Cloning of miR-126 fragment into pISO plasmid and the effect of miR-126 on endothelial development in human ES cells

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12 ECTS thesis submitted in partial fullfillment of a
Baccalaureus Scientiarum degree in Biochemistry and Molecular Biology

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Reykjavík, May 2014

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Abstract

Studying endothelial development and key factors that control growth and specification could help us in the battle against cancer. Anti-angiogenesis treatment could mean that tumors become deprived of oxygen and vital nutrition, inhibiting further growth and malignant tumor formation. To make this possible embryonic stem cell derived endothelial cells need to be investigated further to unravel what affects this process and how it can be manipulated.

In this project the main goal was to study the effect of miR-126 in human ES cell derived endothelial commitment. It is already known that miR-126 plays a big role in angiogenesis. The first part was to clone a 347 base pair sequence around the miR-126 insert into a pISO plasmid. The pISO plasmid containing the miR-126 sequence will later be used to by the research group to study the effect of blocking miR-126 with LNA. The second part was to look at endothelial sprouting in embryoid bodies while overexpressing miR-126 using lentiviral infection in hESCs and adding growth factors that could affect the sprouting process. The first part of the project was successful but the second part was not. Although the second part did not show expected results the protocols were improved and later the research group repeated the project with interesting results. My work was part of a bigger project and added information and material to the ongoing work.

Útdráttur

Rannsóknir á þroskun æðaþels og þeirra þátta sem hafa áhrif á vöxt og sérhæfingu fruma þess gætu hjálpað okkur í baráttunni við krabbamein. Meðferð sem miðar að því að hindra æðavöxt í æxlismyndun, og koma þannig í veg fyrir að súrefni og nauðsynleg næringarefni berist til þess, gætu verið vænlegur kostur. Til þess að það sé raunhæfur möguleiki þarf að útskýra betur hvaðaþættir hafa áhrif á þessi ferli og hvernig væri hægt að nýta það. Megin áherslan í þessu rannsóknarverkefni var að skoða áhrif miR-126 á þroskun stofnfruma úr fósturvísum manna í æðaþel. Það er nú þegar vitað að miR-126 hefur mikil áhrif á myndun æða í fósturþroska músa. Fyrri hluti verkefnisins snýr að því að klóna 347 basaparasvæði í kringum miR-126 röð inn í pISO plasmíð sem seinna væri hægt að nýta af rannsóknarhópnum til að skoða áhrif þess að hindra miR-126 með LNA. Seinni hlutinn snýr svo að því að skoða nýmyndun æða í frumukúlum (e. embryoid bodies) þegar miR-126 er yfirtjáð auk vaxtarþátta sem gætu haft áhrif á æðamyndun. Fyrri hluti verkefnisins tókst mjög vel en seinni hlutinn skilaði ekki niðurstöðu. Þó svo að svo hafi farið með seinni hlutann þá voru verkferlar og vinnuseðlar uppfærðir og endurskrifaðir og hópurinn hefur endurtekið samskonar tilraunir með áhugaverðum niðurstöðum. Verkefnið mitt var því eins og áður sagði hluti af stærra verkefni og bætti upplýsingum og efni í stækkandi gagnabanka þess.

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Abbreviations

AMP	Ampicillin
BMP	Bone Morphogenetic protein
Bp	Base pair
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
EB	Embryoid body
EGFL7	Endothelial growth factor like domain-containing protein 7
etOH	Ethanol
EV	Empty vector
GFP	Green fluorescent protein
HEK	Human emryonic kidney
hESCs	Human embryonic stem cells
LNA	Locked nucleic acid
miRNA	Micro-RNA
mRNA	Messenger-RNA
PBS	Phosphate buffer saline
PBS-T	PBS and 0,05% Tween20
PCR	Polymerase chain reaction
RISC	RNA induces silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
Rpm	Rounds per minute
siRNAs	Small interfering-RNA
TGF-β	Transforming growth factor beta
VEGFR2	Vascular endothelial growth factor receptor 2

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Vinum mínum á ég líka mikið að þakka. Þau eru ómetanleg öll klöppin á bakið, hrós og hvatninguna sem þau veita og vonandi hefur mér tekist að gefa eitthvað af því til baka í þeirra verkefnavinnu.

Að lokum vil ég þakka fjölskyldunni minni sem hefur alltaf stutt mig á skólagöngu minni og ýtt mér áfram þegar þess hefur þurft, án þess þó að ýta of mikið. Þau hafa virt mínar skoðanir á því sem ég vil gera og ég veit að þau munu halda áfram að gera það.

1 Introduction

1.1 Stem cells

When a new individual is made from one sperm cell and one egg cell it is critical that all tissues and organs are formed in the right way. This requires a mass of cells that each play their role in development and function of animals. Stem cell research can give us valuable information when researching cancer, drug testing, tissue replacement and much more. Still, there is so much unclear about lifespan, survival and interactions of stem cells making a big field of study (Passier & Mummery, 2003).

1.1.1 Stem cells in general

Stem cells are undifferentiated and when they divide they produce one cell that is undifferentiated, and has the potential to renew the stem cell, and another cell that differantiates into a certain cell type, depending on the environment. The differentiated daughter cell leaves the stem cell niche while the undifferentiated cell stays there to produce more stem cells and daugther cells (*Developmental Biology, Ninth Edition*, 2010). Two major divisions of stem cells are embryonic stem cells and tissue specific stem cells. Embryonic stem cells are derived from the inner cell mass of mammalian blastocysts while the tissue specific stem cells are found in tissues of organs after maturation. Embryonic stem cells are capable of producing all the cell types of the embryo while the tissue specific cells can only form a subset of cell types used for repair and maintainance of matured organ tissue (Kondo et al., 2003). Totipotent cells can form every type of cell in the embryo and also the trophoblast cells of the placenta. The zygote and the first few blastomeres are the only totipotent cells of the body. Pluripotent cells can form all the cell types of the embryo except for the trophoblast cells. They are usually harvested from the inner cell mass of the blastocyst of mammals and are used in reasearch. Multipotent cells can only differentiate into a limited number of cell types and some adult cells are unipotent stem cells that can only form a particular type of cell (Kondo et al., 2003).

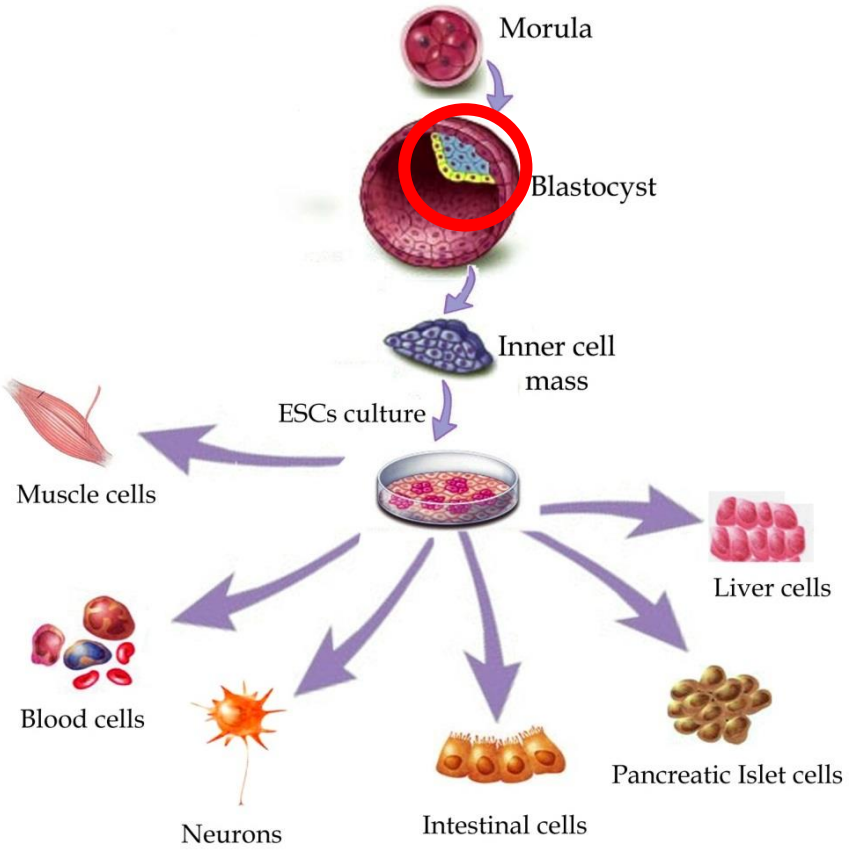

Figure 1: Differentiation of stem cells. The red ring indicates wherefrom stem cells for research are isolated. The stem cells can form various different tissue types and cell types (Mirella Meregalli, 2011).

