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NUCLEAR MICRORNAS: THE ROLE OF MIR-466C IN TRANSCRIPTIONAL GENE ACTIVATION

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Pia Laitinen

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ABSTRACT

Ischemic diseases are a diverse group of diseases that occur due to the lack of oxygen and nutrients reaching the tissues (i.e., ischemia). Ischemic stroke and ischemic heart disease are the most common and the deadliest ischemic diseases, responsible for over 20 % of all deaths globally each year. There are multiple therapies available for ischemic diseases, such as medication with vasodilating drugs or surgery where blocked arteries are bypassed. Nonetheless, these solutions are either not always effective enough or not suitable for all patients. Thus, further studies for novel therapies are required.

MicroRNAs (miRs, miRNAs) are small non-coding RNAs (ncRNAs) that have a crucial role in cellular functions. miRNAs take part in gene regulation by silencing messenger RNAs (mRNAs) in the cytoplasm in an event called post-transcriptional gene silencing (PTGS). In addition to their role in the RNA interference (RNAi) in the cytoplasm, recent studies have indicated a role for miRNAs in the nuclei of cells where they can silence or activate gene function. RNA activation (RNAa) is a phenomenon where a small RNA targets a gene promoter region, thus activating gene transcription in the nuclei. This novel RNAa function of miRNAs has not been extensively studied but it offers an attractive opportunity for the

development of new therapies. This aspect of miRNA biology is also the focus of this thesis.

In this thesis, nuclear and cytoplasmic miRNAs were studied in mouse endothelial C166 cells by sequencing small RNAs in nuclear and cytoplasmic compartments under normoxic and hypoxic conditions. The study identified different miRNA populations enriched in the cell's nucleus or its cytoplasm. Interestingly, most miRNAs were differentially expressed in either nucleus or cytoplasm. Only six miRNAs were found to be differentially regulated in both compartments.

In this thesis, the RNAa for mouse vascular endothelial growth factor A (*Vegfa*) was studied since VEGFA is a major contributor in the pathology of ischemic diseases. We identified potential target sites in the *Vegfa* promoter for both mature arms of mmu-miR-466c. Indeed, as characterized by diverse complementary molecular biology methods, we found that miR-466c upregulated *Vegfa* expression in a mouse endothelial C166 cell line. In addition, the effect of miR-466c was studied *in vivo* in a mouse hindlimb ischemia model. miR-466c was administered either in extracellular vesicles (EV) or delivered using a lentiviral vector (LV). miR-466c treatment increased the limb function (Tarlov score) and body weight of the mice especially with EV delivery. We observed a significant improvement in miR-466c groups (both EV and LV delivery) in the ischemic region of the limb, as measured with the modified ischemia score and functional ultrasound (fUS) intensity levels.

In conclusion, the data in this thesis identified a nuclear population of miRNAs in endothelial cells and characterized one nuclear miRNA, miR-466c, as a finetuner of *Vegfa* expression at the transcriptional level. It is believed that these results will contribute to the development of new therapeutic methods based on nuclear miRNA biology.

Keywords: miRNAs, nuclear miRNAs, ncRNAs, RNAa

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TIIVISTELMÄ

Iskeemiset sairaudet ovat monipuolinen joukko erilaisia sairauksia, jotka johtuvat pääosin hapenpuutteesta kudoksissa, eli iskemiasta. Iskeeminen aivohalvaus ja iskeeminen sydänsairaus ovat yleisimmät ja tappavimmat iskeemisistä sairauksista, sillä ne vastaavat yli 20 % kaikista kuolemista vuosittain maailmanlaajuisesti. Iskeemisiin sairauksiin on olemassa monenlaisia terapiamuotoja kuten verenohennuslääkket ja umpeutuneiden valtimoiden ohitusleikkaus. Nämä vaihtoehdot eivät kuitenkaan sovellu kaikille potilaille tai ne eivät ole aina tarpeeksi tehokkaita. Sen vuoksi uusia tutkimuksia tarvitaan kehittämään tehokkaampia terapiamuotoja näihin sairauksiin.

MikroRNAt (miRNA, miR) ovat pieniä ei-koodaavia RNA:ita, joilla on merkittävä rooli solun eri toiminnoissa. miRNA:t säätelevät geenien toimintaa transkription jälkeisellä geenin hiljentämisellä, missä geenien lähetti-RNA:ita estetään muodostamasta proteiinia solulimassa. Sen lisäksi että miRNA:t hiljentävät geenien toimintaa, viimeaikaiset tutkimukset osoittavat miRNA:iden toimivan myös solujen tumissa. Tumassa miRNA:t voivat joko hiljentää tai aktivoida geenien toimintaa. RNA aktivaatio (RNAa) on uusi ja suhteellisen vähän tutkittu ilmiö, missä pienet aktivoivat RNA:t kohdentuvat geenien säätelyjaksoille, joissa ne saavat aikaan geenien

aktivoinnin. Tämä RNAa toimii myös miRNA välitteisesti, ja se tarjoaa monipuoliset mahdollisuudet uusien terapiamuotojen kehittämiseen. Tämä tutkielma keskittyy miRNA:ihin tästä näkökulmasta.

Tässä tutkielmassa tutkittiin hiiren C166-soluissa tuman ja sytoplasman miRNA:ita normaali- ja vähähappisissa olosuhteissa. Eri solujen osien sekvensointi paljasti miRNA-populaatioita, jotka sijaitsivat joko solujen tumassa tai solulimassa. Suurin osa miRNA:ista myös ilmeni eri tavalla vain tumassa tai solulimassa, riippuen solujen happipitoisuudesta. Huomattavaa oli, että vain kuusi näistä miRNA:ista, joiden ilmeneminen muuttui happipitoisuuden muuttuessa, muuttui sekä tumassa että solulimassa.

Tässä tutkielmassa tutkittiin myös RNAa:ta hiiren vaskulaarisen endoteelin kasvutekijä A (*Vegfa*) -geenille. VEGFA on yksi päätekijöistä iskeemisissä sairauksissa, sillä se lisää angiogeneesiä eli verisuonien uudelleen muodostumista. Tutkimuksissa löydettiin mahdollisia paikkoja *Vegfa*:n säätelyalueella, joihin hiiren miRNA:n mmu-miR-466c:n molemmat kypsät miRNA:t voivat kohdentua. Eri molekyylibiologisten menetelmien avulla tutkimuksissa todellakin havaittiin miR-466c:n lisäävän *Vegfa*:n ilmenemistä hiiren endoteelisessä C166-solulinjassa. miR-466:n vaikutuksia tutkittiin myös hiirissä takaraajaiskemia-mallissa. miR-466c välitettiin iskeemiseen kudokseen joko solun ulkopuolisten vesikkeleiden (EV) välittäminä tai käyttämällä lentivirusvektoria. miR-466c paransi hiirten takaraajojen toimintaa ja nosti hiirten painoa varsinkin EV-välitteisessä miR-466c ryhmässä. Tutkimuksessa huomattiin myös takaraajan verisuonituksen merkittävää parantumista, kun vertailtiin ultraäänikuvia sekä iskemia-arvoja miR-466c-ryhmien ja kontrolliryhmän välillä.

Tässä väitöskirjassa esitetyissä tutkimuksissa löydettiin tumassa olevia miRNA:ita hiiren endoteelisissä soluissa, sekä tunnistettiin miR-466c, joka säätelee *Vegfa*:n ilmenemistä transkriptionaalisella tasolla. Nämä tulokset edesauttavat uusien tuma-miRNA välitteisten terapiamuotojen kehittämistä tulevaisuudessa.

Avainsanat: miRNA, tuma-miRNA, ei-koodaavat RNAt, RNA aktivaatio

“It takes a dream to get started,

desire to keep going and

determination to finish.”

-Eddie Harris Jr.

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Kuopio, 31 May 2023

Pia Laitinen

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I Turunen TA, Roberts TC*, **Laitinen P***, Väänänen M-A, Korhonen P, Malm T, Ylä-Herttuala S and Turunen MP. Changes in nuclear and cytoplasmic microRNA distribution in response to hypoxic stress. Scientific Reports 9:10332, 2019.

II **Laitinen P**, Väänänen M-A, Kolari I-L, Mäkinen P, Kaikkonen MU, Weinberg MS, Morris KV, Korhonen P, Malm T, Ylä-Herttuala S, Roberts TC, Turunen MP* and Turunen TA*. Nuclear microRNA-466c regulates *Vegfa* expression in response to hypoxia. PLoS One 17:e0265948, 2022.

III **Laitinen P**, Väänänen M-A, Shatillo A, Bragge T, Kärkkäinen A-M, Turunen MP* and Turunen TA*. Nuclear microRNA-466c role in mouse hindlimb ischemia. *Manuscript.*

* Authors with equal contribution

CONTENTS

ABBREVIATIONS

3p	3 prime
3'UTR	3'untranslated region
5p	5 prime
5'UTR	5'untranslated region
AAV	adeno-associated virus
Ad	adenovirus
AGO2	Argonaute 2
AMD	age-related macular degeneration
ANRIL	antisense noncoding RNA in the INK4 locus
ASO	antisense oligo
bp	base pair
CAD	coronary artery disease
C/EBP-A	CCAAT/enhancer-binding protein α
cDNA	complementary DNA
ceRNA	competing endogenous RNA
CRISPR	clustered regularly interspaced short palindromic repeats
CVD	cardiovascular disease
Ccnb1	cyclin B1
DGCR8	Di George syndrome critical region 8
ds	double-stranded
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EF1A	elongation factor 1 α
e.g.	*exempli gratia*, for example
eRNA	enhancer RNA
EV	extracellular vesicle
FDA	U.S. Food and Drug Administration

Flt-1	FMS-like tyrosine kinase
fUS	functional ultrasound
FXR1	fragile X mental retardation related protein 1
GRO-seq	Global Run-On-sequencing
H3K4me2	dimethylation of lysine 4 of histone 3
H3K4me3	trimethylation of lysine 4 of histone 3
H3K9ac	acetylation of lysine 9 of histone 3
H3K9me2	histone dimethylation of lysine 9 of histone 3
H3K27ac	acetylation of lysine 27 of histone 3
H3K27me3	trimethylation of lysine 27 of histone 3
HIF	hypoxia-inducible factor
HRE	hypoxia-responsive elements
Hsp	heat-shock protein
HUVEC	human umbilical vein endothelial cells
hypoxamiR	hypoxia regulated microRNA
i.e.	*id est*, that is
i.m.	intra-muscular
IPO8	importin 8
isomiR	miRNA isoform
KDR-1	kinase insert domain-containing receptor
LDL	low-density lipoprotein
LNA	locked nucleic acid
lncRNA	long non-coding RNA
LNP	lipid nanoparticle
LV	lentivirus
Malat1	metastasis associated lung adenocarcinoma transcript 1
MI	myocardial infarction

miR, miRNA microRNA

mRNA messenger RNA

MSC mesenchymal stem cell

MyD88 myeloid differentiation primary response gene 88

ncRNA non-coding RNA

NeuroD1 neuronal differentiation 1

nm nanometre

NPC nuclear pore complex

nt nucleotide

NTA nanoparticle tracking analysis

OSCC oral squamous cell carcinoma

PAD peripheral artery disease

paRNA, pancRNA promoter-associated non-coding RNA

piRNA PIWI-interacting RNA

PLGF placental growth factor

Pol II RNA polymerase II

PTGS post-transcriptional gene silencing

PR progesterone receptor

pre-miRNA precursor microRNA

pre-mRNA precursor messenger RNA

pri-miRNA primary microRNA

RISC RNA induced silencing complex

RNAa RNA activation

RNAi RNA interference

ROI region of interest

RPE retinal pigmented endothelial

RPE65 retinal pigment epithelium 65 kDa

rRNA ribosomal RNA

RT-qPCR reverse transcription quantitative real-time polymerase chain reaction

saRNA small activating RNA

sFlt-1 soluble FMS-like tyrosine kinase

Sfmbt2 Scm-like with 4 Mbt domains 2

shRNA small hairpin RNA

siRNA small interfering RNA

sncRNA small non-coding RNA

snoRNA small nucleolar RNA

snRNA small nuclear RNA

ssRNA single-stranded RNA

ST6GAL ST6-β-galactoside-α-2,6-sialyl-tranferase

TF transcription factor

TGA transcriptional gene activation

TGS transcriptional gene silencing

TNFA tumour necrosis factor–α

tPA tissue plasminogen activator

TRBP Trans-activation response RNA binding protein

TSS transcription start site

tRNA transfer RNA

TNRC6A trinucleotide repeat containing adaptor 6A

VEGF, VEGFA/B/C/D vascular endothelial growth factor A/B/C/D

VEGFR2 vascular endothelial growth factor receptor 2

XIST X-inactive specific transcript

XPO5 exportin 5

ZMYND10 zinc finger MYND-type containing 10

1 INTRODUCTION

Ischemic diseases are a diverse group of diseases that occur due to the lack of oxygen reaching the tissues (i.e., ischemia). This is often caused by the blocking of the vessels (e.g., by plaques), thus impairing the blood flow to the tissue. Ischemic stroke and ischemic heart disease are the most common and the deadliest ischemic diseases, responsible for over 20 % of all deaths globally each year (1).

While unhealthy diets, smoking and an inactive lifestyle are risk factors which play a crucial role in the development of the ischemic diseases, it is known that genetic factors increase the individual's vulnerability to these diseases (2,3). There are multiple therapies available for ischemic diseases, such as medication with vasodilation drugs or surgery where blocked arteries are bypassed (4). Nonetheless, these solutions are either not always effective enough or not suitable for all patients. Thus, there is an urgent need for the development of novel therapies.

As the name indicates, non-coding RNAs (ncRNAs) are RNAs that are not translated into proteins. It has been estimated that over 98 % of the human genome consists of non-coding regions, while under 2 % of the genome is eventually involved in producing proteins (5). ncRNAs are categorised into long and small ncRNAs based on their length, and they take part in the regulation of normal cellular function and thus a malfunction of ncRNAs may evoke detrimental changes and lead to the development of many diseases. Some ncRNAs have already been associated with different ischemic diseases (6,7).

MicroRNAs (miRs, miRNAs) are small ncRNAs (sncRNAs) that play a crucial part in cellular activities. After the discovery of the first miRNA, lin-4, in 1993 (8), there has been a huge expansion in the numbers of studies examining miRNAs. Most miRNAs take part in the gene regulation by silencing messenger RNAs (mRNAs) in the cytoplasm, in an event called post-transcriptional gene silencing (PTGS). In PTGS, miRNAs act by binding

to the 3'untranslated region (3'UTR) of the mRNAs which leads to a degradation of the target mRNAs or inhibition of their translation.

In addition to their RNA interference (RNAi) properties in the cytoplasm, recent studies have indicated a role for miRNAs in the nuclei of the cells. Mature miRNAs are found in the nuclei where they are transported after maturation, since most miRNAs are processed from pre-miRNAs to mature miRNAs in the cell's cytoplasm (9,10). In the nuclei, miRNAs can silence or activate gene function. The exact mechanisms how miRNAs regulate genes in the nuclei have remained elusive but there are some studies indicating that different mechanisms may be exploited (11,12).

In 2006, Li et al. were the first to report on RNA activation (RNAa) (13). In RNAa, miRNAs or other sncRNAs target the promoter region of the gene, leading to the activation of gene transcription. RNAa is a novel, promising field of miRNA studies since it is believed that it may represent a basis for future therapeutics.

Despite the growing evidence of non-canonical miRNA functions, many details remain unclear. This thesis consists of three studies that characterized the nuclear functions of miRNAs. In the first study, a nuclear population of miRNAs was discovered in mouse endothelial C166 cells. In the second study, miR-466c was identified to target the *Vegfa* promoter and induce the expression of *Vegfa*. In the third study, miR-466c was observed to improve limb function in mice subjected to hindlimb ischemia. Together, these studies raise the possibility that in the future, nuclear miRNAs may be developed as therapeutic tools for ischemic diseases.

2 REVIEW OF THE LITERATURE

2.1 ISCHEMIC DISEASES

Ischemic diseases refer to a large group of diverse ailments that occur due to the impaired blood flow to the tissues (i.e., ischemia). The two most common and the deadliest ischemic diseases are ischemic heart disease and ischemic stroke, which together are responsible for over 20 % of total deaths globally each year (**Table 1**). Furthermore, ischemic diseases can take place in many parts of the human body, such as in the limbs (2), eyes (14) and intestines (15). Ischemic diseases are classified under the category of cardiovascular diseases (CVDs) which refers to all the diseases regarding heart and/or blood vessels.

Table 1. Causes of death for the most common ischemic diseases in 2019. The number indicates the percentual amount of each death cause in total deaths in the designated area. The order of each disease in death rates in the designated areas is shown in brackets (1).

2019	Global	USA	Europe	Finland
Ischemic heart disease	16.0 % (1)	17.1 % (1)	23.7 % (1)	17.8 % (2)
Ischemic stroke	5.5 % (4)	3.0 % (6)	8.0 % (2)	5.3 % (3)

The main feature of ischemic diseases is the lack of oxygen and nutrients reaching the tissues. This occurs due to the blockage of the blood vessels, which can be either partial or total. The blocking of vessels occurs when lipids, like cholesterol, and cellular waste start to accumulate on the inner surface of the vessel, forming an obstacle called a plaque (16). When these plaques start to accumulate in the inner surface of the vessels, blood

flow is disturbed, leading to a disorder called atherosclerosis. This is the main cause of cardiovascular (and ischemic) diseases. If the condition is prolonged, the accumulation of plaques blocks the whole vessel which causes infarction in the tissues, leading to the death of the cells. Infarction is a severe condition, which needs immediate medical treatment (17).

The main treatments for ischemic diseases are 1) surgery where the blocked artery is bypassed, or 2) medical treatment with different vasodilative or thrombolytic drugs (4). In addition to traditional treatments, new therapies using stem cells (18,19), gene therapy (20) and cell therapy (21) are currently being evaluated. However, the breakthrough of a new functional treatment has remained unattainable.

Due to the occurrence and lethality of ischemic diseases, more studies investigating the molecular mechanisms behind the conditions, such as hypoxic regulation of the cells, are evidently needed, so that new cures for ischemic diseases can be discovered.

2.1.1 Ischemic heart disease

Ischemic heart disease, also known as coronary heart disease or coronary artery disease (CAD), is the condition that occurs when the heart tissue does not receive enough oxygen and nutrients to allow it to undertake its normal function. As the name implies, the condition takes place in the coronary arteries which are responsible for circulating blood to the cardiac tissues (17).

The normal function of the heart is crucial for the body. The studies that have led to effective ways to treat the malfunctioning heart have saved significant number of lives. Usually, ischemic heart is treated by medication with vasodilators or blood thinning medication (4). It can also be treated by inserting a stent in the blocked artery or by opening the artery with balloon angioplasty and stenting, (20) to physically dilate the vessel. Drug-coated balloons have also been developed (22). However, when blood flow is totally blocked, cells start to die leading to myocardial infarction (MI) i.e., a heart attack. In this case, surgery is conducted, and the blocked arteries are bypassed to ensure the blood flow to the cardiac tissue.

Ischemic heart disease and especially MI are usually experienced by older people but due to the fact that more and more people indulge in an unhealthy lifestyle, it is nowadays becoming increasingly a burden for younger (under 45 years) people (23). Because of smoking, obesity, high alcohol use and inactive lifestyle (2,23), CAD is maintaining its place as the deadliest disease around the world.

Existing therapies to treat CAD do not solve the problem, they mainly relieve the symptoms. Cardiomyocytes do not have a great capacity for renewal, so they recover rather poorly from the infarct. Even though bypass surgery helps some patients, it is not always possible due to several different risk factors i.e., if the person is older and/or has many comorbidities. In addition, despite having undergone surgery or being treated with medication, a cardiac infarction often leads to heart failure (18). The only truly functional cure for severely infarcted heart is heart transplantation and since human hearts are not freely available, there is an urgent need for innovative therapeutics.

2.1.2 Ischemic stroke

Stroke is a suddenly occurring brain malfunction due to problems with blood flow to the brain. Stroke is divided into two types, ischemic stroke and hemorrhagic stroke. An ischemic stroke is the most common type of stroke, responsible for 87 % of all cases. It occurs because of the disturbance in the vascular function due to the presence of plaques or clots in the arteries (3). Hemorrhagic stroke refers to the condition where blood cells leak into the brain tissue from damaged blood vessels. This can occur after an ischemic stroke or due to the increased permeability of the vessels (24).

Similar to the situation in CAD, poor lifestyles, as well as genetic susceptibility are known to play pivotal roles in an individual's probability of suffering from a stroke (2,3). Adherence to a healthy lifestyle is the key to decreasing the probability of all cardiovascular diseases, not just ischemic stroke. For example, if an individual wishes to avoid this

condition, it is especially important to maintain blood pressure within its normal range and not to smoke (2).

Interestingly, many ischemic conditions are linked to each other. For example, a previous ischemic event in the brain (i.e., stroke) leads more often to an ischemic condition in the heart when compared to non-stroke patients. Since the brain and the heart are connected to each other via the blood circulation, the stress factors secreted by the brain reach the heart where they induce stress reactions. Stroke can also affect the hypothalamus and parasympathetic and sympathetic nervous systems, leading to altered regulation of the heart for example by inducing arrhythmias. Importantly, these connected conditions occur also *vice versa*; a cardiac infarction makes the individual more prone to brain ischemia (25,26). For example, it has been claimed that the underlying cardiac defect can lead to an ischemic stroke, but the exact mechanisms behind this association have remained elusive (27).

Medication is the first option to treat ischemic stroke. By using blood thinning medication or thrombolytics like tissue plasminogen activator (tPA), it is possible to restore the blood flow to the brain (28). However, in a severe condition, surgery to remove the blood clot is often needed.

2.1.3 Peripheral artery disease

Peripheral artery disease (PAD) refers to an ischemic condition occurring in the peripheral tissues instead of the heart or brain. This is most often caused by atherosclerosis in the lower limbs (29), so it will be discussed here in this respect.

PAD is a serious condition, and as in other CVDs, it occurs due to atherosclerosis. It develops over time and can exist for a long time without the patient experiencing any symptoms. When the disease becomes more severe, the patient starts to suffer symptoms, such as walking disabilities and leg pain. Older people are more prone to PAD since old age is a major risk factor. If PAD is not treated early enough, then the only treatment is the amputation of the affected limb (2).

PAD itself is rarely lethal but it often indicates the presence of other severe diseases like CAD, MI and ischemic stroke (2). Traditional treatments for PAD are medication like statins that reduce low-density lipoprotein (LDL) levels or inserting stents into the blocked arteries. The optimal way to reduce the risk of ischemic condition of the limb is to improve the lifestyle e.g., by stopping smoking and increasing exercise in the everyday lifestyle (30).

PAD also burdens the health care system especially due to its increased prevalence among older people and as with other ischemic diseases, more studies and better therapeutics for the treatment of PAD are urgently needed.

2.1.4 New therapies for the ischemic diseases

To respond to the growing need for efficient treatments, new treatments have been explored in research labs and clinics over the last years. One example is gene therapy, where a therapeutic gene is inserted into the treated tissue.

As a way of achieving increased gene expression, the messenger RNA (mRNA) can be administered as complementary DNA (cDNA) using naked plasmids or by using vectors carrying the genes, like adenovirus (Ad) (20). While this approach can be utilized for a number of diseases, it is easiest to exploit with diseases based on a malfunction of a single gene that can be then replaced by delivering a functional, correct gene copy to the tissue. For example, Luxturna is a gene therapy drug approved by the U.S. Food and Drug Administration (FDA) to combat the vision loss due to the mutation in the genome of retinal pigment epithelial (RPE) cells that encodes the retinal pigment epithelium 65 kDa (RPE65) protein. This treatment is achieved by inducing a cDNA to code for the functional RPE65 protein via adeno-associated virus (AAV) vector (31). Many studies have indicated that gene therapy, e.g., by delivering small hairpin RNAs (shRNAs), soluble vascular endothelial growth factor receptor 2 (sVEGFR2) or microRNA miR-199a, can have a beneficial outcome also for ischemic diseases (6,32,33).

As a form of cell therapy, newly studied stem cell-based treatments have shown some promise. In this therapeutic model, stem cells, either from the patient or from another donor, are collected, modified and delivered into the infarcted tissues. There are a variety of different stem cells from different sources like adipose- and embryonic derived stem cells as well as induced pluripotent stem cells that have been created by reprogramming of specialized cells. However, mesenchymal stem cells (MSCs) are the most widely used class of stem cells in the treatment of ischemic diseases since they are easy to modify, and they can be collected from several different tissues like bone marrow and muscles. The advantage of stem cell therapy is the low immune reactivity after transplantation but the problem with this method is that the viability and engraftment of stem cells after transplantation is often poor (18).

Despite the poor transplantation, stem cell therapies have achieved beneficial effects in the treatment of a variety of diseases, leading to the idea that the effect behind this treatment method is attributable to the molecules secreted by stem cells. This hypothesis is supported by a number of studies showing that stem cells secrete extracellular vesicles (EVs) that are known to be involved in communication between cells for example by delivering RNAs and proteins to the recipient cells (4,34). It has been shown that MSC derived EVs can exert a beneficial effect on mice hearts subjected to left coronary artery ligation by repressing the extent of inflammation in the ischemic area (35). It has also been demonstrated that IL-35 overexpressing adipose-derived MSCs are able to alleviate the immune reaction after heart transplantation in mice (36), suggesting that stem cells can have a beneficial role also in support of other therapies.

2.1.5 Hypoxia

All human cells and tissues require oxygen to remain viable. Oxygen is a chemical element that is needed for cellular energy metabolism. The term normoxia describes the optimal oxygen level that is required for the normal function and homeostasis of the cells, tissues and body. Instead,

when cells experience hypoxia i.e., low oxygen levels, they cease their growth and if this condition is prolonged, they start to die (37).

The condition when there is a lack of oxygen due to the reduced blood flow to the tissue is called ischemia; it is defined by a lack of both oxygen and nutrients. Hypoxia in the tissues can lead to anoxia which refers to a total lack of the oxygen, clinically translating to a progression of an ischemic condition into an infarction.

When cellular oxygen levels decline, cells start to transmit emergency signals to other cells. In the stress condition, cells cease all functions that are not critical and start to produce factors that are needed to help the cell survive the hypoxia. Hypoxia-inducible factor α (HIFA) is one of these factors; there are three isoforms called HIF1A, HIF2A and HIF3A. HIF1A was the first to be discovered and it has been the most extensively studied form. In normoxic conditions, HIF1A is not needed and therefore it is rapidly degraded in the cells. However, if there is hypoxia, the production of HIF1A is increased, and it is translocated into the nucleus where it binds to HIF1B. This HIF1 complex targets the hypoxia responsive elements (HREs) on gene promoters and upregulates the expression of genes that are beneficial to allow cells to cope with hypoxia (38–40).

The hypoxic condition in the molecular biology laboratory can be replicated by utilizing substances that induce a cellular hypoxia response. For example, cobalt chloride ($CoCl_2$) is used to create hypoxia in cell culture, since it maintains HIF1A and HIF2A levels in the cells (41). If one wishes to achieve a more authentic cellular hypoxia response, it is possible to use devices like hypoxia chambers, in which cells are cultured in an environment where the atmospheric conditions can be controlled. In these experiments, cells are often exposed to 1 % oxygen level; control cells are cultured in a normal incubator where the oxygen level is approximately 18.6 % (in air there is about 21 % oxygen) (42).

However, it is known that the usual oxygen level in a normal functioning tissue is not 18.6 % but usually close to 5 %. When studying tumours, the oxygen levels are normally even lower than in organs. Nonetheless, most studies using cell cultures have been performed closer to the oxygen levels in ambient air than the real physiological condition in the body. It has been

speculated that this detail in the experimental setup may have a huge impact on the obtained results and it may have impaired the translation of results from cell culture to the *in vivo* situation (43).

2.1.6 Vascular endothelial growth factor A

Vascular endothelial growth factor A (VEGF, VEGFA) is one of the main growth factors in the body, responsible both for physiological and pathological angiogenesis. Angiogenesis is the phenomenon where new blood capillaries are formed from the existing blood vessels. Angiogenesis is stringently regulated simply by changing *VEGFA* expression so that it is possible to create a new blood circulation where it is needed or alternatively prevent the formation of functional vessels (39).

VEGFA is a secreted protein, and it acts by binding to its receptors located on the surface of recipient cells. VEGFA receptors are FMS-like tyrosine kinase (Flt-1) and kinase insert domain-containing receptor (KDR-1) (44). These are also known as VEGF receptor 1 and 2, respectively. In addition to the surface-bound receptors, there is a soluble receptor for VEGFA, called soluble Flt-1 (sFlt-1) (45).

The *VEGFA* gene consists of eight exons that form multiple different mRNAs by alternative splicing. These mRNA isoforms are then translated into different proteins named $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ (44).

In addition to different VEGFA isoforms, there are also different VEGF proteins in the VEGF family: vascular endothelial growth factors B, C and D (VEGFB, VEGFC, VEGFD) and placental growth factor (PLGF). Of these, VEGFB and PLGF bind to Flt-1and VEGFC and VEGFD to KDR-1. VEGFC and VEGFD can also bind to the Flt-4 receptor. While all VEGF proteins take part in the regulation of angiogenesis, they can affect the endothelial cells differently based on the specific tissue. At present, the mechanisms underpinning the properties of VEGFA have been the most extensively studied (44,46).

The *VEGFA* gene is activated in hypoxic conditions in many cell types, especially in the endothelial cells that are crucial in the march towards

angiogenesis (44,47). When a tissue suffers from ischemia, as occurs in ischemic diseases, the process of angiogenesis starts (**Figure 1**).

