Regulation by MicroRNAs during Cardiovascular Differentiation of human Embryonic Stem Cells

Arna Rún Ómarsdóttir

Faculty of Life and Environmental Sciences

School of Engineering and Natural Sciences

University of Iceland

2011

Hlutverk microRNA í sérhæfingu stofnfruma úr fósturvísum manna í frumur hjarta- og æðakerfis

Arna Rún Ómarsdóttir

90 eininga ritgerð sem er hluti af
Magister Scientiarum gráðu í Líffræði

Leiðbeinendur
Guðrún Valdimarsdóttir
Zophonías O. Jónsson

Prófdómari / Fulltrúi deildar
Sigríður Rut Franzdóttir

Líf- og umhverfisvísindadeild
Verkfræði- og náttúruvísindasvið
Háskóli Íslands
Reykjavík, október 2011

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Advisors
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Zophonías O. Jónsson

External examiner
Sigríður Rut Franzdóttir

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Faculty of Life and Environmental Sciences
School of Engineering and Natural Sciences
University of Iceland
Askja, Sturlugata 7
101 Reykjavík
Telephone: 525 4000

Bibliographic information:
Arna Rún Ómarsdóttir 2011, Regulation by microRNAs during cardiovascular differentiation of human embryonic stem cells, M.Sc. thesis, Faculty of Life and Environmental Sciences, University of Iceland

Printing: Pixel
Reykjavik, Iceland, October 2011

Útdráttur

Stofnfrumur úr fósturvísum manna (hESC) hafa opnað nýjan rannsóknarvettvang sem veitir ómetanlega innsýn inn í fyrstu stig fósturþroska mannsins og möguleika á því að síðar verði hægt að nota til lækninga á sjúkdómum. Búist er við því að hjartavöðvafrumur og forverafrumur hjarta munu hafa gríðarleg áhrif á meðferð sjúkdóma sem herja á hjarta- og æðakerfi manna sem eru aðal dánarorsök um allan heim. MicroRNA (miRNAs) hafa mikil áhrif á fjölmarga frumuferla, svo sem þroskun og sérhæfingu. Nýlega hafa miRNA verið tengd við þroskun hjarta- og æðakerfisins.

Í þessu verkefni var sett upp skilvirk aðferð til að sérhæfa hESC í hjartavöðvafrumur með því að nota sermislaust æti og vaxtarþætti sem eru þekktir fyrir að ýta undir myndun sláandi hjartavöðvafruma. Skýrt hefur verið frá hlutverki miR-126 í þroskun hjarta- og æðakerfis músa bæði með aukinni tjáningu í hjarta- og lungnavef og með tjáningu miR-126 í forverafrumum hjarta- og æðakerfis úr stofnfrumum músa. Þess vegna kannaði ég hlutverk miR-126 í þroskun hjarta- og æðakerfis í hESC. Í þessari rannsókn náði ég fram sláandi hjartavöðvafrumum bæði með og án notkunar vaxtarþátta sem leggur áherslu á þann mikla mun sem liggur milli frumulína. Enn fremur, með því að sívirkja viðtaka BMP4 náðist að flýta fyrir myndun sláandi hjartavöðvafruma. Yfirtjáning miR-126 í hESC leiðir til aukinnar tjáningar miðlags- og æðaþelsmarkera þegar borið er saman við hjartavöðvamarkera. Eftirfarandi rannsókn gefur betri sýn á mikilvægi upphafsvirkni á ákveðnum innanfrumuboðleiðum í sérhæfingu hESC í hjartavöðvafrumur og hafnar um leið þeirri tilgátu að miR-126 hefur áhrif á sérhæfingarferli hjartavöðvafruma.

Abstract

With human embryonic stem cells (hESCs) a new field has emerged providing invaluable insight into early embryonic development in humans and opening promising possibilities in therapeutics and stem-cell therapy. Stem cell derived cardiomyocytes and cardiac progenitors are anticipated to have a tremendous impact on disease treatment of the leading cause of death worldwide, cardiovascular diseases. MicroRNAs (miRNAs) exert powerful effects on many cellular processes such as development and differentiation. Recently, their role in cardiovascular development and disease was proposed.

The aim of this study was to establish efficient cardiomyocyte differentiation of hESCs by the use of serum-free medium and lineage-specific growth factors, known to facilitate efficient induction of beating cardiomyocytes. The role of miR-126 in mouse cardiovascular development has been indicated with upregulation in both heart and lung tissues and with the presence of miR-126 in cardiovascular progenitors of mouse ESCs. Therefore, I sought to investigate the role of miR-126 in cardiovascular development in hESCs. Here, I show that efficient cardiomyocyte differentiation was established with and without the use of growth factor stimulation underlining the difference in cell line kinetics. Furthermore, by constitutively activating the receptor of a known mesodermal inducer, BMP4, the induction of beating cardiomyocytes was accelerated. Overexpression of miR-126 in hESCs resulted in the upregulation of mesodermal markers and higher induction of vascular markers compared to cardiac markers. Taken together, this study provides further insight into the importance of signal transduction optimization in cardiomyocyte differentiation and rejects our hypothesis of miR-126 function in cardiomyocyte development.

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Abbreviations

ALK	Activin receptor-like kinase
AMP	Ampicillin
AT	Annealing temperature
BMP	Bone morphogenetic protein
bFGF	Basic fibroblast growth factor
Bp	Base pair
CM	Conditioned medium
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
EB	Embryoid body
ECM	Extracellular matrix
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
EHS	Engelbreth-Holm-Swarm
EMT	Epithelial to mesenchymal transition
ESCs	Embryonic stem cells
GF	Growth factor
GFP	Green fluorescent protein
GS	Goat serum
hESCs	Human embryonic stem cells
ICM	Inner cell mass
MEF	Mouse embryonic fibroblast
mESCs	Mouse embryonic stem cells
miRNA	MicroRNA
mRNA	Messenger RNA
Nt	Nucleotide
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
p.i.	Post infection
PS	Primitive streak
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RT	Room temperature
TGFβ	Transforming growth factor beta
UTRs	Untranslated regions

Acknowledgements

Firstly, I would like to thank my supervisors **Guðrún Valdimarsdóttir** and **Zophonías O. Jónsson** for their support, guidance and supervision. I would especially like to thank Guðrún for her patience, encouragement and helpfulness throughout this research and while writing this thesis.

The first part of my research was conducted at Askja were I developed great friendships and had the opportunity to work with some of my best friends, and for that I give special thanks to **Sara Sigurbjörnsdóttir** and **Katrín Briem** for giving their time when I was in need of help, which happened on several occasions! There was one person who trained me in the lab and was always there when I needed help, **Hörður Guðmundsson**, without you this would have been a much harder task.