Both intracellular factors and also external factors such as growth factors determine if the stem cells are pluripotent or not. The best known transcription factors are Oct-4, Sox2 and Nanog but the exact mechanism of how all of them control the pluripotency is not known (Ogony, Malahias, Vadigepalli, & Anni, 2013).

1.1.2 Differentiation of stem cells

Differentiation of stem cells is a process where a stem cell forms a special type of functioning cell in the body. There are different methods available to induce differentiation of stem cells *in vitro*, for example changing the culture, but there are two main methods, spontaneous differentiation and direct differentiation (Passier & Mummery, 2003). Human embryonic stem cells (hESC) in a culture can be kept to proliferate indefinitely and using a directed differentiation can be very useful when studying a certain tissue type. The cells are manipulated to have a stronger tendency to become a certain cell type, often with gene manipulation or growth factors in the medium, whereas in spontaneous differentation they

become a mixture of different cell types. This method has not yet been perfected so even though most cells end up being of a certain cell type, you always end up with a mixture of cell types (Passier & Mummery, 2003).

1.1.3 Endothelial development

After the formation of gastrula in development, it forms the ectoderm, endoderm and mesoderm. The mesoderm cells then go on to form various tissues such as bone tissue, facial muscle and blood cells. In the first phase of vasculogenesis, cells leaving the primative streak in the posterior of the embryo become hemangioblasts, precursors of both blood cells and blood vessels (Sumpio, Riley, & Dardik, 2002). They condense into aggregations that are often called blood islands and the inner cells of these blood islands become blood progenitor cells, while the outer cells become angioblasts. In the second phase of vasculogenesis the angioblasts multiply and differentiate into endothelial cells. Endothelial cells then form the lining of the blood vessels and in the third phase they form tubes to form a network of capillaries (*Developmental Biology, Ninth Edition*, 2010). Besides the essential factor, vascular endothelial growth factor, VEGF, one of the factors that affect endothelial cell fate are bone morphogenetic proteins (BMPs) but they belong to the transforming growth factor-β (TGF-β) superfamily (ten Dijke et al., 2003). Another thing that can affect the development of endothelial cells and angiogenesis is RNA interference, involving micro-RNA, (S. Wang et al., 2008). Modern cancer chemotherapy has been increasingly successful but there are still some drawbacks and furthermore advanced tumors often develop multiple drug resistance (K. Wang, Wu, Wang, & Huang, 2013). Researchers are looking into blocking growth and proliferation pathways in tumors and finding better methods, possibly by using anti-angiogenic methods to inhibit the growth of tumors by cutting off blood flow to the tumor (Li & Li, 2014).

1.2 Small RNAs

There are several short RNAs that repress or silence expression of genes. They do so with genes that are homologous to those short RNAs. This interference or silencing is called RNA interference or RNAi. These small RNAs have different names; small interfering RNAs (siRNAs), that are made artificially or produced *in vivo* from double stranded RNA, and microRNAs (miRNAs), that are derived from precursor RNAs. miRNAs can regulate

gene expression by binding to the mRNA and either inhibit translation of the mRNA or degrade the mRNA but either way their presence downregulates protein production. Here, the focus is mainly on miRNAs and the role they have in hESC development towards endothelial cells.

1.2.1 MicroRNA

miRNAs are short molicules that are found both in plants and animals and take part in regulating gene expression. miRNAs are generated by special enzymes that cleave them from longer double stranded RNAs (*Molecular Biology of the Gene, Sixth Edition*, 2007). The first miRNA was discovered in 1993 while scientists were studying the role of the *lin-4* gene in *C. Elegans* (Lee, Feinbaum, & Ambros, 1993). Since their discovery they have been found in many other species including plants, algea, viruses and animals (*Introduction to Genetic Analysis*, 2010). In general, RNAs can be grouped into two classes, messenger RNAs and functional RNAs. Functional RNAs have many different roles and are of many classes including transfer RNAs, ribosomal RNA and miRNAs (Wienholds & Plasterk, 2005).

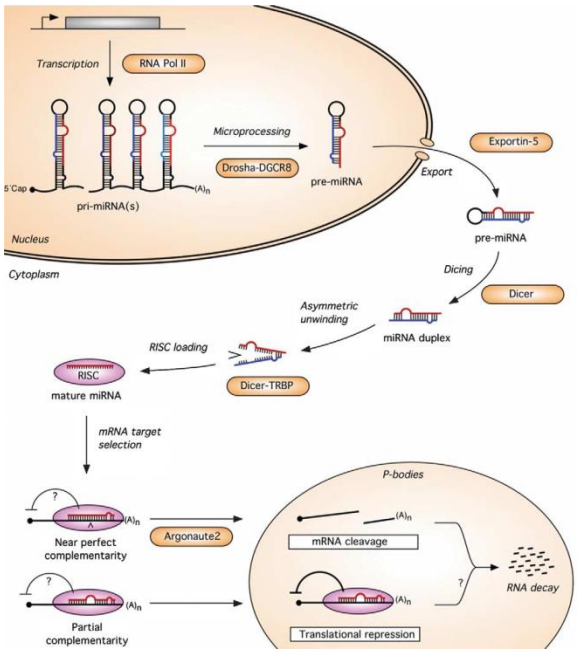

Figure 2: Synthesis of miRNA in the cell. miRNA is formed from a pre-miRNA in the cytoplasm of the cell. Dicer cuts the hairpin loop off the pre-miRNA to form a miRNA duplex that is loaded on to the RISC complex (Wienholds & Plasterk, 2005).

miRNA affect the stability and translation of mRNA and through that have an effect on many different pathways in the cell. The regulation takes place either by silencing parts of the RNA or by speeding up breakdown of RNA strands by removing the poly-A tail (Filipowicz, Bhattacharyya, & Sonenberg, 2008). The mechanism between these different methods of silencing are remarkably similar and includes a complex called RNA-induced silencing complex or RISC. RISC contains proteins of the Argonaute family, Ago proteins that the miRNA guide on to the translation location to stop the translation of the mRNA by pairing between the miRNA and the mRNA involved (Agudo et al., 2014). With these actions miRNAs regulate the amount of protein that is produced by the cell involved and that can have a dramatic effect on the cell function and its survival.