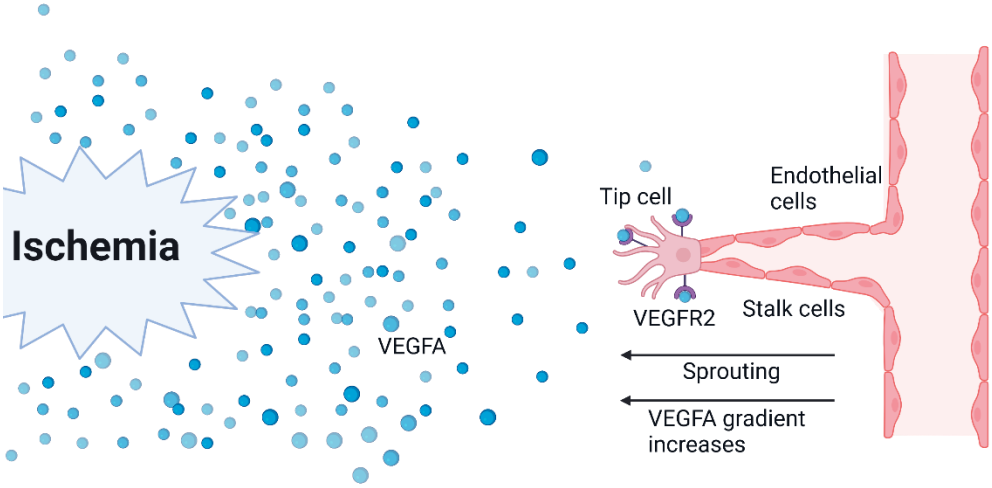

Figure 1. VEGFA-induced angiogenesis. HIF1A induces *VEGFA* expression at the ischemic site. This upregulation of *VEGFA* promotes new capillary formation. The endothelial cells targeted by VEGFA are transformed into tip cells that start to express higher amounts of VEGFR2. Numerous signalling pathways are triggered in these cells, leading to sprouting to the ischemic site by following the higher VEGFA concentration. The tip cells are followed by other endothelial cells, called stalk cells, leading to the angiogenesis and formation of a new capillary in the ischemic site. VEGFA= vascular endothelial growth factor A, HIF1A = hypoxia-inducible factor 1α, VEGFR2= VEGFA receptor 2. Created with BioRender.com.

As *VEGFA* is a master regulator of hypoxic conditions, its expression is regulated by many transcription factors, such as the earlier mentioned HIF1A. When there is hypoxia, HIF1A induces *VEGFA* expression in the ischemic area and VEGFA, as a soluble factor, is secreted from the cells and transported further from the ischemic area. Angiogenesis starts when endothelial cells in an already existing nearby vessel recognize the elevated VEGFA levels. These endothelial cells become tip cells, after VEGFA has induced multiple different signalling cascades. These tip cells are transient

cell types, and they express an abundance of VEGFR2, enabling efficient activation by the binding of soluble VEGFA molecules. Tip cells start to follow the VEGFA gradient i.e., they sprout towards the ischemic site where there is a higher concentration of VEGFA molecules available. Tip cells are followed by other endothelial cells called stalk cells, which then produce a new vasculature. As the tip cells reach the ischemic site, they produce a new capillary to the ischemic site to ease the ischemic effect in the tissue by allowing the passage of oxygen and nutrients to the ischemic site (48,49).

2.2 NON-CODING RNAS

Non-coding RNAs (ncRNAs) are RNAs that are transcribed from the genome but not translated into proteins and in this respect they differ from mRNAs. It has been estimated that only 2 % of the human genome is transcribed into protein-coding mRNAs and most of the remaining non-coding regions are transcribed into ncRNAs (5). ncRNAs have been widely studied and their expression has been linked in the homeostasis of the cells, tissues and organs as well as with many pathophysiological conditions. ncRNAs are expressed by all the cells in the body. They also include translational machinery RNAs transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) that are not discussed further since it is transcriptional regulation which is the focus of this thesis.

In general, ncRNAs can be divided into two groups: long non-coding RNAs (lncRNAs) and small non-coding RNAs (sncRNAs). ncRNAs that are over 200 nucleotides (nt) in length are considered as lncRNAs and ncRNAs below the 200 nt limit are termed sncRNAs. However, since the lengths of the ncRNAs can vary within the group, these classifications are somewhat arbitrary. For example, small nucleolar RNAs (snoRNAs) are considered to be sncRNAs, although their size can vary from 60 nt to 300 nt (50,51).

ncRNAs are transcribed from the different parts of the genome, from the intergenic regions but also from gene regulatory elements and gene introns. ncRNAs are found both in the nuclei and in the cell's cytoplasm, as well as in the extracellular space and in the secreted EVs which transport

these ncRNAs from cell to cell. As they are expressed by diverse cell types, ncRNAs also have various functions like gene silencing and activation. These functions can then lead to a variety of functional outcomes like cell proliferation and angiogenesis, cell-to-cell communication, development and even cell death (52). Overall, ncRNAs are important factors in maintaining the homeostasis and normal function of the cells.

As ncRNAs are important in a cell's normal function, abnormal expressional changes of the ncRNAs may lead to malfunctions. Abnormally expressed ncRNAs then regulate their target genes incorrectly leading to undesirable functional outcomes and possibly the triggering of some disease. For this reason, ncRNAs are thought to hold a significant therapeutic potential.

2.2.1 Long non-coding RNAs

As stated, in contrast to mRNAs, lncRNAs are RNA transcripts that do not serve as templates for protein synthesis. There are also other differences between mRNAs and lncRNAs. lncRNAs can be found in both the nucleus and cytoplasm of the cell, whereas mature mRNAs are cytoplasmic since that is the location where translation takes place. Like mRNAs, also lncRNAs are transcribed by RNA polymerase II (Pol II), and they can have a polyA tail and 5'cap. In addition to differences in their protein coding function, there are also differences in their expressional levels. mRNAs are usually expressed at quite high levels while ncRNAs can be expressed at relatively low or high levels, depending on the ncRNA. Moreover, ncRNAs are usually more tissue and cell specific than mRNAs (53,54). lncRNAs can be further classified into different categories based on their expression or function. However, since the different lncRNAs were only discovered rather recently, their classification system is constantly evolving.

lncRNAs have been linked to many cellular functions and they regulate their targets at both the transcriptional and post-transcriptional level. For example, the lncRNA X-inactive specific transcript (*XIST*) participates in X chromosome silencing (55) and metastasis associated lung adenocarcinoma transcript 1 (*Malat1*) regulates angiogenesis by binding

VEGFR2 in mouse hindlimb ischemia (56). lncRNAs have also been associated with ischemic stroke; for example, *MALAT1* and the antisense noncoding RNA in the INK4 locus (*ANRIL*) promote beneficial effects in the ischemic tissue. The amount of *ANRIL* is increased in ischemia, and it induces angiogenesis by targeting the *VEGFA/Flt-1* pathway. The level of *MALAT1* is also increased in ischemic stroke and its expression has been linked to a reduction in the extent of inflammation and cell death in ischemia (5).

Promoter-associated ncRNAs (paRNAs, pancRNAs) are lncRNAs that are transcribed near to the transcription start site (TSS) region of the genes (57,58). paRNAs, like RNAs in general, are produced by Pol II. paRNAs can either activate or inhibit their target genes by acting as scaffolds for other ncRNAs and proteins to enable the regulatory function (58).

Enhancer RNAs (eRNAs) are ncRNAs that take part in gene regulation by acting as scaffolds for transcription factors (TFs). eRNAs can be both short or long ncRNAs, and their size varies extensively depending on the tissue or cell type. The median size of a human eRNA has been proposed to be 346 nt (59). eRNAs regulate their target gene expression by working together with gene promoters to activate gene expression. Many enhancers upregulate their target genes by inducing the levels of epigenetic markers for open chromatin, such as acetylation of lysine 27 in histone H3 (H3K27ac), on target gene promoters. In this way, eRNAs regulate gene expression by affecting the 3D structure of chromatin by looping chromatin to their target genes and are therefore able to regulate distant genes (60).

2.2.2 Small non-coding RNAs

sncRNAs are ncRNAs that are generally under 200 nt in size and they have important cellular regulatory roles. For example, sncRNAs have been linked to normal cellular functions like proliferation and metabolism. However, sncRNAs participate also in the development of many diseases, such as many cancer types and ischemic diseases (51,61).

Small ncRNAs are classified into subgroups; the main groups are snoRNAs, small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs) and microRNAs (miRNAs). Each of these groups has its own specific features (**Table 2**) and together with lncRNAs, they comprise a cascade of RNAs functioning together to ensure optimal cellular function. This network of RNAs regulates gene expression in a complex fashion. For example, lncRNA AK131850 acts as a sponge for miR-93-5p, thereby inhibiting the binding of this miRNA to its target *VEGFA* mRNA, thus leading to an increase in *VEGFA* expression (62). lncRNAs can therefore compete with mRNAs for miRNA binding and it is the balance of these different ncRNAs that directly affects the gene expression level.

Table 2. The most common small non-coding RNA (sncRNA) groups. The main features of the snoRNAs, piRNAs, siRNAs and miRNAs. rRNA=ribosomal RNA, snRNA=small nuclear RNA, ssRNA=single-stranded RNA, TGS=transcriptional gene silencing, PTGS=post-transcriptional gene silencing, TGA=transcriptional gene activation.

sncRNA	Size (nt)	Specific feature	Found in	Main functions	Reference
small nucleolar RNA (snoRNA)	60-300	C/D box and H/ACA box families	eukaryotic cells, nucleoli	rRNA and snRNA modifications	(50)
PIWI-interacting RNA (piRNA)	24-32	2'O'methyl at 3'end and 5'end uracil of precursor ssRNA	germ line cells, nucleus, and cytoplasm	epigenetic regulation, transposon silencing	(63)
small interfering RNA (siRNA)	19-24	Perfect base-pairing in duplex	nucleus and cytoplasm	PTGS, TGS	(64)
microRNA (miRNA)	20-23	target recognition by seed sequence, a lot of isomiRs	nucleus and cytoplasm	PTGS, TGS, TGA	(65)

In addition to sponging, other types of lncRNA-sncRNA co-operation have been examined. It has been shown that miRNAs can actively degrade lncRNAs in the nucleus. This was studied in the Hodgkin lymphoma cell line L428 where *MALAT1* was downregulated by the miR-9. In that experiment, no sponging effect was proposed since there was no significant change in the expression of miR-9 even when *MALAT1* was knocked-down (66). miRNA co-operation has been also identified with enhancers. Nuclear activating miRNAs can bind to the enhancers and promote their function to activate their target genes (67).

2.3 MICRORNAS

MicroRNAs (miRNAs, miRs) are sncRNAs that have been widely studied since the first miRNA, lin-4, was discovered in *Caenorhabditis elegans* in 1993 (8). Subsequently, most of the research has focused on studying the canonical role of miRNAs, i.e., post-transcriptional gene silencing (PTGS). This RNA interference (RNAi) is a crucial method to silence gene expression by degrading the target mRNA or blocking the translation of mRNA.

However, in recent years, new information has appeared about the non-canonical role of miRNAs including activation of mRNA translation in the cytoplasm (68) as well as functions in the cell's mitochondria (69). Nonetheless, the most fundamental property of miRNAs relates to their function in the nuclei of the cells, either silencing or activating the expression of their target genes. All in all, miRNAs have a pivotal role in gene regulation and their malfunction has been linked to many diseases like CVD (11).

2.3.1 Biogenesis of microRNAs

miRNAs are widely conserved in animals. This means that the same miRNAs and/or their targets can be found in different organisms. For example, there is 94 % miRNA target complementarity between humans and chimpanzees (70). Especially, the miRNA seed sequence, which refers to nucleotides 2-7 of miRNA from the 5'end, is often conserved between

mammals (71). In addition to mammals, miRNAs are found in other animals, plants, fungi and even in some viruses (72). The biogenesis of miRNAs is somewhat similar in different organisms, however some differences e.g., in processing enzymes trans-activation response RNA binding protein (TRBP) and Di George syndrome critical region 8 (DGCR8) have been detected (73). In this chapter, the biogenesis of miRNAs is described in the manner it occurs in mammals (**Figure 2**).

miRNAs are transcribed from multiple regions in the genome. They can be located in the non-coding areas between genes, as well as in the introns and exons of the genes. miRNAs can also be transcribed from genomic repetitive elements, and they can arise from the duplication of the gene or the transcription of an antisense transcript of mRNA (71). When specific miRNAs are needed, TFs gather at the specific loci of the genome (promoter of the miRNA) to start the transcription (52). miRNAs originating from intronic and exonic regions of the genes are produced when the mRNAs of these genes are produced. These genes that co-express miRNAs are called the host genes of these miRNAs. Alterations in the host gene expression therefore directly affect the expression changes in the simultaneously transcribed miRNAs (65).

Transcription starts when Pol II enzyme begins the transcription of primary miRNA (pri-miRNA) (65). pri-miRNAs consist of miRNA precursor (pre-miRNA) i.e., loop-structured hairpin, a cap in the 5' end and sometimes a polyA tail in the 3'end (74). pri-miRNAs containing one pre-miRNA are called monocistronic miRNAs and pri-miRNAs coding for multiple pre-miRNAs are called polycistronic miRNAs.

As pri-miRNA is transcribed, the next step is its cleaving to one or multiple pre-miRNAs. In pre-miRNA loop-structure, there are gaps, mismatches and bulges which are needed to make pre-miRNA unstable, so they are processed properly (75). pre-miRNAs are processed in either a DROSHA dependent or a DROSHA independent way. DROSHA works together with DGCR8 to create a pre-miRNA. DROSHA is an RNase III endonuclease that cleaves the pri-miRNA in a specific position. DROSHA independent pre-miRNAs are called mirtrons since they originate from the intron of precursor mRNA (pre-mRNA) and are processed by spliceosomes.

In addition, it is possible that miRNAs can originate from snoRNA and tRNA precursors (11).

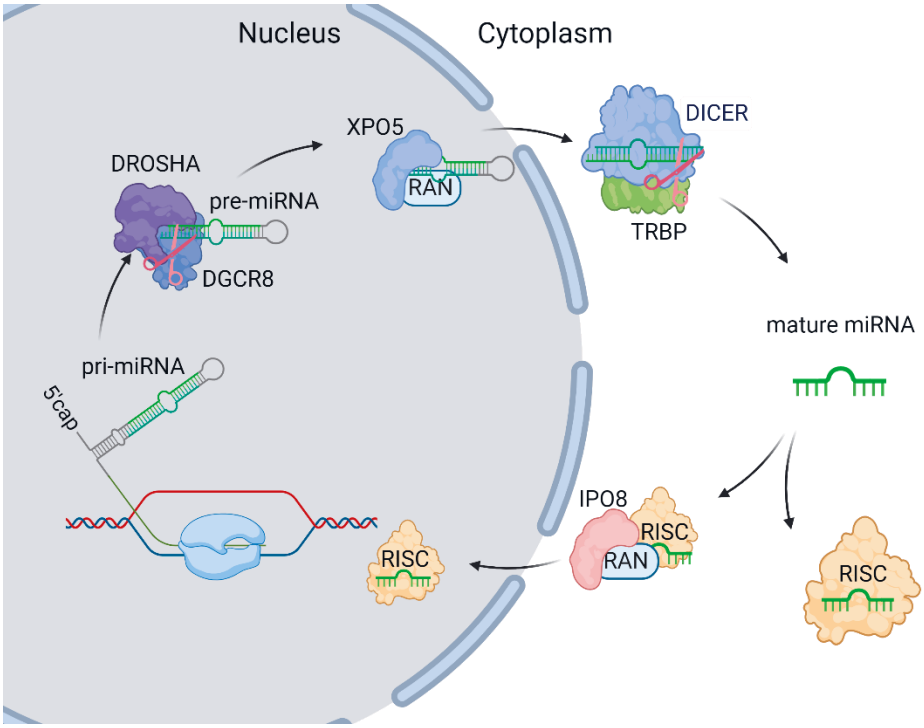

Figure 2. Mammalian microRNA biogenesis. miRNAs are transcribed as pri-miRNAs which are then processed into pre-miRNAs by DROSHA and DGCR8 in the nucleus. XPO5 transports pre-miRNAs into the cytoplasm where they are cleaved into duplex miRNAs by DICER and TRBP. miRNAs are loaded into the RISC which they can lead to their targets in the cytoplasm (PTGS) or in the nucleus (TGS/TGA) by IPO8/XPO5 mediated transportation. DGCR8= Di George syndrome critical region 8, XPO5= exportin 5, pri-miRNA=primary microRNA, pre-miRNA= precursor microRNA, TRBP= trans-activation response RNA binding protein, RISC= RNA-induced silencing complex, PTGS= post-transcriptional gene silencing, TGS= transcriptional gene silencing, TGA= transcriptional gene activation, IPO8= importin 8. Created with BioRender.com.

After cleaving, pre-miRNAs are exported from the nucleus to the cytoplasm with the help of Ran-GTPase and Exportin 5 (XPO5) (73). pre-miRNAs are recognized by their 2 nt overhang in their 3' end which is the

signal for XPO5 for their transportation (11,74). In the cytoplasm, pre-miRNAs are further processed by the DICER enzyme. DICER is an RNase III endonuclease but unlike DROSHA, it works together with TRBP to cleave the loop-structure of the pre-miRNAs, thus creating a double-stranded (ds) miRNA structure. This ds-miRNA consists of two miRNA strands, a 5 prime (5p) arm starting from the 5' end and a 3 prime (3p) arm in the opposite direction. Traditionally, the strand that is produced more is considered as the guide strand and the other one is the so-called passenger strand. After the ds-miRNA is separated into two miRNA strands by a helicase (76), the passenger strand is degraded, and the functional guide strand is bound to Argonaute 2 protein (AGO2). Nowadays it has become evident that both the guide and the passenger strand can be functional, even at the same time (52), and it has been speculated that the strand selection is due the orientation of the ds-miRNA. This selection has been thought to favor the strand that has a more suitable 5' end when binding to AGO2 (73). One other explanation to account for strand selection is related to thermodynamics. Some studies have shown that the strand having a more unstable 5' end would be more likely to be selected as the functional, AGO2 incorporated miRNA strand (11,75).

The diversity of miRNAs is increased by miRNA isoforms (isomiRs). isomiRs originate from the same pre-miRNA strand but are different in their sequences in comparison to mature miRNAs. The differences tend to be modest, such as single nucleotide polymorphism or deletion or addition of one nucleotide in the 3' or 5' end of the mature miRNA. These changes in the mature sequence occur during the processing of mature miRNA, for example due to the shift of DICER or DROSHA cleaving sites (77).

The miRNA strand becomes functional when it is incorporated in the RNA induced silencing complex (RISC). AGO2 leads miRNA to RISC that consist of proteins like TRBP, and trinucleotide repeat containing adaptor 6A (TNRC6A) that assist in miRNA function. The miRNA leads the complex to the target (e.g., 3'untranslated region (3'UTR) of mRNA in the cytoplasm) by recognizing the sequence complementarity of the target. Usually, the miRNA seed is the main recognition site while the remaining miRNA sequence can have mismatches for the target (78). After target recognition,

the proteins associated with the RISC will ensure the silencing or activation of the target.

After miRNAs have served their purpose, they are degraded by different ribonucleases. In general, miRNAs are stable and long-lived. The exact mechanisms underpinning the regulation of their stability remain elusive, but studies have described that 3' end uridylation and low sequence specificity with their target mRNAs could account for their longevity. In addition, mature miRNAs are protected by the bound RISC proteins (79). Nonetheless, the lifespan of miRNAs differs significantly between cell types e.g., neuronal miRNAs have been estimated to have half-lives only between one to three and a half hours (80), whereas miRNAs in general can have half-lives lasting for multiple days (73).

2.3.2 Post-transcriptional gene silencing

Canonical miRNAs act in the cytoplasm in an event called PTGS to inhibit the function of their target gene. Most miRNAs target 3'UTR of mRNAs and inhibit their function either by decreasing the amount of mRNAs or totally preventing their translation. miRNAs find and bind their targets in a sequence-specific manner. As earlier described, the miRNA seed is the most critical sequence of mature miRNA that determines the miRNA's targets. In contrast to siRNAs which bind to their targets with perfect sequence complementary, miRNAs can bind their targets imperfectly. This means that one miRNA is capable of targeting many different targets. The more perfect is the target-binding of miRNA, the more likely the target mRNA is to be degraded, and not only inhibited (81,82).

miRNAs are the guides for RISC proteins to the specific targets. AGO2 is one of the key proteins participating in gene silencing since it is the only AGO (humans have AGO1-4) possessing an endonuclease activity (83). In addition to AGO2, DICER, TRBP and TRNC6A are common RISC proteins participating in PTGS (84).

In addition, miRNA PTGS in the cytoplasm can occur via the targeting of the 5'untranslated region (5'UTR) of mRNAs instead of the 3'UTR (85). Some investigators have also described miRNA targeting for the coding regions of

target mRNAs. For example, it was revealed that several miRNAs can target the same mRNA on both the coding regions and the 3'UTR at the same time (86). In addition to these properties, miRNAs have been linked to the alternative splicing of the pre-mRNA by targeting splicing factors leading to changes in the different isoforms of target mRNA (87). These observations highlight the extensive network exploited by miRNAs in the regulation of PTGS.

2.3.3 Nuclear transportation of microRNAs

As miRNA mediated PTGS takes place in the cytoplasm, the function of miRNAs in that location has been widely studied. However, recent studies have revealed that mature miRNAs are found also in the nuclear fraction of cells (88). In addition, RISC proteins like DICER (89) and AGO2 (90), have been detected in the nuclei, indicating also functional roles for miRNAs in the nucleus.

DICER can cleave pre-miRNAs into mature miRNAs not only in the cytoplasm but also in the nucleus. However, most of the factors that are needed for miRNA loading to AGOs, are found in the cytoplasm and not in the nucleus (84). In addition, Ohrt et al. demonstrated that the miRNA-AGO2 complex is initially loaded in the cytoplasm and only then transported to the nucleus (10). Indeed, there is convincing evidence that miRNAs are first matured in the cytoplasm and then transported into the nucleus. However, more studies are needed to unravel miRNA transportation between subcellular locations.

Importin 8 (IPO8) is a protein that is known to transport miRNAs from the cytoplasm to the nucleus through the nuclear pore complex (NPC) via a Ran-GTPase mechanism. IPO8 binds AGO2, which is linked to the mature miRNA, and the complex is subsequently transported to the nucleus (91). For miRNAs, the AGUGUU sequence at the 3' end and the uridylation of nucleotide 10 of mature miRNA have been suggested to act as a nuclear transportation signal. This was studied in human HeLa cells, where miR-29a was found to be cytoplasmic and miR-29b nuclear enriched (92). However, this was not observed in human colon cancer HCT116 cells (93).

In another study conducted in HeLa cells miR-29b and miR-29a were both found in the nucleus (94). This indicates that nuclear enrichment of miRNAs may be cell type specific or perhaps dependent on environmental conditions (72).

In addition to AGUGUU, other sequences have also been proposed to enhance the nuclear import of miRNAs. Nonetheless, there are studies that have not identified any specific sequence among nuclear transported miRNAs (11). In addition to the sequence, different isomiRs i.e., different miRNA lengths, have been proposed to determine the miRNA's localization (95). It has also been proposed that RISC binding proteins can determine the complex to be formed, and therefore the localization of the miRNA. For example, TNRC6A contains both nuclear import and export signals, and it can transport miRNAs into the nucleus (96).

2.3.4 Nuclear microRNAs

Nuclear miRNAs are mature miRNAs that are present and active in the cell nuclei. As earlier described, mature miRNAs are likely to be transported from the cytoplasm to the nucleus. Cytoplasmic miRNA function has gained more attention in the scientific community, but nuclear miRNA functions are now garnering attention, thus revealing more profound aspects on the functionality of these miRNAs.

Many recent studies have revealed the presence of mature miRNAs in the nucleus, and identified many miRNAs that are even more abundant in the nucleus than in the cytoplasm (84,88). In addition to mature miRNAs, RISC proteins have been found to be active in the nucleus. From the four human AGO proteins, only AGO2 and AGO1 are found in the nucleus (97). Nuclear RISC seems to be much smaller than its equivalent in the cytoplasm (10). This has been proposed to be due to the different composition between the two RISCs. AGO2 and TNRC6A are part of the RISC in both the nucleus and in the cytoplasm, while DICER and TRBP are associated with RISC only in the cytoplasm (82).

In the nucleus, miRNAs have been associated with many functions; they take part in the gene regulation by transcriptional gene silencing (TGS) or

by transcriptional gene activation (TGA). For example, while miRNAs have been described to activate or repress the ncRNA transcript on the promoter or induce epigenetic changes on gene promoters (11), they can also activate the expression of other miRNAs. This phenomenon was studied in mouse cells (L929, NIH-3T3 and 3T3-L1) where miR-709 inhibited pri-miR-15a/16-1 by direct binding in the nucleus, preventing its processing into a pre-miRNA (98). Indeed, while many interactions have been identified, the exact mechanism for each miRNA type has remained largely elusive.

The most intriguing hypothesis behind nuclear miRNA localization relies on the target abundance. This hypothesis suggests that miRNAs are located in those cell compartments where there are more of their targets. It is assumed that miRNAs can constantly shuttle between the cytoplasm and the nucleus but that they tend to remain in the compartment where their targets are more abundant (**Figure 3**) (78,99). Since changes in target abundance differ between different stress stimuli like hypoxia, this property also changes where miRNAs are localized in the cell.

miRNAs take part in cellular homeostasis since their transcription and maturation is rapid, so they represent a quick way to allow cells to react to a cellular stress (100). Nuclear miRNAs can rapidly respond to different conditions by directly affecting the gene transcription.

miRNA localization can also be determined by the transcript from which the miRNA is derived. Mitochondrial and tRNA transcript derived miRNAs are known to be located more in the cytoplasm, whereas snoRNA-derived miRNAs are more abundant in the nucleus (88). Nonetheless, the factors and mechanisms behind miRNA localization need to be clarified.

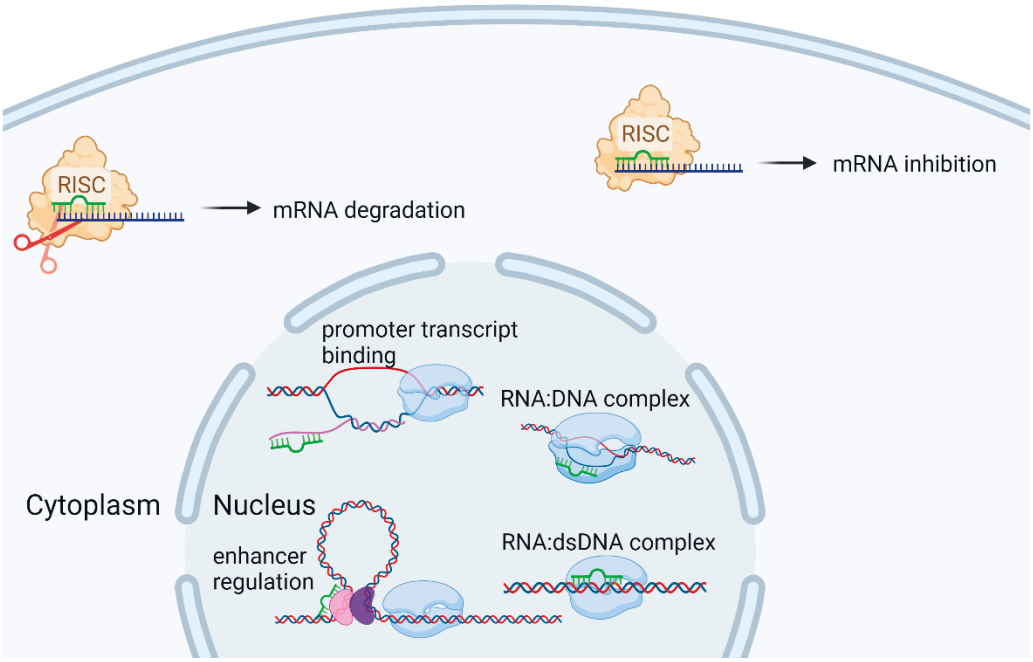

Figure 3. MicroRNAs regulate gene expression levels in cells. miRNAs regulate gene expression in the nucleus (TGS/TGA) by targeting the promoter transcript of a target gene or binding to the single-stranded or double-stranded DNA at the promoter region or they can act by binding to the enhancer. In the cytoplasm, miRNAs inhibit their target mRNA function either by degrading their target mRNAs or by inhibiting their translation. TGS= transcriptional gene silencing, TGA=transcriptional gene activation. Created with BioRender.com

2.3.5 Hypoxia regulated microRNAs

Hypoxia regulated miRNAs (hypoxamiRs) are miRNAs that are differentially expressed if there are hypoxic conditions. There are multiple networks of miRNAs participating in the regulation of hypoxia; for example they are linked to angiogenesis and proliferation, as well as to cellular apoptosis. Hypoxia regulation by miRNAs is a fast process, in fact miRNAs are one of the first agents to react not only to hypoxia, but also to other cellular stress conditions. The assay of hypoxia miRNAs can be then used as biomarkers of different conditions like CVDs (100).

The hypoxia-induced regulation of miRNAs can be either transcriptional or post-transcriptional. HIF1A is one of the key factors regulating hypoxamiR transcription in hypoxic conditions (38,101). For example, hypoxamiR miR-210 is transcriptionally activated by HIF1A (102). There are multiple studies showing different miR-210 target genes; in human umbilical vein endothelial cells (HUVEC), miR-210 has been associated with the angiogenesis induced via the *VEGF* pathway (103) and to enhanced endothelial cell migration by directly targeting and silencing the 3'UTR of Ephrin-A3 mRNA (104). It has also been shown that miR-210 can upregulate NOTCH1 protein levels and increase angiogenesis in HUVECs (105).

miRNAs can be either upregulated or downregulated under hypoxic conditions. For example, miR-133a has been demonstrated to be downregulated in MI tissue. Interestingly, it has been shown to be upregulated in the patient's serum after MI (106). It has been proposed that elevated miR-133a levels in MI tissue leads to better cardiac function. This was studied with MSCs where miR-133a increased the survival of bone marrow-derived rat cells cultured under hypoxia (107).