In Læknagarður I joined forces with my good friend **Lena** who helped me freshen up my cell culturing skills and was my only buddy for several months until Freebe and Flala showed up. Over the months I have developed a great and long-lasting friendship with **Svala** and **Jói** that can be described with great laughter, a lot of fun and let´s not forget good food! They are always there when a need help or when I´m in the need of a good talk and uplifting and for that they have made the writing of this thesis bearable.

Special thanks go to **Guðrún Jónsdóttir** and **Pétur Henry Petersen** for both good friendship and guidance during qRT-PCR experiments. I thank **Sigríður Rut Franzdóttir** and **Sævar Ingþórsson** for their help during my lentiviral research and my good friends at SCRU, who are always willing to lend a helping hand. I am grateful for the help **Sigríður Jónsdóttir** gave me by reading this thesis and for help and support in the assembling of my lecture.

I thank my parents for their endless faith, support and pride in all my endeavors, without them I wouldn´t be able to do half of what I´m doing today. To my little family I give special thanks, for their endless support and patience, especially during the writing of this thesis.

The project was funded by the Postgenomic Biomedicine Grant (Markáætlun) from the Icelandic Center for Research, Rannís.

1 Introduction

Throughout the life of every organism stem cells serve as a fundamental source for tissue regeneration. During growth they provide the body with cells and they are also responsible for regeneration following disease or injury in the adult body (Passier and Mummery, 2003). Researching stem cell biology provides useful information to applications such as tissue replacement, disease modeling and drug testing. At present, scientists deal with the question whether stem cells are capable of producing enough quantity of specific cell types for use in transplant surgeries (Passier and Mummery, 2003).

1.1 Stem cells

Stem cells possess the ability to self-renew and they are able to differentiate into other cell types of the organism (Chambers and Smith, 2004). All developing tissues retain cells with stem cell properties. Certain tissues of the body, such as the skin and hematopoietic system are capable of self-renewal but it remains to be clearly demonstrated whether this is the case throughout the entire adult body (Steele and Steele, 2006). When undergoing cell division, stem cells have two possibilities; the daughter cell can either self-renew and remain undifferentiated or differentiate to a terminally differentiated cell, for example cardiomyocyte.

Different types of stem cells exist, depending on the ability to maintain stem cell-like properties and how variable their derivatives are. **Unipotent stem cells** undergo self-renewal and are able to generate one mature cell type. **Multipotent stem cells** give rise to two or more differentiated cell types. An example of multipotent stem cells can be found in the adult organism, for example hematopoietic stem cells (HSC) which can differentiate into numerous blood cell types (Kondo et al., 2003). **Pluripotent stem cells** have the unique ability to differentiate into and form all derivatives of the three germ layers; endoderm, ectoderm and mesoderm (Chambers and Smith, 2004).

1.2 Embryonic stem cells

Embryonic stem cells (ESCs) are derived from early embryos as the name implies. They are pluripotent cells under *in vitro* culturing conditions and maintain the capacity to differentiate to the majority of cell types found throughout the whole body (Passier and Mummery, 2003). ESCs can in

principal form around 220 different cell types which are present in the adult body (Dambrot et al., 2011). The first embryonic stem cells were isolated from pre-implated, blastocyst-stage, mouse embryos over 30 years ago. The background to the discovery lay in studies of teratocarcinomas, spontaneously grown tumours of the gonads in mice and humans. They consist of tissues as diverse as hair, muscle, bone and teeth (Mummery and van den Eijnden-van Raaij, 1999). In the mid-1970s it was observed that adult mice that had been grafted with early mouse embryos into extra-uterine sites produced teratocarcinomas. These teratocarcinomas contained undifferentiated stem cells that could be isolated and grown in culture without losing the capacity to differentiate. Meanwhile, biologists pondered the question whether it could be possible to isolate stem cells directly from mouse embryos (Passier and Mummery, 2003). In 1981, it was reported that efficient isolation of mouse embryonic stem cells (mESCs) had been achieved and a mouse embryonic stem cell lines derived directly from mouse blastocysts established (Evans and Kaufman, 1981).

1.2.1 Derivation of human embryonic stem cells

Typically, human embryonic stem cells (hESCs) are isolated from surplus embryos after *in vitro* fertilization treatment (Pal, 2009). Several cell divisions after fertilization cells rearrange to form a hollow sphere of around 100 cells surrounding a fluid-filled cavity (blastocyst). The outer layer of the blastocyst (trophoectoderm) becomes the fetal contribution to the placenta while the inner cell mass (ICM) gives rise to the embryo proper. Around days 5-8 of development the trophoectoderm is removed, the ICM isolated and plated on to a feeder layer of mouse or human embryonic fibroblasts (Stojkovic et al., 2004a; Stojkovic et al., 2004b; Thomson et al., 1998), which is essential for ICM survival (Cowan et al., 2004) (Figure 1). The ICM then flattens producing a compact colony that can be mechanically dissociated and re-plated several times producing a stable cell line. Cells can then be held undifferentiated or they are differentiated to various cell types (Terskikh et al., 2006).

It was predicted that ESCs could be isolated from humans in view of the similarities between mice and human teratocarcinomas. In the mid-1980s the first attempts of hESC isolation were made with no success. Many discontinued this research (Passier and Mummery, 2003) but Thomson and co-workers went forward becoming the first to demonstrate pluripotent cells isolated from non-human primates possessing some of the expected ESC features (Thomson et al., 1995). Then three years later, in 1998, this was extended to derivation of cell lines from human blastocysts creating the first hESC lines (Thomson et al., 1998). To identify hESC lines and examine their capacity, undifferentiated hESCs are transplanted into an animal model where they form teratomas containing cells from all three germ layers. Their ability to form teratomas is the

accepted criterion for identifying hESC lines, a crude but necessary test of pluripotency (Terskikh et al., 2006).