1.2.2 MicroRNA-126

miRNA-126 (miR-126) is a short RNA molecule that is located inside intron 7 of the EGLF7 gene, which is located on chromosome 9 in humans. miR-126 expression is regulated by binding of two transcription factors, ETS1 and ETS2, which by binding initiate the transcription of miR-126 pre-miRNA. This causes a loop formation on the hairpin pre-miRNA and this hairpin miRNA is then marked for cleavage. The product of cleavage is the miR-126 and a the complementary miR-126* (Meister & Schmidt, 2010). It is known that miR-126 is expressed in endothelial cells, in capillaries and bigger veins where it affects many different transcription processes that are related to angiogenesis (Meister & Schmidt, 2010). Studies have shown that in fetal development, expression of miR-126 is of great importance for angiogensis and formation of a healthy circulatory system. Is is also important for vascular integrity (Fish et al., 2008). In a paper published in Developmental Cell in 2008 the authors stated that knocking out miR-126 in mice lead to leaking veins and internal bleeding. Roughly 40% of the mice with no miR-126 expression died in embryonic stages while the wild type littermates were all born healthy with normal heart and cardiovascular system. Studies from the same group also showed that TGFβ, Epidermal Growth Factor-Like domain 7 (EGFL7) and miR-126 play a key role in the development of the mesoderm, specially the cells of the circulatory stystem (S. Wang et al., 2008). In a more recent study miR-126 was linked to the innate immunity system. miR-126 regulated the survival and homeostasis of plasmacytoid dentritic cells and regulates the expression of genes involved in innate immunity response (Agudo et al., 2014). The miR-126/VEGFR2 axis is identified as an important regulator of innate response and the first

response to pathogen-associated nucleic acids (Agudo et al., 2014). In the relatively short time since the discovery of miR-126 many studies have focused on the function of this miRNA and it is certainly a hot research topic today.

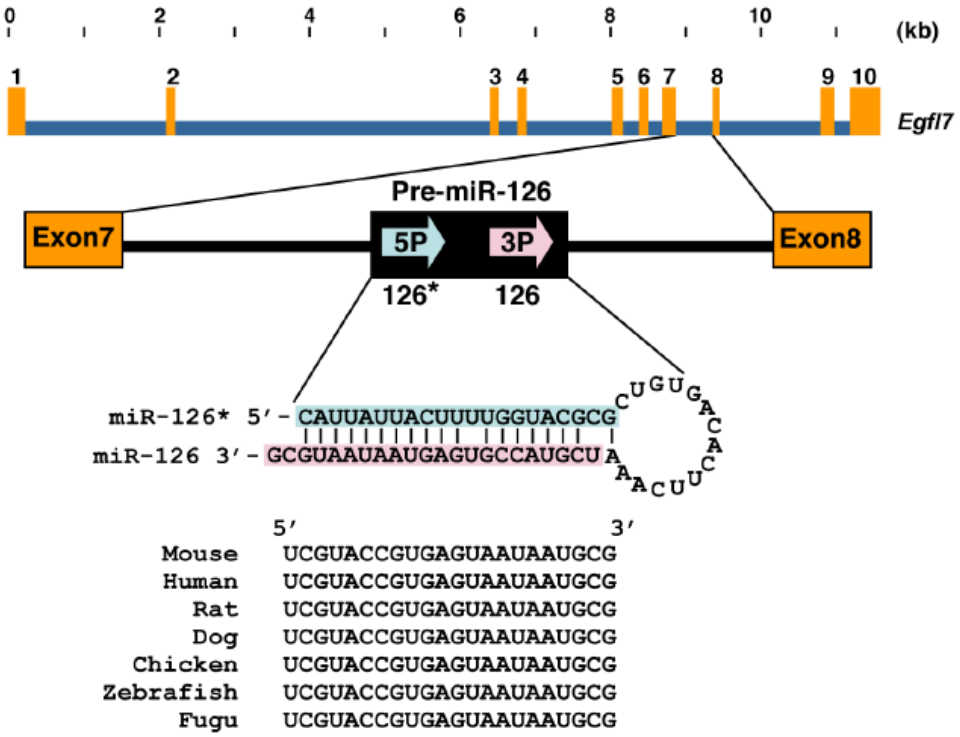

Figure 3: Structure of mouse EGLF7 gene. miR-126 is generated as stem loop encoded by intron 7 as is the miR-126* (S. Wang et al., 2008).

2 Aim of this project

This project had two main objectives. The first one was to clone the miR-126 insert into pISO plasmid and then transform it in *E.coli* cells. My project is a part of a bigger project of the research group to look at linkage between the TGFβ superfamily, EGLF7 and miR-126 in development of endothelial cells from hESC. To look at the effect of miR-126 on endothelial development the plan is to block miR-126 with LNA (locked nucleic acid) from Exiqon. Since nothing is known about the target genes of miR-126 in hESC a 347 bp pri-miR-126 fragment will be inserted into the pISO luciferase plasmid that stops emitting light if the LNA has worked. Moreover I hope to make a tool that can verify the LNA efficiency and the knockdown of miR-126 in later studies by the research group.

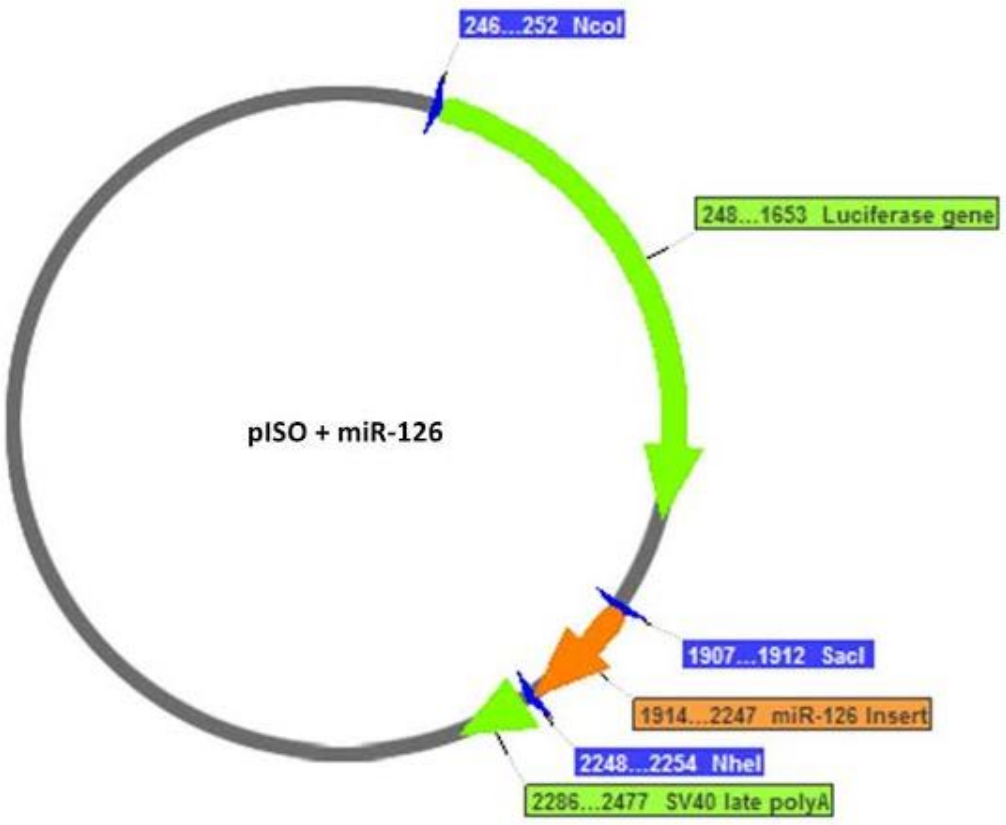

Figure 4: pISO plasmid with miR-126 insert. pISO plasmid showing miR-126 insert, digestion enzymes used and the luciferase gene. This should be the final product of the first part of the project.

The second part was to lentivirally infect hESC with a pLVTHM2/GFP plasmid carrying a miR-126 and look at the expression of different growth factors that likely relate to the

development of endothelial cells with over expression of miR-126. The hESC were then grown and their differentiation in embryoid bodies was examined and RNA extracted for further analysis. The following figure shows the process.