HypoxamiRs are crucial regulators of a hypoxia response. They have been observed to regulate the expression of HIF1A and several other genes participating in angiogenesis. For example, miR-424 is upregulated in response to hypoxia and it works as an inhibitor of Cullin 2 which leads to the destabilization of HIF1A. Interestingly, HIF1A induces the expression of many miRNAs, which in turn can regulate HIF1A expression by either a negative or positive feedback loop (101).

2.3.6 MicroRNA families in the *Sfmbt2* intron 10

Scm-like with 4 Mbt domains 2 (*Sfmbt2*) is a Polycomb group gene that is located on chromosome 2 in the mouse genome (108). Mouse *Sfmbt2* contains a large miRNA cluster in intron 10, which is interestingly not found in other mammals than in rodents (109). miRNAs are often expressed from clusters which are conserved between species. The miRNA clusters that are located within host genes are usually orientated in the same orientation as

their host gene so they are co-transcribed under the host gene promoter (52,110).

Sfmbt2 is a maternally imprinted gene which means that *Sfmbt2* is active from the allele inherited from the father. This imprinting takes place in the early mouse embryos, and it takes part in the placental development phase in the placental growth and function (108). miRNAs found in intron 10 of *Sfmbt2* are also important for development in mice, since deletion of this cluster led to placental problems and therefore impaired fetal development (109).

miRNAs are divided into miRNA families according to sequence similarities, especially those sharing the same seed sequence. Usually, these miRNAs originate from the same ancestor. Having the seed similarity means that they tend to have the same targets and therefore this often leads to shared functions of these miRNAs (73,92).

miRNAs that derive from intron 10 of *Sfmbt2* gene can be divided in the four families: miR-297, miR-466, miR-467 and miR-669 families (**Figure 4**) (111). This division is based on their sequence similarities. The miR-467 family members mainly have the seed AAGUGC, whereas the miR-297 family miRNAs exhibit the UGUAUG seed sequence. The miR-466 family members have shifted seeds UGUGUG, AUGUGU and GAUGUG; this family of miRNAs is considered to be a miRNA superfamily as these miRNAs have highly similar sequences extending beyond the seed sequence. In addition to these miRNAs, the miR-669 family has variable seeds among its miRNAs. It has been proposed that miR-297 and miR-467 are the newest families in the cluster, originating from the miR-466 family which has arisen from the miR-669 family (112).

These miRNA families have been investigated in a variety of ways; for example, *in silico* analysis of miR-466 and miR-467 showed 1704 and 956 potential target sites, respectively, on mouse integument genes, suggesting that they may play a role in hair development (113). In conditions of glucose deprivation, *Sfmbt2* gene expression is upregulated, together with the expressions of miR-466h-5p and miR-669c (114). In arsenic treated cells, the miR-466-669 cluster was upregulated. In this study as well, *Sfmbt2*

levels were shown to be elevated, suggesting that miRNAs and their host genes are transcribed simultaneously (115).

Neurogenic transcription factors are potential targets for miRNAs from these families. Indeed, the miR-466/669 family miRNAs were shown to inhibit neurogenic transcription factor neuronal differentiation 1 (*NeuroD1*) expression (115). Furthermore, the amounts of miR-669c-3p, a member of the miR-669 family, have been shown to be increased in mice subjected to an ischemic stroke. The overexpression of miR-669c-3p decreased the brain injury by targeting the myeloid differentiation primary response gene 88 (*MyD88*) mRNA and decreasing its expression (116).

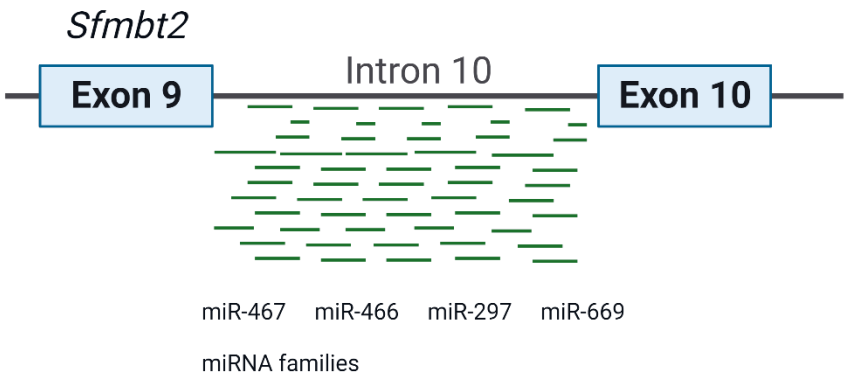

Figure 4. Intron 10 of *Sfmbt2* gene in mouse chromosome 2. There is a large miRNA cluster in the intron 10 of *Sfmbt2* gene. These miRNAs can be divided into miRNA families: miR-467, miR-466, miR-297 and miR-669. Created with BioRender.com.

2.4 TRANSCRIPTIONAL ACTIVATION BY SMALL RNAS

The discovery of this novel way to transcriptionally regulate genes has revolutionized the whole field of molecular biology by offering a new way of modifying cellular functions. TGS has been long known and described previously in plants (117). Transcriptional silencing of the genes in human cells was first described by Morris et al. in 2004. In that study, siRNAs were used to target the elongation factor 1 α (*EF1A*) promoter transcript which led to a silencing of *EF1A* transcription and therefore its function was

terminated (118). RNA activation (RNAa) is a phenomenon where small activating RNAs (saRNAs) mediate gene upregulation at the transcriptional level. RNAa was first described in a publication by Long-Cheng Li, showing that dsRNAs were targeted to gene promoters, activating their expression by inducing epigenetic changes on chromatin (13). Subsequently, different types of saRNA-mediated target activation have been described.

In addition to RNA activation, it has been shown that miRNAs can upregulate their target mRNAs in a post-transcriptional manner. For example, hsa-miR-369-3 was shown to be required for the activation of tumour necrosis factor–α (*TNFA*) expression in HEK293 cells. This study was done in serum-starved conditions when cells were in non-proliferative stage i.e., in cell cycle arrest. It was demonstrated that hsa-miR-369-3 recruited different proteins, such as AGO2 and fragile X mental retardation–related protein 1 (FXR1), to the AU-rich elements in the 3'UTR of the *TNFA* mRNA which activated the translation of TNFA (68).

miRNA mediated activation is not restricted to non-proliferative cells, it has also been observed in dividing HEK293T cells. It was stated that most miRNAs targeting ST6-β-galactoside-α-2,6-sialyl-tranferase-1 and -2 (ST6GAL1and ST6GAL2) enzymes upregulate their expression by direct binding to their 3'UTRs. AGO2 and FXR1 were required also in this experiment for the miRNA-mediated 3'UTR activation (119).

miRNAs act in different ways to upregulate genes. In addition to the studies that have demonstrated lncRNA competitive binding where miRNAs are captured and prevented from binding to their mRNA targets, miRNAs can also directly activate target gene expression. Examples of different mechanisms how miRNAs and other saRNAs may directly activate their target transcription are described in the following sections.

2.4.1 Promoter targeting

Gene promoters are the regions of the genome that serve as transcriptional regulatory units for the genes. Transcription of the gene starts when the gene promoter is activated. The realization that there are potential miRNA target sites on gene promoter sequences, as there are in

3'UTRs in mRNAs in the cytoplasm, served as a starting point for experiments which revealed the nuclear gene regulatory functions for miRNAs. Already a decade ago, Younger et al. postulated that there would be 800 000 miRNA seed matches in 27 345 gene promoters (120).

In *Drosophila melanogaster*, Ago2 and Dicer2 are required to evoke the functional response of heat shock proteins (Hsp) Hsp23 and Hsp70 after a heat-shock. Importantly, it was shown that Ago2-bound miRNAs were enriched on the promoters of these genes after a heat-shock, leading to the conclusion that there can be miRNA-mediated regulation of these genes (121).

One of the earliest studies showing that miRNA could target promoter sequence of genes was when miR-373 was found to target E-cadherin and cold-shock domain-containing protein C2 gene promoters in human PC-3 cells. miR-373 was shown to induce transcription of both genes, which was seen as an elevated level of Pol II at the TSS of these promoters (122).

RNAa has been witnessed between paRNAs and sncRNAs; it has been demonstrated in human breast cancer cells that artificial small double-stranded RNAs (dsRNAs) can directly target the antisense transcript on the promoter of the progesterone receptor (*PR*) and activate or inhibit the *PR* gene expression (123). In a recent report, this paRNA activation has been revealed to act in a miRNA-mediated manner. In that study, zinc finger MYND-type containing 10 (*ZMYND10*) expression was induced by miR-34a by targeting the lncRNA on the *ZMYND10* promoter. This has been found to occur in many human cell lines, for example in airway epithelial cells (124).

As another example of the properties of RNAa, saRNA was shown to target the *PR* promoter. In that experiment, five out of 20 different promoter-targeted dsRNAs were able to activate *PR* mRNA expression. Interestingly, these investigators postulated that in addition to promoter transcript targeting, the same saRNA could also bind directly to genomic single-stranded DNA on the *PR* promoter (125). The computational analysis conducted by Paugh et al. suggested that miRNA could directly bind to double-stranded DNA (dsDNA) with the formation of a triplex (126). However, miRNA let-7i was shown to upregulate interleukin 2 transcription by direct binding to its promoter TATA-box both in human and mouse T-

lymphocytes. It was discussed by the authors that miRNA may be able to bind to the open, single-stranded TATA-box instead of dsDNA (72).

Potential mechanisms underpinning the promoter targeting described in the above studies are illustrated in **Figure 3**. One mechanism was due to targeting of the promoter ncRNA transcript, another possibility was that saRNA targets unwound, single-stranded DNA, and finally, there may have been direct binding of saRNA into a dsDNA. Often the exact mechanisms of actions have not been described or studied further. More detailed studies on saRNA regulation are needed to reveal the exact mechanisms of saRNA functions and to increase our understanding of RNAa.

2.4.2 Epigenetic alterations

Promoter targeted saRNAs have often been associated with epigenetic changes on the gene promoter. Epigenetic modulation refers to heritable modifications on chromatin that do not alter the base composition of dsDNA. Epigenetic alterations regulate which genes are, and which genes are not, actively transcribed. This is achieved by different modifications on specific proteins called histones which are located on the chromatin, or with direct DNA modifications. Histones can pack the DNA into a tighter chromatin structure; it is the modification on the histone tail parts that determines the openness of the chromatin. Many epigenetic modifications have been described, often containing acetylation or methylation of histone tails or direct methylation of genomic DNA. These modifications are either activating or silencing and their combination determines the overall effect on gene expression (**Figure 5**) (127).

The first study of RNAa already presented data that dsRNAs could induce changes in the epigenetic markers on the chromatin as it noted that artificial dsRNAs with total sequence complementary induced E-cadherin, *p21* and *VEGF* expression in human HeLa cells. dsRNAs decreased the amount of histone dimethylation of lysine 9 of histone 3 (H3K9me2) in E-cadherin promoter leading to an upregulation of the gene (13).

Janowski et al. demonstrated that dsRNA targeted the *PR* promoter, inducing the transcription of both isoforms of PR protein. This treatment

increased tri- and di-methylation of lysine 4 of histone 3 (H3K4me3, H3K4me2) on the *PR* promoter in human MCF7 cells (128). Mouse miRNAs miR-744 and miR-1186 have been shown to target the cyclin B1 (*Ccnb1*) promoter in mouse NIH/3T3 cells and thus upregulate *Ccnb1* expression. This was associated with increased Pol II levels and an open chromatin H3K4me3 mark on the TSS on the *Ccnb1* promoter (129).

Furthermore, *Vegfa* promoter has been shown to be targeted by artificial shRNAs. The upregulation with shRNA was studied both *in vitro* and *in vivo* in the mouse hindlimb ischemia model. In this experiment, shRNA-451, which targeted the *Vegfa* promoter region, induced the presence of open histone marks of H3K4me2 and H3K4me3 and decreased the amount of the silencing histone marker H3K9me2 on the TSS and on the targeted region of the *Vegfa* promoter. This promoter activation via epigenetics led to an upregulation of *Vegfa*. Interestingly, shRNA-856 targeting the *Vegfa* promoter at a different locus was found to silence *Vegfa* expression by decreasing open histone markers H3K4me2 and acetylation of lysine 9 of histone 3 (H3K9ac) and increasing silencing histone markers H3K9me2 and trimethylation of lysine 27 of histone 3 (H3K27me3). Therefore, it seems that *Vegfa* can be regulated with either TGA or TGS, depending on the location to where the shRNA is targeted (130).

Vegfa upregulation was also observed in the mouse MI model with the activating shRNA. shRNA mediated upregulation of *Vegfa* evoked a significant therapeutic effect in this animal model, as observed by a decreased infarct size in the mice after MI (32). As described by Janowski et al. with RNAa for the *PR* receptor (128), all *Vegfa* isoforms were also upregulated in the endothelial C166 cells.

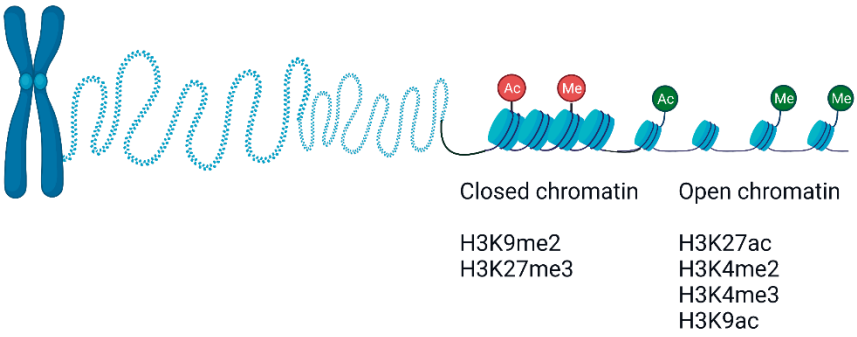

Figure 5. Epigenetics. Epigenetic modifications of the cells are heritable changes that do not include changes in the DNA nucleotide composition. Epigenetic changes include DNA methylation as well as the acetylation and methylation of the histones attached to the chromatin. Examples of the histone modifications are shown that lead to the active i.e., open chromatin or inactive forms i.e., closed chromatin. H3=histone 3, K=lysine, me=methylation, ac=acetylation. Created with BioRender.com.

2.5 THERAPEUTICS FOR ISCHEMIC DISEASES

2.5.1 Delivery methods for gene therapy

One of the most crucial aspects of gene therapy is how to determine the best delivery method of the therapeutic gene since this influences the success rate of the gene therapy. Therefore, this step needs to be thoroughly evaluated and optimized carefully. There are different delivery approaches that can be roughly divided into viral and non-viral delivery methods.

Viral delivery methods include viral vectors like lentiviruses (LVs), adenoviruses (Ads) and adeno-associated viruses (AAVs) that are used to transport the beneficial gene into the tissue. Viral vectors can be further subdivided into transient and permanent vectors, depending on the time that the introduced gene will be expressed. LVs integrate into the host genome, and thus they provide a permanent expression of the introduced gene. This gene is constantly expressed unless the promoter of the gene is epigenetically modified to be closed. Ads and AAVs do not integrate into

the host genome so they infect the cells without affecting the contents of the genome. Ad and AAV -mediated gene expression is therefore transient, and the gene expression achieved is diluted when cells divide (131).

There are also a variety of different non-viral delivery vectors, such as polymers, lipid nanoparticles (LNPs) and inorganic nanoparticles. These can be either natural or synthetic in their nature. Polymers are positively charged substances that are commonly used to transfect cells since they bind to negatively charged DNA and RNA and can transport these acidic compounds into the cells. LNPs are also positively charged particles which consist of a lipid surface that can bind nucleic acids. Inorganic nanoparticles are inorganic i.e., they are not composed of a carbon chain like organic materials but can be formulated from other materials for example from gold or silica particles (132).

As already mentioned in Chapter 2.1.4., EVs are small particles that cells naturally secrete to communicate with each other. They are found in every fluid in the body. The structure of EVs is a lipid layered vesicle that contains proteins, RNAs, lipids and other cellular molecules. As EVs function as carriers of the messengers between cells, the release of EVs by cells changes in response to different stress stimulus like in hypoxia (133). Not only the amount but also the content of the EVs can be altered in hypoxic conditions (134). EVs offer a great potential for gene therapy since as natural particles they evoke a low immunogenic reaction when administered into the body. In addition, the size of the EVs favor their therapeutic potential. It has been described that the nanoparticles with a size close to 100 nanometres (nm) would be optimal for delivering products via the blood circulation, since particles over 200 nm are cleared by liver and spleen while those under 5 nm particles are filtered in the kidneys (132). For example, it has been speculated that exosomes as one subclass of EVs with a size between 30-150 nm (135) would offer a great potential for use as delivery vehicles. Importantly, modification of EV content is possible with different loading methods and these modified EVs can be manufactured as therapeutic agents. The importance of the EVs has recently been recognized and more and more studies using EVs as non-viral therapeutic delivery vehicles have appeared (4,136).

Non-viral vectors are tempting tools for use in gene therapy since in general they are less immunogenic than viral vectors. However, their efficacy on gene transport can vary extensively and changing their properties is often harder than is the case with viral vectors. Nonetheless, it is important to note that the experiments with viral vectors have been on-going already for decades while the potential of non-viral vectors has only lately been acknowledged (132).

There is not one golden standard for gene delivery since it depends on the target tissue, gene of interest and other conditions, including whether the desired therapy should be transient or permanent. The positive side of permanent expression e.g., with a lentiviral vector, is that the gene needs to be introduced only once. However, as the gene can be integrated into multiple sites on the genome, this can lead to problems in normal cellular functions (137). One consideration with non-permanent delivery of gene therapy is whether the dosing will need to be administered multiple times to achieve the desired therapeutic effect. Already there are many good and functional gene delivery tools available. Nonetheless, there is a need for an optimization of the current approaches as well as a search for new and better tools to ensure a more efficient, safer and a more practical way to deliver these therapies.

2.5.2 Therapies on the market

Since *VEGFA* expression regulates the whole vasculature, *VEGFA* is an important study subject also with respect to therapies intended for ischemic diseases. As VEGFA induces angiogenesis, most of the research that has proceeded to clinical trials has been aimed at inhibiting *VEGFA* expression but there are also some studies which have examined the therapeutic properties of *VEGFA* activators.

Inhibition of angiogenesis is considered to be beneficial in the treatment of cancers and some other diseases. Many anti-VEGFA therapies have been successfully trialled and today there are commercially available drug therapies. For example, Macugen (previously known as Pegaptanib) is an aptamer that is used to treat wet age-related macular degeneration (AMD).

Macugen binds and blocks the $VEGF_{165}$ isoform, which is the isoform most responsible for AMD development, and inhibits the spreading of the disease (138). Today, there are different VEGFA antibody treatment on the market. For example, Lucentis and Eylea are used as VEGFA inhibitors in the treatment of AMD as they are capable of binding to all the isoforms of VEGFA (139,140). In addition to treating AMD, Avastin is an antibody binding to all VEGFA isoforms and it is being used to treat different cancers (141).

Drugs that affect the alternative splicing of the pre-mRNAs have also been developed. For example, Borreliding 1 is a chemical that is used in RPE cells to affect alternative splicing of the *VEGFA* pre-mRNA. This chemical triggers a specific upregulation of the VEGFA isoforms that are less angiogenic (142).

In addition to drugs that inhibit *VEGFA* expression, there have been clinical trials aiming to treat diseases like ischemic diseases by inducing VEGFA and therefore evoking angiogenesis in ischemic tissues. However, only one of these clinical trials has received a marketing licence. In 2011, Neovasculgen was approved for the treatment of PAD in Russia. In that trial, a DNA plasmid coding $VEGF_{165}$ was administered intramuscularly into the calf muscles of patients suffering for chronic lower limb ischemia. Administration was repeated after two weeks, and neovascularisation was witnessed after 6 months after the first treatment (143). Another similar approach was involved in the drug being tested by Moderna and AstraZeneca, which was trialled in a clinical Phase 2a study for the treatment of CAD with $VEGF_{165}$ being administered to the heart after bypass surgery to trigger beneficial angiogenic effects (144). However, this trial was terminated by AstraZeneca in 2022 (145).

Even though the clinical trials have focused on the regulation of *VEGFA*, there are other new studies and possibly also novel drugs emerging in the field of RNA. This is evident from the clinical trials website (146) where on-going clinical trials are registered. Searching with terms “ischemic stroke” and “RNA”, yielded 37 trials at different phases (16 of those as recruiting; search on 24.2.2023). For “ischemic heart disease” and “RNA”, 116 hits were found (25 of those as recruiting; search on 24.2.2023).

2.5.3 RNA therapeutics

As RNAa has emerged as a new mechanism for the gene regulation, it has opened the door for novel medicines. However, since the RNA network is complex with multiple different types of RNAs regulating not only gene expression but also each other, the task of developing new RNA therapies is far from straightforward.

Nowadays, there are RNA drugs that utilize RNAi as an approach to treat diseases. These are small RNAs that regulate gene expression, such as antisense oligos (ASOs). ASOs act to inhibit the target gene with variety of mechanisms. The first oligonucleotide based ASO to proceed to being marketed was Fomivirsen; this took place already in 1998. Fomivirsen blocks the viral mRNA in patients suffering from cytomegalovirus retinitis. Since then, at least seven other ASOs have obtained an FDA licence (147).

In addition to ASOs, there are three siRNA medications (patisiran, givosiran and lumasiran) approved for the US market. The first siRNA drug, patisiran, was approved in 2018 to treat peripheral nerve disease (polyneuropathy). In the following years, givosiran was approved to treat acute hepatic porphyria and lumasiran for treating the primary hyperoxaluria type 1. Patisiran and lumosiran work by inhibiting their target mRNAs by binding the 3'UTR of those mRNAs, while interestingly, givosiran's silencing effect is due to its binding to the coding sequence of its target mRNA (147).

At present, only one RNAa medication has entered into clinical trials. MiNa Therapeutics has developed a saRNA for the treatment of hepatocellular carcinoma by the activation of CCAAT/enhancer-binding protein α (*C/EBP-A*) transcription. C/EBP-A is known to be a key regulator in liver homeostasis, angiogenesis and cell proliferation and its production has been found to be deregulated in many tumours. This saRNA medicine is an artificial, totally complementary sncRNA designed to target the *C/EBP-A* promoter and is delivered with LNPs (148). In addition to the therapy of hepatocellular carcinoma, MiNa Therapeutics is developing treatments for other diseases, for example a combination treatment of C/EBP-A with other drugs (149).

miRNAs have been explored especially for the use as disease biomarkers since they can be easily measured from the blood samples (150). However, they may have a more important potential as gene regulators, a property that has not yet been exploited to its full extent. There are some clinical trials already underway, with anti-miRNA drugs. Miravirsen is a locked nucleic acid (LNA) molecule targeting miR-122 used in the treatment of hepatitis C virus (151). In addition to anti-miRNAs, miRNA mimics are being developed. For example, targeted bacterial minicells delivering miRNA mimics (TargomiRs) are in Phase I for the treatment of thoracic cancer patients (152). Cardior Pharmaceuticals has also developed miRNA treatments for MI patients. They are also investigating ASOs to target and silence miR-132-3p expression, leading to the recovery of the heart after MI. This trial is in Phase II (153).

sncRNAs have already entered the clinics and there are a great number of candidates awaiting testing in future clinical trials. As we gain more experience in using siRNAs, ASOs and artificial saRNAs, we will also obtain new insights into future miRNA therapies.

3 AIMS OF THE STUDY

miRNAs have been a focus of wide interest and research for decades. Nonetheless, most of the studied functions have concentrated on the mechanisms for the PTGS in the cytoplasm. In addition to the cytoplasmic role of miRNAs, it has now become evident that these cytoplasmic functions alone do not encompass all of the miRNAs' mechanisms. Recent studies have described some nuclear functions of the miRNAs, although the exact mechanisms have remained unclear. The aim of this thesis is to widen our knowledge of the nuclear miRNAs and their actions in that location. Specifically, the aims of this thesis were:

I To characterize the differences in miRNA populations between the cytoplasm and the nucleus and to investigate if the localization is affected by hypoxia

II To identify *Vegfa* promoter targeting miRNAs and to examine their functions

III To evaluate the therapeutic potential of nuclear miRNA miR-466c using the mouse hindlimb ischemia model

4 SUBJECTS AND METHODS

4.1 SUBJECTS

4.1.1 Cell lines

Cell lines used in this thesis are described below (**Table 3**).

Table 3. Cell lines.

Cell line	Description	Source	Used in
C166	Mouse endothelial cell line, yolk-sac derived	ATCC: CRL-2581	I, II
C2C12	Mouse myoblast cell line, muscle derived	ATCC: CRL-1772	I
MS1	Mouse endothelial cell line, pancreas/islet of Langerhans derived	ATCC: CRL-2279	I
MOVAS	Mouse spindle-shaped smooth muscle cell line, heart/aorta derived	ATCC: CRL-2797	I
HEK293T	Human endothelial cell line, embryonic kidney derived	ATCC: CRL-11268	II, III
466-del	miR-466c-1 deleted from genome of C166 cell line	Modified from ATCC CRL-2581 with CRISPR	II

4.1.2 CRISPR oligos

CRISPR oligos used in this thesis are described below (**Table 4**).

Table 4. CRISPR oligos.

Gene		Oligo	Source	Used in
466crispr_del, guide RNA 1	Forward	GATCCGCATATACACATACATAG	Integrated DNA Technologies	II
	Reverse	AAACCTATGTATGTGTATATGCG	Integrated DNA Technologies	II
466crispr_del, guide RNA 2	Forward	GATCCGTGAGCACACAGACACAG	Integrated DNA Technologies	II
	Reverse	AAACCTGTGTCTGTGTGCTCACG	Integrated DNA Technologies	II
466crispr_del, PCR primers	Forward	TCAGGAGTGCAAGTTCATGGT	Integrated DNA Technologies	II
	Reverse	GGATTGATGAGTGCCATTCCC	Integrated DNA Technologies	II

4.1.3 Taqman Assays

Taqman microRNA and Taqman Gene Expression assays used in this thesis are described below (**Table 5**).

Table 5. Taqman assays.

Gene	Taqman Assay	Source	Used in
hsa-miR-27a-5p	ID: 002445	Thermo Fisher Scientific	I, II
mmu-miR-3535	ID: CTEPR23	Thermo Fisher Scientific	I, II
mmu-miR-210-3p	ID: 000512	Thermo Fisher Scientific	I
mmu-miR-466c-3p	ID: 464896_mat	Thermo Fisher Scientific	II, III
mmu-miR-466c-5p	ID: 463771_mat	Thermo Fisher Scientific	II, III
Vegfa	ID: Mm00437306_m1	Thermo Fisher Scientific	II, III
Sfmbt2	ID: Mm00616783_m1	Thermo Fisher Scientific	II
Gapdh	ID: Mm99999915_g1	Thermo Fisher Scientific	II, III
Actb	ID: Mm00607939_s1	Thermo Fisher Scientific	II

4.1.4 Primer and probe sequences

Primers and probes used in this thesis are described below (**Table 6**).

Table 6. Primers and probes.

Gene	Type	Primer Sequence/ Probe Sequence (5'→ 3')	Source	Used in
Malat1	Forward	GCTGGTAACCGCTGCTATAA	Thermo Scientific	I
	Reverse	CAGAGAATCCAGACCCAGTAAG	Thermo Scientific	I
Neat1	Forward	CGCTACTGACCACAGACTTTAC	Thermo Scientific	I
	Reverse	GTTGGATTGGGTCTCCTTCTAC	Thermo Scientific	I
tRNA-Lys-TTT-1-3	Forward	GCCCGGATAGCTCAGTCG	Thermo Scientific	I
	Reverse	CGCCCGAACAGGGACTTG	Thermo Scientific	I
tRNA-Met-CAT-1-2	Forward	GCCTCGTTAGCGCAGTAG	Thermo Scientific	I
	Reverse	TGCCCCGTGTGAGGATCG	Thermo Scientific	I
Vegf-a pre-mRNA	Forward	AGGAGAGATGAGCTTCCTACAG	Integrated DNA Technologies	II
	Reverse	GGACCCACACTAACACATGTAAC	Integrated DNA Technologies	II
	Probe	56-FAM/TCTGGCCTC/ZEN/ACCTGCATTCACATC/3IABkFQ	Integrated DNA Technologies	II

mVegf-a promoter -485 to -317 from TSS	Forward	CGTAACTTGGGCGAGCCG	Integrated DNA Technologies	II
	Reverse	GGTTGGAAGGCGGAGAGC	Integrated DNA Technologies	II
	Probe	56-FAM/GAGGGAGGACGCGTGTTTCAATGTGAGTG/3BHQ-1	Integrated DNA Technologies	II
mVegf-a promoter -675 to -477 from TSS	Forward	GCTTCCGAGGTCAAACAC	Integrated DNA Technologies	II
	Reverse	CAAGTTACGATCTCCCCG	Integrated DNA Technologies	II
	Probe	CGCAATTATTTGGGAGCTCAAAGTCTGCCG	Integrated DNA Technologies	II
mVegf-a promoter -910 to -717 from TSS	Forward	GTTTCCACAGGTCGTCTC	Integrated DNA Technologies	II
	Reverse	GGGGAGTATGCTTATCTG	Integrated DNA Technologies	II
	Probe	56-FAM/ACTTCCCAGAGGATCCCATTCACCCCAG/3BHQ-1	Integrated DNA Technologies	II

4.2 METHODS *IN VITRO*

In vitro methods used in this thesis are briefly introduced in the following table (**Table 7**). A more detailed explanation of methodology is found in the publications and manuscript attached to this thesis.

Table 7. *In vitro* methods.

