppressed members of the miR-30 family in KLEC. To validate the target prediction observations, the inventors transfected synthetic miR-30b and miR-30c mimics into LEC and measured DLL4 expression. DLL4 mRNA levels were significantly reduced in LEC expressing either miR-30b or miR-30c mimics (Figure 2A) and this corresponded to a significant decrease in DLL4 protein expression (Figure 2B). Levels of an unrelated mRNA (E-selectin) were unchanged in miR-30b- or miR-30c-expressing cells, suggesting that the suppression of DLL4 mRNA is not a non-specific effect on global mRNA levels in response to the mimics (Khan et al., 2009). The effect of miR-30b and miR-30c mimics on DLL4
levels could be titrated, but their co-expression, at an equivalent total concentration, did not increase DLL4 repression, suggesting that there are no additive or synergistic effects between these miR-30 family members (Figure S2A). These findings correspond with the target prediction studies, which indicate only one miR-30 target site on the DLL4 3'UTR. The inventors also generated lentiviruses expressing miR-30b or miR-30c by cloning 500bp fragments surrounding the pre-miRNA sequence of each miRNA from LEC genomic DNA (Lagos et al., 2010). They confirmed expression of mature miR-30b and miR-30c in LEC transduced with these viruses and a corresponding suppression of DLL4 (Figure 2C and S2B); these effects could be titrated with increasing copy number of virus per cell (Figure S2B). Conversely, transfection of hairpin inhibitors against miR-30b and miR-30c into LEC led to an increase in DLL4 protein levels (Figure 2D). Taken together these data indicate that the miR-30 family may play a role in endogenous DLL4 regulation.

To confirm that these miRNAs act through the DLL4 3'UTR the inventors utilised a vector (MT01) expressing this 3'UTR downstream of the luciferase coding sequence (DLL4 3'UTR_wt), and expressed this construct in the presence of miR-30b- or miR-30c-mimics (Figure 2E). They observed a 50% reduction in luciferase activity in cells co-expressing this construct with exogenous miR-30; in agreement with their previous data, no additive repression of luciferase activity was observed in cells expressing both miR-30b and miR-30c. The inventors mutated the predicted miR-30 target site in the DLL4 3'UTR (Figure S2C) to prevent miRNA association (DLL4 3'UTR_mut) which significantly increased luciferase activity to near-baseline levels (Figure 2D). The original MT01 vector maintained its luciferase activity in the presence of exogenous miR-30, suggesting that the changes in activity were due to the effects of miR-30 on the DLL4 3'UTR. Taken together, these data suggest that miR-30 can influence the expression of DLL4 in LEC by targeting a predicted site in its 3'UTR.

**miR-30 targeting of DLL4 influences endothelial cell behaviour in vitro**

DLL4 expression influences angiogenic pathways in endothelial cells (Harrington et al., 2008) and contributes to the angiogenic signature of KSFTV-infected endothelial cells (Wang et al., 2004). The inventors used KSHV-infection of LEC to investigate the effect of miR-30 on DLL4 levels in an angiogenic model (Figure 3A). They expressed exogenous miR-30 in LEC and observed a significant decrease in DLL4 levels compared to control cells.
Expression of DLL4 in KLEC was increased 3-fold compared to LEC and this induction was attenuated to 2-fold in KLEC expressing exogenous miR-30, suggesting that miR-30 can suppress DLL4 levels in a dynamic system where it is normally upregulated (Figure 3A). This partial decrease in DLL4 in the presence of miR-30 reflects the predominant role of the viral protein, vGPCR, in inducing DLL4 in KLEC (Emuss et al., 2009). Expression of vGPCR, which is a known activator of MAPK/ERK signaling (Sodhi et al., 2000), did not influence miR-30 levels in LEC (Figure S3A). Taken together, these data suggest the reduction of miR-30 by KSHV contributes to increased DLL4 in KLEC and is required for its full induction, but miR-30 suppression is achieved independently of vGPCR. The inventors also observed that miR-30 levels in LEC were unaffected by hypoxia, another stimulus known to induce DLL4 in endothelial cells and that increases DLL4 expression 2.5-fold in LEC in their experiments (Figure S3B). This suggests that the transcription of miR-30 is induced through mechanisms distinct to those that increase DLL4 levels.