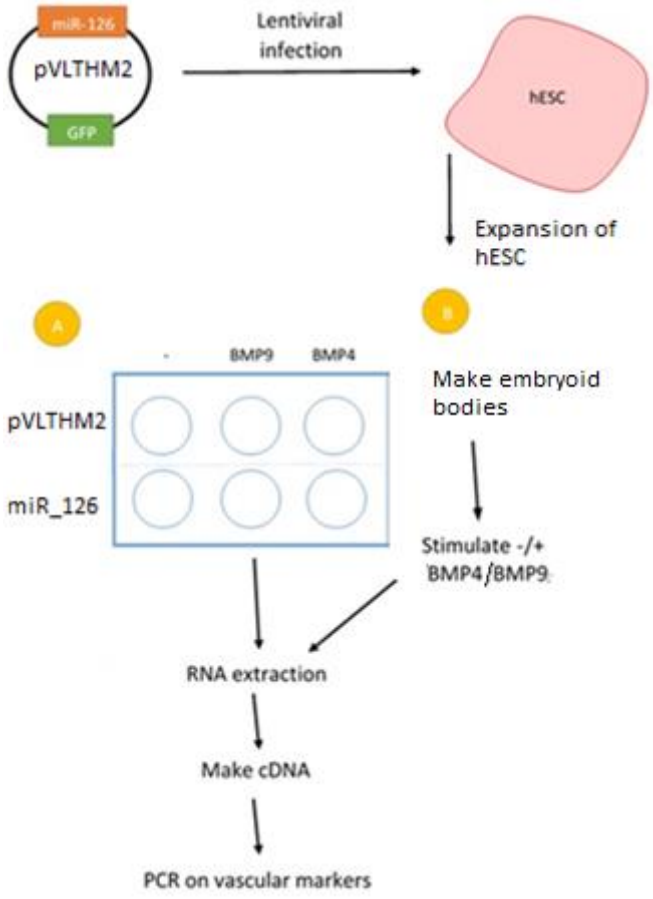

Figure 5: Schematic overview of the miR-126 overexpression in hESCs: Each step is explained in the methods chapter and more information given on the cell line and vector.

Embryoid bodies were also put in collagen to look at sprouting, and for further research with Immunofluorescent staining which is not shown in the figure 5 as it was later added to the project.

3 Methods

3.1 Cloning

3.1.1 PCR to amplify miR-126 insert

PCR reaction was performed to amplify the miR-126 strand. The primers, forward (fw) and reverse (rev), were pre-designed as shown in Table 2. Water was used as a control.

Table 1: PCR reaction solution

Reaction solution	Volume (µl)
Genomic DNA (150 ng/µl)	1
10x Buffer + $MgSO_4$	5
dNTPs (2 mM)	5
Primer fw	1
Primer rev	1
Pfu polymerase (2,5 U/µl)	0,75
Water	36, 25
Total	50

Table 2: Primers for PCR reaction

Name	Sequence: 5‘ – 3‘
Id1 F185	aaa cgt gct gct cta cga ca
Id1 R337	gat tcc gag ttc agc tcc aa
mir126_SacI_fw	ggt ggt gag ctc gag gat agg tgg gtt ccc ga
mir126_NheI-rev	ggt ggt gct agc cag agg tct cag ggc tat gc
CD31 FW	atc att tct agc gca tgg cct ggt
CD31	att tgt gga ggg cga ggt cat aga

Annealing temperature for the primers can be seen in Table 3. Two seperate programs were used for the PCR.

Table 3: PCR programs

Step	Temperature (°C)	Time	Temperature	Time
1	95	5 min	95	5 min
2	95	45 sec	95	45 sec
3	Start at 55 + 0,5 each cycle	45 sec	63	45 sec
4	72	45 sec	72	45 sec
	Cycle to step 2 for 22 times		Cycle to step 2 for 22 times	
5	72	10 min	72	10 min
6	4	∞	4	∞

Both samples were run on 0,8% agarose gel, along with a 100 bp ladder, with 8,3 µl 6x loading buffer in each sample. The band containing the miR-126 fragment was cut out and the DNA isolated with Nucleo Spin® Gel and PCR Clean-Up kit according to manufacturer‘s instructions.

3.1.2 Restriction digest of miR-126 and pISO vector

Restriction endonucleases were used to cut the PCR product and the pISO vector before ligating the two fragments together to make a whole plasmid containing the miR-126 insert. NheI and SacI cut out a 23bp fragment of the pISO vector, leaving sticky ends for the miR-126 insert.

Table 4: Digestion of insert and vector

Reaction solution	Insert (µl)	Vector (µl)
DNA	16	3,5
NheI	2	2
SacI	2	2
NEB4 (10X)	6	6
Water	34	46,5
Total	60	60

DNA was digested at 37°C for 3 hours. The vector was then treated and the phospate ends of the digested vector were cut off to prevent self adherance.

Table 5: Dephosphorylation of vector

Reaction solution	Volume (µl)
Antarctic Phosphatase buffer	7
Antarctic Phosphatase	2
Vector	60
Total	69

The sample was incubated at 37°C for 30 minutes and then heat inactivated at 65°C for 20 minutes. Both the digested vector and the miR-126 fragment were again run on 0,8% agarose gel with 6x loading buffer, cut out of the gel and cleaned with Nucleo Spin® Gel and PCR Clean-Up kit and the protocol used and followed.

3.1.3 Ligation of fragment and vector

The fragment was ligated into the vector to make a plasmid in two different ratios, 1:1 and 1:3 Vector:Insert. Two different ratios were used because previous work suggested that the ratio could affect the outcome.

Table 6: Ligation

Reaction solution	1:1 (µl)	1:3 (µl)
Vector DNA (33,34ng)	1	1
Insert DNA (100,02 ng)	2,4	7,15
T4 ligase	2	2
T4 10x buffer	3	3
Water	21,6	16,85
Total	30	40

Samples were ligated at 16°C over night in a heat block and subsequently stored at -20°C.

3.1.4 Transformation of *E.coli* DH5 alfa bacteria

E.coli DH5 alfa bacteria were used to transform the pISO plasmid and plated on LB agar with ampicillin for the colonies to grow on. The following protocol was used for the transfection of the *E.coli* bacteria:

- Thaw bacteria on crushed ice.
- Add 5 µl of DNA from ligation reaction to 50 µl of bacteria.
- Incubate on crushed ice for 30 minutes.
- Heat shock at 42°C for 45 sec.
- Place on crushed ice for 2 minutes.
- Add 950 mL LB medium = 1 mL total volume.
- Incubate at 37°C for 1 hour in a shaker.
- Spin down the bacteria at 3.000 rpm for 5 minutes.
- Take off 800 µl af medium and resuspend the bacteria in the left over medium.
- Plate on LB agar + amp, 25 µl of culture and 125 µl of culture on seperate plates for each ratio (1:1 and 1:3).
- Incubate at 37°C over night.

pGL3 vector (50 ng/µl) was diluted 1:20 and used as a control, containing no miR-126 insert. Colonies were then picked from the plates with a pipette tip and grown further in 2,5 mL LB medium + amp in 37°C over night in a shaker to maximize the DNA that could be extracted from each culture. A NucleoSpin® Plasmid miniprep was then performed to extract the plasmid DNA from the bacteria and stored at -20°C.

3.1.5 Verification of pISO with miR-126 insert

To identify colonies containing recombinant plasmids that had incorporated the miR-126 plasmid, but not just the plasmid without the insert, a series of digestions were performed with different digestion enzymes. The enzymes used are marked in green on this figure of pISO plasmid.

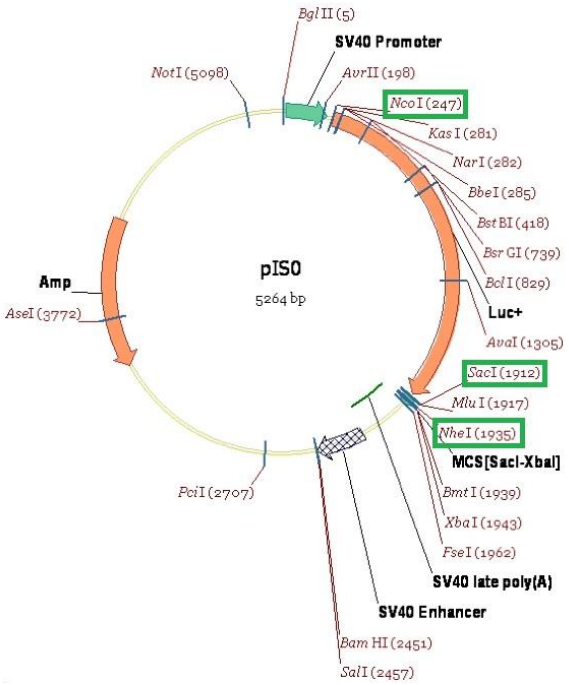

Figure 6: pISO vector and digestion enzymes. Indicated in green are the digestion enzymes used in the digestion of the purified DNA.