Method	Purpose	Used in
Cell culture	To culture cells for the experiments	I, II, III
Target selection	Analysis and visualization of miRNA target sites	II
Target prediction	Promoter targets of studied miRNAs are selected	II
Plasmid cloning	Cloning specific sequences in plasmids for lentiviral production	II
Lentiviral transductions	To create a cell line that overexpresses cloned products	II, III
Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)	To create a cell line from which miR-466c-1 is removed	II
EV collection, fractionation, and concentration (nanoparticle tracking analysis (NTA))	To produce EVs for PAD animal experiment	III
Nuclear/cytoplasmic fractionation	To produce nuclear and cytoplasmic fractions of the cells	I, II

RNA extraction	To extract RNA from the cells	I, II, III
RNA sequencing (small)	To quantify small RNAs from the sample	I
RNA sequencing (total transcriptome)	To quantify long RNAs from the sample	II
Global Run-On-sequencing (GRO-seq)	To quantify active transcription sites on the genome	II
complementary DNA (cDNA) synthesis	To reverse transcribe RNA into complementary DNA	I, II, III
Reverse Transcription quantitative real-time Polymerase Chain Reaction (RT-qPCR)	To quantify cDNA of the sample	I, II, III
Biotin-labeled miRNA pulldown	To quantify specific targets of miRNAs	II
miRNA Fluorescence in situ hybridization (FISH)	To qualify miRNA location in the cells	I, II
Western blot	To quantify specific protein of the cells	I, II
Enzyme-Linked Immunosorbent Assay (ELISA)	To quantify specific protein of the cells	II
Luciferase assay	To elucidate miRNA binding to its targets	II

4.3 METHODS *IN VIVO*

In vivo methods were used in Manuscript III in this thesis and they are briefly introduced in the following sections. A more detailed explanation of methodology is found in the manuscript attached to this thesis.

4.3.1 Experimental setup for PAD

Ten week old BALB/c AnNCrl were used in this study. Twelve mice were utilized in each of three treatment groups i.e., mice treated with EV-466, LV-466 and vehicle (PBS). The left femoral artery of the mice was ligated in two sites and treatments were administered by a single intra-muscular (i.m.) injection. The day of surgery and when treatment was initiated is called day 0 (D0). Functional ultrasound (fUS) imaging was done on D9 and the mice were sacrificed and hindlimbs were collected on D10. In addition to fUS, the weight and limb function of the animals were monitored.

4.3.2 Tarlov and modified ischemia scoring

The wellbeing and limb functions of the mice were visually scored one to three times a day between D0-D9. The average of each mouse was calculated and scores represented by the groups. Tarlov and modified ischemia scores were visually evaluated between 0-7 (i.e., no movement – full and fast walking) and between 0-7 (i.e., auto-amputation of the limb – no necrosis).

4.3.3 Functional ultrasound imaging

Functional ultrasound imaging (fUS) was used to image the blood vessels of mice limbs on D9. Whole legs of the treated (left) and non-treated (right) legs of the studied mice were imaged with both 2D and 3D. Signal intensity levels (i.e., the higher the signal, the more a vascular system was present) were evaluated.

5 RESULTS

5.1 MATURE MICRORNAS ARE FOUND IN THE NUCLEI OF THE CELLS

5.1.1 Nuclear and cytoplasmic enriched microRNA populations

As described previously in Chapter 2.3.4, mature miRNAs have been found in the nuclei of cells. However, their expression and localization seem to be very cell type specific (38,72). As endothelial cells are known to respond to hypoxia (47), we wanted to examine in detail how miRNAs would react to hypoxia in different subcellular compartments of these cells. Therefore, we performed a nuclear-cytoplasmic fractionation for mouse endothelial C166 cells treated with normoxia and hypoxia. Fraction purity was confirmed using western blot for specific nuclear and cytoplasmic proteins (**Publication I, figure 1a**) as well as using reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) for known lncRNAs and tRNAs enriched in the nucleus or in the cytoplasm, respectively (**Publication I, figure 1b**). This validated the chosen method for producing pure nuclear and cytoplasmic fractions of C166 cells.

After fractionation, small RNA sequencing was performed to identify the mature miRNAs present in these cells. We detected miRNAs in both fractions (91 out of total 196). In addition, we identified specifically nuclear enriched miRNAs (46 out of 196) and cytoplasmic enriched miRNAs (59 out of 196) (**Publication I, figure 2a-d**). The most nuclear enriched miRNA was found to be miR-3535, with an over 27-fold change in expression between the fractions. miR-27a-5p was cytoplasmic enriched with a 48-fold change difference between cytoplasm and nucleus. In order to validate the sequencing data with a complementary method, we checked the levels of these miRNAs also with RT-qPCR. The nuclear or cytoplasmic enrichment of these miRNAs was also observed with RT-qPCR, confirming that these miRNAs were indeed compartment-specific in their expression (**Publication I, figure 2e-f**). Importantly, the levels of these miRNAs were

analysed also in other mouse cell lines. miR-3535 was shown to be nuclear enriched and miR-27a-5p cytoplasmic enriched also in mouse muscle derived myoblast cells (C2C12), pancreas-derived endothelial cells (MS1), and aorta-derived smooth muscle cells (MOVAS), indicating that at least these miRNAs were not endothelial cell specific in their localization (**Publication I, supplementary figure S4**).

Interestingly, we also identified some well-known miRNAs that have been widely studied previously in a cytoplasmic PTGS context to be more enriched in the nuclei of the cells. This occurred for example with the known hypoxamiR, miR-210. This sequencing result was also confirmed with RT-qPCR (**Publication I, figure 3e-f**). We observed that miR-210-3p was nuclear enriched, and further confirmed its localization with confocal microscopy (**Publication I, figure 4**).

5.1.2 MicroRNAs are differentially expressed in the nucleus and in the cytoplasm upon hypoxia

Next we aimed to see if miRNAs in the different subcellular fractions would be differentially expressed upon hypoxia. As expected, there were nuclear and cytoplasmic miRNA populations in the C166 cells that changed their expression in the presence of hypoxia (**Publication I, figure 3a**).

When looking into the sequencing data, there were 48 miRNAs in nuclear fraction and 29 miRNAs in cytoplasmic fraction that significantly changed their expression when comparing 24 h hypoxia to normoxia. Most of these miRNAs were downregulated upon hypoxia (59 out of 77) (**Publication I, figure 3c-d**).

Again, we used miR-210 for validation and applied RT-qPCR to analyse the expression changes occurring in response to hypoxia. Indeed, we observed that this known hypoxamiR was significantly upregulated upon hypoxia and its expression increased in both fractions. However, the increase was more distinct in the nuclear fraction (**Publication I, figure 3e-f**).

Interestingly, most of the miRNAs altering their expression due to hypoxia changed only in one cellular compartment. Only six miRNAs were

found to significantly change their expression due to hypoxia in both cellular compartments. Two of these miRNAs were upregulated (miR-210-3p, miR-669c-5p) in both fractions, and four were decreased (let-7a-5p, miR-200c-3p, miR-193a-5p, miR-203-3p) (**Publication I, figure 3b**).

In addition to known miRNAs, small RNA sequencing resulted in the identification of new, not previously annotated small RNA sequences. These putative miRNA sequences were located in either nuclear or cytoplasmic fraction or found in both fractions irrespective of the conditions (**Figure 6**). Interestingly, the size distribution of putative small RNAs was between 19-23, indicating that these sequences could be mature miRNAs (**Figure 7**).

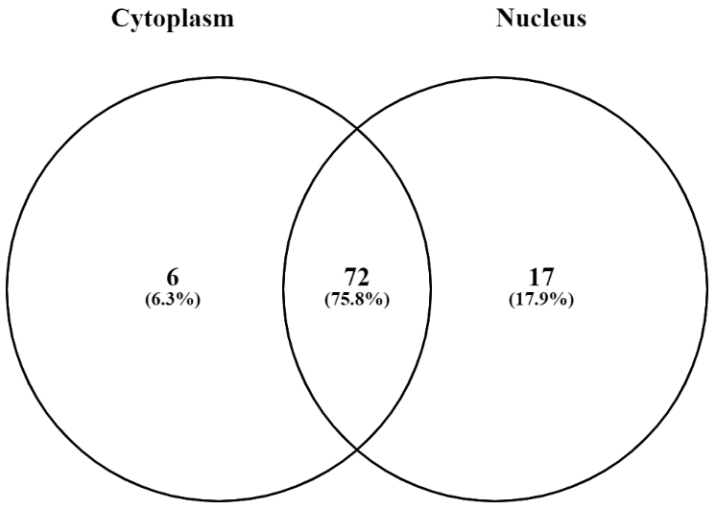

Figure 6. Putative small RNA sequences in the cytoplasm and/or in the nucleus of the mouse C166 endothelial cells. Putative sequence data from normoxia and hypoxia was combined and then analysed for nuclear and cytoplasmic presence. Under 5 copies/small RNA sequence was filtered out. Created with Venny 2.1 (154).

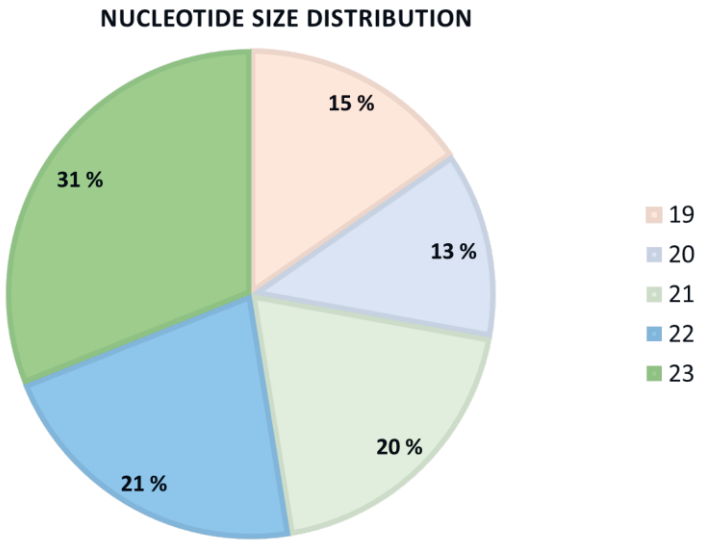

Figure 7. Size distributions of the putative sequences. Percentages of total number of sequences are presented in the diagram for each length. The numbers of putative sequences were: 19 nt= 87, 20 nt= 71, 21 nt= 111, 22 nt= 122, 23 nt= 176. Created with PowerPoint.

5.2 MIR-466C REGULATES *VEGFA* EXPRESSION UPON HYPOXIA

5.2.1 miR-466c is predicted to target mouse *Vegfa* promoter and is able to bind promoter-associated lncRNA

To further study nuclear miRNAs in the endothelial C166 cells, a target prediction was done for the *Vegfa* promoter as that gene has been previously shown to be regulated by an artificial promoter targeting shRNAs (32,130) and it is a crucial factor in angiogenesis as described earlier. Potential target sites for miRNAs on *Vegfa* promoter were predicted using the RegRNA 2.0 target prediction tool (155). Indeed, the *Vegfa* promoter was observed to have potential target sites for both of the mature arms of mmu-miR-466c (**Publication II, figure 1a-b**).

As many previous studies have shown that the promoter targeting occurs via binding to the promoter-associated lncRNA, we next analysed whether paRNA would be expressed at the *Vegfa* promoter and could serve as binding site for miR-466c. We performed Global Run-On-sequencing

(GRO-seq) for the C166 cells. GRO-seq revealed the locations where DNA was actively transcribed, by identifying active Pol II binding sites. Indeed, we observed that in C166 cells, there was an antisense transcript on the *Vegfa* promoter (**Publication II, figure 4a**). To validate this sequencing result, we performed strand-specific RT-qPCR for *Vegfa* promoter transcript by using specific promoter targeted primer sets and confirmed that there was a paRNA on the *Vegfa* promoter on the loci where miR-466c arms were predicted to be targeted (**Publication II, figure 4b**).

Lentivirus (LV) is a commonly used tool to administer therapeutic genes in gene therapy. LVs integrate the desired gene into the host's genome and therefore they result in a constant expression of the integrated gene (156). To further study the *Vegfa* promoter transcript, we cloned the promoter region from -671 to -115 base pair (bp) of the genomic sequence from the TSS into a lentiviral vector (LV-prox). We validated the functionality of the cloned construct by transducing it into the cells; the expression of promoter lncRNA was observed to be upregulated as expected (**Publication II, figure 4c**). After LV-prox transduction, nuclear and cytoplasmic fractionation was conducted for the transduced C166 cells and LV-prox transcript levels were checked with RT-qPCR. The LV-prox transcript was enriched in the nuclear fractions of the cells as compared to the cytoplasmic fraction (fold changes of 27.7 in the nucleus and 2.8 in the cytoplasm, in comparison to the non-treated nuclear fraction). As the ncRNA was observed to be mainly upregulated and retained in the nucleus, whole cell lysates were used in the following experiments to improve the feasibility of the studies.

The luciferase assay is used in molecular biology to study the interaction between sequences by monitoring the luciferase expression level that is co-expressed with the target sequence. In this study, the luciferase assay was exploited to examine whether miR-466c associated directly with the predicted targets. The promoter area (from -538 to -344 starting from the TSS) of *Vegfa* was cloned into a luciferase plasmid. This plasmid produced a construct where the promoter region and luciferase were expressed together. We co-transfected cells with luciferase-promoter plasmid and miR-466c mimics. As miR-466c was binding to the promoter sequence, it

downregulated its expression and therefore also decreased the luciferase expression. In conclusion, we demonstrated that miR-466c is indeed capable of directly binding to the *Vegfa* promoter transcript (**Publication II, figure 4g**).

To further confirm the direct binding of miR-466c to the *Vegfa* promoter transcript, we performed a biotin-pulldown assay. Cells that were transfected with an LV-prox construct were also transfected with biotinylated miR-466c-3p and miR-466c-5p mimics. By pulling down the biotinylated mimics using streptavidin beads, we were able to analyse the bound RNA that was pulled down with the miRNA sequence. Indeed, as in the luciferase assay, we observed that miR-466c-3p was capable to bind to the lncRNA of the *Vegfa* promoter (**Publication II, figure 4e**).

5.2.2 miR-466c is hypoxia regulated and found in the nuclei of endothelial cells

After showing that miR-466c was able to bind directly to the *Vegfa* paRNA and therefore could act in the transcriptional regulation of *Vegfa*, we evaluated further the function of miR-466c. As *Vegfa* is known to be hypoxia regulated, we examined if miR-466c would respond to hypoxia. We observed that miR-466c changed its expression upon hypoxia, with increases evident both in the cytoplasm and in the nucleus (**Publication II, figure 1e**).

In addition to the RT-qPCR analysis, we also used confocal microscopy to confirm the localization of miR-466c. Indeed, as with RT-qPCR, we noticed that miR-466c-3p was expressed more in hypoxic conditions (24h) than in normoxia and it seemed to be more abundant in the nucleus than in the cytoplasm (**Publication II, figure 1g-h**).

miR-466c was expressed on chromosome 2 from the intron 10 of *Sfmbt2* gene. We also confirmed that *Sfmbt2* and miR-466c were both upregulated in hypoxia, indicative of their co-regulation (**Publication II, Figure 2e, 2i**).

5.2.3 miR-466c regulates *Vegfa* expression in endothelial cells

To study whether miR-466c has a role on *Vegfa* regulation in hypoxia, we produced a LV containing miR-466c hairpin sequence as a 255 bp of genomic sequence from intron 10 of *Sfmbt2*. After confirming the efficacy of the LV-vector to overexpress mature miR-466c in cell culture, *Vegfa* levels were also checked from the same cells. *Vegfa* mRNA expression was upregulated upon miR-466c overexpression in the C166 cells. Interestingly, also the pre-mRNA of *Vegfa* was upregulated, indicating that miR-466c upregulation had induced the transcription of *Vegfa* (**Publication II, figure 2b**). Therefore it seemed that the regulation of *Vegfa* by miR-466c occurred through a transcriptional mechanism and not by a post-transcriptional action e.g., by stabilizing existing mRNA transcripts.

As overexpression of miR-466c led to the upregulation of *Vegfa*, we next tested if miR-466c downregulation would affect *Vegfa* expression. This was studied by creating a cell line where one miR-466c transcript was removed from the C166 cell genome by using clustered regularly interspaced short palindromic repeats (CRISPR) technology. After miR-466c deletion, miR-466c-3p and -5p arms were significantly downregulated in the CRISPR cell line, named 466-del, both in normoxia and in hypoxia (**Publication II, figure 2e**).

To test the possible off-target effects of CRISPR modifications of our 466-del cell line, we performed a total transcriptome sequencing for C166 and 466-del cell lines in normoxia and in hypoxia. When comparing the sequencing results between these cell lines, we noted that most of the transcripts that were differentially expressed between 24 h hypoxia and normoxia, were changed in both cell lines (**Publication II, figure 3b**). When examining the *Vegfa* regulation pathway, we observed that gene expression followed the same trend in both cell lines e.g., the same genes were upregulated in both cell lines but to different extents (**Publication II, figure 3e**). With RT-qPCR, we measured *Sfmbt2* mRNA levels, and we observed that also *Sfmbt2* expression was downregulated in the 466-del cell line, indicating that miR-466c and its host gene *Sfmbt2* were being co-expressed and affected each other's expression (**Publication II, figure 2i**).

In the 466-del cell line, *Vegfa* expression was reduced compared to the parental C166 cell line. This was observed especially upon hypoxic conditions, where *Vegfa* was significantly less upregulated in the 466-del cell line in hypoxia than in the unmodified C166 cell line (**Publication II, figure 2g**). This implies that miR-466c is needed for the functionality of *Vegfa* under hypoxic conditions.

This change was observed also in the pre-mRNA levels, where pre-*Vegfa* was less upregulated in hypoxia in 466-del cells than in C166 (**Publication II, figure 2f**). As pre-mRNA and mRNA levels were downregulated, we also analysed the protein levels of VEGFA. There was a significant difference in the VEGFA protein levels between the 466-del and C166 cell lines (**Publication II, figure 2h**). This highlights the significant impact of miR-466c on the regulation of *Vegfa*, where transcriptional regulation results in a different level of the protein being produced.

When adding miR-466c back to 466-del cell line using LV-466, in normoxic conditions we observed an upregulation of *Vegfa*. However, in hypoxia, LV-466 did not significantly increase *Vegfa* in the 466-del cell line i.e., the hypoxia response could not be completely rescued by external miR-466c expression (**Publication II, figure 2j**).

5.3 MIR-466C INCREASES VASCULARITY IN HINDLIMB ISCHEMIA MOUSE MODEL

Our *in vitro* studies indicated that miR-466c directly regulated *Vegfa* expression. Thus, next we wanted to evaluate the therapeutic potential of miR-466c *in vivo* in the mouse hindlimb ischemia model. This surgical procedure models the human PAD and it is achieved by ligation of the mouse femoral artery from the two sites. Here, three treatment groups were used, lentivirally delivered miR-466c (LV-466), EV-loaded miR-466c (EV-466) and PBS as a control (vehicle).

EVs are natural carriers of RNAs and proteins into the cells since they work as mediators for cell-to-cell communication. EVs can be produced in cell cultures and their content can be modified (157). In this study, EVs were used to deliver miR-466c into the mouse hindlimbs. We produced the

miR-466c loaded EVs by modifying the producer cell line HEK293T with lentiviral transduction of LV-466. The cells, now overexpressing miR-466c, secreted EVs into the cell culture medium. Next, the collection of the medium and isolation of the EVs with size exclusion chromatography resulted in purified, miR-466c loaded EVs. The purified EV-466 was analysed with nanoparticle tracking analysis (NTA) for the size distribution and particle concentration. We observed the expected EV size with a main peak of 50-150 nm and a good concentration of particles (3.13x10^11 particles/ml) (**Manuscript III, figure 1d**). The EV-466 miRNA content was analysed with RT-qPCR from EVs. The EV-466 group had more miR-466c-3p/-5p than the control group (EVs from unmodified producer cells) (**Manuscript III, figure 1e**).

After surgery, the mice were monitored, and their ischemia severity was assessed by Tarlov scoring one to three times a day. The score was given based on the walking ability of the mice. By taking a closer look at the score values of each treatment group, it was evident that the mice in the EV-466 group showed beneficial effects as compared to the vehicle group. In addition, LV-466 showed the same trend when compared to the vehicle, although the difference between the groups was not significant (**Manuscript III, figure 2a**).

The severity of the ischemia in the animals was also scored with ischemia scores where the ischemic leg of the mice was assessed visually, and the score was given based on visible necrosis in the mouse leg, for example as observed by dislocation of nails or toes. In comparison to the vehicle group, both EV-466 and LV-466 groups showed beneficial effects on the ischemia score (**Manuscript III, figure 2b**).

Functional ultrasound imaging (fUS) of both LV-466 and EV-466 groups showed promising values in the fluorescence intensity of the treated legs, indicating that the treatments had promoted blood capillary formation in the ischemic area (**Manuscript III, figure 3b**). This was scored from the three different region of interests (ROIs) called distal, middle, and proximal ROIs, referring to the area of the induced ischemia. Interestingly, both EV-466 and LV-466 increased the fluorescence signal (i.e., more capillary formation) but the intensity was different in the three ROIs. LV-466 showed

best effect in the middle section, and EV-466 in proximal section as compared to the injection area and normalized to the untreated right leg (**Manuscript III, figure 3a**).

The lethality was assessed by monitoring the survival of the mice after ischemia and the treatments. Hindlimb ischemia itself rarely is fatal, and this was the case also in the vehicle group. Lethality was most prominent in the LV-466 group as compared to the vehicle and the EV-466 groups (4 dead mice vs. 0 for vehicle and 2 for EV-466) (**Manuscript III, figure 1f**). Since LV is integrated into the target gene, in this case miR-466 had been integrated to multiple sites in the genome and produced a permanent effect of constant miR-466c expression. This may explain why it may have been more lethal than the transient miR-466c expression achieved by EV delivery. Indeed, EVs have been shown to have a half-life of under 30 min in mice (158), thus limiting the exposure to the therapeutics to a narrow time window. In addition to better survival, EV-466 treated mice gained weight the most during the monitoring period (**Manuscript III, figure 1g**) suggesting that mice in this group were the healthiest as they were best able to eat and move, leading to a gain in their body weight. The vehicle group showed the lowest body weight suggesting that the healing process in this group of mice was worst in comparison to the LV-466 and EV-466 groups.

Even though the observations *in vivo* showed promising results, these beneficial effects were not seen at the molecular level. With RT-qPCR, we confirmed that miR-466c was indeed upregulated in the muscles when administered with LV-466 but there were no effects observed on VEGFA mRNA or protein levels. In the EV-466 group, we were not able to detect miR-466c nor any *Vegfa* upregulation in the mouse hindlimbs. Furthermore, no miR-466c was detected in the blood, most likely reflecting the fast degradation of EV-466 particles (**Manuscript III, figure 4**).

6 DISCUSSION

6.1 NUCLEAR MICRORNAS

6.1.1 Compartment specific microRNAs

RNAi started a new era of research where previously non-appreciated ncRNAs were identified and recognized for their role in the cellular functions. Decades of research have paid off and nowadays we are realizing the importance of ncRNA in both health and disease. Since siRNAs have been already actively developed as therapeutics and used every day in biomedical research, it can be proposed that the more recent discovery of nuclear miRNAs and other nuclear ncRNAs will be in turn as groundbreaking as PTGS were previously. The discovery of nuclear sncRNAs led to the question of what functions they performed in the cell's nucleus. The first studies performed by Li et al. 2006 and Place et al. 2007 on RNAa were unprecedented in this field and opened the door for this new research topic. RNAa showed that nuclear sncRNAs really could activate gene expression transcriptionally.

In this thesis, nuclear and cytoplasmic miRNA populations were studied in the mouse endothelial cells both in the normoxic and under hypoxic conditions. It was demonstrated that there are populations of miRNAs in the cytoplasm but also in the nucleus. Some of the miRNAs responded to a hypoxic stimulus, suggesting that they have a role in the regulation of the response of the cells to hypoxia.

Interestingly, while some miRNA populations did not respond to the hypoxic stimulus, other miRNAs significantly changed their expression upon hypoxia. It has been proposed that more of these miRNAs are produced during hypoxia since they are needed to regulate the cell's response to hypoxia.

One other possible explanation is that for some miRNAs, more miRNAs are not produced i.e., the total amount of the miRNAs is stable in the cells but the mature miRNAs that are in the cells, are transported from one

fraction to another location where they are needed. This shuttling of the mature miRNAs is an intriguing hypothesis based on the concept of target abundance (78,99). In this case, target abundance, as explained in Chapter 2.3.4., means that mature miRNAs are transported and functioning where their targets are located. It proposes that miRNAs will shuttle from the cytoplasm to the nucleus and back, screening for potential targets and then functioning where the target, based on the matching target sequence, is located. If there is an external stimulus, like hypoxia, cells respond quickly to ensure cellular homeostasis and survival. The cell initiates the signalling cascades that are needed for its survival. It makes sense that the cell will take advantage of the miRNAs already existing e.g., by opening more promoter targets for the miRNAs they can achieve a prompt activation of the transcripts by miRNAs. On the other hand, it is well known that miRNAs are important in the cellular feedback response e.g., even though HIF1A is an important regulator of the hypoxia, its expression still needs to be regulated and eventually silenced, in order to maintain the balance of the regulatory pathways.

While we observed that some miRNA populations preferred to locate in one compartment and did not respond to the hypoxic stimulus with a change in their localization, there were miRNA populations that changed both their expression and localization in the presence of hypoxia. Interestingly, we discovered only two miRNAs that were upregulated both in the nucleus and in the cytoplasmic fractions, possibly indicating that the change in their expression would be explained by increased transcription. These two miRNAs, miR-669c-5p and miR-210-3p, revealed both nuclear and cytoplasmic activity in the studied cells.

Previous studies by others have shown that miR-669c-5p is associated with a low intensity magnetic field in mouse spermatocyte-derived GC–2 cells (159). In another study, this miRNA has been proposed to be a potential biomarker for a traumatic brain injury in mice (160). miR-669c-5p has also been shown to be upregulated in the glucose-deprivated mouse cell line B/CMBA.Ov (114). Until now, the nuclear localization of this miRNA had not been published.

miR-210, a known hypoxamiR as discussed in Chapter 2.3.5., was found to be more abundant in the nucleus than in the cytoplasm. This leads to the speculation of whether also other miRNAs known to be important in modulating different gene functions may also be nuclear enriched and having additional functions in that location. In fact, some investigators have already proposed a role for miR-210 in the activation of genes like *Vegfa* and *Notch1*, although the mechanism behind this regulatory has not been described (103,105). These new findings of nuclear localization and possible nuclear actions are not contradictory to the previously studied functions of miR-210 in PTGS. Instead, they provide a new aspect of miRNA regulation that has not been characterized before and may shed new light on its complementary roles in gene regulation. To underline the complexity of miRNAs, the same miRNA may be able to either activate or inhibit its targets, depending on the environment and whether the targets are on PTGS or on a promoter.

In addition to the already recognized miRNAs, as categorized in miRBase (161), our sequencing resulted in the identification of previously uncharacterized small RNAs. At least some of these putative sncRNAs are likely to be miRNAs since they are of a suitable size i.e., between 19 nt and 23 nt (**Figure 7**). Some of these miRNAs are likely to be isomiRs since their sequence clearly has one parental sequence from which they are most likely derived. Some of these putative miRNAs responded to the hypoxic stimulus, evidence that there may still be unidentified, uncharacterized novel miRNAs that participate in the hypoxia regulation pathways. These putative miRNAs may well be cell type specific and therefore have remained previously uncharacterized. Some of the putative miRNAs were expressed in low copy numbers, and therefore they may have been discarded in other studies. However, nuclear promoter targeting mechanisms may not require as many copies of miRNAs as these lncRNAs are often themselves expressed at very low levels, so these putative miRNAs may have important nuclear roles and should be evaluated in more detail in the future.

In our study, we identified miR-3535 as the most nuclear enriched miRNA according to the small RNA sequencing. This miRNA has been

evaluated before in the ApoE deficient mouse model where atherosclerosis was induced by feeding a high-fat diet to the animals. The study group received Icariin, the effective molecule in a traditional oriental herbal medicine Epimedium used to treat atherosclerosis. After 12 weeks, it was shown that the atherosclerotic plaques were smaller in the Icariin treated mice than in the control group. Interestingly, the miRNA pathways were studied between these groups and mmu-miR-3535 was shown to be widely downregulated in the Icariin treated mice when compared to the vehicle group. The Gene Ontology analysis conducted in the same study proposed that miR-3535 was participating in the *PI3K-Akt* signalling pathway that is known to induce cellular proliferation and angiogenesis. It was therefore suggested that miR-3535 was able to silence important factors for angiogenesis, and Icariin treatment silenced miR-3535 to induce angiogenesis (162). With our new data, we can propose that this miRNA can have also a nuclear, perhaps transcriptionally activating, functions since its levels were increased in hypoxia in our model.

Even though nuclear miRNAs offer a new intriguing therapeutic potential, the path towards future applications is not going to be straightforward. Since there are multiple groups of RNAs shuttling between the nucleus and the cytoplasm, affecting to the protein coding genes, proteins and even each other, it is evident that the RNA network is a diverse and complex phenomenon. Yet, many studies have taken the effort to solve the mystery behind the complex network, unravelling this network and its properties piece by piece.

6.1.2 Nuclear miR-466

In this thesis, nuclear miRNAs were studied in mouse endothelial cells. We discovered miR-466c in the nuclei of these cells. In subsequent studies, miR-466c was found to upregulate *Vegfa* transcription in the nucleus.

Some investigators have previously studied miR-466c or its close relatives; for example, mmu-miR-466c-3p has been shown to take part in the Renin-Angiotensin-Aldosterone signalling system in immortalized mouse cortical collecting duct cells. In that experiment, miR-466c-3p was

upregulated after aldosterone administration and it inhibited mineralocorticoid receptor gene by targeting the 3'UTR of its mRNA (163). In another study, mmu-miR-466b-3p was found to be downregulated in the soleus muscle of active mice as compared to the same muscle in less active mice. This study suggested that miR-466b-3p was upregulated and promoting signalling pathways in low active mice (164).