During pathological and physiological angiogenesis, endothelial cells expressing DLL4 stimulate signaling in adjacent cells. These signal-generating cells are specified as "tip" cells and localise to the tip of the developing sprout; the signal-receiving cells are excluded from the tip of the angiogenic sprout and contribute to the body of the developing vessel. Suppression of Notch signaling, such as through loss of ligand expression, leads to excessive sprouting and multiple vessel branches because the tip cell phenotype is not restricted. These vessels have aberrant morphology and are non-functional. The inventors utilised an in vitro model of sprouting angiogenesis to investigate whether DLL4 targeting by miR-30b could affect normal tip cell specification (Weber et al., 2008). Human umbilical vein endothelial cells (HUVEC) were transfected with either NTC or miR-30b mimics and then induced to form spheroids (Alajati et al., 2008; Korff and Augustin, 1998; Korff et al., 2004) which were subsequently embedded in matrigel. Spheroids comprised of miR-30b overexpressing HUVEC displayed an increased propensity to form sprouts which was maintained for 5 days (Figure 3B). Furthermore, the sprouts in the miR-30b expressing spheroids were significantly longer. This indicates that miR-30 overexpression disturbs endothelial tip cell specification.

Exogenous expression of miR-30 induces aberrant branching and intersegmental vessel development in zebrafish
The developing zebrafish vasculature is an established model of angiogenic processes and previous work has shown that expression of both miR-30b and miR-30c homologs is induced during development and in the adult fish (Chen et al., 2005; Wienholds et al., 2005). The inventors confirmed these results by qRT-PCR using RNA from embryos at different timepoints (hours) post-fertilisation (hpf, Figure S4A). Zebrafish dll4 regulates the correct development of functional intersegmental vessels (ISV) and dll4 expression is detectable from 8hpf. They measured dll4 expression by qRT-PCR in the same zebrafish embryos and observed induction between 6hpf and 12hpf (Figure S4A).

The inventors observed that there is a correlation between expression of miR-30 and dll4 between 18hpf and 30hpf. dll4 levels decrease between 18hpf and 24hpf as miR-30 expression increases; at 30hpf, dll4 levels return to 18hpf levels coincident with a drop in miR-30 expression (Figure 4A and S4A). This fluctuation corresponds with the temporal window during which the primary wave of angiogenic sprouting occurs, and the sharp increase in dll4 observed corresponds to the initiation of the secondary wave where ISV formation is consolidated and strict controls of endothelial branching are required (Ellertsdottir et al., 2010).

These coincident changes in dll4 and miR-30 suggest a functional interaction during zebrafish development that may contribute to the tight control of dll4 expression during this time. The inventors investigated this relationship by disrupting miR-30 expression by microinjection of miR-30 mimics. To reduce non-specific effects of exogenous miR-30, they titrated the amount of microinjected mimic to levels where miR-30 expression was detectable, but aberrant embryo morphology and head necrosis was minimised (Figure S4B). dll4 expression titrated with levels of miR-30 and was reduced by 20-30% compared to control embryos at the lowest mimic concentrations (Figure S4C). They confirmed suppression of dll4 mRNA levels in the developing zebrafish vasculature by in situ hybridization compared to uninjected wild-type (wt) and non-targeting control (NTC)-injected embryos (not shown). These data indicate that dll4 mRNA levels can be disrupted by exogenous expression of miR-30.

Silencing of dll4 with morpholinos (MO) induces aberrant ISV branching (Leslie et al., 2007; Siekmann and Lawson, 2007). The inventors investigated the effect of reduced dll4 expression by miR-30 on endothelial cell behaviour in Tg(kdrl:GFP) zebrafish embryos by
visualizing endothelial cells under UV light, using dll4 MO-injected embryos as a positive control (Figure 4B). Compared to uninjected, control embryos, they observed aberrant migration of endothelial cells at 25hpf (left panels, white arrowheads) in embryos expressing miR-30b and miR-30c mimics. This phenotype has not been previously reported in response to dll4 silencing but was also present in the dll4 MO-injected embryos, suggesting that this effect of miR-30 may result from decreased dll4 levels. The inventors also observed that the size of the dorsal aorta (DA) was significantly reduced at 25hpf in all embryos where dll4 expression was suppressed compared to wt embryos (Figure 4C). This effect was comparable in the case of both dll4 MO and miR-30 mimic injection. This reduction in vessel diameter suggests that dll4 could affect vessel stability and concurs with previous work showing that DLL4 upregulation in tumors correlates with vessel maturation and size (Li et al., 2007; Patel, 2006).