Digestion enzymes used were NheI, SacI and NcoI along with SmaI that should only cut the miR-126 insert but no the pISO plasmid as can be seen in Figure 7.

Figure 7: Cutting site of SmaI in miR-126: SmaI cuts the miR-126 insert at one site, 27 bp are to the left of the cut and 320 bp to the right of the cut.

Table 7: Digestion of purified plasmid with different digestion enzymes

Reaction solution	SmaI+NcoI (µl)	NheI+SacI (µl)	NehI (µl)
DNA	2,5(500 ng)	0,5(100 ng)	0,5(100 ng)
Tango buffer 10x	2	1,5	2
SmaI (10 U/µl)	1	-	-
NcoI (10 U/µl)	0,5	-	-
SacI (10 U/µl)	-	0,5	-
NehI (10 U/µl)	-	0,5	0,5
Water	14	10	17
Total	20	13	20

pISO plasmid without the insert was used as a control, both undigested and also the digested pISO from previous digestion. All the samples were run on a 0,8% agarose gel with controls and λHindIII ladder and 100 bp ladder.

3.2 Stem cells

3.2.1 Transfection in HEK cells

Human embryonic kidney (HEK) cells were used for transfection. The cells were around 60% confluent at the time. 0,5 mL of OPTI-MEM medium was put in to two tubes, one for emtpy pLVTHM2 vector (EV) cells and another for miR-126 insert cells. Medium was taken off HEK cells and the cells washed with 2,5 mL PBS. pMd2 (1,25 μg) and pax (3,75 μg) were used as packaging plasmids for the EV and the vector with miR-126 insert. 5 μg were used of both vectors for the transfection. 2-3 hours after the transfection the tranfection medium was replaced with HES medium, harvested after 24 hours and after 36 hours and used to infect hESC. The lentivirus is then dispensed into the medium and the medium used to infect hESC.

3.2.2 Cell culture and maintenance of hESCs

One line of hESC was used, HES2, that was obtained from Gordon Keller´s lab, Toronto (Reubinoff, Pera, Fong, Trounson, & Bongso, 2000). The HES2 cells were grown in hES medium with mTeSR medium on matrigel from BD Bioscience (1:100 dilution) in 6 well plates, one plate for each vector.

Table 8: hES medium

	Stock concentrations	Final concentration
Dulbecco‘s modified eagle medium, DMEM/F-12 + GlutaMAX (31331-028)(Invitrogen)		
KO serum replacement (Invitrogen)		20%
Non-Essential Amino Acids (Invitrogen)	100x	100x dilution
Penicillin (Invitrogen)	5000 U/ml	50 U/ml
Strepomycin (Invitrogen)	5000 μg/ml	50 μg/ml
GlutaMAX (Invitrogen)	200 mM	2 mM
2-Mercaptoethanol	55 mM	77 μmol/ml
mTeSR		25%

Table 9: mTeSR medium

	Volume
mTeSR™1 Basal Medium (StemCell Technlogies)	400 ml
ADD	
mTeSR™1 5x Supplement	100 ml
• Bovine Serum Albumin	
• Rh bFGF	
• Rh TGFβ	
• Lithium Cloride	
• Pipecolic acid	
• GABA	

mTeSR medium is a feeder-independant maintenance medium used for hESC. It is a defined serum-free medium and does not require addition of other growth factors.

3.2.3 hESC differentiation

For differentation and embryoid body (EB) formation, one 12 well non-attachment plate was used for both EV cells and miR-126 cells. After washing once with PBS, cells were dissociated with Dispase for 30 minutes (1 mg/ml). The cells were scraped off with the medium and pooled in seperate 15 mL Falcon tubes, one for EV and one for miR-126. Cells were left to sink to the bottom, medium removed and new medium added, BPEL medium (Ng, Davis, Stanley, & Elefanty, 2008). Cells were distributed in the wells with 3 mL of medium in each well and the top line of wells contained no growth factors. BMP4 (10 ng/µl) and BMP9 (1 ng/µl) was added to the other two lines of wells accordingly. These cells were grown to study the effect of miRNA-127 overexpression in the EBs and different growth factors used.

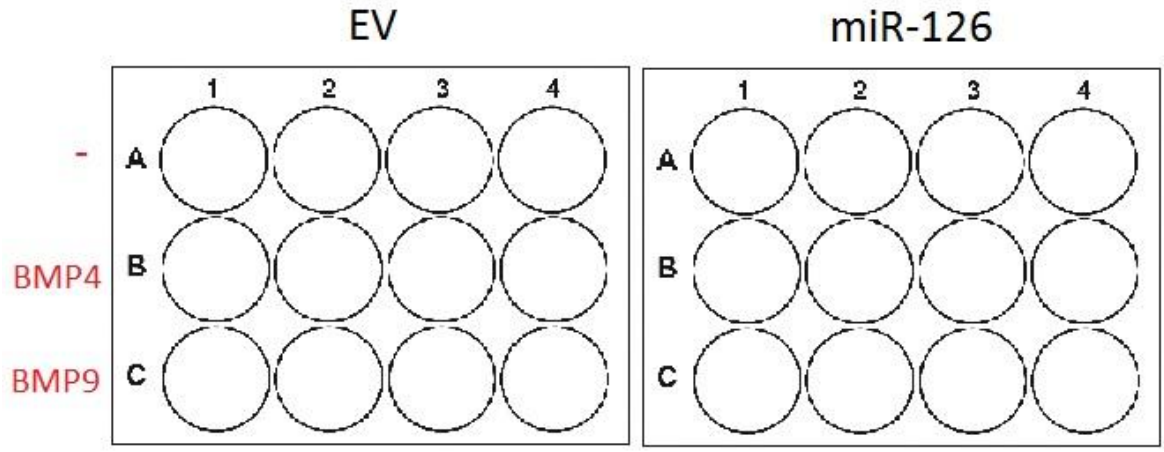

Figure 8: Embryoid bodies with or without miR-126 overexpression and growth factors. One plate was used for each vector and three wells were used for BMP4, BMP9 or no growth factor on each plate.

The same method was used to generate EBs for sprouting research in collagen (see part 3.2.4), 12 well plate with 6 wells for EV and 6 wells for miR-126, 2 wells for each growth factor and the control. EBs were grown to look at endothelial sprouting formation.

3.2.4 Collagen mixture and Immunofluorescent staining.

To look at sprouting, EBs were pooled together in tubes, one for each vector and growth factor, and 0,5 mL of BPEL medium added. EBs were left to sink to the bottom, growth factors were added and the tubes were filled to 1 mL with a collagen mixture and distributed in a 48 well plate.

Table 10: Collagen mixture

Solution	Volume (µl)
CollagenI	2000
10x M199 medium	400
HEPES	800
1x M199 medium	800
Total	4000

After 10 days the EB culture in the collagen were transfered to glass slides and dried.