While previously miR-466c has been linked to a silencing of its targets, this thesis describes a novel alternative to explain how miR-466c can regulate other genes. To date, the results emerging from this thesis project have provided the first glimpse of the nuclear functions of miR-466c.

Here, we studied how miR-466c downregulation affected *Vegfa* expression in the studied cells. By using CRISPR, we deleted only one copy of miR-466c from mouse endothelial cells. The mouse genome has a total of three copies of this miRNA. Importantly, the deletion of this miRNA copy was crucial since we observed a significant downregulation of miR-466c in 466-del cells (**Publication II, figure 2e**). This miRNA deletion also affected the VEGFA mRNA and protein levels in these cells. This suggests that miR-466c is an important regulator of *Vegfa*. Interestingly, as the RNA sequencing data shows, deletion of miR-466c did not significantly affect other pathways than the *Vegfa* pathway. The differences observed between the cell lines were due to differential regulation of *Vegfa* and related downstream processes, which was expected as *Vegfa* expression was altered by the deletion of miR-466c.

This miRNA was shown to be nuclear localized and able to function by transcriptionally activating *Vegfa* expression in mouse cells. miR-466c is expressed from intron 10 of the *Sfmbt2* miRNA cluster and it belongs to the family of miR-466. In this thesis, it was confirmed that *Sfmbt2* and miR-466c were both upregulated in hypoxia, indicating their co-regulation. Other genes and miRNAs in their introns have been previously shown to be co-transcribed (110).

In addition to the evident significance of miR-466c, the role of other miRNAs belonging to the same miRNA family as miR-466 could be equally important, at least in mice. This is because this miRNA family is considered as a superfamily, indicating that in addition to the seed sequence, in

general these miRNAs display very similar sequences. As there are so many of these miRNAs that are so similar to each other, this may represent an indication of their importance in cellular functions. Having multiple copies of very similar sequences in miRNAs probably indicates similar functionality and the vast number of family members would reflect their importance to the cell.

Even though miR-466c is a rodent specific miRNA and it is not found in the human genome, some human miRNAs have very similar sequences as miR-466c and therefore a similar regulation mechanism may occur in humans. **Figure 8** illustrates the sequence similarities between miR-466c-5p and its close relation in humans, hsa-miR-297.

hsa-miR-297 has been studied in human oral squamous cell carcinoma (OSCC) cells collected from patients; it was proposed that lncRNA *INC00668* is upregulated in the OSCC cells where it acted as a competing endogenous RNA (ceRNA) for hsa-miR-297, therefore preventing its targeting of *VEGFA* mRNA. Therefore, *VEGFA* was upregulated in these cells leading to a worsened survival of these patients (165). It has also been proposed from *in silico* experiments that hsa-miR-297, which was claimed to be upregulated after statin treatment (simvastatin), could target the Rho-associated protein kinase in HUVEC (166).

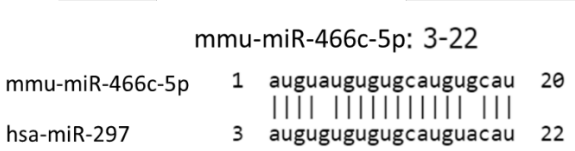

Figure 8. Sequence similarities between mmu-miR-466c-5p and hsa-miR-297. Sequences and alignment have been obtained from the miRBase (161).

6.2 MECHANISMS OF RNA ACTIVATION

6.2.1 Processing is important for microRNA function

It has been previously observed that the shRNAs targeting the *Vegfa* promoter can either activate or inhibit *Vegfa* expression (32,130). Interestingly, when we studied the *Vegfa* promoter in greater detail, we found potential target sites for miR-466c-3p and miR-466c-5p on the same chromosomic loci where the activating shRNA-451 is known to target. The data in this thesis provides evidence that these miRNAs will be able to target near to the shRNA-451 target site i.e., they are able to activate *Vegfa* by targeting the same region.

As shRNA-451 is an artificial RNA, we believe that it is mimicking the natural endogenously expressed miR-466c. This is considered to be due to the similarities between their target sites since there is only a 7 bp difference between shRNA-451 and miR-466c binding sites on the *Vegfa* promoter. In addition, there are also similarities between these RNA types as they both have a hairpin structure, most likely leading to a similar processing of these RNAs.

miRNA processing has been determined in many studies to be crucial for the function of miRNAs. During the studies we performed on miR-466c, we discovered that pre-miRNA processing was indeed required for miRNA function. We observed that by cloning pre-miRNA (255 bp) into an LV-vector that more *Vegfa* was induced than could be achieved by only cloning the mature miRNA sequence into the LV-vector (data not shown). This could mean that the pre-miRNAs that are naturally processed by the cells may be more functional than the mature mimics. This is also supported by the data of Turunen et al. (32) where it was shown that the hairpin structure of the shRNA-451 was needed to obtain the beneficial effects of shRNA.

In the studies with shRNA-451, the mechanism proposed for the action of shRNA was that it exerted its functions through epigenetics. Although epigenetic changes were not studied in this thesis, the similarities

explained above suggest that a similar function could be also shared between shRNA-451 and miR-466c.

It is concluded that the structure and processing of small RNAs are important for their function. During the maturation process, the appropriate factors are associated to the miRNAs so they can function in the RISC. If no processing takes place, or if it occurs incorrectly, these important factors like Dicer, TRBP and Ago2 will not form the correct complex with miRNAs, which hinders the endogenous maintenance of cellular functionality. Interestingly, it has been shown recently that nuclear miRNAs can regulate the promoter activity by directing Pol II so that a desired transcript will be transcribed (124). Thus, in addition to the miRNAs' function of mediating the collection of the correct factors to the promoters, miRNAs can also directly "push" the transcriptional machinery in the desired direction.

6.2.2 MicroRNA targets promoter associated non-coding RNA on gene promoters

As described earlier, many investigators have proposed different mechanisms for the function of saRNA. The main mechanisms involve a saRNA-paRNA interaction as well as saRNA binding to either single-stranded or double-stranded DNA, with the latter forming a RNA:DNA:DNA triplex on chromatin.

Many of the studies on RNAa have only revealed saRNA upregulation to lead to the TGA of the genes. In fact, the studies that propose some mechanism for the saRNA mediated upregulation of the genes have often described the presence of a promoter transcript on the upregulated genes. This is the model that was considered also in this thesis to be the most likely mechanism to account for miR-466c's properties. We identified an antisense transcript on the *Vegfa* promoter and miR-466c was found to be able to bind to this promoter transcript sequence (**Publication II, figure 4e, 4g**).

We have hypothesised that these miRNAs would be located in the nuclear fraction since regulation of the lncRNA transcript of *Vegfa* would

potentially take place in the nuclear fraction of the cells. The process of PTGS is well-known, and it has been detected in the numerous of the studies that miRNAs can direct the PTGS by targeting cytoplasmic mRNA and silencing its function. It would make sense that the processing mechanisms of the nuclear enriched miRNAs would work in the same way as occurs in the RNA-RNA binding phenomenon. As some miRNAs are found both in the nucleus and in the cytoplasm of the cells, they likely have targets in both fractions. Therefore, the same miRNA can target both the mRNA in the cytoplasm and the promoter transcript in the nucleus i.e., it would be able to take part in the PTGS and in the TGA, depending on where it finds its targets. This would suggest that miRNAs function via RNA:RNA interaction in all cellular compartments.

It is well known that these paRNAs are often expressed in a low copy number, at least when compared to mRNAs or even other lncRNAs. However, as paRNAs are located in the promoters of their target genes and they promote the TGA of these genes, it is quite logical that the copy numbers of these paRNAs should be rather low. In the same way, we can speculate that also the nuclear miRNAs that activate genes by targeting the promoter would still be very effective even if they are not expressed in huge copy numbers. This phenomenon was evident also in our study. The expression of the antisense transcript on the *Vegfa* promoter was needed to be increased by LV-prox transduction before conducting the pulldown experiment. We also observed that the miR-466c copy numbers were quite low in the C166 cell line. When targeting the paRNA on the *Vegfa* promoter, an effective response was evident even with a lower copy amount when these miRNAs regulated the transcriptional expression of *Vegfa* in the nucleus.

This thesis' data suggests that miR-466c activated *Vegfa* expression by targeting the paRNA on the *Vegfa* promoter, inducing its transcription, most likely by inducing an epigenetic modification on the promoter. It can be proposed that miRNA finds its target in a sequence specific manner, this time, it would be paRNA on the *Vegfa* promoter. Proteins that are attached to miRNA in RISC are then participating on promoter regulation by recruiting more proteins to this site. This RNA-protein complex then

expectedly induces the triggering of epigenetic modifications on the *Vegfa* promoter, leading to the activation of the *Vegfa* gene. It can be hypothesized that based on the sequence specificity; miRNA therefore acts by bringing the correct proteins to the appropriate sites to allow them to function. We can assume that miRNA is the guide allowing the proteins to reach their targets i.e., targets either needing to be activated or silenced, depending on the situation.

6.3 RNA ACTIVATION THERAPY

RNAa has enlarged the RNA field; for example, it is now opening the possibility of exploiting saRNAs in future therapies. However, the RNAa field requires still more basic research so that the mechanisms of saRNA and their functions can be truly understood and correctly utilized. This field is rather complex, but very promising.

Hypoxia treatment of stem cells has been found to alter their functions in a fashion that was beneficial for heart function when the cells were subsequently administered into rodent or human hearts (18). Therefore, hypoxia could be a key aspect that needs to be borne in mind when new treatments for ischemic diseases are being developed. Although not always considered by researchers, hypoxic conditions are the normal conditions within many tissue and cell types i.e., the oxygen levels within the body are lower in almost every tissue than the atmospheric oxygen levels, which are considered to be normoxic. As a result, the treatments are currently being evaluated under different conditions than where they would be eventually administered, and this may lead to the enormous gap between results obtained under *in vitro* conditions and *in vivo* studies. This aspect could also explain some of the differences between promising pre-clinical outcomes and failures in clinical trials.

In our mice model, we observed that miR-466c showed promise in achieving a beneficial effect on the hindlimbs of the mice after ischemia. miR-466c was administered in either an LV-delivered or an EV-mediated way. In general, both groups showed beneficial effects in terms of their ischemia and Tarlov scores as well as in fUS imaging. However, there were

more deaths in the LV-466 treated animals than in mice receiving EV-466. In addition, the EV-466 treated mice gained more weight than the animals in either the LV-466 or vehicle groups.

Since EVs are naturally occurring mediators in the body, they represent a very attractive option for delivery vehicles for administration of therapeutic molecules. Whereas LVs achieve a permanent expression of the genes they are expressing, EVs are degraded very rapidly. It was demonstrated in a mouse model that EVs have half-lives of less than 30 min after their administration (158). As EV-mediated gene induction is transient, there are many variables that need to be considered and resolved before this form of delivery can reach the clinics. For example, it still must be examined how different doses can affect the outcome of the response. More research is also needed to define whether EVs need to be administered multiple times, and if so, what timepoints would be optimal. Overall, even though there are many questions still to be answered, EVs do seem to offer a promising delivery method for biological drugs, for example for miRNAs.

One can speculate that also in our hindlimb ischemia study, we would have obtained more beneficial results if the EV administration could have been repeated and more timepoints used to monitor mice. This might also explain why we were not able to observe any upregulation of *Vegfa* expression in the muscles of the mice at 10 days after the ischemia; we may not have used the optimal timepoint at which to analyse the mRNAs. In addition, it is possible that we were not able to observe *Vegfa* expression since we analysed the whole muscle in the wet lab studies. In addition to the endothelial cells that express VEGFA, muscle contains many other cell types that could have obscured the *Vegfa* expression. It may also be possible that the levels of *Vegfa* had been increased only in a small part of the injured muscle and thus by not restricting the analysis to only the ischemic site, we could have overlooked the actual differences between treatments.

Unlike artificial shRNAs, miRNAs are endogenously produced by cells and thus they can likely achieve a more natural response when they gain access to the cells. miRNAs are attractive tools for the activation of gene

expression since they are RNAs which have a natural origin in the tissues. For this reason, they are less likely to trigger an intense immune response and may induce natural reactions after administration to the body. Interestingly, it has been found recently that mmu-miR-466e-3p, a member of the same miRNA family as miR-466c, is naturally enriched inside the EVs of SVEC endothelial cells. In addition, the same study identified miR-669c-5p, also belonging to the same *Sfmbt2* gene miRNA family cluster, to be packed into EVs even more universally i.e., it was detected in every cell line tested in that study (167). This discovery is interesting as it indicates that these miRNAs could well be delivered inside EVs since their biology promotes their packing into these structures.

In comparison to mRNA drugs, miRNAs may also offer several advantages. Since miRNAs are small RNAs, they tend to be more stable than mRNAs which are more prone to RNA degradation. In addition, miRNAs that regulate their target genes by transcriptional activation, induce the whole repertoire of gene whereas the mRNA drugs that only give rise to the one isoform that is administered into the body. Especially *in vivo* this may result in a better therapeutic response in the tissue. Furthermore, transcriptional gene regulation occurs through epigenetic changes in the promoter, which evoke a more long-lasting effect in the cell. This mechanistic pathway allows the cell to regulate the transcriptome in response to environmental changes, in other words, the epigenetic modifications induced by the miRNA drug may be terminated when the stress stimuli has ended, and the cell no longer needs that gene to be activated. Therefore, this kind of miRNA-mediated activation has the potential to deliver a more extensive, while still natural, effect on gene regulation.

It has been proposed that miRNAs regulate over 60 % of the genes by targeting their mRNA in the cytoplasm (66). In fact, this value only takes into account the cytoplasmic function of the miRNAs. One can only speculate how high this number will reach if the properties of nuclear miRNAs are also included. Ultimately, miRNA therapeutics offer a real chance for finding new therapies most probably for a wide spectrum of diseases, many of which are currently incurable.

6.4 FUTURE DIRECTIONS

As discussed throughout this thesis, RNA therapeutics possess an interesting therapeutic potential; in the future their exploitation may become more common. Even though the studies in this thesis highlight the potential nuclear role of miR-466c in the transcriptional activation of *Vegfa*, they offer only a glimpse of the complex function of nuclear miRNAs. More studies on this form of miRNA regulation will be needed before these miRNAs can enter into the clinics and truly release their great potential as RNA-based drugs. Both *in vitro* and *in vivo* experiments will need to be performed; these should include: a clarification of their exact mechanism of action in order to better understand what changes occur in the cells and tissues when nuclear miRNAs are administered. In addition, the therapeutic effect of miR-466c should be optimized by undertaking more *in vivo* studies.

An improved understanding of the mechanisms of action would expand both our knowledge of basic cellular biology and help to develop safe and specific drugs based on nuclear miRNAs. For example, we need to be able to answer several questions e.g., where exactly do miRNAs interact in the promoter (newly transcribed paRNA or in association with chromatin) and what proteins are involved in this process. Another question to be resolved is the miR-466c targeting to the promoter of *Vegfa;* this could be verified by conducting a CRISPR-mediated modification of the target site on chromatin in order to observe whether mechanisms other than promoter targeting play a role in the regulation process. miRNAs naturally have multiple targets in the cells as these are mostly directed by the binding of their seed sequence, and they can bind either mRNAs or ncRNAs within the cells. It would be important to clarify whether changes in other miRNA sequences, outside the seed sequence, could generate a more specific targeting to the desired target e.g., by inclusion of some of the *Vegfa* promoter sequence into the miR-466c.

In addition to improving our mechanistic understanding, the optimization of *in vivo* process should also proceed. The studies performed

in muscle cells in this thesis also need to be done in other tissues to evaluate the biodistributions of the treatments. In addition, it would be advantageous to undertake histological studies of the muscles to identify the exact cell population where the beneficial effect is taking place. This would give us a better understanding of the cell type specificity of miR-466c. Furthermore, the optimal mode of delivery needs to be determined. There are more options for delivery than we have tested, for example LNPs are often used for drug delivery of RNA. Are the EVs the best option for this or would LNPs be a better and more feasible delivery approach? In the *in vivo* experiment performed here, only one dose of miR-466c in EVs was used and the results were analysed at one timepoint. EVs are degraded quickly and therefore it may be beneficial to administer them repeatedly to increase the amount of miR-466c in a tissue. Shorter timepoints may be needed to reveal the effects in the muscles e.g., by determining *Vegfa* expression levels, in a hindlimb ischemia animal model.

Once both *in vitro* and *in vivo* studies regarding miR-466c and other nuclear miRNAs have been successfully completed, we will have gained a better and more comprehensive view of the function of nuclear miRNAs. The experiments performed in this thesis can be considered as the foundation stones from which therapies based on nuclear miRNA can be built in the future.

7 CONCLUSIONS

7.1 SUMMARY AND CONCLUSIONS

Ischemic diseases are diseases attributable to a failure of the tissues to receive an adequate suppy of oxygen and nutrients. They are serious diseases, being responsible for over 20 % of deaths globally each year. miRNAs are small ncRNAs that have been shown to have diverse functions in the cells under both physiological and pathological conditions. In addition to the canonical miRNAs that function as gene silencers in the cytoplasm in a process called PTGS, miRNAs are nowadays also known to exist and function in the nuclear fraction of the cells. In this thesis, miR-466c was found to target the *Vegfa* promoter in the mouse endothelial cells leading to an upregulation of *Vegfa*. miRNAs act as guides of the active RISC in subcellular compartments, leading to the formation of complexes with their targets, inducing either their upregulation or downregulation. Because miRNAs represent a diverse and natural gene regulatory technique, they are promising targets for future therapy models. In this thesis, miR-466c was found to be a nuclear, hypoxia regulated miRNA with the ability to activate its target gene i.e., the expression of *Vegfa*. In addition to the positive results in the *in vitro* experiments, miR-466c induced beneficial effects in the hindlimb ischemia model in mice. Therefore miR-466c shows good potential as a drug to be administered in the treatment of ischemic diseases in the future (**Figure 9**).

The following conclusions can be made based on the studies included in this thesis.

I miRNAs are present in the nucleus and in the cytoplasm of endothelial C166 cells and there are miRNA populations that change their expression in hypoxic conditions

II The expression of miR-466c changes in hypoxic conditions and miR-466c upregulates *Vegfa* expression at the transcriptional level

III EV-delivered miR-466c shows promising and beneficial effects on mouse hindlimbs subjected to ischemia

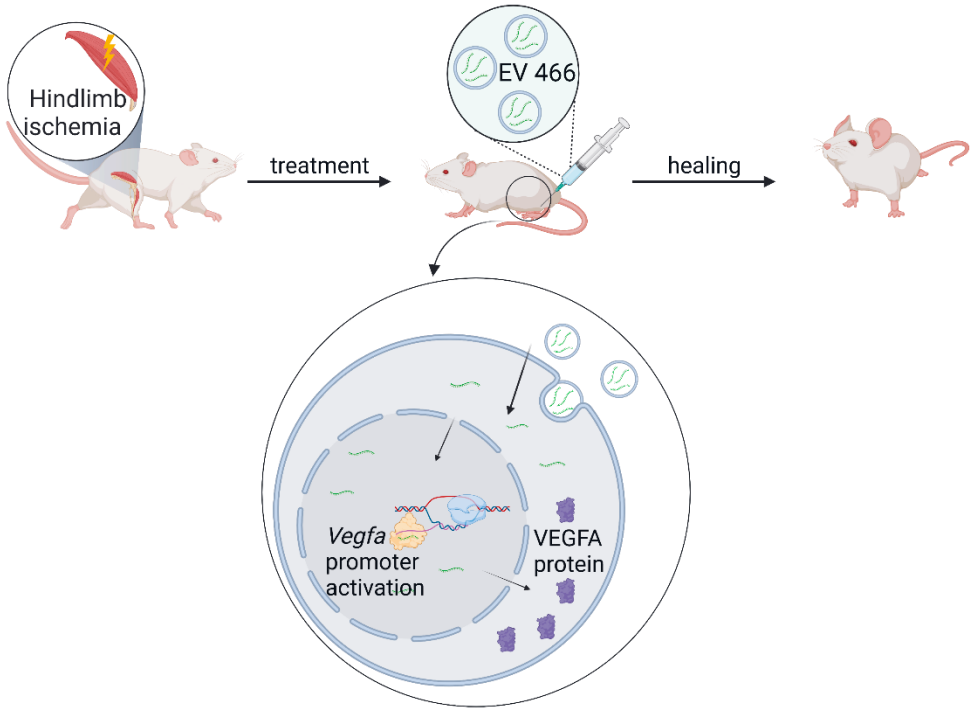

Figure 9. Summarizing the function of miR-466c *in vitro* and *in vivo*. EV-mediated miR-466c induces *Vegfa* expression by targeting the promoter transcript of *Vegfa* in the nucleus. This induction increases VEGFA in the cytoplasm and represents the basis for the therapeutic effects seen in mice subjected to hindlimb ischemia. Created with BioRender.com.

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ORIGINAL PUBLICATIONS (I – III)

I

Changes in nuclear and cytoplasmic microRNA distribution in response to hypoxic stress

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Changes in nuclear and cytoplasmic microRNA distribution in response to hypoxic stress

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MicroRNAs (miRNAs) are small non-coding RNAs that have well-characterized roles in cytoplasmic gene regulation, where they act by binding to mRNA transcripts and inhibiting their translation (i.e. post-transcriptional gene silencing, PTGS). However, miRNAs have also been implicated in transcriptional gene regulation and alternative splicing, events that are restricted to the cell nucleus. Here we performed nuclear-cytoplasmic fractionation in a mouse endothelial cell line and characterized the localization of miRNAs in response to hypoxia using small RNA sequencing. A highly diverse population of abundant miRNA species was detected in the nucleus, of which the majority (56%) was found to be preferentially localized in one compartment or the other. Induction of hypoxia resulted in changes in miRNA levels in both nuclear and cytoplasmic compartments, with the majority of changes being restricted to one location and not the other. Notably, the classical hypoxamiR (miR-210-3p) was highly up-regulated in the nuclear compartment after hypoxic stimulus. These findings reveal a previously unappreciated level of molecular complexity in the physiological response occurring in ischemic tissue. Furthermore, widespread differential miRNA expression in the nucleus strongly suggests that these small RNAs are likely to perform extensive nuclear regulatory functions in the general case.

The canonical view of microRNA (miRNA) function is that these small RNA molecules typically repress gene expression by targeting the mRNA 3′ untranslated region (3′ UTR) in order to induce post-transcriptional gene silencing (PTGS)[1]. A growing body of evidence suggests that miRNA functionality may be much more complex than this canonical paradigm, and recent studies have suggested multiple novel modes of action for small RNAs[2–5]. For example, small RNA-mediated transcriptional gene activation (TGA) has been reported in some cases, whereby synthetic small RNAs or endogenous miRNAs targeted to gene promoters can induce increases in gene expression[6,7]. TGA involves an epigenetic mechanism, although a detailed understanding of this phenomenon has thus far remained elusive[8].

We have previously shown that short hairpin RNA (shRNA)-mediated transcriptional activation of the *Vegfa* promoter is of therapeutic benefit in murine models of hindlimb ischemia[9] and myocardial infarction[10]. These observations suggested that shRNAs may function by mimicking endogenous miRNAs. To date, intracellular miRNA localization has been largely overlooked, and only a handful of publications have performed global miRNA analysis in nuclear and cytoplasmic fractions. Conversely, the majority of citations focusing on miRNA function have not addressed the issue of subcellular localization. Previous reports have analyzed the subcellular distribution of small RNAs in 5-8F[11] and HTC116 cells[12] and showed that the majority of miRNAs, regardless of their sequence, are shuttled to nucleus. Some sequence motifs have been demonstrated to affect miRNA subcellular distribution[13,14], but other factors may also direct miRNA localization patterns. For example, Khudayberdiev *et al.*, observed that nuclear accumulation of miRNAs may be associated with global down-regulation of miRNA levels occurring during neuronal development[15]. Importantly, components of the RNA interference machinery have been observed to be functional in the nucleus, although small RNA loading is restricted to the cytoplasm[16]. While

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several studies have profiled miRNA expression in the nucleus, no study has to date applied this analysis to cells undergoing physiological transitions (i.e. during cellular differentiation, or in response to environmental stimulus).

Hypoxia is a severe pathophysiological condition observed in multiple diseases (e.g. myocardial infarction and cerebral ischemia) where it impairs tissue function, resulting in a rapid and complex response at the molecular and cellular level. In the present study, we selected hypoxia as a prototypical, medically-relevant, pathophysiological stimulus that is known to lead to changes in gene and miRNA expression[17]. To investigate the extent to which mature miRNAs are present in the nucleus, we performed high throughput sequencing of small RNAs (sRNA-seq) in nuclear and cytoplasmic fractions derived from endothelial cells after the induction of hypoxia. This analysis has identified multiple miRNAs that are enriched in either the nucleus or cytoplasm. Furthermore, we show for the first time, that specific miRNAs are differentially expressed in a subcellular location restricted manner in response to hypoxic stimulus. These findings suggest that miRNAs likely execute nuclear functions in addition to their cytoplasmic PTGS activity, and reveal a previously unappreciated level of complexity in the cellular response to hypoxia.

Results

Isolation of nuclear and cytoplasmic fractions from endothelial cells. The murine C166 endothelial cell line was cultured under normal conditions or exposed to hypoxia (1% oxygen) for 24 h. Cells were separated into nuclear and cytoplasmic fractions according to the protocol described by Gagnon *et al*., and RNA and proteins were extracted from each sample[18] (Supplementary Fig. S1). Fraction purity was confirmed by western blot using antibodies against nuclear (histone H3) and cytoplasmic (β-tubulin) marker proteins (Figs. 1a and S2). Similarly, the nuclear-enriched long non-coding RNAs (lncRNAs) *Malat1* and *Neat1*[19] were found to be 37- and 125-fold enriched in the nucleus respectively, as determined by RT-qPCR (Fig. 1b). Conversely, transfer RNAs (tRNA) tRNA-Lys-TTT and tRNA-Met-CAT were found to be 12- and 17-fold enriched in the cytoplasm, respectively (Fig. 1b), consistent with successful subcellular fractionation.

Identification of nuclear and cytoplasmic enriched miRNAs. C166 cells were cultured in either normoxic or hypoxic conditions (for 2 h and 24 h) and RNA from nuclear and cytoplasmic fractions analyzed by sRNA-seq ($n = 2$) (Supplementary Fig. S1). 214 million reads were generated in total, and library sizes were found to be comparable between all samples (Supplementary Fig. S3). The data were filtered to remove low abundance miRNAs (<10 reads in each library) after which 350 miRNAs remained. Statistical differences between samples were tested using DESeq2[20] and libraries were visualized by Principal Component Analysis (PCA) (Fig. 2a) which showed tight clustering of biological replicates. Nuclear and cytoplasmic samples were clearly separated in principal component one (which contained 74% of the variation in the dataset), whereas the response to hypoxic stress was reflected in principal component 2 (12% of the variance).

Nuclear and cytoplasmic enrichment was determined by differential expression analysis of all nuclear libraries against all cytoplasmic libraries and visualized by hierarchical clustering (Fig. 2b) and a volcano plot (Fig. 2c). 196 miRNAs (56% of all miRNAs) were significantly different ($P < 0.01$) between nucleus and cytoplasm, suggesting that the majority of miRNAs are preferentially enriched in one of the compartments. Of these, 105 miRNAs were enriched in either compartment by ≥ 2-fold (i.e. 46 enriched in the nucleus, and 59 enriched in the cytoplasm). MA plot analysis showed that differentially enriched miRNAs were found across a broad range of absolute expression (i.e. counts) values (Fig. 2d). The most strongly location-enriched miRNAs were miR-3535 (27.9-fold enriched in the nucleus) and miR-27a-5p (48-fold enriched in the cytoplasm) (Fig. 2e). Differential nuclear-cytoplasmic distribution of these miRNAs was further confirmed by RT-qPCR, where additional replicate cultures were analyzed to increase statistical power and confirm reproducibility of these findings (Fig. 2f). RT-qPCR data were normalized to the levels of miR-186-5p which was determined to be the most stably expressed miRNA across all conditions using the NormFinder method[21].

miRNAs are differentially expressed in distinct subcellular compartments. We next sought to identify miRNAs that were differentially expressed in response to hypoxic stress.

Nuclear and cytoplasmic libraries were considered separately, and differential expression determined between time points. Using a fold-change cut-off of 2, and a stringent statistical cut-off of $P < 0.01$ (Benjamini-Hochberg adjusted), 54 and 35 significantly different miRNAs were called in the nuclear and cytoplasmic libraries respectively for the 24 h vs 0 h comparison. In contrast, no significant differences were called in the 2 h vs 0 h comparison for either nuclear or cytoplasmic fractions.

Expression ratios for nuclear and cytoplasmic libraries were not correlated (Pearson $r = 0.0488$, Spearman $r = 0.0912$, $P > 0.05$) (Fig. 3a), indicating that differential miRNA expression typically occurred exclusively in one fraction and not the other. Interestingly, the majority of differentially expressed miRNAs were down-regulated under hypoxic conditions. Only 6 miRNAs were identified as exceptions that were differentially expressed in both nucleus and cytoplasm after 24 h of hypoxic stress (Fig. 3b). These included 2 up-regulated miRNAs (miR-210-3p and miR-669c-5p) and 4 down-regulated (let-7a-5p, miR-200c-3p, miR-193a-5p and miR-203-3p). In the cytoplasm, 29 miRNAs were differentially expressed (Fig. 3c) (5 up-regulated and 24 down-regulated). In the nucleus, 48 miRNAs were differentially expressed (Fig. 3d) (13 up-regulated and 35 down-regulated). Surprisingly, miR-210-3p, a well-described hypoxamiR[22], was observed to be highly up-regulated in the nuclear fraction in the sRNA-seq data (Fig. 3e). This finding was confirmed by RT-qPCR (Fig. 3f). Fluorescent *in situ* hybridization (FISH) was used to analyze miR-210-3p localization (Fig. 4). Detected signal in confocal microscopy for miR-210-3p was punctate and could be observed in the nuclei of C166 cells both in normoxia and hypoxia (24 h). Qualitative assessment of miR-210-3p puncta also correspond with the increase in miR-210 levels observed by RT-qPCR. miR-210-3p is known to have a role in several pathways related to the low cellular oxygen and has been shown to regulate *EFNA3*, *E2F3*, *HOXA3*, *HIP1*, *BDNF*, *KCMF1* and *NDUFA4P1* by canonical PTGS[23,24].