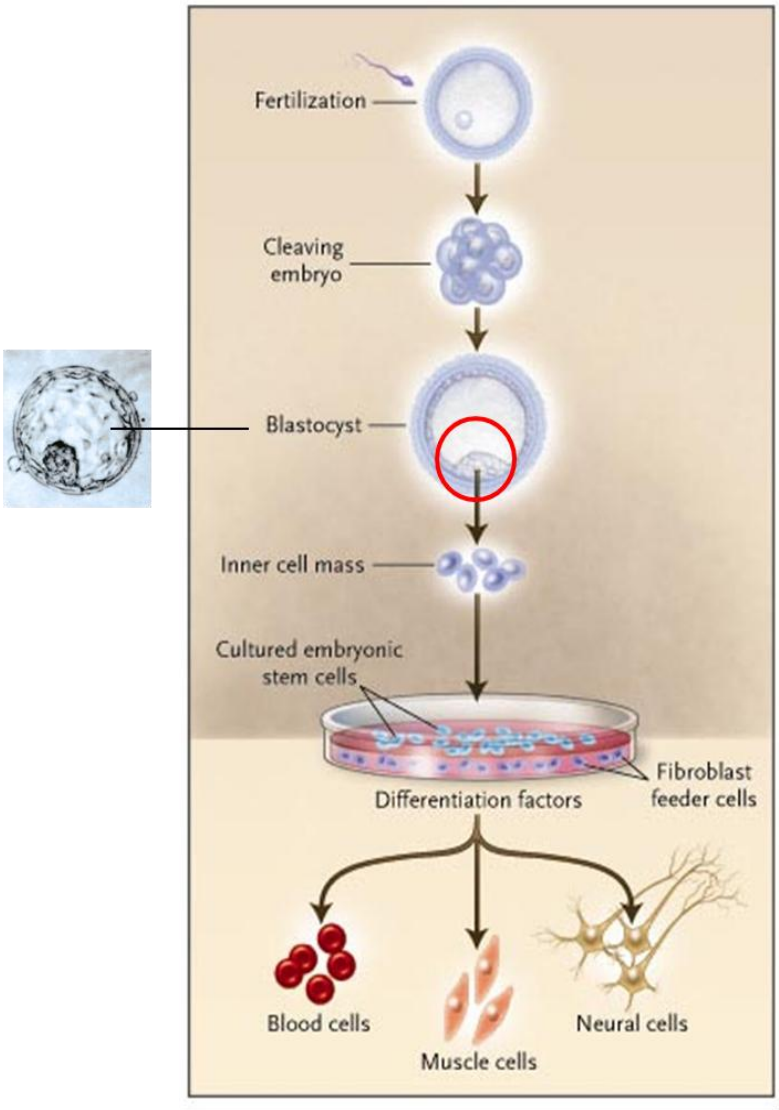


Figure 1: Human embryonic stem cell origin.
Blastocyst forms containing the inner cell mass which can be isolated around days 8-9 of development, forming embryonic stem cells. Embryonic stem cells can be kept pluripotent or differentiated into various cell types (endoderm, mesoderm, ectoderm).

1.3 Maintenance of human embryonic stem cells

When cultured in the laboratory, undifferentiated hESCs possess a distinct morphology appearing as a tightly packed monolayer and forming colonies with defined borders (Rosler et al., 2004) (Figure 2). To support self-renewal, hESCs are usually cultured on mouse embryonic fibroblast (MEF) feeder cells which are isolated from mouse embryos. Before MEFs can be used as feeder cells they must be treated with irradiation or mitomycin C to prevent cell division (Terskikh et al.,

2006). MEFs release factors into the medium that are not fully characterized but it is known that these factors support proliferation and self-renewal of hESCs (Xu et al., 2001). hESCs require a growth medium with specific properties to maintain pluripotent state (Yao et al., 2006) and it has been shown that medium supplemented with basic Fibroblast Growth Factor (bFGF) together with MEFs is sufficient to maintain hESCs in their pluripotent state (Xu et al., 2005).

hESC colonies are routinely passaged for maintenance of self-renewal, expansion and for experimental purposes (Zwaka and Thomson, 2003). They must remain within a narrow size during passaging procedures to prevent differentiation so it is clear that cell-cell interactions, as well as paracrine and autocrine signals, play essential roles within colonies in maintaining an undifferentiated state (Vazin and Freed, 2010). During passaging, colonies are dissociated with enzymatic solutions and replated onto new tissue culture plates. Enzymatic dissociation with trypsin solution (0,05% trypsin / ethylene diamine tetraacetic acid (EDTA)) is widely used but advantages with the use of dispase or collagenase have been shown with reduced cell death and greater karyotypic stability (Zwaka and Thomson, 2003).

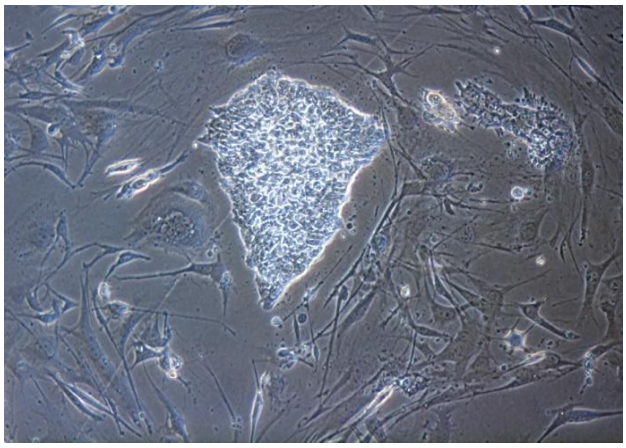


Figure 2: Human embryonic stem cells in culture.
hESCs form colonies with defined borders cultured on MEF feeder cells.

1.3.1 Feeder-free culturing systems

In certain hESC experiments, depletion of feeder cells is necessary. Although the use of MEF feeder cells is sufficient for many research applications, it was recognized early on that co-culture of hESCs and feeder-cells is not optimal for transplantation applications (Xu et al., 2001) in regard to pathogen transmission and infection (Klimanskaya et al., 2005). This knowledge prompted the development of a feeder-free system that has developed fast the last few years.

In 2001, new technology emerged when Xu et al showed that hESCs could be maintained pluripotent in the absence of feeder cells. They used both Matrigel^{TM} (BD Biosciences) as substrate and natural laminin (Xu et al., 2001). Matrigel is a gelatinous protein mixture, secreted by

Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, that resembles extracellular environment. Feeder-free culturing is usually performed in conditioned medium (CM) collected from MEF feeder-cells but it is optimal to eliminate completely the requirement of MEFs. The first fully defined culture medium was described recently and then marketed as mTeSR®1 (STEMCELL Technologies). This medium was shown to be effective when hESCs were cultured in combination with Matrigel (Ludwig et al., 2006).

Many research groups have made great effort in developing simpler and safer methods for hESC culturing. These methods are coming forth making stem cell therapy a closer reality. Many of these methods involve culturing under feeder- and serum-free conditions which is important to avoid various infection risks (Mannello and Tonti, 2007).