The inventors also visualized the embryos at 72hpf, once ISV formation is established (Figure 4B, right panels, white arrows). Concurring with previous work, dll4 MO injection caused aberrant branching of the ISV (arrows) and the inventors observed aberrant branching in embryos expressing miR-30b and miR-30c mimics.

To further investigate these phenotypes, embryos were co-injected with a target protector (TP) morpholino designed to bind to a region within the dll4 3'UTR containing the miR-30 target site (dll4-TP$^{\text{miR-30}}$) (Choi et al., 2007) (Figure S4D). Co-injection with dll4-TP$^{\text{miR-30}}$ led to a partial rescue of the DA and ISV phenotypes described (Figure 4D), indicating that dll4 downregulation, rather than downregulation of another miR-30 target, is the major contributing factor to these phenotypes.

These data suggest that aberrant expression of miR-30 during zebrafish development influences endothelial cell behaviour in vivo and that the targeting of dll4 by miR-30 is functionally relevant during vascular development.

References


Ref Type: Abstract


Claims

1. An isolated polynucleotide comprising a nucleotide sequence having substantial homology to the nucleotide sequence ACAAAUGU for use in the modulation of angiogenesis.
2. The polynucleotide of claim 1, wherein the polynucleotide contains the nucleotide sequence CAAAUG.
3. The polynucleotide of claim 1 or claim 2, wherein the polynucleotide comprises one of the following sequences:
   ACAAAUGU; CCUACAAAUGU; UCCUACAAAUGU; CCCUACAAAUGU;
   AXUCCUACAAAUGU; AXXCCCUACAAAUGU; UXXXACAAAUGU;
   CXXXACAAAUGU; and AXXXXXXXXACAAAUGU, wherein X is any nucleotide.
4. The polynucleotide of claim 3, wherein the polynucleotide comprises one of the following sequences:
   ACAAAUGU; CCUACAAAUGU; UCCUACAAAUGU; CCCUACAAAUGU;
   AXUCCUACAAAUGU; AXXCCCUACAAAUGU; UXXXACAAAUGU;
   CXXXACAAAUGU; and AXXXXXXXXACAAAUGU, wherein X is any nucleotide, and wherein the sequence is found at the 5' end of the polynucleotide.
5. A polynucleotide according to any preceding claim, wherein the polynucleotide is a RNA molecule, particularly a miRNA.
6. A polynucleotide according to any preceding claim, wherein the polynucleotide is one of miR-30 a, b, c, d and e, especially miR-30c.
7. A vector comprising a polynucleotide comprising a nucleotide sequence having substantial homology to the nucleotide sequence ACAAAUGU for use in the modulation of angiogenesis.
8. The vector of claim 7, wherein the polynucleotide contains the nucleotide sequence CAAAUG.
9. The vector of claim 7 or 8, wherein the polynucleotide comprises one of the following sequences:
   ACAAAUGU; CCUACAAAUGU; UCCUACAAAUGU; CCCUACAAAUGU;
   AXUCCUACAAAUGU; AXXCCCUACAAAUGU; UXXXACAAAUGU;
   CXXXACAAAUGU; and AXXXXXXXXACAAAUGU, wherein X is any nucleotide.
10. The vector of any of claims 7 to 9, wherein the polynucleotide comprises one of the following sequences:
    ACAAAUGU; CCUACAAAUGU; UCCUACAAAUGU; CCCUACAAAUGU;
    AXUCCUACAAAUGU; AXXCCCUACAAAUGU; UXXXACAAAUGU;
CXXXACAAAUGU; and AXXXXXXACAAAUGU, wherein X is any nucleotide, and wherein the sequence is found at the 5’ end of the polynucleotide.