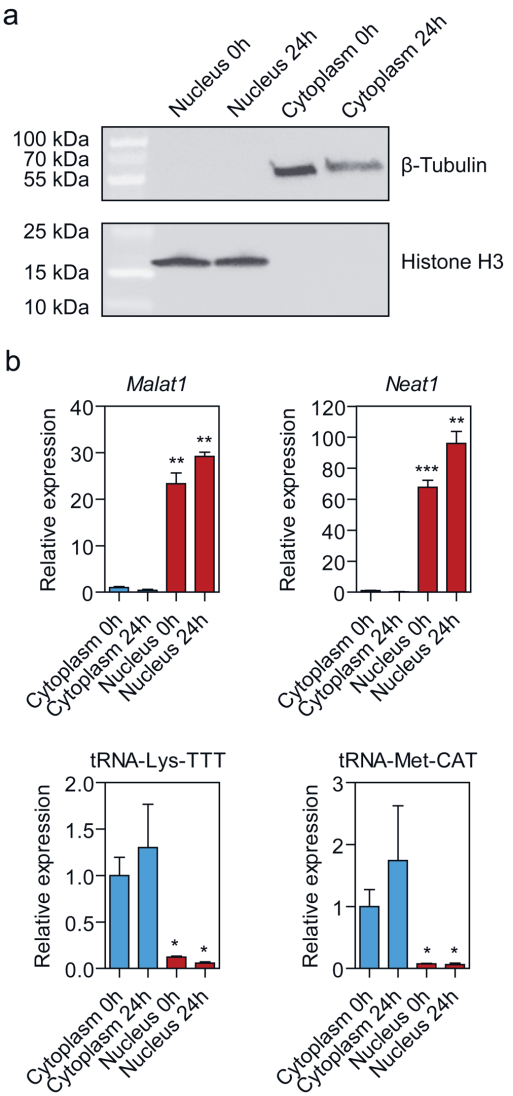

Figure 1. Isolation of nuclear and cytoplasmic fractions. (**a**) Western blot analysis of cytoplasmic (β-tubulin) and nuclear (histone H3) marker protein expression. (**b**) RT-qPCR analysis of nuclear-enriched lncRNAs (*Malat1* and *Neat1*) or cytoplasm-enriched tRNAs (tRNA-Lys-TTT and tRNA-Met-CAT). Values are mean ± SEM, $n = 3–4$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (*t*-test with Welch correction for unequal variance, comparisons are to the matched group-matched Cytoplasmic fraction).

Furthermore, miR-210-3p was previously shown to interact with is *XIST*, a lncRNA located in the nucleus which is responsible for the initiation of X chromosome inactivation[25]. Together, these data suggest that miR-210-3p likely regulates nuclear processes, in addition to well-established cytoplasmic PTGS mechanisms.

To complement the analysis in C166 cells, we validated the most interesting miRNAs in other mouse cell lines by RT-qPCR. The expression of the miRNAs with nuclear and cytoplasmic localization, miR-3535 and miR-1291 (nuclear) and miR-27a-5p (cytoplasmic), was also observed to be similar in the cell lines MS1 (pancreatic endothelial), MOVAS (smooth muscle) and C2C12 (skeletal muscle) (Supplementary Fig. S4). In addition, miR-210-3p was upregulated in hypoxia in the cytoplasmic and nuclear fractions of these cells in a similar fashion as to that observed in C166 cells. Overall, these results demonstrate that the nuclear-cytoplasmic distribution of certain

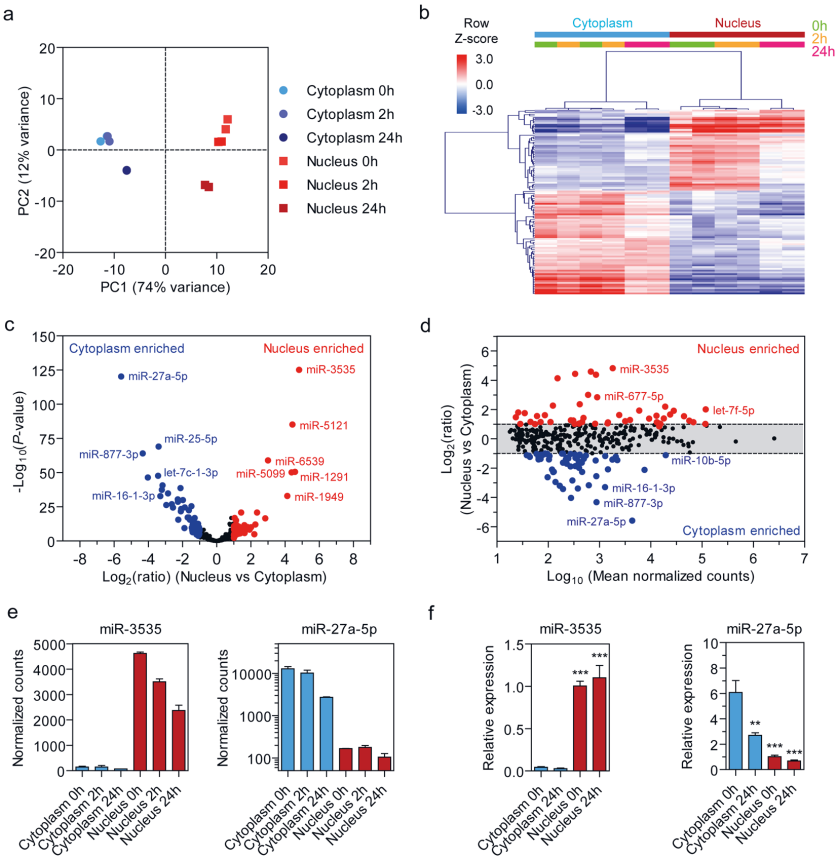

Figure 2. Nuclear and cytoplasmic small RNAs. (**a**) Principal component analysis (PCA) shows tight clustering of biological replicates. Principal component one (PC1) shows the separation between nuclear and cytoplasmic samples (74% of variation), whereas PC2 depicts the response to hypoxic stress (12% of variation). (**b**) Hierarchical clustering of differential expression between nuclear and cytoplasmic samples (adjusted $P < 0.01$, fold change $\geq |2|$). Scale bars show mean-centered $\log_2$ normalized counts (row Z-score) where red and blue indicate higher and lower than mean abundance respectively. (**c**) Volcano plot depicting nuclear and cytoplasmic miRNA enrichment. (**d**) MA plot analysis of location-enriched miRNAs showing that differentially nuclear/cytoplasm-enriched miRNAs were observed over a broad range of absolute expression values. (**e**) The two most location-enriched miRNAs across the dataset were miR-3535 (in the nucleus) and miR-27a-5p (in the cytoplasm). (**f**) miRNA-expression levels of nuclear (miR-3535) and cytoplasmic (miR-27a-5p) miRNAs identified by miRNA-seq were validated by RT-qPCR. miRNA levels were normalized to miR-186–5p and the mean value of the Nucleus 0 h group scaled to a value of one. Values are mean ± SEM, $n = 2$ (sRNA-seq), $n = 3$ (RT-qPCR), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA with Bonferroni *post hoc* test, comparisons are to the Cytoplasm 0 h group).

location-enriched miRNAs, and the nuclear up-regulation of miR-210-3p are similar between different cell lines derived from distinct lineages.

sRNA-seq analysis was performed using equal amounts of input RNA for each sample, and the RT-qPCR analysis performed above normalized according to a stable reference gene identified from the sequencing dataset. These experiments therefore assume that the amount of RNA in the nuclear and cytoplasmic fractions is equal. To address this potential confounding factor, RT-qPCR validation experiments were repeated using new C166 cultures in which a synthetic spike oligonucleotide (the *C. elegans* miRNA: cel-miR-39) was added to the nuclear and cytoplasmic fractions at the phenol extraction phase. This technical modification allowed for assumption-free RT-qPCR normalization independent of any difference in total RNA or total miRNA content between the nuclear and cytoplasmic fractions. Results for the miRNAs-of-interest (miR-27a-5p, miR-3535, miR-210-3p and miR-1291) were found to be very similar irrespective of whether the data were normalized to the exogenous spike (cel-miR-39)

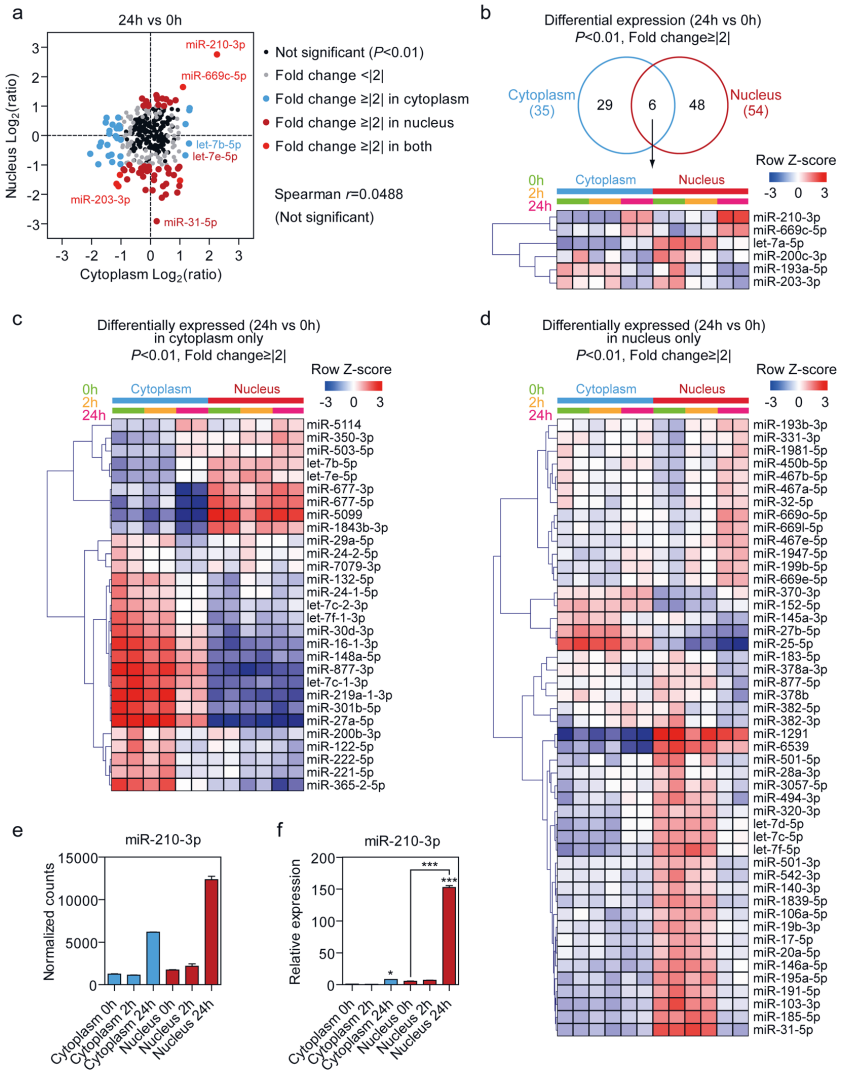

Figure 3. Differential expression of nuclear and cytoplasmic miRNAs upon hypoxic stimulus. (**a**) Comparison of expression ratios for nuclear and cytoplasmic libraries (0 h vs 24 h hypoxia). No significant correlation was observed between the nuclear and cytoplasmic expression ratios. (**b**) Heatmap of common differentially expressed miRNAs upon hypoxia in both nuclear and cytoplasmic samples. (**c**) Heatmap of the differentially expressed miRNAs in cytoplasmic fraction only. (**d**) Heatmap of the differentially expressed miRNAs in nuclear fraction only. Scale bars show mean-centered $\log_2$ normalized counts (row Z-score) where red and blue indicate higher and lower than mean abundance respectively. (**e**) miR-210-3p was identified to be highly up-regulated upon hypoxia in the nuclear fraction. (**f**) miR-210-3p expression in nuclear and cytoplasmic extracts was validated with RT-qPCR. miRNA-levels were normalized to miR-186-5p levels and the mean value of the Cytoplasm 0 h group scaled to a value of one. Values are mean ± SEM, $n = 2$ (sRNA-seq), $n = 3$ (RT-qPCR), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA and Bonferroni *post hoc* test, statistical comparisons are to the Cytoplasm 0 h group unless otherwise indicated).

or the endogenous reference (miR-186-5p) (Supplemental Fig. S5). miRNA expression levels and changes upon hypoxia in cytoplasmic and nuclear compartments were similar with both normalization methods used, indicating that the expressional changes observed are not related to possible different total RNA amount of different samples.

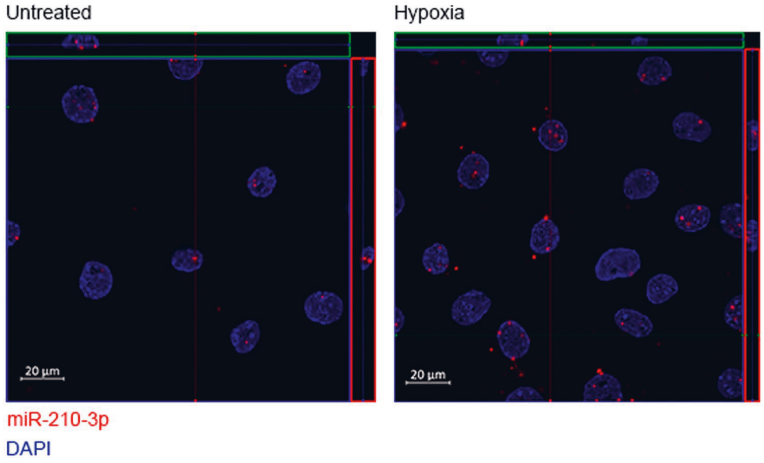

Figure 4. miR-210-3p localization upon hypoxic stimulus. miR-210-3p localization in C166 cells in normoxia and hypoxia (24 h) using fluorescent *in situ* hybridization (FISH). miR-210-3p is found in nucleus in normoxia and hypoxia. DAPI was used to stain nuclei of the cells. miR-210-3p is stained in red. To better display the co-localization of nuclear and miR-210-3p staining, areas within green and red rectangles show the view to the confocal microscopy image stack from the side.

Analysis of sequence motifs associated with enrichment in nuclear and cytoplasmic fractions. Given that it has been previously reported that a hexanucleotide motif (5′-AGUGUU-3′) located at the 3′ terminus of miR-29b-3p was capable for directing this miRNA to the nucleus[14] we sought to determine the nucleocytoplasmic distribution of miR-29 family members in our sRNA-seq data. miR-29b-3p was found to be significantly enriched in the nucleus by 2.4-fold (Fig. 5a). However, miR-29a-3p and miR-29c-3p (which lack the hexanucleotide motif) were similarly found to be significantly nuclear-enriched, although to a lesser extent; 1.8-fold and 1.5-fold respectively. The minor arm of miR-29a (i.e. miR-29a-5p) was also detected in our sequencing data, and was not significantly enriched in either fraction (Fig. 5a). We next searched for other detected miRNAs containing the hexanucleotide motif. Only two such miRNAs were identified; miR-199a-5p and miR-199b-5p, in which the motif was located towards the 5′ end, starting at position 4 (Fig. 5b). These miRNAs were modestly nuclear-enriched by 1.4-fold and 1.5-fold respectively, but dynamically changed their nucleocytoplasmic distribution upon hypoxic stimulus. Furthermore, in the unperturbed normoxic state (time 0 h) miR-199a-5p and miR-199b-5p were expressed at equivalent levels in both compartments (Fig. 5c). Taken together, these findings suggest that the miR-29b-3p hexanucleotide motif is not a *bone fide* nuclear-enrichment signal, at least in this context. Notably, several other studies have failed to detected enrichment of this motif in nuclear sRNA libraries, consistent with the findings reported here[11,12].

We next used *de novo* motif analysis to identify potential signals that might confer enrichment in either the nucleus or the cytoplasm. Two motifs were significantly enriched in nuclear-localized miRNAs, whereas four motifs were enriched in cytoplasm-localized miRNAs (Fig. 5d,e). Upon inspection of motif annotation results, it became apparent that in many cases, motifs were commonly identified in related miRNA family members. This effect was most strongly apparent for the let-7 family (Fig. 5f). As such, the empirically-determined nuclear enrichment signals 5′-UUGCAUAGU-3′and 5′-AGGUUGKSUG-3′(these motifs also have the potential to overlap) where found to be present in almost all of the let-7 family members that exhibited nuclear enrichment. Interestingly, nuclear enrichment was observed only for the 5′ arm (i.e. the dominant arm) of the let-7 family members. In contrast, 3′ arm let-7 family members (i.e. the minor arm that is expressed with ~10 times fewer read counts than the dominant arm) were almost exclusively enriched in the cytoplasm, and contained many of the empirically-identified cytoplasmic enrichment motifs. These findings demonstrate that the 5′ and 3′ arms of let-7 family miRNAs exhibit opposite nucleocytoplasmic localization. The detection of multiple related miRNAs is therefore likely to have skewed the motif enrichment analysis. It is consequently non-trivial to determine if the identified nuclear enrichment motifs are directing their associated miRNAs to distinct subcellular locations, or if they are identified as a consequence of multiple co-regulated miRNAs with high sequence similarity biasing the motif enrichment analysis. Specific details of the *de novo* motif enrichment analysis are described in Supplementary File S1.

Discussion

In this study we have profiled the subcellular localization of miRNAs in endothelial cells, and identified a set of miRNAs which exhibit nuclear enrichment. Furthermore, induction of hypoxia resulted in differential miRNA expression changes that were spatially restricted to either the nucleus or cytoplasm, or a small number of changes that occurred simultaneously in both compartments. This is the first study to profile subcellular

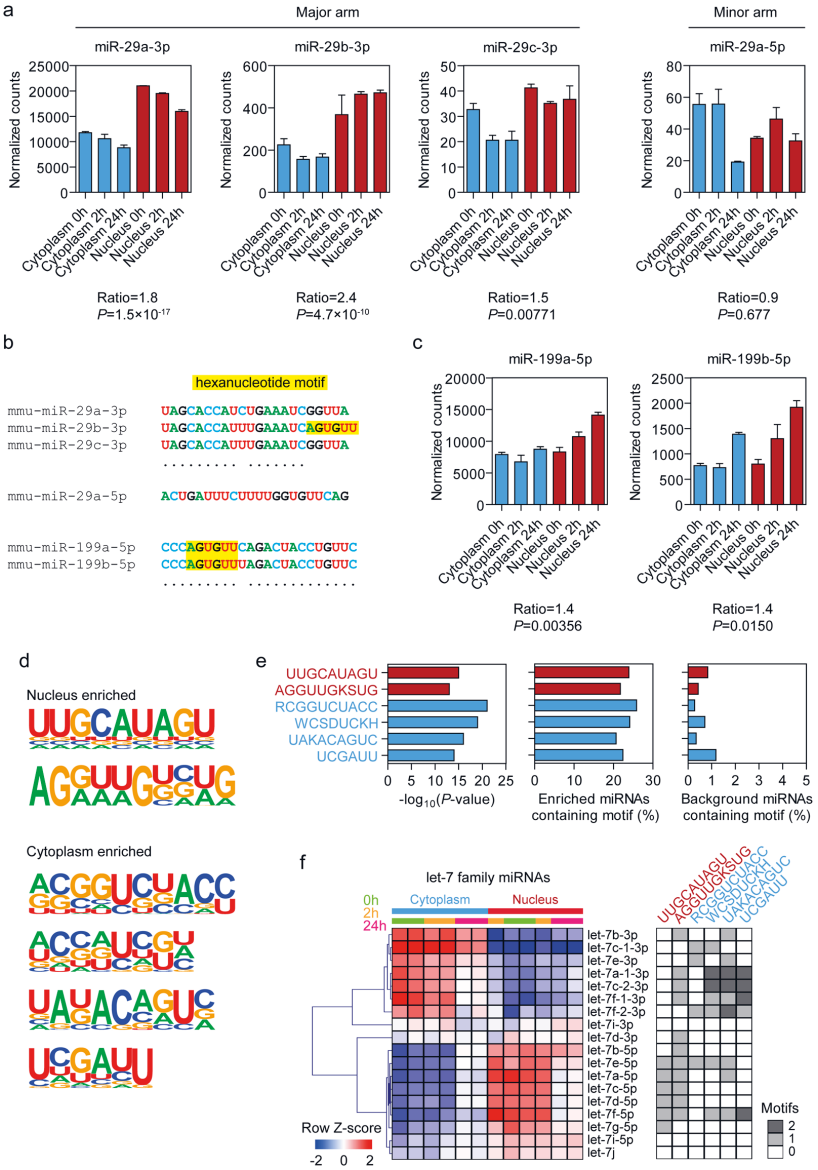

Figure 5. Analysis of putative miRNA sequence motifs affecting subcellular localization. (**a**) sRNA-seq normalized counts values for C166 nuclear and cytoplasmic fractions, with and without hypoxic stress, for the major arms of miR-29 family, and the minor arm of miR-29a-5p. (**b**) Alignments of all detected miR-29 miRNA family members, and miR-199a/b-5p. The positions of the 5′-AGUGUU-3′ hexanucleotide motif are indicated in yellow. (**c**) Normalized sRNA-seq counts for miR-199a/b-5p. (**d**) Nuclear and cytoplasmic enrichment sequences identified by *de novo* motif enrichment. (**e**) Output statistics for *de novo* motif discovery; enrichment *P*-values, percentage of enriched miRNAs in containing each motif, and percentage of non-enriched miRNAs (i.e. background) containing each motif. (For ambiguous nucleotides; K = U or G, S = G or C, R = A or G, W = A or U, D = A, G, or U, and H = A, C, or U). (**f**) Heatmap of sRNA-seq data for let-7 family members. Scale bars show mean-centered $\log_2$ normalized counts (row Z-score) where red and blue indicate higher and lower than mean abundance respectively. Occurrences of each enrichment motif in each family member are indicated in the greyscale heatmap. All values are mean ± SEM, $n = 2$ (sRNA-seq).

compartment-specific differential miRNA expression, and provides a strong rationale for the investigation of nuclear and cytoplasmic miRNA levels in other biological contexts.

Hypoxia-associated changes in miRNA levels in the nucleus are highly suggestive of functional significance. However, the mechanisms-of-action for nuclear miRNAs are at present incompletely understood. Studies of TGS and TGA mediated by exogenous small RNAs provided early clues that endogenous miRNAs might regulate transcription in a similar manner. Indeed, there have now been multiple studies that have demonstrated miRNA-mediated transcriptional regulation[7,26–33]. It is generally thought that in these instances, the miRNA directs an Argonaute-containing protein complex to non-coding, promoter-associated transcripts[26,27,29]. Exogenous small RNAs have also been shown to regulate pre-mRNA splicing decisions[34–36], although such effects have yet to be demonstrated for endogenous miRNAs. The observation that siRNAs are capable of silencing nuclear-localized target transcripts implied a similar activity for miRNAs[16,37,38]. Indeed, a recent study by Sarshad *et al*., showed that AGO2 protein is highly expressed in the nuclei of stem cells, where it mediates the silencing of nuclear transcripts, such as pre-mRNAs[39]. Aside from the regulation of gene expression, the miRNA processing machinery has also been implicated in the cellular response to DNA damage[40,41], the regulation of 3-dimensional chromatin structure[42], and regulation of the production of intron-included, truncated proteins as a consequence of direct interactions between the miRNA and genomic DNA[43].

Relative to the enormous research effort directed towards canonical miRNA activity, the nuclear targets and functions of specific miRNAs have so far been largely neglected. Further studies are needed to elucidate the mechanisms through which nuclear miRNAs execute their functions in different cell types and physiological conditions. Given the diversity of functional possibilities this presents a significant task. Analogous to the manner by which the post-transcriptional regulation of mRNA expression and translation can be fine-tuned by multiple miRNAs binding on multiple co-acting sites[44], multiple non-canonical mechanisms mediated by multiple miRNA may be acting on a gene in the nucleus at any one time. (For example, miRNAs also have the potential to target mRNA coding regions[45] and intronic sequences in newly transcribed pre-mRNA[39] simultaneously). One approach is to predict miRNA target sequences in regions that are considered non-canonical targets[46]. However, this strategy does not take into account the transcriptional landscape of the cell of interest, which is expected to be highly lineage specific (e.g. a gene expressed at very high levels might not be susceptible to gene activation). Predictions are also expected to have a high false positive rate, and so extensive experimental validation is required to identify *bona fide* miRNA-target interactions. Alternatively, crosslinking immunoprecipitation techniques can be used to empirically determine miRNA binding events[47,48]. These techniques suffer from the limitation of having relatively low resolution, and of being technically challenging.

miRNAs constitute a promising class of therapeutic target molecules that can be inhibited using antisense oligonucleotides or mimicked using synthetic siRNAs, shRNAs or artificial miRNAs[49]. Our group has previously demonstrated transcriptional regulation of *Vegfa* expression by promoter-targeted shRNAs *in vitro* and *in vivo*[9,10]. Depending on their target site, these shRNAs are able to either up-regulate (TGA) or down-regulate (TGS) *Vegfa* expression[9]. These shRNAs were shown to exert positive therapeutic effects in both hindlimb ischemia and myocardial infarction models[9,10]. Similarly, an siRNA which activates CEBPA expression[50] is being explored as a treatment for hepatocellular carcinoma with patients currently being enrolled for a phase I clinical trial sponsored by MiNA Therapeutics[51]. As such, nuclear miRNA functions offer new possibilities for the manipulation of gene expression, or may be novel targets in their own right.

Given that altered miRNA expression in hypoxia gives rise to multiple gene expression changes associated with canonical miRNA function[17], our data suggest that nuclear miRNAs may similarly regulate a wide variety of hypoxia-associated genes through non-canonical mechanisms. This is exemplified by the well-established hypoxamiR miR-210-3p, which was significantly increased in the nucleus of the cells upon hypoxic stimulus, and therefore likely regulates a plethora of nuclear targets. This notion is supported by a previous report showing the interaction between miR-210-3p and the nuclear lncRNA *XIST*[25]. Indeed, interaction between small RNAs and lncRNAs is a common theme. For example, miR-9 has been shown to downregulate *MALAT1* in the nucleus[52], and promoter-associated transcripts have been shown to regulate transcription of the *VEGFA* locus by acting as targets for TGA-inducing small RNA[53].

Notably, hypoxic conditions can vary in different physiological contexts with corresponding differences in the cellular gene expression response. This was demonstrated in a recent study in the context of the tumor microenvironment where the commonly-used, continuous, long-term hypoxia condition was compared to a shorter cyclic hypoxia protocol[54]. Previously-described hypoxamiRs were identified in the long-term hypoxia model, but cyclic hypoxic conditions resulted in a different expression pattern, with only 31 common miRNAs between these groups (including miR-210-3p). These observations illustrate the complexity of ncRNA regulation in hypoxia, an issue compounded when considering the possible functions of miRNAs in the nucleus.

Components of the RNAi machinery such as AGO2, Dicer, and GW182 (TRNC6A) have been shown to be present in the nucleus[16]. Given that Dicer processing and RISC loading are generally considered to be restricted to the cytoplasm[16], this would necessarily require mechanisms for the re-import of miRNAs to the nucleus. The karyopherins exportin-1 (XPO1)[55] and importin-8 (IPO8)[56] have been implicated in the transport of miRNA shuttling across the nuclear envelope. Recently, it was also shown the stress-induced response complex (SIRC) (consisting of AGO1, AGO2, YB1, CTCF, FUS, SMAD1, SMAD3, and SMAD4 proteins) is involved in nucleocytoplasmic trafficking of miRNAs in a cellular stress-dependent manner[57]. As such, RNA binding proteins and complexes like SIRC may regulate the subcellular distribution of miRNAs, as we observe also in response to hypoxic stimulus. Some studies have suggested that short sequence motifs might act as nuclear localization signals, as in the case of the 3′ terminal hexanucleotide motif in miR-29b-3p, presumably by recognizing specific RNA binding proteins such as ANT2 (ADP/ATP translocase 2)[58]. However, while we did observe a modest nuclear enrichment of miR-29b-3p, the related miRNAs miR-29a-3p and miR-29c-3p which lack the hexanucleotide motif were similarly nuclear-enriched. We have previously suggested an alternative hypothesis whereby

miRNAs are non-specifically shuttled between nucleus and cytoplasm and then become enriched in the compartment where their target transcripts are most concentrated[2,59,60].

In conclusion, this study demonstrates that miRNAs are differentially expressed in the nucleus and cytoplasm in response to hypoxic stress. These findings offer new insights into the molecular response to hypoxia, and for the function of miRNAs in general. Studies of ncRNA networks in gene regulation by our group and others significantly expand our understanding of the role of miRNAs beyond their canonical PTGS functions, and suggest that a vibrant world of ncRNA biology and regulatory potential resides within the nucleus.

Materials and Methods

Cell culture. C166 (yolk-sac-derived mouse endothelial cells, ATCC:CRL-2581), C2C12 (skeletal myoblast-derived mouse muscle cells, ATCC:CRL-1772), MS1 (pancreas/islet of Langerhans-derived mouse endothelial cells, ATCC:CRL-2279) or MOVAS (aorta-derived smooth muscle cells, ATCC:CRL-2797) cells were maintained under normal conditions (37 °C, 5% CO_2). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). For hypoxia experiments, cells were cultured in a hypoxia chamber with 1% O_2, 5% CO_2 (Baker Ruskinn).