1.3.2 Transcriptional networks in hESCs

The molecular mechanism underlying hESCs self-renewal and pluripotency is poorly understood and is yet to be deciphered. There are both external factors, such as growth factors, and also various intracellular factors that determine whether hESCs maintain their pluripotency or differentiate. Many known transcription factors contribute to maintaining hESCs in a pluripotent state, the most essential ones being Oct-4, Sox2 and Nanog (Deb, 2007; Gan et al., 2007). Oct-4 (POU5F1) is a POU domain-containing transcription factor that regulates downstream genes by binding to Sox2, and in turn Sox2 contributes to pluripotency by regulating Oct-4 levels. Common to Oct-4, Sox2 and Nanog is the ability to bind to their own promoters to maintain their expression. They can also occupy the same target genes and activate genes to promote ESCs self-renewal while silencing important factors that otherwise induce differentiation (Jaenisch and Young, 2008). Recent data suggesting that self-renewal and pluripotency in hESCs is mediated upon TGFβ-Smad2/3 activation that in turn leads to Smad2/3-Nanog interaction (Xu et al., 2008). However, the exact mechanism of how Nanog regulates pluripotency still remains unclear (Liu et al., 2007) along with plenty of other factors and interactions that need to be both discovered and/or studied.

1.4 Differentiation of hESCs

Differentiation of a cell is the process by which a pluripotent ESC forms a functionally specialized cell type, for example cardiomyocyte or endothelial cell. This can primarily be achieved by changing the growth conditions with addition of lineage-specific growth factors (Deb, 2007). Knowledge of differentiation methods that direct hESCs into certain directions is quiet limited but differentiation can be achieved by two main methods; spontaneous differentiation or directed differentiation. Cells

are not directed towards a certain lineage in spontaneous differentiation so the outcome is a mixture of random cell types. In a directed differentiation the cells are however manipulated so that they have a stronger tendency to differentiate into certain cell types (Mummery et al., 2003; Passier and Mummery, 2003).

1.4.1 Spontaneous differentiation

To obtain hESC differentiation it is common to use a method where complex, three-dimensional, multi-layered structures are formed called embryoid bodies (EBs). Differentiation proceeds in a manner reminiscent of early embryonic development where blastocyst environment is simulated resulting in a range of differentiated cell types from the three germ layers (Khoo et al., 2005; Passier and Mummery, 2003).

Most of the early differentiation protocols were based on EB formation (Passier and Mummery, 2003) which is accomplished by detaching pluripotent ESCs from feeder-cells, dissociating them into single-cells and transferring them into media containing no substances facilitating pluripotency (Heo et al., 2005). The close association of cells in these cultures promotes the generation of EBs and after culturing for several days they can by transferred to standard liquid cultures to complete their differentiation (Keller, 1995). In “hanging-drop” method known number of dissociated cells are in each drop that is placed on the lid of a culture dish. The culture dish is then closed, leaving drops suspended imitating blastocyst environment. This method is not considered to have a successful yield of cell type of interest because of the low survival rate of the dissociated ES cells (Mogi et al., 2009) (Figure 3).

Another way to trigger spontaneous differentiation is to form cell aggregation in suspension by culturing them on non-adherent surfaces. There, ESC colonies are dissociated into clumps, not single cells, so the colony organization is preserved (Mogi et al., 2009) (Figure 3). Because of the stochastic and unpredictable nature of EB composition many researchers have been trying to develop protocols for directly controlling the differentiation (Terskikh et al., 2006). In directed differentiation previous methods are also used in combination with predicted lineage-specific growth factors.

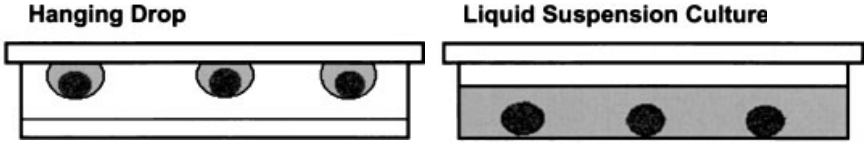


Figure 3: Spontaneous differentiation of hESCs.
In the „hanging-drop“method, ESCs are cultured in a drop of medium simulating blastocyst formation (left panel). In liquid suspension, ESCs are cultured on non-adherent surfaces (right panel). Figure modulated from (Dang et al., 2002).

1.4.2 Directed differentiation

Differentiation of hESCs can be directed *in vitro* towards a certain lineage with several different methods. The most frequently used are: The addition of lineage-specific growth factors to the medium, gene manipulation and co-culturing of hESCs with other cell types. However, these differentiation methods have not been completely evolved so the end result is not a pure culture but a mixture of cell types (Passier and Mummery, 2003).

Co-culturing of hESCs and gene manipulation methods pose a problem for therapeutic use making the addition of growth factors to the medium one of the most successful differentiation approach used. In directed differentiation, EB methods are commonly used with growth factors supplemented in the medium. These methods include suspension culture described above. When culturing EBs in suspension, variables occur that influence the precision of the differentiation, such as the size of hESC clumps that initiate EB formation. A recently described method for differentiation of hESCs addresses a number of shortcomings in differentiation methods. In this method differentiation is initiated by aggregating a known number of undifferentiated hESCs in a 96-well low-attachment plate resulting in the formation of EBs of uniform size in each well. With this method a new defined serum-free medium was introduced that is 'neutral' and permissive for the effects of exogenously added growth factors (Ng et al., 2008).

In order to achieve success in stem cell therapy, pathogen-free differentiated cells need to be generated with high ratio and quality of a desired cell type (Ng et al., 2008; Passier and Mummery, 2003). For that to become a reality, methods for directed differentiation need to be more efficient. To promote effective cardiovascular differentiation it is necessary to clarify and control signaling pathways that regulate the establishment of this lineage during embryonic development (Zhu et al., 2009).

1.5 Cardiac differentiation

One of the first functional organs established in the vertebrate embryo is the heart. At the onset of gastrulation, epiblast cells in the posterior part of the embryo undergo an epithelial to mesenchymal transition (EMT) forming the primitive streak (PS) from which the mesoderm emerges. The newly formed mesoderm then migrates away from the PS patterning into various subpopulations with distinct developmental fates. The first mesodermal cells generated contribute mainly to hematopoietic and vascular lineages of the blood islands in the yolk sac (Figure 4; A). Around embryonic day (*E*) 7.75 of development, mesoderm cells migrate to the anterior region of the embryo forming the cranial border known as the cardiac crescent or primary heart field. The cardiac crescent then fuses at the midline forming the heart tube which harbours progenitors of the

atrial, ventricular and outflow tract lineages together with endothelial cells (Figure 4; B) (Kattman et al., 2007; Pal, 2009).