11. A vector comprising a polynucleotide encoding one of miR-30 a, b, c, d and e, or a promotor or repressor of one or more of miR-30 a, b, c, d and e for use in the modulation of angiogenesis.

12. A pharmaceutical composition comprising a polynucleotide comprising a nucleotide sequence having substantial homology to the nucleotide sequence ACAAAAUGU; or a vector comprising a polynucleotide comprising a nucleotide sequence having substantial homology to the nucleotide sequence ACAAAAUGU, or comprising a polynucleotide encoding one of miR-30 a, b, c, d and e, or a promotor or repressor of one or more of miR-30 a, b, c, d and e; and a pharmaceutically acceptable carrier or excipient.

13. An agent which increases the expression of one or members of the miR-30 family for use in the modulation of angiogenesis.

14. A method of modulating angiogenesis comprising administering one or more of an isolated polynucleotide comprising a nucleotide sequence having substantial homology to the nucleotide sequence ACAAAAUGU; or a vector comprising isolated polynucleotide comprising a nucleotide sequence having substantial homology to the nucleotide sequence ACAAAAUGU, or comprising a polynucleotide encoding one of miR-30 a, b, c, d and e, or a promotor or repressor of one or more of miR-30 a, b, c, d and e; or an agent that alters the expression of one or more member of the miR-30 family; or a pharmaceutical composition according to claim 12 to a subject.

15. An agent that reduces the expression of one or more members of the miR-30 family or interferes with the interaction of one or more members of the miR-30 family and DLL4 mRNA for use in the modulation of angiogenesis.

16. A target protector polynucleotide comprising at least 22 nucleotides, including the nucleotide sequence TGTAACA.

17. A target protector polynucleotide according to claim 16, comprising 25 contiguous nucleotides found in the following sequence:

ACCCATCCAGGATGCAATGTAAACAATGCAGAAGGAAGGTC.

18. An anti-miRNA oligonucleotide specific to one or more members of the miR-30 family or an antibody against one or more members of the miR-30 family for use in the modulation of angiogenesis.
19. A method of modulating angiogenesis comprising administering an agent that reduces expression of one or more members of the miR-30 family or interferes with the interaction between one or more members of the miR-30 family and DLL4 mRNA to a subject.

20. A polynucleotide, vector, agent or method according to any preceding claim, wherein the modulation of angiogenesis is for the treatment of cancer or of a vascular disorder or ischemia related condition.
Figure 3

A

Fold change Dll4 mRNA

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B

20h

![Images](image3)

42h

![Images](image4)

120h

![Images](image5)
Supplementary Figure 1

A

Fold change pre-miRNA

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<tr>
<td>pre-miR-30c-2</td>
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** and * indicate significant differences compared to LEC.
Supplementary Figure 2

A

Fold change DL4 mRNA

NTC    miR-30b    miR-30c    miR-30b & 30c

B

Fold change mRNA

pSIN miR-30b    miR-30c

C

Fold change DL4 mRNA

5 c/c    50 c/c    120 c/c    240 c/c
Supplementary Figure 3

A

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Supplementary Figure 4

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Fold change

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D

Danio rerio dll4 3'UTR/miR-30 site target protector morpholino (dll4-TPmirR-30)
5'-TGTAACAAATCCAGAAAAAAAGATT-3'
Designed so that the 5' end binds to the target site while the 3' region binds the upstream flanking sequences.

B

Fold change mRNA

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Fold change dll4 mRNA

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WT NTC mir-R-30b mir-R-30c
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/113 A61K31/713

ADD.

According to International Patent Classification (IPC) and to 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, BIOSIS, EMBASE, Sequence Search, EMBL, WPI Data, CHEMABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>wo 2009/137807 A2 (ASURAGEN INC [US]; SHEN JI KUI [US]; KELNAR KEVIN [US]; SHELTON JEFFREY) 12 November 2009 (2009-11-12) claims 1,16; examples 8,9; tables 2,3</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A" document member of the same patent family

Date of the actual completion of the international search

16 May 2012

Date of mailing of the international search report

30/05/2012

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Bucka, Alexander

Form PCT/ISA/210 (second sheet) (April 2005)
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