Western blot. Protein samples were extracted from nuclear and cytoplasmic fractions according to protocol by Gagnon *et al.*,[18] and with TRI reagent (Molecular Research Center) according to the manufacturer's instructions. For western blot, equal volumes of extracted protein were loaded on precast gels (Mini-Protean TGX Stain-free Precast Gel, 4–20%, Bio-Rad) and transferred to nitrocellulose membranes (Trans-Blot Turbo Bio-Rad Midi, 0.2 μm nitrocellulose, Bio-Rad). The membranes were blocked with 5% milk for 1.5 hours at room temperature, washed with TBST (0.15 M sodium chloride, 0.050 m TRIS-HCl buffer; 0.05% Tween 20; pH 7.6) and incubated with antibodies against a known nuclear protein (anti-trimethyl-histone H3 (Lys27), Millipore, 1:2,500) and a cytoplasmic protein (anti-β-tubulin, Sigma Aldrich, 1:1,000) overnight at 4 °C. The membranes were washed with TBST and incubated with secondary antibodies (goat anti-rabbit IgG (H + L), HRP-conjugated, Invitrogen; anti-mouse IgG, HRP-conjugated, R&D systems; both 1:5,000) for 1 hour at room temperature. Membranes were analyzed using ECL Plus Western Blotting Substrate (Pierce) and imaged with the ChemiDoc Imaging System (Bio-Rad).

RNA extraction and small RNA-sequencing. For sRNA-sequencing samples, C166 cells were cultured in hypoxic conditions for 0, 2 or 24 hours and cells were separated into nuclear and cytoplasmic fractions, as described previously[18]. Briefly, the cells were collected from 15 cm plates by scraping and washed once with cold PBS. Cells were pelleted by centrifugation at 700 *g* for 5 minutes and lysed with hypotonic lysis buffer (10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM MgCl2, 0.3% (vol/vol) NP-40, 10% (vol/vol) glycerol) to collect the cytoplasmic fraction. Cytoplasmic RNA was obtained by ethanol precipitation overnight at −20 °C followed by re-extraction using TRI reagent. The remaining nuclear pellet was washed three times with the hypotonic lysis buffer, followed by extraction with TRI reagent according to the manufacturer's instructions. Libraries were prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina kit (New England Biolabs). Equimolar quantities of each library were pooled and small RNA sequencing performed as a service by Exiqon A/S (Vedbaek, Denmark). FASTQ files were trimmed using Trimmomatic[61] and aligned to reference genome (miRBase release 20) using bowtie2 (v2.1.0)[62]. Differential expression was tested using DESeq2 [20] which utilizes the Wald test for determining statistical significance. (Benjamini-Hochberg adjusted *P*-values are reported). Counts data for the full dataset are provided in Supplementary Data S1. sRNA-seq data were analyzed using the NormFinder method[21] which identified miR-186-5p as being stably expressed between all experimental groups. This miRNAs was subsequently selected as a reference gene for RT-qPCR data normalization. Alternatively, 15 fmol of cel-miR-39 ssRNA was added to the samples during phenol extraction phase and was used as spike-in to calculate relative expression of miRNAs.

RT-qPCR. Total RNA samples were treated with DNase I, RNase-free (Thermo Fisher Scientific) in order to eliminate genomic DNA contamination. For miRNA analysis, cDNA synthesis was performed using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) according to manufacturer's protocol and analyzed using miRNA-specific TaqMan assays (mmu-miR-27a-5p ID: 002445; mmu-miR-3535 ID: CTEPR23; mmu-miR-186-5p, ID: 002285, mmu-miR-210-3p ID: 000512; mmu-miR-1291, ID: 466942_mat; cel-miR-39-3p, ID: 000200; Thermo Fisher Scientific).

For expression analysis of lncRNAs and tRNAs, cDNA was synthesized using RevertAid Reverse Transcriptase (Thermo Scientific) and random hexamer primers (lncRNA) or gene-specific primers (tRNA) (reverse primers, Supplemental Table 1) and quantified using Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific). Thermal cycling was performed using a LightCycler480 (Roche) with the following program: 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 60 s at 60 °C. Primers for lncRNA and tRNA analysis are shown in Supplemental Table 1. RT-qPCR data were analyzed using the ΔΔCq method where normalization was available, or the ΔCq method for un-normalized data (validation of nuclear-cytoplasmic fractionation only). Statistical significance was assessed by one-way ANOVA with Bonferroni *post hoc* correction or *t*-test with Welch correction as appropriate (GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA)).

miRNA fluorescent *in situ* hybridization (FISH). For miRNA FISH, C166 cells were seeded on 8-well chamber slides and grown in normoxia or hypoxia (24 h). ViewRNA miRNA ISH Cell Assay Kit (Thermo Fisher Scientific) was used for the hybridizations according to the manufacturer's protocol. ViewRNA Cell Plus Probe Set (Thermo Fisher Scientific) was used to detect miR-210-3p (assay ID: VM1-10263-VCP, detection label Alexa

Fluor 546). Cell nuclei were visualized using DAPI stain. Pictures were taken using ZEISS LSM700 confocal microscope using 40× oil objective and analyzed with ZEN lite blue 2.6 software (ZEISS).

***de novo* motif enrichment.** Sequence motifs were identified in lists of miRNA sequences (enriched in either nucleus or cytoplasm) using HOMER (v4.9.1)[63]. Motifs were restricted to between 6 and 10 nucleotides, and analysis run in strand-specific mode using the findMotifsGenome.pl function. miRNA sequences were subsequently annotated to identify those which contained one or more HOMER-identified motifs using the annotatePeaks.pl function.

Data Availability

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus[64] and are accessible through GEO Series accession number GSE125390 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE125390).

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Author Contributions

M.P.T. and T.C.R. designed the study. T.A.T., P.L., M.-A.V. and P.K. performed the experiments. T.C.R. analyzed sequencing data. T.A.T., T.C.R., S.Y.-H. and M.P.T. wrote the paper. T.M., M.P.T. and S.Y.-H. acquired funding.

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RESEARCH ARTICLE

Nuclear microRNA-466c regulates *Vegfa* expression in response to hypoxia

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Abstract

MicroRNAs are well characterized in their role in silencing gene expression by targeting 3´-UTR of mRNAs in cytoplasm. However, recent studies have shown that miRNAs have a role in the regulation of genes in the nucleus, where they are abundantly located. We show here that in mouse endothelial cell line (C166), nuclear microRNA miR-466c participates in the regulation of vascular endothelial growth factor a (*Vegfa)* gene expression in hypoxia. Upregulation of *Vegfa* expression in response to hypoxia was significantly compromised after removal of miR-466c with CRISPR-Cas9 genomic deletion. We identified a promoter-associated long non-coding RNA on mouse *Vegfa* promoter and show that miR-466c directly binds to this transcript to modulate *Vegfa* expression. Collectively, these observations suggest that miR-466c regulates *Vegfa* gene transcription in the nucleus by targeting the promoter, and expands on our understanding of the role of miRNAs well beyond their canonical role.

Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs that typically repress genes by targeting the 3′-untranslated region (3′ UTR) of mRNAs via post-transcriptional gene silencing (PTGS), and can be considered endogenous triggers of RNA interference (RNAi) [1]. However, miRNA biology appears to be much more complex than traditionally thought, and RNAi machinery components have been found to be active in both the cytoplasm and nucleus [2]. We recently showed by small RNA-sequencing that miRNAs are abundant in the nucleus, that hypoxia induces changes in the miRNA levels both in the nucleus and cytoplasm, and that

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the majority of these changes occur preferentially in specific cellular compartments [3]. Recent studies have also identified nuclear roles for miRNAs [4]. Nuclear actions of small RNAs have been more extensively studied in other organisms such as plants, where early reports showed that non-coding RNAs were able to modulate DNA methylation [5]. However, recent studies in mammalian cells have suggested multiple novel modes of action for small RNAs, such as binding to long non-coding RNAs (lncRNAs) or regulating alternative splicing [4, 6]. For example, miR-9 has been shown to target a known lncRNA MALAT1 in the nucleus, leading to its degradation [7]. Small interfering RNAs (siRNAs) and double-stranded RNAs (dsRNAs) have been shown to regulate alternative splicing of mRNAs [8, 9] and similarly acting antisense oligonucleotides (ASOs) are already used clinically for exon skipping therapy [10]. To date, no studies on the regulation of alternative splicing by miRNAs have been published. However, this may likely be one of the nuclear roles of miRNAs, as splicing events occur quickly after transcription in the nucleus [11]. In addition, synthetic small RNAs and natural miRNAs have been shown to target gene promoters [12–17]. Interestingly, this promoter targeting can either repress or induce transcriptional gene expression [18]. It has been proposed that the targeted promoters express promoter-associated lncRNAs that are bound by the small RNAs. This recruits chromatin modifying factors to the site, inducing changes in the gene expression [19, 20]. Nonetheless, the ability to induce transcriptional gene activation (TGA) provides interesting therapeutic opportunities. We were the first to show this phenomenon occurs *in vivo* in a mouse model of hindlimb ischemia [18], and since that study, progress in therapeutic field has been made. TGA-based small activating RNA targeting C/EBP-α has been developed by MiNa Therapeutics [15] and was the first to enter phase I clinical trials for treatment of liver cancer [21].

Regulation of Vascular Endothelial Growth Factor A (VEGFA) levels is important for many gene therapy applications and has been under development for the treatment for conditions such as myocardial infarction and peripheral arterial disease. We have previously shown that TGA by shRNA targeting *Vegfa* promoter results in efficient treatment of hindlimb ischemia [18] and myocardial infarction [22]. Our sequencing study also identified a wide population of nuclear-localized endogenous miRNAs in endothelial cells [3]. Here we expand on our previous observations and investigate whether naturally occurring miRNAs endogenously target the *Vegfa* promoter and regulate its expression. We find that mmu-miR-466c targets the *Vegfa* promoter to regulate its expression through interactions with a promoter lncRNA, and that this interaction is important for *Vegfa* expression in hypoxia. Our observations suggest that in addition to artificial small RNAs, endogenous miRNAs are capable of also targeting and regulating gene promoters. Further, we find that these endogenous miRNAs function to upregulate gene transcription in the nucleus, as we show here with endogenous miR-466c.

Results

miR-466c is predicted to target the *Vegfa* promoter and is induced in the nucleus by hypoxia

We first performed a bioinformatic screen to identify putative miRNA target sites contained within the murine *Vegfa* promoter. A 700 bp fragment of DNA upstream of the annotated *Vegfa* transcription start site was retrieved and searched for target sites in both sense and antisense orientations using RegRNA 2.0 [23], which itself uses the miRanda algorithm [24] for target site prediction. Multiple members of miR-466c family were predicted to target *Vegfa* promoter (Fig 1A and 1B). Interestingly, both miR-466c-5p and miR-466c-3p were predicted to target sites on the promoter near sites previously described to be susceptible to shRNA mediated TGA [18] (Fig 1A). To analyze the expression of miR-466c in our experimental

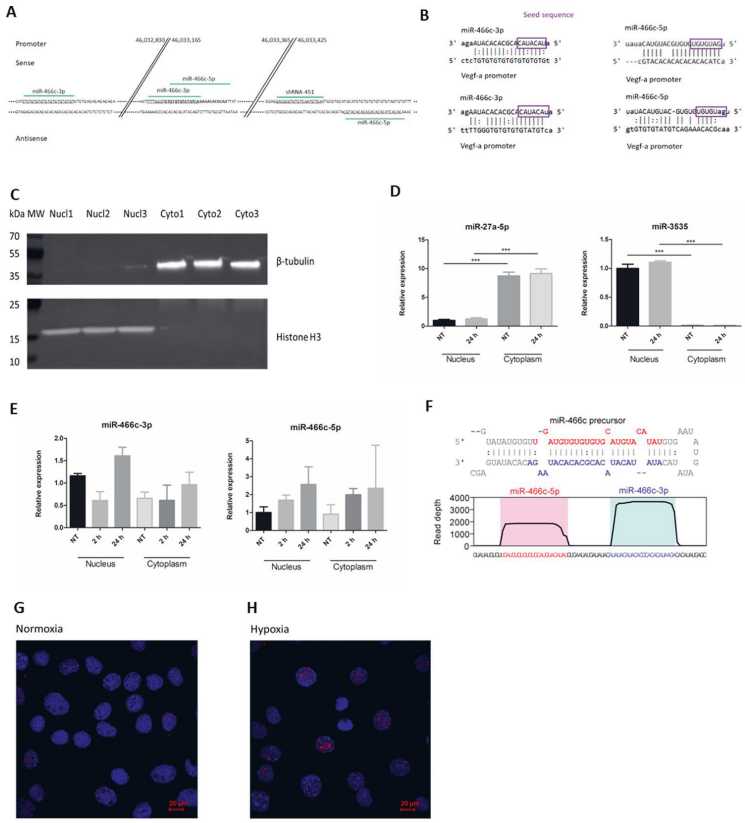

Fig 1. mmu-miR-466c is predicted to bind *Vegfa* gene promoter and is found in the nucleus of endothelial cells. A. Schematic figure showing the predicted targeted loci of mmu-miR-466c on *Vegfa* promoter. Numbers refer to locus on chromosome 17. shRNA-451 is published previously for targeting *Vegfa* promoter and upregulating its expression [18]. miR-466c targets were predicted using RegRNA 2.0. B. Sequence alignment showing miR-466c binding to promoter transcripts. Seed sequence of miR-466c is marked with purple box. C. Mouse endothelial cells (C166 cell line) were fractionated to nuclear and cytoplasmic fractions to study the compartmentalization of miRNAs. Purity of fractions was verified with western blot against cytoplasmic and nuclear markers β-tubulin and histone H3, respectively. n = 3. D. Purity of fractions was also verified by qPCR of cytoplasmic and nuclear miRNAs miR-27a-5p and miR-3535, respectively. NT is normoxic control sample, 24h is sample treated with hypoxia for 24h. n = 3, ANOVA with Bonferroni correction was used for determining the statistical significance, data is represented as mean ± SD. E. Both miR-466c-3p and miR-466c-5p were detected from both nucleus and cytoplasm of C166 cells by RT-qPCR. miR-466c expression was increased upon hypoxia, especially miR-466c-3p was induced in the nuclear fraction of the cells. NT is normoxic control sample, 2h and 24h are samples treated with hypoxia for 2h or 24h, respectively. n = 3–4, data is represented as mean ± SD. F. miR-466c precursor structure, from which both miR-466c-5p and miR-466c-3p are processed. miRBase graph shows how deep sequencing reads of the mature miRNAs are distributed and miR-466c-3p is the more abundant mature miRNA. G. miRNA-FISH for miR-466c-3p confirms the nuclear localization of the mature miRNA both in normoxia and hypoxia (24h timepoint). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

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setting, we next cultured mouse endothelial C166 cells in normoxia or hypoxia and collected samples at 2h or 24h for nuclear-cytoplasmic fractionation followed with RNA and protein isolation. Successful separation of nuclear and cytoplasmic fractions was confirmed by western

blot for histone H3 (nuclear marker) and β-tubulin (cytoplasmic marker) (Figs 1C and S1), and RT-qPCR for miR-3535 (nuclear) and miR-27a (cytoplasmic), consistent with our previous study [3] (Fig 1D). The expression of miR-466c, both 3p and 5p arms, was found to be increased upon hypoxia in C166 endothelial cells as analyzed by RT-qPCR (Fig 1E). The 3p arm was observed to be the most abundant form, both in nucleus and in the cytoplasm. This strand bias was also observed in publicly available small RNA-seq data taken from miRbase [25] (Fig 1F). We used RNA *in situ* hybridization (miRNA FISH) to visualize the localization of miR-466c-3p in normoxic and hypoxic C166 cells (Fig 1G and 1H). With miRNA FISH, we verified that miR-466c is observed in the nucleus both in normoxia and hypoxia.

miR-466c regulates *Vegfa* expression in endothelial cells

We next sought to see if manipulation of miR-466c levels in C166 cells would lead to changes in *Vegfa* expression using lentiviral vector overexpression system (LV-466). Transduction of C166 cells resulted in elevated levels of both miR-466c-3p and miR-466c-5p (Fig 2A). We analyzed the expression of both pre-*Vegfa* and mature mRNA *Vegfa* from cells transduced with LV-466 to determine any changes in transcription. Overexpression of miR-466c increased both pre-*Vegfa* and mature *Vegfa* levels 1.5-fold in these cells, which is consistent with previous observations with *Vegfa* upregulating shRNA [18]. These data indicate that miR-466c over-expression functionally activates *Vegfa* gene transcription (Fig 2B).

Removal of miR-466c significantly reduces *Vegfa* expression in hypoxic endothelial cells

To analyze the role of miR-466c in the regulation of *Vegfa* in hypoxic response, we used CRISPR-mediated gene editing to remove 97 bp region from the *Sfmbt2* intron 10 which contains the miR-466c hairpin from the parental C166 cell line (Fig 2C). Clonal cell populations were generated and screened for miR-466c expression, as well as miR-669c-3p, which is expressed from the same miRNA cluster in *Sfmbt2* intron 10, expression. *Sfmbt2* intron cluster contains two other miR-466c pre-miRNA hairpins, but removal of this one copy (mmu-mir-466c-1) was sufficient to dramatically reduce miR-466c expression, whereas miR-669c-3p expression was not changed, indicating that the CRISPR removal of miR-466c was not affecting the other miRNAs in this cluster (Fig 2D). After removal of miR-466c (466-del cell line), the hypoxia-mediated induction of miR-466c was lost (Fig 2E). *Vegfa* expression is known to be upregulated upon hypoxia [26]. Importantly, when miR-466c was removed, the upregulation of *Vegfa* expression in response to hypoxia was also diminished as determined by RT-qPCR of pre-*Vegfa* (Fig 2F) and mature *Vegfa* mRNA (Fig 2G). In addition, Vegfa protein expression was decreased after removal of miR-466c (Fig 2H). The expression of the parent gene, *Sfmbt2*, was also not found to be induced by hypoxic stimuli (Fig 2I). Rescue of miR-466c expression in the 466-del cell line via lentiviral overexpression was sufficient to increase *Vegfa* levels both in normoxic and hypoxic conditions, but hypoxia did not further induce *Vegfa* expression to the normal extent (Fig 2J).

To further analyze the changes in global gene expression, we performed whole transcriptomic sequencing from nuclear and cytoplasmic fractions of C166 and 466-del cell lines grown in normoxia and hypoxia. As depicted by PCA plot, gene expression profiles between the two cell lines differ remarkably both in cytoplasm and nucleus (Fig 3A). When looking at all differentially expressed genes in the cell lines, we observe that majority of the genes differentially regulated in the cell lines are shared (680 genes), whereas the mutation induces changes in expression of 305 genes that are not deregulated in parental cell line, and 264 genes are not differentially expressed in 466-del cell line even though they are found in the parental line

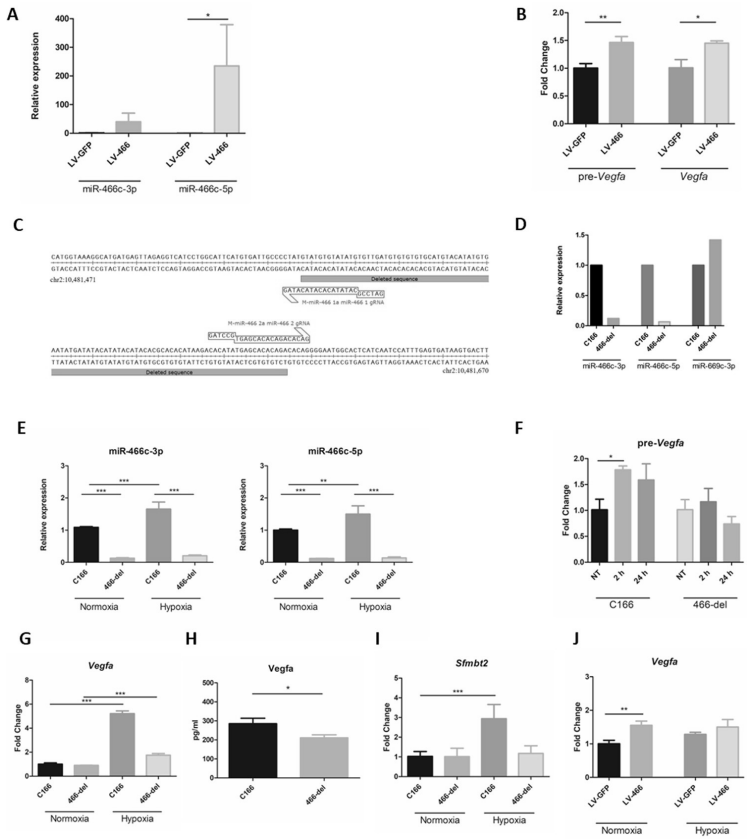

Fig 2. miR-466c participates in the regulation of *Vegfa* expression. A. Lentiviral expression of miR-466c increased both miR-466c-3p and miR-466c-5p levels in C166 cells. Cells were transduced with MOI 10 and samples collected 3d after transduction. C166 cells transduced with lentiviral vector expressing GFP only (LV-GFP) were used as control. n = 3, ANOVA with Bonferroni correction was used for determining the statistical significance, data is represented as mean ± SD. B. Overexpression of miR-466c (LV-466) in C166 cells resulted in increased transcription of pre-*Vegfa* and mature *Vegfa* as compared to control GFP only vector (LV-GFP), using RT-qPCR. n = 3, t-test was used for determining the statistical significance, data is represented as mean ± SD. C.To study the effects of knockdown of miR-466c, CRISPR-Cas9-mediated deletion of miR-466 from the C166 genome was performed. Schematic figure illustrates the deletion position in Sfmbt2 gene intron 10 in chromosome 2. D. Deletion of miR-466c resulted in a significant drop in miR-466c levels in the deletion cell line. qPCR for miR-669c, a miRNA expressed from the same cluster as miR-466c from Sfmbt2 gene intron 10, showed that CRISPR-Cas9-deletion of miR-466 precursor does not decrease the levels of miR-669c in the cells, whereas both strands of miR-466c are repressed. n = 1 E. Deletion of miR-466c resulted in a significant drop in miR-466c levels in the 466-del deletion cell line compared to parental C166 cells, and hypoxia did not induce miR-466c levels. n = 4, ANOVA with Bonferroni correction was used for determining the statistical significance, data is represented as mean ± SD. F. pre-*Vegfa* expression increases upon hypoxia in C166 cell line but does not change upon hypoxia in 466-del cell line. n = 3, ANOVA with Bonferroni correction was used for determining the statistical significance, data is represented as mean ± SD. *G. Vegfa* gene expression increases in hypoxic conditions in parental C166 cell line, but not to same extent in 466-del cell line. n = 4, ANOVA with Bonferroni correction was used for determining the statistical significance, data is represented as mean ± SD. H. Vegfa protein expression is decreased in 466-del cell line compared to parental C166 cells. n = 3, t-test was used for determining the statistical significance, data is represented as mean ± SD. I. Parental gene *Sfmbt2* expression is upregulated upon hypoxia in parental C166 cells, but not in miR-466-deletion cell line 466-del. n = 4, ANOVA with Bonferroni correction was used for determining the statistical significance, data is represented as mean ± SD. J.

Overexpression of miR-466c with lentiviral vector in miR-466c-deletion cell line does not return the hypoxia responsiveness to parental cell level. n = 4, t-test was used for determining the statistical significance, data is represented as mean ± SD. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

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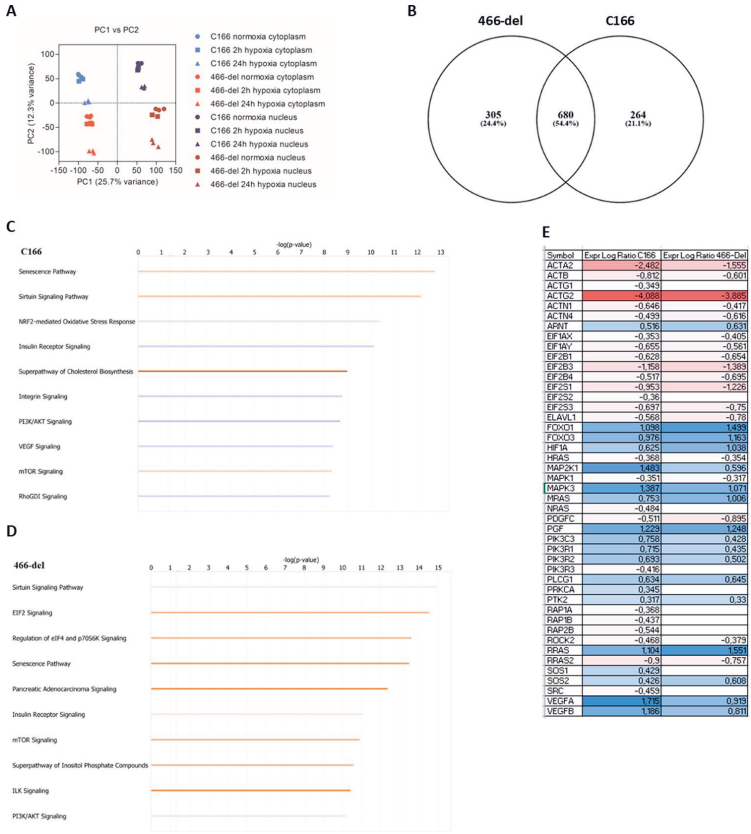
(Fig 3B). We analyzed the changes more closely using ingenuity pathway analysis (IPA). Many pathways where changes were associated were shared between the cell lines (sirtuin signalling, senescence, insulin receptor signalling, PI3K/AKT signalling, mTOR signalling), but some



Symbol	Expr Log Ratio C166	Expr Log Ratio 466-Del
ACTA2	-2,482	-1,555
ACTB	-0,812	-0,601
ACTG1	-0,349	
ACTG2	-4,088	-3,885
ACTN1	-0,646	-0,417
ACTN4	-0,439	-0,616
ARNT	0,516	0,631
EIF1AX	-0,353	-0,405
EIF1AY	-0,655	-0,561
EIF2B1	-0,628	-0,654
EIF2B3	-1,158	-1,383
EIF2B4	-0,517	-0,635
EIF2S1	-0,953	-1,228
EIF2S2	-0,36	
EIF2S3	-0,697	-0,75
ELAVL1	-0,568	-0,78
FOXO1	1,096	1,439
FOXO3	0,976	1,163
HIF1A	0,625	1,038
HRAS	-0,368	-0,354
MAP2K1	1,483	0,596
MAPK1	-0,351	-0,317
MAPK3	1,387	1,071
MRAS	0,753	1,006
NRAS	-0,484	
PDGFC	-0,511	-0,895
PGF	1,225	1,248
PIK3C3	0,758	0,428
PIK3R1	0,715	0,435
PIK3R2	0,693	0,502
PIK3R3	-0,415	
PLCG1	0,634	0,645
PRKCA	0,345	
PTK2	0,317	0,33
RAP1A	-0,368	
RAP1B	-0,437	
RAP2B	-0,544	
ROCK2	-0,468	-0,379
RRAS	1,104	1,551
RRAS2	-0,9	-0,757
SOS1	0,429	
SOS2	0,426	0,608
SRC	-0,453	
VEGFA	1,715	0,318
VEGFB	1,186	0,811

Fig 3. Total transcriptome sequencing of parental and miR-466c deletion cell lines shows significant differences on gene expression profiles. A. Both C166 and 466-del miR-466c deletion cell lines were subjected to hypoxia (2h or 24h), fractionated to nuclear and cytoplasmic samples and analyzed by RNA-sequencing. PCA plot of all sequenced groups shows that the samples are grouped by difference in nuclear and cytoplasmic transcripts, but also by cell line. B. Venn diagram of differentially expressed genes in parental C166 and 466-del deletion cell line shows that most changes in gene expression (680 differentially expressed genes) are still shared in both cell lines. C. Comparison analysis of 24h hypoxia vs. normoxia in cytoplasm of parental C166 cell line. Top 10 canonical pathways altered are shown in the figure, red colour indicates positive z-score, blue indicates negative z-score. D. Comparison analysis of 24h hypoxia vs. normoxia in cytoplasm of 466-deletion cell line. Top 10 canonical pathways altered are shown in the figure, red colour indicates positive z-score, blue indicates negative z-score. E. VEGF signalling pathway members were differentially regulated in C166 and 466-deletion cell line in same fashion, but to different extent.

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pathways were different (e.g. EIF2 signalling in mutation cell line and NRF2-mediate oxidative stress response in parental cell line) (Fig 3C and 3D). We also analyzed the individual members of VEGF signalling pathway, and noticed that most of the genes were still regulated in similar fashion after miR-466 removal (e.g. downregulated in both cell lines), but the extent of regulation was notably different (Fig 3E). The most differentially regulated VEGF signalling pathway member was MAP2K1, which was significantly downregulated following miR-466 deletion.

Identification of a non-coding transcript on the mouse *Vegfa* promoter

Previous studies demonstrated that transcriptional gene silencing (TGS) and transcriptional gene activation (TGA) may mechanistically require bidirectional transcription and/or promoter associated transcripts [16, 20, 27]. Promoter-associated transcripts for mouse *Vegfa* have not been previously characterized. Therefore, we performed Global Run-On-sequencing (GRO-seq) in order to determine if there is bidirectional transcription at the *Vegfa* promoter. Notably, the promoter was found to have an antisense transcript located up to ~27 kb upstream from its reported transcription start site (Fig 4A). Utilizing publicly available GRO-seq data, we can see that the promoter of *Vegfa* is transcribed also in other murine cell lines (3T3L1, AtT20, B-cell, Liver, MEF, Muscle, Neuron) (S2 Fig). For RT-qPCR confirmation of the transcript, cDNA was synthesized using antisense directional primers located at different promoter loci (nc-1800, nc-1500, nc-1300 and nc-500, where the number indicates the distance upstream of *Vegfa* TSS). qPCR was then performed with primer pairs at three different loci (-910 – -717, -675 – -477 and -485 – -317, relative to TSS) and confirmed the presence of antisense transcript on the proximal promoter of *Vegfa* (Fig 4B). These observations suggest that the promoter targeted shRNAs identified previously [18] and the miRNAs identified here may exert their action on the *Vegfa* promoter via interactions with this antisense transcript. The promoter sequence of *Vegfa* was further cloned into LV-vector, and the expression of the promoter non-coding RNA produced by the lentivirus was confirmed by RT-qPCR (Fig 4C). Interestingly, transduction of this non-coding RNA to C166 cells did not significantly affect *Vegfa* expression (Fig 4D). These data suggest that the ncRNAs may require the correct chromosomal context.