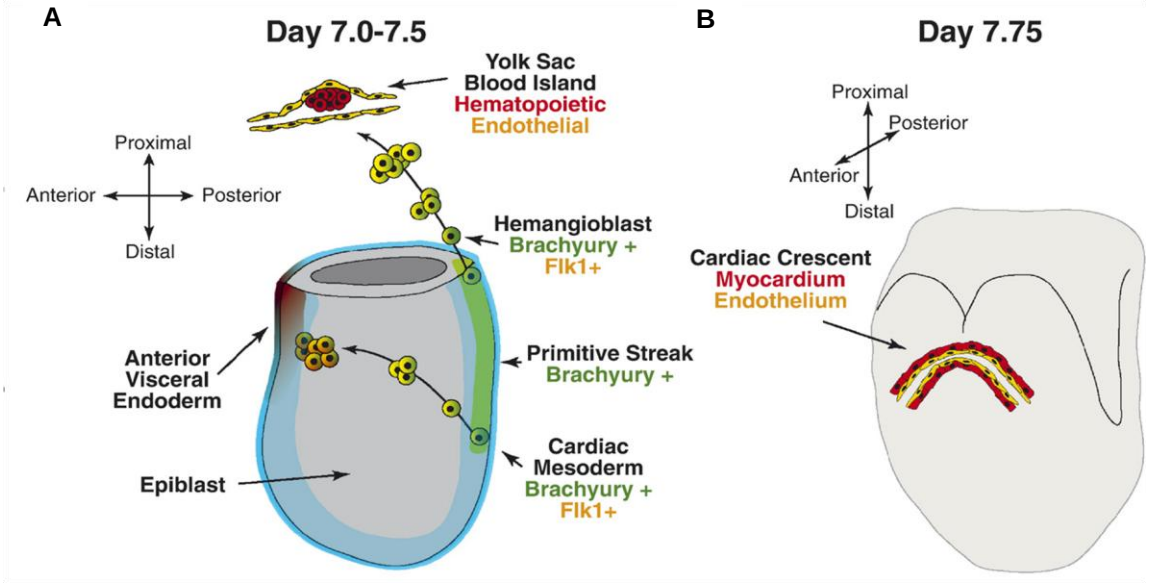


Figure 4: Formation of the cardiac crescent in embryonic development.
(A) The epiblast cells (*gray*) traverse through the PS region expressing *brachyury* (*green*). Cells from the proximal PS Flk1 (*yellow*) migrating into the yolk-sac to contribute to the hematopoietic and vascular lineages. At a later time-point and more distal on the PS, cardiac mesoderm upregulates Flk1 and migrates to the anterior of the embryo. (B) When relocated to the anterior region of the embryo, the cardiac mesoderm commits to the cardiovascular lineages forming the cardiac crescent (Kattman et al., 2007).

One of the earliest markers defining the mesoderm lineage is the T-box transcription factor brachyury (T) which is expressed in the mesoderm and downregulates when cells undergo patterning and specification. One of the next genes to be expressed on different mesodermal subpopulations as they undergo patterning is fetal liver kinase-1 (Flk-1) which encodes the vascular endothelial growth factors receptor-2 (VEGFR2). Flk-1 is also known as KDR (kinase insert domain receptor) and it has been demonstrated to be a broad mesodermal marker which is expressed in cells that will give rise to the hematopoietic, vascular, cardiomyocyte and skeletal myocyte lineages (Kattman et al., 2007; Pal, 2009; Yang et al., 2008). Mesp1 is the earliest marker of cardiovascular development *in vivo* and almost all cells of the future heart are derived from cells expressing Mesp1 at one point (Bondue et al., 2011) (Figure 5).

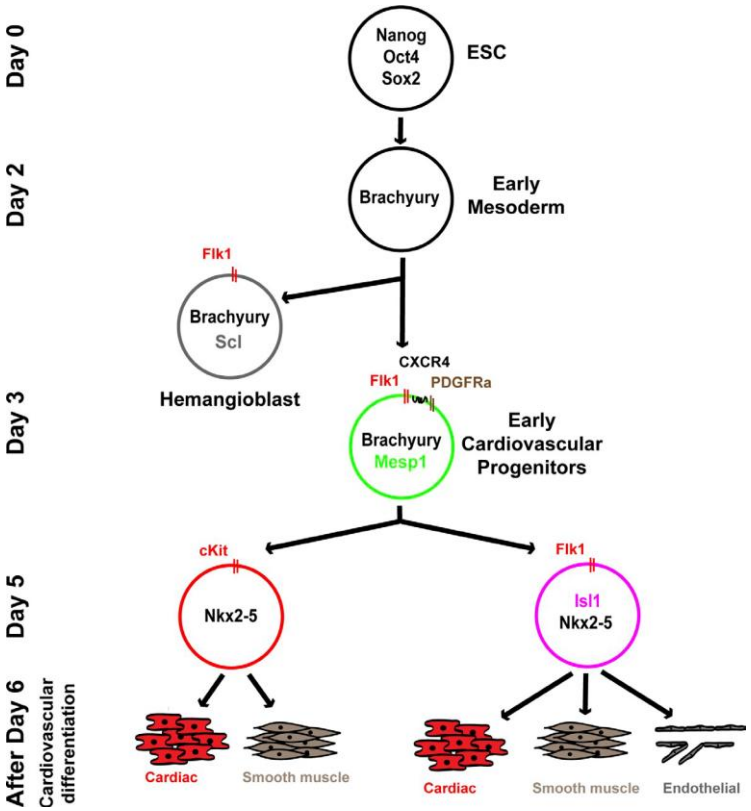


Figure 5: hESC cardiovascular differentiation.
During hESCs differentiation into the cardiovascular lineage. Early cardiovascular progenitors express Flk-1 and brachyury and are able to further differentiate into all cardiovascular lineages (Bondue et al., 2011).

It is now well established that the heart develops from at least two separate progenitor pools or heart fields, the primary heart field and the secondary heart field, identified at the cardiac crescent stage. The secondary heart field is derived from pharyngeal mesoderm cells that contribute to the formation of the right ventricle and outflow tract. Numerous transcription factors function in the establishment of the cardiovascular lineages. Transcription factors involved in the onset of myocardial commitment are first expressed in the cardiac crescent, one of the most conserved being the homeodomain factor tinman/Csx/Nkx2.5 which is essential for appropriate heart formation. The primary and secondary heart fields can be distinguished based on their expression of molecular markers. For example, the transcription factor Tbx-5 (T-box family) is expressed in the primary heart field whereas the secondary heart field expresses Islet-1 (Isl1), a LIM homeodomain transcription factor, and fibroblast growth factor-10 (FGF-10) (Kattman et al., 2007; Yang et al., 2008; Zhu et al., 2009). At ~*E*8.25 the heart tube goes through series of morphogenetic changes, ultimately forming the four-chambered heart (Pal, 2009).