Immunofluorescence procedure:

- EBs on slides fixed in 4% PFA for 25 min at room temperature (RT).
- Wash 3x with 1xPBS.
- Permeabilise for 10 min at RT with 0,5% Triton X-100 in PBS.
- Wash 3x with 1xPBS.
- Block for 1 hour at RT with 4% goat serum in PBS + IgG (1:500)
- Incubate over night at 4°C with primary antibody in 4% goat serum in PBS.
- Wash 3x with PBS and 0,05% Tween20 (PBS-T)
- Incubate 1 hour at RT with second antibody in 4% goat serm in PBS.
- Wash 3x with PBS-T.
- Wash 5x with dH_2O + 1:500 TO-PRO®-3.
- Embed in Fluoromount.
- Store at 4°C light protected.

The primary antibody used was anti-Endoglin/CD105 (BD Pharmingen). TO-PRO®-3 stain is a carbocyanine monomer nucleic acid stain with far-red fluorescence. It is useful as a nuclear counterstain and dead cell indicator, and is among the highest-sensitivity probes for nucleic acid detection.

3.2.5 RNA isolation

Cells were harvested in 15 mL Falcon tubes and spun down at 1200 rpm for 5 min. Medium was removed and 250 µl of Tryzol added to each, transported to eppendorf tubes and stored at -80°C. A protocol was then followed for RNA isolation.

- Add 50 µl chloroform and shake vigorously (15 sec)
- Incubate for 2-3 min at 15-30°C.
- Centrifuge at 4°C and 15000 rpm for 15 min.
- Take off aqueous RNA solution and add 125 µl isopropanol.
- Incubate for 10 min at 15-30°C.
- Centrifuge for 10 min and take off isopropanol.
- Wash by adding 250 µl 75% etOH, mix.
- Centirfuge 10000 rpm for 5 min.

- Take off supernatant and air dry for 5-10 min.
- Dissolve RNA in 10 µl H_2O and heat at 55°C for 10 min.

The concentration of RNA was then measured with a nano drop spectrometer.

4 Results

4.1 Cloning

4.1.1 Restriction digest of miR-126 and pISO vector

To make sure the right plasmid and insert was being used, both were digested and then run on agarose gel to confirm the size of both bands. The expected size for the pISO vector was 5241 bp and the size of the miR-126 insert was expected to be 347 bp with the primers. As can be seen in Figure 9, where a 100 bp ladder was used, the miR-126 insert band is located between the 300 and 400 bp bands on the ladder and pISO, which is considerably thicker, is located far above the ladder, indicating much larger band, agreeing with expected sizes.

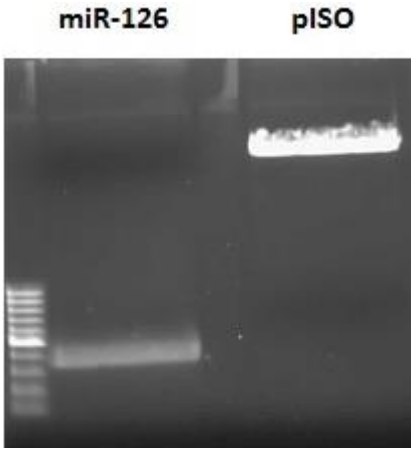

Figure 9: miR-126 insert and pISO vector on agarose gel. The miR-126 band on the left is located between the 300 bp and 400 bp bands of the ladder while the pISO band is much larger.

4.1.2 Transformation of *E.coli* DH5 alfa bacteria

5 LB agar plates were made for the transformation of *E.coli* and after the transformation the cells were spread on the plates. One plate was made with pISO as control without the miR-126 insert, two plates were made for the 1:1 ratio and two plates for the 1:3 ratio.

Table 11: *E.coli* plate organizing and volume

Plate	Ratio	Volume (µl)
A	1:1	25
B	1:1	125
C	1:3	25
D	1:3	125
E	control	125

Colonies grew on all plates, in total 18, except for the control. Colonies were then picked from the plates with a pipette tip and grown further in 2,5 mL LB medium + amp in 37°C over night in a shaker to maximize the plasmid DNA that could be extracted from each culture. They were marked according to the plate they grew on and after the miniprep the concentration was measured in each sample.

Table 12: Concentration of purified DNA

Sample	ng/ul	260/280	260/230	Sample	ng/ul	260/280	260/230
A1	254,54	1,89	2,34	D2	221,43	1,88	2,35
A2	230,18	1,88	2,33	D3	148,63	1,92	2,35
B1	224,70	1,89	2,34	D4	187,49	1,88	2,32
B2	138,67	1,90	2,33	D5	118,24	1,91	2,31
B3	225,79	1,88	2,35	D6	99,61	1,93	2,33
B4	196,89	1,89	2,33	D7	95,73	1,89	2,30
B5	213,44	1,89	2,33	D8	116,13	1,89	2,32
B6	167,66	1,89	2,31	D9	78,57	1,91	2,25
D1	190,61	1,89	2,33	D10	107,13	1,85	2,28

In further work samples A2, B1, B5, D2 and D4 were used.

4.1.3 Digestion of purified DNA

To verify successful plasmid isolation the DNA of the samples was cut with different digestion enzymes.

Table 13: Digestion enzymes and expected bands

Digestion enzymes	Wells	Band 1(bp)	Band 2 (bp)
NehI + SacI	5-8	5241	347
NehI	9-13	5588	-
SmaI + NcoI	14-19	1692	3896

Table 14: Placement of samples in wells in agarose gel

Well	Sample	Well	Sample
1	λHindIII ladder	9-13	Sample digest with NheI
2	pISO undigested	14-18	Sample digest with SmaI+NcoI
3	pISO digested	19	λHindIII ladder
4	Sample undigested	20	100 bp ladder
5-8	Sample digest with NheI+SacI		

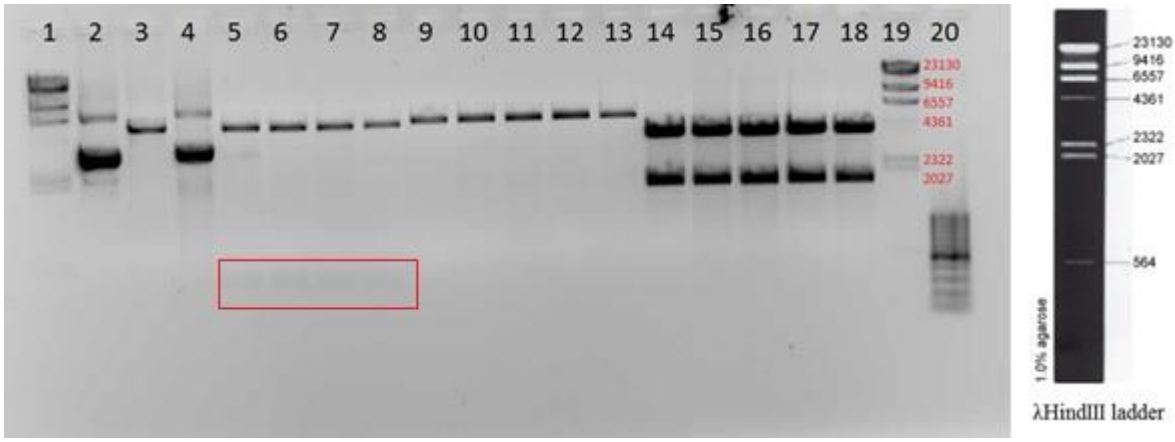

Figure 10: All digested samples, controls and ladders: The well order and ladders are explained in the text. Two types of ladders were used, λHindIII ladder and 100bp ladder.

Wells 5-8 show two separate bands, one clearly visible between the 4361 bp band and the 6557 bp band of the ladder which matches the 5241 bp it should be. The other band is not as clear but has been marked in for all the samples on Figure 10 and is located between the 300 bp and 400 bp band of the 100 bp ladder, which matches the 347 bp it should be. Wells 9-14 show just one band, slightly larger than the bands in wells 5-8, which matches that it should be 5588 bp. In wells 14-18 there are again two bands, the larger one just below the 4361 bp band of the ladder and the smaller one just below the 2027 bp band. That is in contrast with the bands being 3896 bp and 1692 bp. According to this all the

bands are the of correct size and the overall cloning of the pri-miR-126 insert into the pISO plasmid was successful.