To show the miR-466c targeting of promoter-associated transcript on *Vegfa* promoter, we performed biotin pulldown assay using biotinylated miR-466c mimics transfected to C166 cells. Since the promoter transcript was detected at very low levels in RT-qPCR, we first increased its expression by lentiviral vector (LV-prox). RT-qPCR analysis showed that miR-466c-3p binds the proximal promoter transcript, and we detect the pulled down transcript at locus -485 – -317 and -675 – -477 bp upstream from TSS (Fig 4E), consistent to the bidirectional RT-qPCR analysis of LV-prox induced transcript (Fig 4B). We also performed luciferase reporter assay to analyze the binding of miR-466c to the promoter transcript. Promoter sequence (S1 Table) was cloned into luciferase reporter plasmid and transfected to HEK293T cells together with miR-466c-3p, miR-466c-5p or control mimic. miR-466c-3p showed significant reduction in luciferase activity compared to control and is therefore able to bind the transcript on *Vegfa* promoter.

Discussion

Previous studies have shown that, in addition to well described role in silencing of gene expression by targeting 3′-UTR of mRNAs in cytoplasm (post-transcriptional gene silencing, PTGS), miRNAs have a role in the regulation of gene expression in the nucleus by TGS and TGA. We showed recently that vast number of miRNAs are preferentially located in the nuclear fraction of endothelial cells and that their distribution between cytoplasm and nucleus is affected by

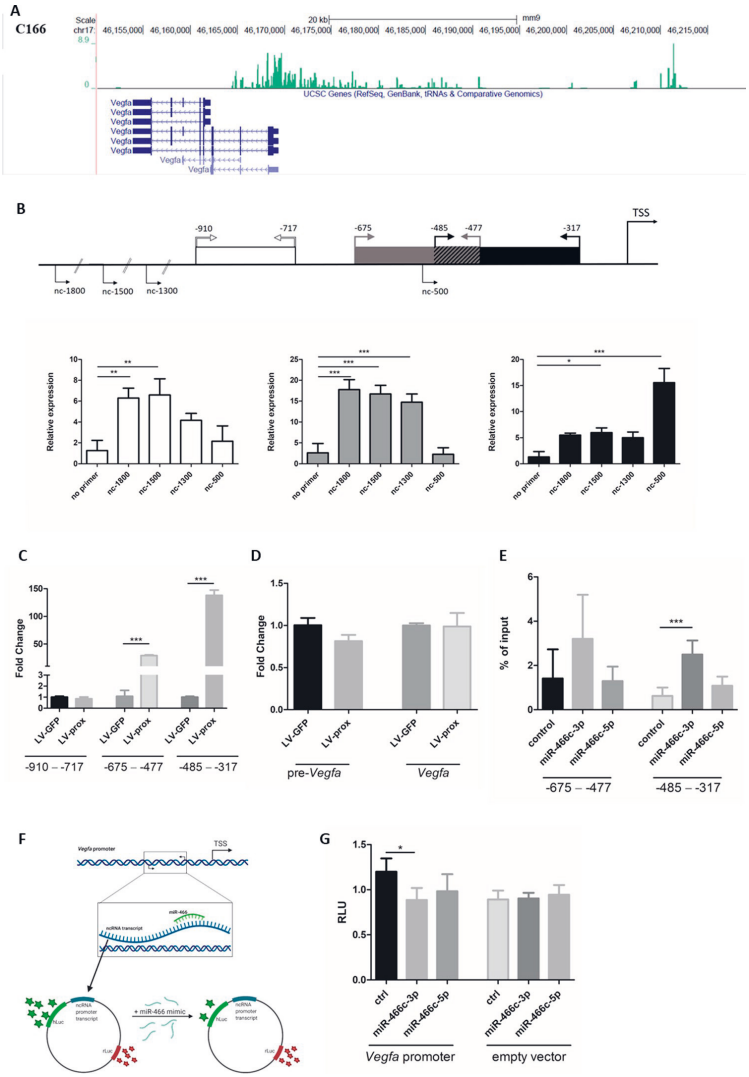

Fig 4. Murine *Vegfa* promoter is associated with antisense non-coding transcript, which is the target for miR-466c. A. GRO-seq shows antisense lncRNA at *Vegfa* promoter in C166 cells. B. Antisense ncRNA is detected from *Vegfa* promoter using strand-specific qPCR. ncRNA is found from the regions that were predicted for miR-466c targeting. n = 3, ANOVA with Bonferroni correction was used for determining the statistical significance, data is represented as mean ± SD. C. qPCR of ncRNA shows increased levels of antisense ncRNA after transduction with lentiviral vector encoding the proximal promoter region (LV-prox). n = 3, t-test was used for determining the statistical significance, data is represented as mean ± SD. D. Overexpression of *Vegfa* promoter-associated ncRNA with lentiviral vector (LV-prox) does not affect expression levels of *Vegfa*. n = 3, data is represented as mean ± SD. E. Biotinylated miR-466c mimic was used to pulldown RNA transcripts binding to miR-466c. *Vegfa* promoter associated ncRNA was detected from the pulldown samples by RT-qPCR. n = 5, ANOVA with Bonferroni correction was used for determining the statistical significance, data is represented as mean ± SD. F. Schematic illustration of luciferase

assay design. Figure was created with BioRender.com. Republished from BioRender.com under a CC BY license, with permission from BioRender.com, original copyright 2022. G. Direct interaction between miR-466c and *Vegfa* promoter-associated ncRNA was confirmed using luciferase assay, where miR-466c-3p transfection resulted in significant regulation compared to control. Empty luciferase vector showed no difference with any of the transfected mimics. n = 4, t-test was used for determining the statistical significance, data is represented as mean ± SD. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

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stimulus such as hypoxia [3]. Non-canonical roles of miRNAs are still largely unknown and less studied than their action in cytoplasmic RNAi. However, since miRNAs are abundantly present as mature miRNAs in the nucleus, it thus seems likely that they participate in nuclear functions.

We have previously demonstrated regulation of *Vegfa* expression by transducing cells with lentiviral vectors encoding promoter-targeted shRNAs. Depending on their target site, shRNAs are able to upregulate (TGA) or downregulate (TGS) *Vegfa* expression [18]. The shRNAs were shown to have efficient therapeutic function both in hindlimb ischemia and myocardial infarction models [18, 22]. These observations suggested that shRNAs may function by mimicking structurally similar endogenous miRNAs that could act in a same manner *in vivo* and potentially regulate *Vegfa* expression by targeting *Vegfa* promoter. In the study presented here, such nuclear miRNA with target sites in the murine *Vegfa* promoter was identified. Some miR-466c predicted targets were close to the previously described site that is subjectable to TGA by shRNA [18]. The shRNA was designed to be fully complementary to the promoter sequence, whereas for endogenous miRNAs, binding is more partial although the binding of seed sequence is considered often critical. Our analysis shows that miR-466c exhibits partial complementarity, also from other parts than seed sequence, to the *Vegfa* promoter at several sites.

As seen from miRBase, miR-466c-3p is more abundantly expressed across studies. However, miR-466c-5p, although expressed at lower levels, is found both in our analysis by RT-qPCR in C166 cells, as well as in other studies by sequencing, as represented by miRBase data. The processing of miRNAs and miRNA strand selection may occur differently in different cell types or conditions. In many cases, processing of miRNA leads to one functional "active strand" which is incorporated into RISC and the other "passenger strand" is degraded. However, both strands of miRNA may be equivalently functional and participate in gene regulation, possibly by coordinated pathways [28]. We speculate that this may also be the case with miR-466c in hypoxic endothelial cells, where both strands could have a regulatory and compensatory role in the regulation of *Vegfa*. This might even occur by targeting both promoter via TGA and mRNA 3′UTR via traditional PTGS and thus creating a regulatory network loop to fine-tune the expression of essential genes.

The miR-466c precursor resides within intron 10 of the polycomb gene *Sfmbt2* [29]. *Vegfa* is known to be regulated in response to hypoxia and the observations presented here demonstrate that *Sfmbt2* expression is similarly increased upon hypoxia, as does the expression of miR-466c. Overexpression of miR-466c using lentiviral vector (LV-466) increased pre- and mature mRNA levels of *Vegfa*. *Vegfa* is regulated on multiple levels post-transcriptionally, including increased stability of mRNA upon hypoxia [30], but our result clearly indicates increased transcription of the gene in case of overexpression of miR-466c, thereby indicating potential TGA by miR-466c. Removal of miR-466c by CRISPR decreased Vegfa protein levels but did not induce any changes in *Vegfa* mRNA expression at basal levels, but this may be due to the longer timepoint (4d) used in protein experiments and accumulation of Vegfa protein to the cell culture medium. However, deletion of miR-466c impaired the upregulation of *Vegfa* typically observed following hypoxic stimulus. It is possible that other compensatory

mechanisms regulate basal level expression of *Vegfa*, but miR-466c is essential for the induction of gene expression followed by hypoxic stimulus. When miR-466c was returned to the miR-466c-deleted cell line by lentiviral overexpression, we detect upregulation in *Vegfa* expression already at normoxia, corresponding to the overexpression experiment in parental intact C166 cells. However, complete restoration of hypoxia-induced upregulation does not occur in the absence of endogenous miR-466c.

Previous research has shown that TGS and TGA often require the presence of non-coding transcripts on the targeted promoter [20]. Studies on the human *VEGFA* promoter have identified promoter-associated antisense and sense transcripts which would be bound by promoter targeted small RNAs [31, 32]. We performed GRO-seq and located an antisense RNA transcript on the mouse *Vegfa* promoter in C166 endothelial cells and confirmed it by directional RT-qPCR. This promoter-associated RNA transcript is most likely the target of the miRNAs and shRNAs found to target and regulate *Vegfa* promoter and affect hypoxia induced modulation of *Vegfa*. This was verified with biotin pulldown and luciferase reporter assays, which both showed miR-466c to target and directly bind the promoter transcript. It is possible that the target abundance determines the localization of miRNA, as has been observed with cells transfected with siRNA that selectively shuttle to those compartments containing the small RNA target [33]. Importin-8 has been shown to transport the mature miRNAs from cytoplasm to the nucleus together with Ago2 [34]. Sequencing experiments have shown that miRNA abundance overlaps between nucleus and cytoplasm, suggesting that nuclear miRNAs are shuttled to and from the cytoplasm [2, 35].

The location in the promoter where shRNA was designed to target and where miR-466c was predicted to target *Vegfa* is a "hot spot" for *Vegfa* activation and contains Hif1α and Yy1 binding sites and a cAMP response element (CRE). It is well known that hypoxia induces Hif1α and Yy1 expression [36–38]. Both of these transcription factors have predicted target loci in the *Sfmbt2* promoter as well and therefore could be involved in the regulation of *Sfmbt2* induction in hypoxia, which as shown here results in increased miR-466c expression. It is likely that pre-miRNAs expressed from the genome are processed in multiple steps both in nucleus (Drosha/DGCR8) and in cytoplasm (Dicer) [39] and some mature miRNAs are directed and shuttled back to nucleus. However, some publications have shown the existence of nuclear Dicer and RNAi factors, so it is possible that nuclear miRNAs are processed completely in the nucleus [2, 40]. *Vegfa* promoter harbours a ncRNA transcript, which can be bound by miR-466c to recruit TGA-associated chromatin modifying proteins to the site [18]. We have previously shown that preventing CBP-CREB interaction inhibited the shRNA-mediated Vegfa activation [22], which is likely due to the conserved CRE [41] located at the shRNA target site. Also, the predicted target sites of miR-466c on the *Vegfa* promoter are in proximity to the CRE and therefore the molecular mechanism of miR-466c could be similar to the action of the shRNA. A putative model for the *Vegfa* regulation by miR-466c in hypoxia based on these observations is presented in Fig 5.

Collectively, the observations presented here regarding the nuclear functions of miR-466c, along with several previous reports, suggest that small non-coding RNAs, both endogenously and exogenously expressed, modulate gene activation in the nucleus by a naturally occurring biological mechanism that also involves interactions with promoter associated long ncRNAs. This hypoxia induced ncRNA network of transcriptional regulation significantly expands our understanding of the role of miRNAs well beyond their canonical role in regulating PTGS and ultimately suggests that a vibrant world of ncRNAs and regulatory potential resides in the nucleus.

miR-466c is expressed from *Sfmbt2* intron 10 and processed to mature miRNAs, miR-466c-3p and miR-466c-5p. Mature miRNAs are transported back to the nucleus, where they re-

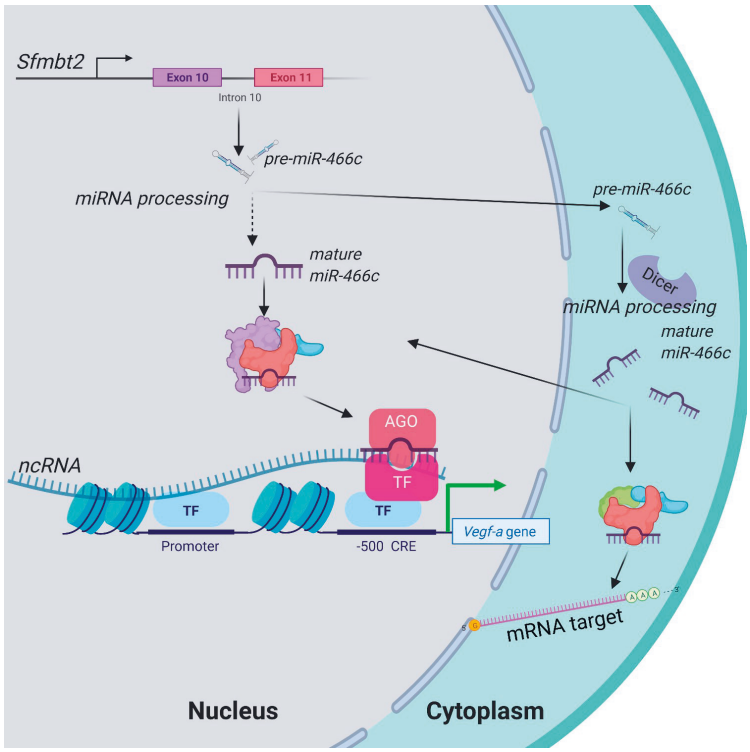

Fig 5. Schematic illustration depicting miR-466c targeting of murine *Vegfa* promoter.

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locate to their target sites in non-coding RNA on *Vegfa* gene promoter. Binding of miR-466c to the promoter-associated ncRNA recruits transcription factors and epigenetic modifiers to the chromatin locus, thus resulting in upregulation of *Vegfa*. Figure was created with *BioRender.com*. Republished from BioRender.com under a CC BY license, with permission from BioRender.com, original copyright 2022.

Materials and methods

Cell culture

C166 (yolk-sac derived mouse endothelial cell line, ATCC:CRL-2581), 466-del and HEK293T (ATCC: CRL-11268) cells were maintained under normal conditions (37˚C, 5% CO_2) in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Germany) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 1% penicillin-streptomycin (PS) (Thermo Fisher Scientific). In hypoxia experiments, cells were cultured for 2h or 24h in a hypoxia chamber with 1% O_2, 5% CO_2 (InvivO2, Baker Ruskinn, UK). The nuclear-cytoplasmic fractioning of the cells was performed as described previously [42].

miRNA target predictions

Mouse *Vegfa* promoter sequence encompassing 700 bp of upstream promoter sequence of the *Vegfa* gene was retrieved from Genome Browser [43] (https://genome.ucsc.edu/; Mouse genome Dec. 2011 (GRCm38/mm10). Promoter sequence was submitted to RegRNA 2.0 [23] (http://regrna2.mbc.nctu.edu.tw/detection.html) tool to predict targeting miRNAs.

Western blot

Protein samples were extracted from nuclear and cytoplasmic fractions according to protocol by Gagnon et al. [42] and with TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. For western blot, equal volumes of extracted protein were loaded on precast gels (Mini-Protean TGX Precast Gel, 4–15%, Bio-Rad, USA) and transferred to nitrocellulose membranes (Trans-Blot Turbo Midi 0.2 μm Nitrocellulose Transfer Packs, cat. #1704159, Bio-Rad). The membranes were blocked with 5% milk for 1 hour at room temperature, washed with TBST (0.15 M sodium chloride, 0.050 M TRIS-HCl buffer; 0.05% Tween 20; pH 7.6) and incubated with antibodies against a known nuclear protein (anti-trimethyl-histone H3 (Lys27), 1:2,500 dilution, cat. 07–449, Merck, Germany) and a cytoplasmic protein (anti-β-tubulin, 1:1,000 dilution, cat. T5201, Sigma-Aldrich) overnight at 4˚C. The membranes were washed with TBST and incubated with secondary antibodies (alexa fluor 488 goat anti-rabbit IgG (H + L), 1:3,000 dilution, cat. A11034, Invitrogen by Thermo Fisher Scientific; alexa fluor 488 goat anti-mouse IgG, 1:2,500 dilution, cat. A11001, Invitrogen by Thermo Fisher Scientific) for 1 hour at room temperature. Membranes were washed with TBST and imaged with the ChemiDoc MP Imaging System (Bio-Rad).

RNA extraction, cDNA synthesis and RT-qPCR

RNA was extracted from nuclear and cytoplasmic fractions according to protocol by Gagnon et al. [42] using TRI reagent (Sigma-Aldrich). Total cellular RNA was extracted with TRI reagent (Sigma Aldrich) according to the manufacturer's instructions and treated with DNase I, RNase-free (Thermo Fisher Scientific). For miRNA analysis, cDNA synthesis was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific) according to manufacturer's protocol and analyzed with qPCR using miRNA-specific TaqMan assay (mmu-miR-466c-3p ID: 464896_mat; mmu-miR-466c-5p ID: 463771_mat, mmu-miR-669c-3p ID: 464620_mat, hsa-miR-27a-5p ID: 002445; mmu-miR-3535 ID: CTEPR23; Thermo Fisher Scientific). For gene expression analysis, cDNA was synthesized using RevertAid Reverse Transcriptase (Thermo Fisher Scientific) and random hexamer primer (Thermo Fisher Scientific). In experiments detecting non-coding RNA transcript on *Vegfa* promoter, specific primers (nc-500, nc-1300, nc-1500 and nc-1800; listed in S1 Table, all from Integrated DNA Technologies IDT, USA) were used in cDNA synthesis. cDNA quantification was performed using TaqMan Gene Expression Assays (*Vegfa* ID: Mm00437306_m1; *Sfmbt2* ID: Mm00616783_m1; endogenous control *Gapdh* ID: Mm99999915_g1; *Actb* ID: Mm00607939_s1, Thermo Fisher Scientific) and custom designed primers (pre-*mVegfa* and *Vegfa* promoter primers, Integrated DNA Technologies IDT) (S1 Table). Samples were quantified by using Maxima Probe/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific). Thermal cycling was performed using a LightCycler® 480 Instrument II (Roche, Switzerland) with the following program: 10 min at 95˚C, followed by 50 cycles of 15 s at 95˚C and 60 s at 60˚C. miRNA RT-qPCR was started with an additional step of 2 min at 50˚C. RT-qPCR data were analyzed using the ΔΔCq method where normalization was available, or the ΔCq method for un-normalized data.

miRNA fluorescent *in situ* hybridization (FISH)

For miRNA FISH, C166 cells were seeded on 8-well chamber slides and grown in normoxia or hypoxia (24h). ViewRNA miRNA ISH Cell Assay Kit (Thermo Fisher Scientific) was used for the hybridizations according to the manufacturer's protocol. ViewRNA Cell Plus Probe Set (Affymetrix/Thermo Fisher Scientific) was used to detect mmu-miR-466c-3p (miR-466p-3p, assay ID: VM1-21572, detection label Alexa Fluor 546). Cell nuclei were visualized using DAPI stain. Pictures were taken using ZEISS LSM700 confocal microscope using 40× oil objective and analyzed with ZEN lite blue 2.6 software (ZEISS, Germany).

Lentiviral transductions

For overexpression of miR-466c, 255 bp locus of genomic sequence from Sfmbt2 intron 10, which contains the miR-466c hairpin (S1 Table), was cloned into third generation human immunodeficiency virus 1 (HIV-1)–based LV-PGK-GFP-U6-RNA vector (LV-466). For overexpression of *Vegfa* promoter ncRNA, genomic sequence from *Vegfa* promoter region between -671 – -115 bp relative to TSS was cloned into the lentiviral vector (S1 Table). As a control, we used lentivirus (LV) encoding only GFP (LV-GFP). The vectors were prepared by standard calcium phosphate transfection method in 293T cells [44]. C166 cells were transduced with lentiviral vector expressing either mmu-miR-466c (LV-466) or proximal non-coding RNA (LV-prox) using MOI 10 and samples were collected 3 days after the transduction (LV-466) or after three weeks of culturing (LV-prox).

Deletion of miR-466c with CRISPR

In order to remove miR-466c from intron 10 of *Sfmbt2*, two guide RNAs were cloned into separate expression plasmids (pcDNA-H1-sgRNA) (S1 Table) and transfected into C166 cells along with Cas9 plasmid co-expressing GFP (PX458, Addgene, USA) using Nucleofector I (Amaxa/Lonza Bioscience, Germany). Based on GFP positivity, single cells were sorted into 96-well plate wells using sorting FACS (BD FACSARIA III Cell Sorter, BD Biosciences, USA) and clonal cell populations established. Cultures were genotyped by PCR (primers listed in S1 Table) to identify cells that contained the desired deletion, and positive clones further confirmed by Sanger sequencing.

Enzyme-linked immunosorbent assay (ELISA)

C166 and 466-del cells were split to 6-wells (30 000 cells/1 ml/6-well; three replicates each cell line) in no phenol red DMEM with 10% FBS and 1% PS. After four days, medium samples were collected and centrifuged at +4 C 18 000 g for 20 min. Supernatants were discarded and samples resuspended to 100 ul NP-40 buffer (150 mM NaCl, 0,1% Triton X-100, 50 mM Tris-HCl, 1x protease inhibitor). Total protein amount of the samples was measured using BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer's protocol. 10 ug total protein was used for mVegf ELISA assay (Quantikine ELISA, mouse VEGF, Cat. No.: MMV00) according to manufacturer's protocol. Victor^2 WALLAC 1420 Multilabel counter (PerkinElmer) was used to measure wavelength 450nm and background wavelength 544nm was subtracted from those values.

Whole transciptome sequencing and analysis

Parental C166 cells or miR-466c-deletion cell line (466-del) were cultured in normoxia or hypoxia for 24h. Cells were fractionated to nuclear and cytoplasmic fractions and RNA was isolated as described earlier. Whole transcriptome sequencing was performed as a service from

Exiqon A/S (Vedbaek, Denmark). The library preparation was done using TruSeq® stranded total RNA sample preparation kit with rRNA depletion (Illumina, USA). The starting material (300 ng) of total RNA was rRNA depleted using biotinylated, target-specific oligos combined with Ribo-Zero rRNA removal. The isolated mRNA was subsequently fragmented using enzymatic fragmentation. Then first strand synthesis and second strand synthesis were performed and the double stranded cDNA was purified (AMPure XP, Beckman Coulter, USA). The cDNA was end repaired, 3' adenylated and Illumina sequencing adaptors ligated onto the fragments ends, and the library was purified (AMPure XP). The mRNA stranded libraries were pre-amplified with PCR and purified (AMPure XP). The libraries size distribution was validated and quality inspected on a Bioanalyzer high sensitivity DNA chip (Agilent Technologies, USA). High quality libraries were quantified using qPCR, the concentration normalized and the samples pooled according to the project specification (number of reads). The library pool (s) were re-quantified with qPCR and optimal concentration of the library pool used to generate the clusters on the surface of a flowcell before sequencing on Illumina HiSeq2500 instrument using hiSeq v.4 reagents (51 cycles) (Illumina, USA.). Experiment setting was 30 million 50 bp paired-end reads. Exiqon data analysis pipeline based on the Tuxedo software package, employing software developed internally at Exiqon to interpret and improve the readability of the final results. The components of our NGS RNA seq analysis pipeline include Bowtie2 (v. 2.2.2), Tophat (v2.0.11) and Cufflinks (v2.2.1). Annotation was done using Mus musculus Reference genome GRCm38, Annotation reference: Ensembl_81. The data is available in NCBI's Gene Expression Omnibus64 [45] accessible through GEO Series accession number GSE174483 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE174483).

Global Run On -sequencing (GRO-seq)

Global Run On -sequencing was performed for C166 cells according to previously published protocol [46]. For the analysis of *Vegfa* promoter transcription, publicly available GRO-seq datasets from seven other cell types were used (3T3L1 [47], GEO: GSE56747; AtT20 [48], GEO: GSE64515; B-cell [49], GEO: GSE62296; Liver [50], GEO: GSE59486; MEF [51], GEO: GSE27037; Muscle [52], GEO: GSE26512; Neuron [53], GEO: GSE66703).

Biotin-labeled miRNA pulldown

C166 cells were transduced with LV-prox and cultured for three weeks before beginning of biotin pulldown experiment. Cells were cultured to 10 cm plates (1,5x 10^6 cells/plate) on day 1. On day 2, medium was changed and transfections with biotin-labeled miRNAs (mmu-miR-466c-3p ID: HRIZN-009055; mmu-miR-466c-5p ID: HRIZN-009047; cel-miR-67 (negative control siRNA) ID: HRIZN-009049; Dharmacon/Horizon Discovery, UK) were done by using TransIT-TKO® Transfection Reagent (Mirus Bio, USA) according to the manufacturer's instructions using final concentration of miRNAs 40 nM (280 pmol) and five replicates. On days 3 and 4, pulldown protocol was done according to the protocol of Wani & Cloonan [54]. Apart from the protocol, 50 μl of Dynabeads MyOne Streptavidin C1 magnetic beads (Thermo Fisher Scientific) were used per one sample. RNeasy Mini Kit (Qiagen, Germany) was used to extract RNA. Elution was done by eluting twice with 30 μl of molecular biology water. 8 μl of each sample was used for DNase treatment and cDNA synthesis like previously described.

Luciferase assay

Vegfa promoter sequence (-538 – -344 bp relative to TSS) was ordered as duplex DNA oligo from IDT (S1 Table) and was cloned into miTarget™ miRNA 3' UTR Target Clone plasmid (MmiT028449-MT06, GeneCopoeia, USA). HEK293T cells were seeded at a density of 100

000 cells/well on 12-well plate. The following day, cells were co-transfected with mimics (mmu-miR-466c-3p ID: MIMAT0004878; mmu-miR-466c-5p: MIMAT0004877; negative control siRNA cat. C-121964-00-20; Dharmacon) and miTarget™ miRNA 3' UTR Target Clone plasmid containing the *Vegfa* promoter (S1 Table) or negative control vector plasmid (CmiT000001-MT06, GeneCopoeia). Transfection was done using co-transfection protocol from TransIT-TKO transfection reagent according to manufacturer's instructions (TransIT-TKO (Mirus Bio) for miRNA, TransIT-2020 (Mirus Bio) for plasmid), where 1 μg of plasmid DNA was transfected to the cells by using 3 μl of TransIT-2020 transfection reagent. After 24h, cells were washed once with PBS, collected, and used for luciferase assay. Luciferase assay was performed using Luc-Pair™ Duo-Luciferase Assay Kit 2.0 (GeneCopoeia) according to manufacturer's instructions. Luminescence measurements were performed using CLARIOstar plate reader (BMG Labtech, Germany) with emission wavelength of 580 nm for Firefly Luciferase and 480 nm Renilla Luciferase. Results are presented as the ratio of RLU(Firefly): RLU (Renilla).

Statistics

Statistical significance was assessed by one-way ANOVA with Bonferroni post hoc correction or unpaired two-tailed t-test with Welch's correction as appropriate (GraphPad Prism 5 (GraphPad Software, USA)). Outliers were tested using GraphPad's Outlier calculator (https://www.graphpad.com/quickcalcs/Grubbs1.cfm). Data are presented as mean±SD. Differences were considered significant when $P \leq 0.05$.

Supporting information

S1 Fig. Unmodified blots. Uncropped and unadjusted images of western blots (used to generate data blots in Fig 1C). Blots were imaged with the ChemiDoc MP Imaging System (Bio-Rad).
(TIF)

S2 Fig. GRO-seq of promoter ncRNA. GRO-seq data from other mouse cell lines show promoter-associated ncRNA at *Vegfa* promoter in other cell lines as well.
(TIF)

S1 Table. Primers and oligos. Primers and oligos used in this paper are listed on the table.
(XLSX)

S1 Raw images.
(PDF)

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III

Nuclear microRNA-466c role in mouse hindlimb ischemia

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Ischemic diseases are a group of diseases like coronary artery disease and ischemic stroke, which occur due to the lack of adequate supply of oxygen and nutrients to the tissue. In these hypoxic conditions, rescue of the tissue could be achieved by triggering growth factors like VEGFA. In this thesis, novel miRNAs, which are located in the nucleus and regulate transcription, were studied. One nuclear miRNA, miR-466c, was identified to increase VEGFA expression. miR-466c has the potential to rescue circulation in ischemic tissue both *in vitro* and *in vivo*, thus providing a potential new RNA drug in the future.

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