Studies on both mouse and human research models suggest that cardiomyocyte differentiation *in vitro* mimics events during normal embryonic development and that it is regulated by factors provided by adjacent tissue structures (Zhu et al., 2009). These effects can by imitated at some extent by the use of growth factors, including several factors belonging to the TGFβ superfamily, such as Activin A, TGFβ and BMPs. Fibroblast growth factor (FGF) has also been implemented in cardiomyocyte formation by both inducing the formation of functional cardiomyocytes and proliferation of precardiac mesoderm enhancing cardiac development (Kattman et al., 2011; Zhu et al., 2009).

Even though much is known about the differentiation of hESCs to cardiomyocytes, better strategies are needed to overcome the high degree of heterogeneity of differentiated populations. If hESCs are to have a future in cell-based cardiac repair, efficiency of cardiomyocyte formation must be substantially improved (Pal, 2009; Zhu et al., 2009).

1.5.1 Potential uses of hESC-derived cardiomyocytes

There are a number of possibilities regarding the use of hESC-derived cardiomyocytes in the future, both as research tools and for clinical applications. These include drug screening, disease modeling and gene targeting, and regenerative medicine (Dambrot et al., 2011).

One of the major cause of mortality and morbidity in the world are cardiovascular diseases. For the heart to be able to function properly, contraction of cardiomyocytes are essential and disturbances in these processes may lead to serious conditions. Unfortunately, owing to the limited capacity of residing cardiomyocytes, the myocardium is particularly vulnerable to irreversible injury and poor outcome. Our knowledge of the genetic basis of cardiac diseases has increased enormously over the past two decades and recently stem cell-based therapy has become a realistic option to replace damaged cardiomyocytes. However, this is still complex in practice with many aspects still unclear. Among the most important issues being considered is to identify the most suitable strategy for directed cardiac differentiation that results in a homogenous preparation of cardiomyocytes for *in vivo* applications because a great deal of cardiomyocytes is needed in stem cell-based therapy because of a low yield percentage. The challenge for the coming years is to generate *in vitro* models by using hESC-derived cardiomyocytes that harbor disease-associated mutation that mimics in part the cardiac disease phenotype as it manifests in patients (Dambrot et al., 2011; Pal, 2009; Zhu et al., 2009).

1.6 MicroRNA

MicroRNAs (miRNAs) were first discovered in the worm *Caenorhabditis elegans* when characterization of genes that control larval developmental timing revealed two small regulatory RNAs, known as *lin-4* and *let-7 (*Lee et al., 1993*)*. However, miRNAs were not recognized as a distinct class of regulatory elements with conserved function until *lin-4* and *let-7* were reported to represent small endogenous RNAs found in worms, flies and mammals (Lau et al., 2001; Lee and Ambros, 2001). Since then, miRNAs have been found in numerous other species such as plants, green algae, viruses and more deeply branching animals (Griffiths-Jones et al., 2008). Genetic deletions of miRNAs in organisms have shown that few developmental processes are completely dependent on single miRNAs (Small and Olson, 2011) but functional studies indicate that they engage in almost every cellular process regulation that has been investigated and changes in their expression are observed in human pathologies (Filipowicz et al., 2008).

1.6.1 microRNA biogenesis and function

miRNAs are small single-stranded, non-coding RNAs approximately 18-24 nucleotides (nt) in length (Jovanovic and Hengartner, 2006). They are regulators of gene expression inhibiting the expression of specific mRNA targets (Filipowicz et al., 2008). This is most often facilitated through binding of the miRNA seed region with sequences usually located in the 3´untranslated regions (UTRs) (Bartel, 2004). They typically exert modest inhibitory effects on many mRNAs that often encode proteins managing the same biological process (Small and Olson, 2011). The human genome is estimated to encode up to 1.000 miRNAs (Bartel, 2004) where about half of them have already been cloned and confirmed. miRNAs are classified as intergenetic, intronic and exonic based on their location in the genome (Liu and Olson, 2010). Intergenetic miRNAs stem from their own transcriptional units in the intergenetic regions of the genome. Intronic and exonic miRNAs are however located within the introns and exons of host genes whether they are protein-coding genes or non-protein coding (Bartel, 2004).

Biogenesis and effector pathways are similar in most animal miRNAs. They are transcribed by RNA polymerase II as precursor molecules called pri-miRNAs, which can then encode both single and multiple miRNAs (Bartel, 2004; Liu and Olson, 2010). The pri-miRNAs fold back on themselves into imperfect dsRNA-like distinctive hairpin structures containing imperfectly base-paired stems. They are then further processed in the nucleus by RNase III type endonuclease Drosha and its cofactor DGCR8 into 60-100 nt hairpins called pre-miRNAs The pre-miRNA is then

exported out to the cytoplasm by exportin5 where it is further processed by the endonuclease Dicer to yield about 21 bp miRNA duplexes. One strand is then selected to function as a mature miRNA, while most often the complementary strand is degraded. Following their processing, the mature miRNA strand is incorporated into the RNA induced silencing complex (RISC) where it guides the complex to specific targets inducing post transcriptional gene silencing (Figure 6).