4.2 Stem cells

4.2.1 Transfection of HEK cells

Earlier, a master student in the lab cloned miR-126 into a lentiviral vector to study the effect of miR-126 overexpression in hESCs. The results suggested that miR-126 promoted vascular differentiation as vascular markers were upregulated in miR-126 infected hESCs. Therefore, I wanted to investigate if miR-126 infected cells promoted EB sprouting, a typical assessment of angiogenesis during early development. Infection of lentiviral vectors is used to introduce foreign DNA into cells. The foreign DNA becomes stably introduced into the host cell genome. First, HEK cells were transfected with lentiviral packaging plasmids and either with pLVTHM2 plasmid (including GFP) containing miR-126 or just pLVTHM2 without an insert and after 2 days the cells were examined for GFP expression before infecting hES2 cells (Ómarsdóttir, 2011).

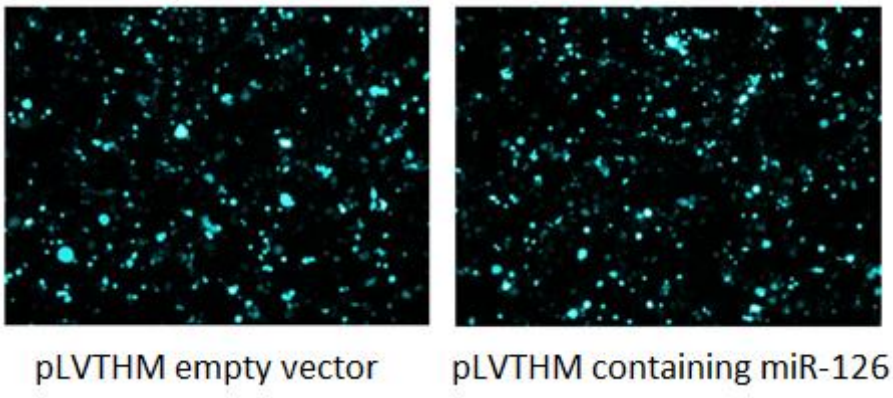

Figure 11: HEK cells transfected with pLVTHM containing miR-126 or an empty vector. Cells containing an emtpy vector are on the left and cells containing miR-126 are on the right. The pictures are taken at 100x magnification.

No major differences can be seen between the GFP expression of the pLVTHM2 transfected HEK cells and the miR-126 transfected HEK cells.

4.2.2 Infection of hES2 cells

hES2 cell were infected with the lentiviral medium produced by the HEK cells. They were examined for GFP expression after infection and pictures were taken on the 6th day.

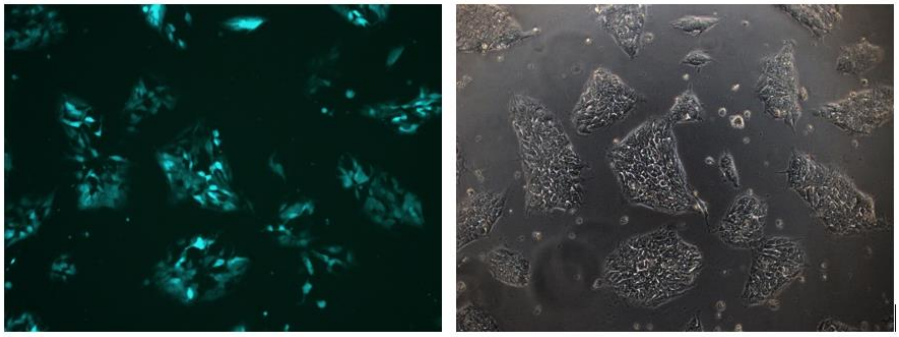

Figure 12: EV infected hES2 cells 6 days after infection. hES2 cells were infected with lentiviral medium from HEK cells, containing an empty pLVTHM2 vector. The pictures are taken at 10x magnification.

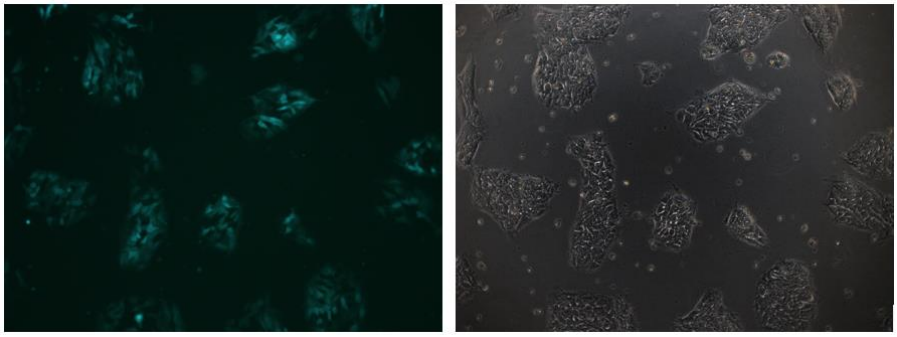

Figure 13: miR-126 infected hES2 cells 6 days after infection. hES2 cells were infected with lentiviral medium from HEK cells, containing a pLVTHM2 vector with miR-126 insert. The pictures are taken at 10x magnification.

As can be seen in figures 12 and 13 hES2 cells were successfully infected. No clear difference can be seen after 6 days in GFP expression of the EV infected hES2 cells and the miR-126 infected hES2 cells.

4.2.3 hESC differentiation

Attempts were made to induce differentiation of the hES2 cells to see if miR-126 expression had any effect on the differentiation process and specifically endothelial sprouting of the EBs. Each cell group, both EV and miR-126 insert, were treated with the growth factors BMP4 (known mesodermal inducer) or BMP9 (a possible vascular inducer)

or untreated as control. GFP expression, EB formation and sprouting was examined after treatment. EBs were embedded in collagen on differentiation day 5 to examine further the sprouting and pictures taken a week after the cells were embedded in collagen.

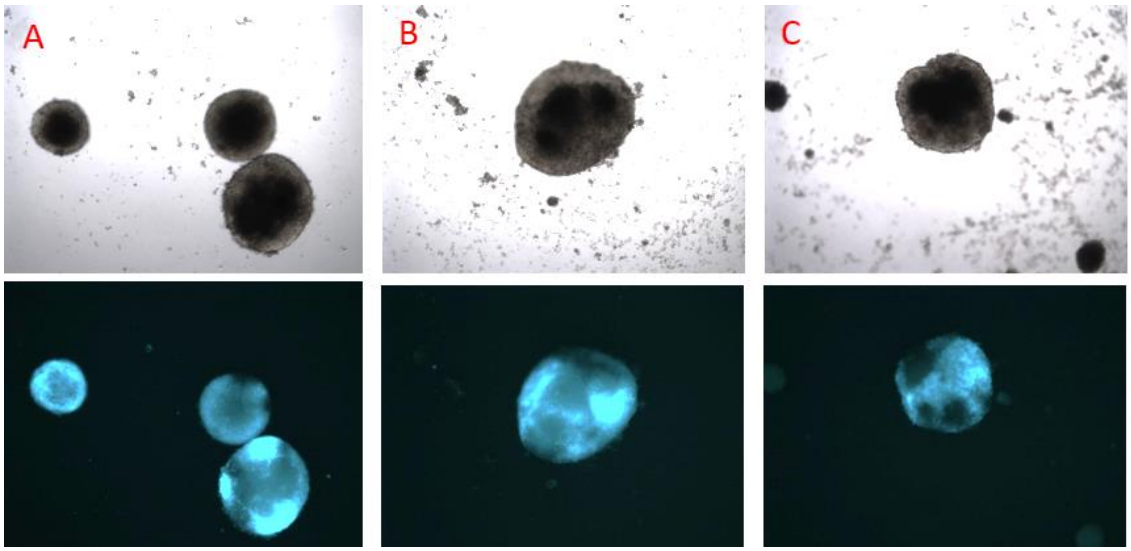

Figure 14: hEBs expressing empty vector, untreated (A), with BMP4 (B) and BMP9 (C). Upper panel shows phase contrast pictures. Lower panal shows GFP. The pictures are taken at 5x magnification.

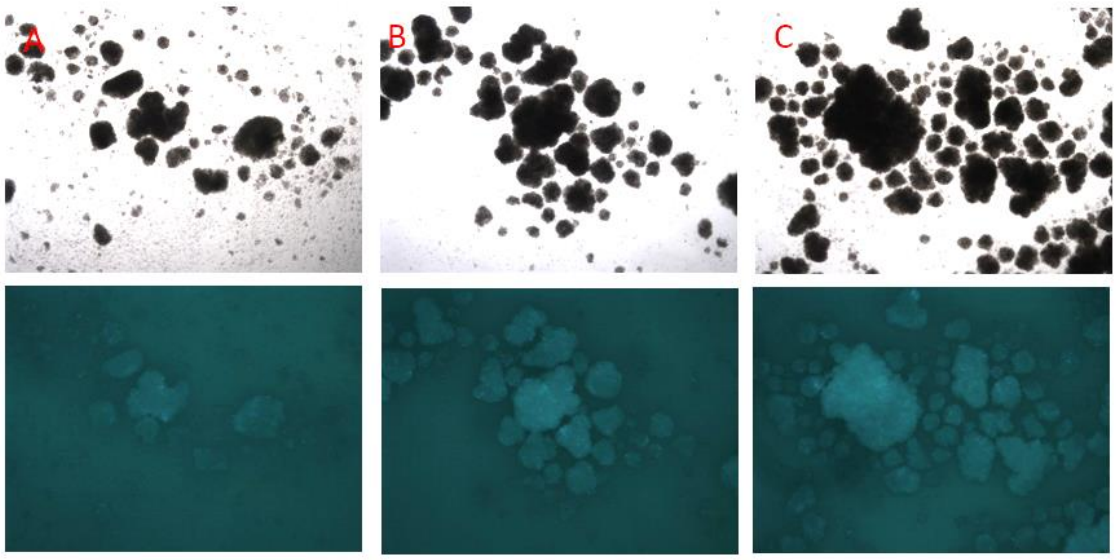

Figure 15: hEBs expressing miR-126 insert, untreated (A), with BMP4 (B) and BMP9 (C). Upper panel shows phase contrast pictures. Lower panal shows GFP. The pictures are taken at 10x magnification.

Here, a clear difference can be seen between the EV and the miR-126 insert regarding GFP expression and EB formations. The EV cells formed much more round and compact EBs while the miR-126 insert cells formed smaller and weaker looking EBs but in greater

quantities. Previous studies from the research group had shown that miR-126 cells formed larger EBs with better structure. These results were not in agreement with that. The EBs in the collagen were transfered to glass plates and immunofluorescence was used to examine the EBs.

4.2.4 Immunofluorescence

During the Immunofluorescence procedure the samples got mixed up so there was no way to tell which sample was EV or miR-126 and which contained the BMP4 and BMP9 growth factors. Only two EBs were visible in the confocal.

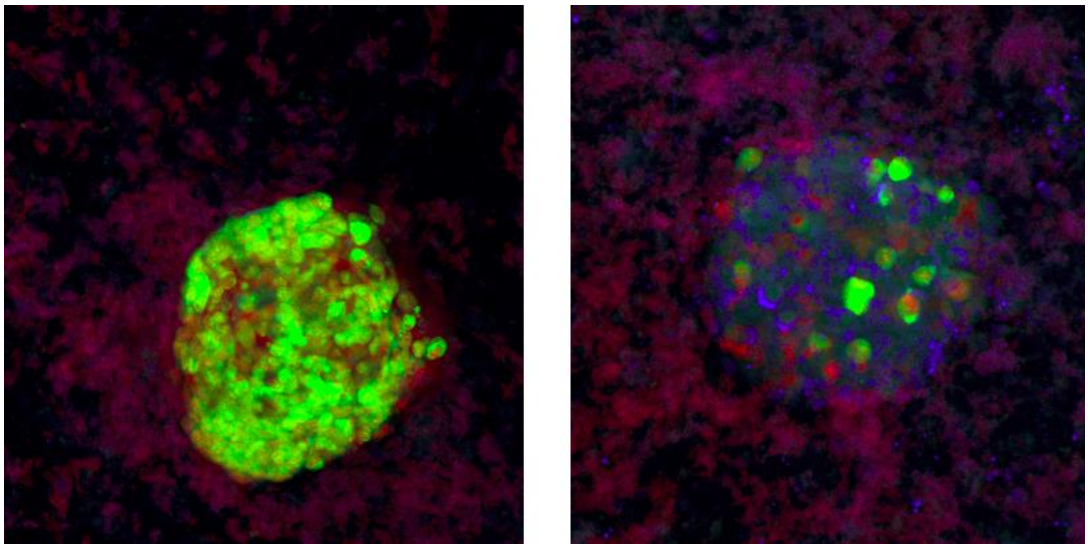

Figure 16: Immunoflurescent staining on hEBs. In these confocal pictures green is GFP expression, blue is Topro expression and red is Endoglin expression. The pictures are taken with magnification of 20x.

Both EBs expressed GFP as can be seen by the green color in the confocal pictures. Blue color indicated Topro expression, which marks the nucleus of the cell. Both cells show nuclear activity. Endoglin, red, colors endothelial markers so both of the samples showed endoglin expression.

4.2.5 RNA isolation

The RNA from the hEBs was isolated and the concentration measured.

Table 15: RNA concentration

Sample	ng/µl	260/280	260/230	Sample	ng/µl	260/280	260/230
EV-	12,61	2,01	0,03	Mir-	17,44	1,81	0,04
EV+BMP4	42,33	1,85	0,09	Mir4	7,85	1,89	0,02
EV+BMP9	10,70	2,03	0,06	Mir9	16,67	1,70	0,04

As can be seen the concentrations were not acceptable and because of that no further experiments on the RNA expression were conducted.

5 Discussions

5.1 Cloning

The first part of this study involved an insert containing miR-126 cloned into a plasmid for later use in stem cell culture. At first the cloning was not successful and the tranformation of *E.coli* did not produce any positive clones. After 3 months with no success we decided to try new primers and a different plasmid, the previous plasmid was pGL3-promo and was replaced with the pISO vector that has many more restriction sites and therefore opening more possibilities. After that everything went according to plan and in the end all the colonies that grew were positive, and should therefore contain the plasmid with the insert. Nevertheless, it is necessary to analyze the sequence to make sure it is in fact the right plasmid and insert. Another thing that could have affected the results in the first part was that we used restriction enzymes that cut the plasmid in one place and the insert was supposed to go in that place. In the second try we used two seperate digestion enzymes that cut out a 23 bp part of the plasmid, leaving sticky ends, and the insert was ligated into that gap. The end result was very good and this plasmid can be used by the research group for further studies on with LNA to verify the LNA efficiency and the knockdown of miR-126.

5.2 Stem cells

The second part of the study was to overxpress miR-126 in hES2 cells using the lentiviral vector pLVTHM2 and look at sprouting and RNA expression of different endothelial markers. The first cell culture infected with the lentivirus from the HEK cells grew very well but the miR-126 insert cells were not responding the way we expected them to do. Previous projects had suggested that miR-126 insert resulted in bigger and more condense embryoid bodies while in my case that was not the result. BMP4 and BMP9 growth factors did not seem to affect the EBs. miR-126 overexpressing were smaller and looked weaker then the EBs with the empty vector. There was not enough time to repeat the study but after my lab work ended, the protocol for the collagen mixture and immunofluorescence procedure was improved and the group got some interesting results. Although the second part of my project was not successful it contributed to the improvement of the method for the whole group.

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