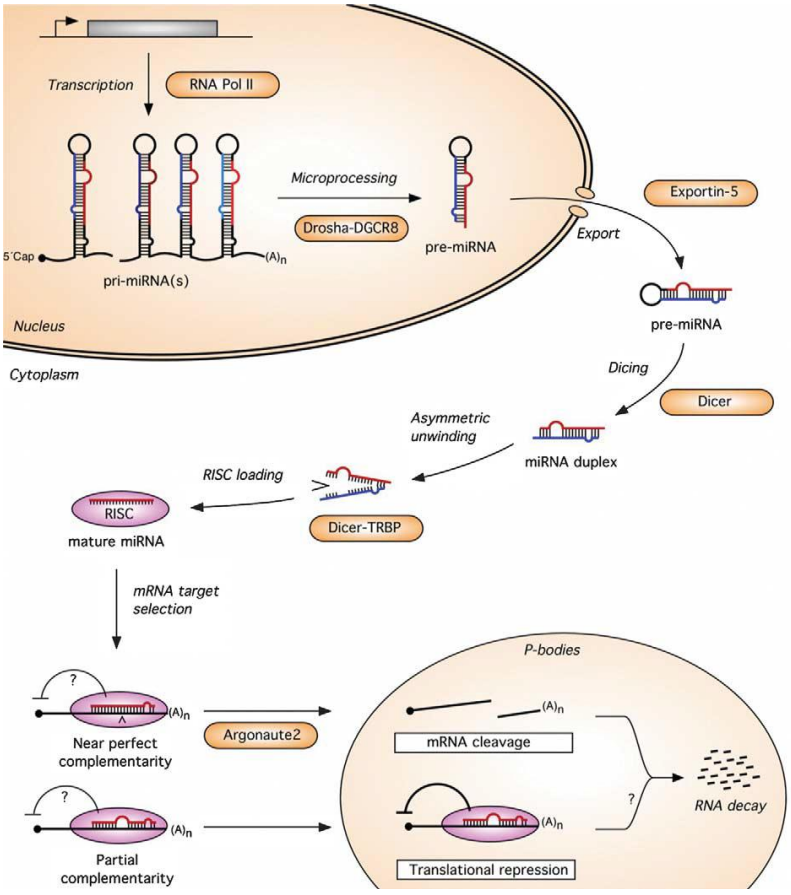


Figure 6: Schematic picture of microRNA biogenesis.
miRNAs are transcribed as long precursors (pri-miRNAs), which are processed in the nucleus by Drosha into hairpins called pre-miRNA. Pre-miRNAs are then exported out of the nucleus to the cytoplasm where Dicer forms mature ~21 bp miRNA heteroduplex. miRNAs are then incorporated into RISC where they act on target mRNA as shown (Wienholds and Plasterk, 2005).

There are several mechanisms that have been proposed for the fate of target mRNA; miRNAs can inhibit translation initiation, mark target mRNAs for degradation or sequester mRNAs into P bodies (Bartel, 2004; Filipowicz et al., 2008; He and Hannon, 2004; Liu and Olson, 2010). In most cases miRNAs act to repress their mRNA targets but in rare cases they have also been reported to stimulate mRNA translation (Vasudevan et al., 2007).

It remains a major challenge to pinpoint how miRNAs identify their mRNA targets. miRNAs, for example in plants, usually base pair to mRNAs with nearly perfect complementarity and trigger endonucleolytic mRNA decay (Filipowicz et al., 2008). However, metazoan miRNAs form in most cases imperfect base pairs with their target sequences and therein lies the target identification difficulties (Bartel, 2009). With only few exceptions, miRNA-binding sites in metazoan mRNAs lie in the 3´UTR where they are usually present in multiple copies for effective repression of translation (Filipowicz et al., 2008) but this is also contradicted (Kozak, 2008).

miRNAs often operate through feedback loops in addition to regulation downstream mRNA targets. This is done to ensure precise control of both their own expression and of their targets (Figure 7). In feed-forward loops gene expression can be enhanced through repression of negative regulators. miRNAs can also repress activators of miRNA/mRNA expression in negative feedback loops (Chen et al., 2006).

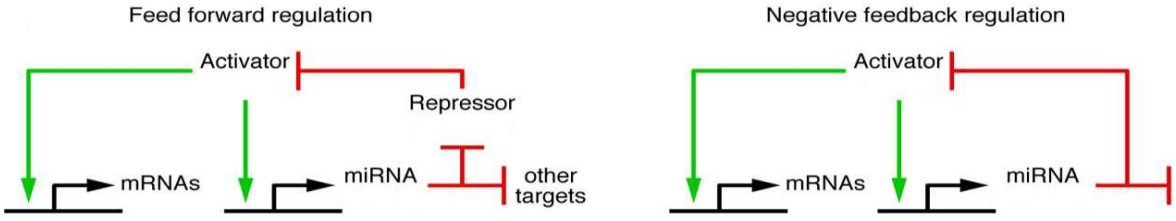


Figure 7: MicroRNAs regulation overview.
MicroRNAs inhibit a repressor in feed-forward regulation leading to activation of transcription factors that then in turn activate miRNAs expression (left panel). In negative feedback loops miRNAs repress transcription factors that are required for miRNA expression which leads to decreased miRNA expression (right panel) (Liu and Olson, 2010).

1.6.2 miRNAs in cardiac development and diseases

The crucial roles of the miRNA pathway in both ESCs and early embryogenesis have been demonstrated in several model organisms by loss-of-function genetic analysis of several components. With genetic ablation of *Dicer,* extensive defects in ESCs are induced and effects can also be seen in early development. Homozygous mutations of the same gene in animals result in lethality or infertility (Wang et al., 2009; Wienholds and Plasterk, 2005). miRNA-deficient ESCs develop both defects in normal proliferation and in differentiation.

During mammalian development, ESCs must shift to alternative molecular programs to inhibit self-renewal and promote differentiation into highly specialized cell types (Gangaraju and Lin, 2009). These dramatic changes are accompanied by the upregulation of many miRNAs. Tissue development depends on the correct spatio-temporal expression of particular miRNAs (Thum et al., 2008) and among the first miRNAs identified as major regulators in lineage determination were those promoting the formation of muscle (Zhao et al., 2005).

To form the heart, cells from multiple cell lineages must differentiate into unique regions, each possessing different electric, physiological and anatomic properties. By expression profiling it has been shown that the 18 most abundant miRNAs in the heart account for over 90% of all cardiac miRNAs (Small and Olson, 2011) were two widely conserved miRNAs, miR-1 and miR-133, play a key role in cardiac- and skeletal muscle proliferation and differentiation (Cordes and Srivastava, 2009; Ivey and Srivastava, 2010; Thum et al., 2008). miR-1 is the most abundant miRNA in cardiomyocytes being the first implicated in heart development. Related to miR-1 is miR-133 which arises from a common precursor RNA. They seem to function together, promoting mesoderm differentiation of ESCs, but later in the cardiac lineage they have opposing roles when miR-1 promotes cardiomyocyte differentiation while miR-133 works as an inhibitor (Small and Olson, 2011).

The heart is very sensitive to both physiological stimuli and pathological states where even slight perturbations can lead to severe cardiac remodeling, often with detrimental outcomes (Thum et al., 2008). Dysregulation of miRNAs by several mechanisms has been described in various disease states including cardiac disease. Today, diseases of the cardiovascular system are the most common congenital birth defects and cause of adult morbidity and mortality. Cellular mechanisms and gene mutations responsible for numerous cardiovascular disorders have been studied a great deal in the last few years and it has become apparent only recently that miRNAs play key roles in cardiovascular diseases. Under certain conditions of stress miRNA function becomes especially pronounced which underscores their role in disease (Small and Olson, 2011). It is of fundamental importance to identify and validate miRNA targets to achieve a comprehensive understanding of miRNA function and potential therapeutic use in heart disease (Thum et al., 2008).

1.6.3 MicroRNA-126

In light of recent studies revealing important roles for miRNAs in the response of the cardiovascular system to injury and stress, two research articles were published in 2008 implicating miR-126 to cardiovascular development in mice (Fish et al., 2008; Wang et al., 2008). miR-126 is encoded by intron 7 of the epidermal growth factor-like domain 7 (*EGFL7*) gene (Small and Olson, 2011) which encodes the biologically active miR-126 and miR-126* in vertebrates (Wang et al., 2008). The location of *mir-126* gene in intron 7 is conserved in mouse, human and zebrafish making it evolutionary conserved between species (Wienholds and Plasterk, 2005) (Figure 8). miR-126 has been found to regulate the response to the growth factor VEGF in part by directly repressing negative regulators of the VEGF pathway. These negative regulators include the Sprouty-related protein Spred-1. Thus, in the absence of miR-126, increased expression of Spred-1 diminishes the transmission of intracellular signals by VEGF. With studies on mouse embryonic stem cells

(mESCs) and mouse embryos it was shown that miR-126 is expressed in a broad range of tissues, with the highest expression in both lung and heart (Fish et al., 2008; Wang et al., 2008).

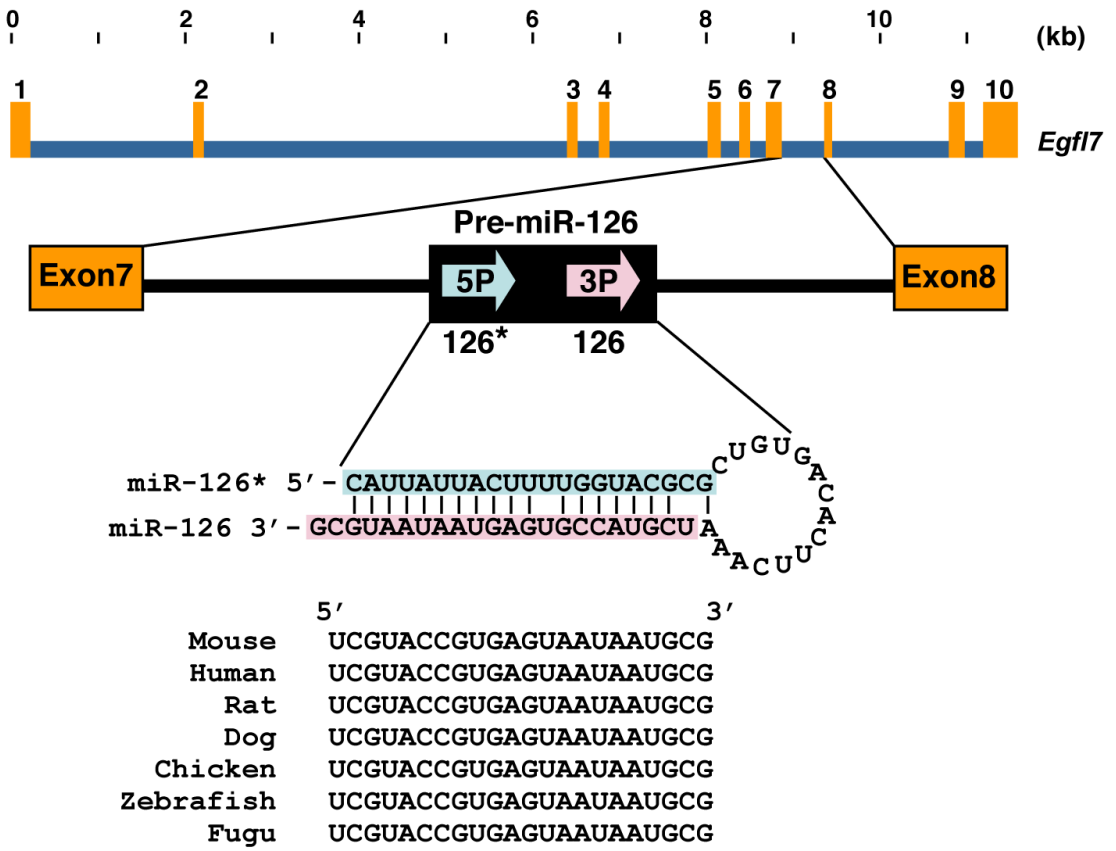


Figure 8: Structure of the mouse *EGFL7* gene.
miR-126 (miR-126-3p) and miR-126* (miR-126-5p) are generated as stem loops encoded by intron 7. Evolutionary conservation of miR-126 is very high as shown (Wang et al., 2008).

Significant advances have been made in our understanding of cardiac differentiation from mouse and human experimental models. Findings from these studies have demonstrated that these three lineages develop from a common cardiovascular progenitor that can be monitored by expression of kinase insert domain protein receptor (Flk-1/KDR). This progenitor represents one of the earliest stages in mesoderm specification to the cardiovascular lineages (Kattman et al., 2011; Yang et al., 2008). Furthermore, Fish et al. demonstrated that Flk-1 positive cells generated from mESCs in a 4 day old EB model were enriched of miR-126 together with endothelial cells ($CD31^{+}$) suggesting a role in the cardiovascular lineage (Fish et al., 2008). miR-126 and miR-126* are both relevant for the development of the cardiovascular system and have been implicated in both cardiovascular diseases and formation of cancer (Nikolic et al., 2010).

2 Project aim

Controling the formation of cardiomyocytes *in vitro* from human embryonic stem cells (hESCs) has until now been difficult. However, with increasing knowledge it is becoming a procedure which can be manipulated and directed to a certain extent. Although much research remains, new and improved protocols for guiding pluripotent stem cells to the cardiomyocyte lineage are accumulating in the scientific literature using both serum-free and feeder-free culturing conditions, favouring further use in stem cell therapy.

The thesis is divided into two main parts:

1. Differentiation of human embryonic stem cells into cardiomyocytes

The first aim of this project was to establish efficient cardiomyocyte differentiation method of hESCs in our lab using published reports describing various procedures and the use of serum-free medium and lineage-specific growth factor for cardiomyocyte facilitation. Morphology and gene/protein expression were determined in cells undergoing differentiation.

2. Role of microRNAs in cardiovascular differentiation of hESCs

The second aim of this study was to investigate the role of microRNAs in hESC cardiovascular development because of their importance in cardiovascular disease and development. MicroRNA-126 has been implicated to the cardiovascular system in mouse development and mESCs. Therefore, the role of miR-126 in cardiovascular development was investigated in hESCs by monitoring morphological changes and relative gene expression.

3 Materials and methods

3.1 Cell culture and maintenance of hESCs

Three different hESC lines, called HUES9, H1 and HES2 were used in this study. HUES9 was obtained from Douglas A. Melton at Howard Hughes Medical Institute, Harvard University (Cowan et al., 2004) and the H1 cell line from WiCell (Thomson et al., 1998). The HES2 cell line was obtained from ES Cell International (ESI) (Reubinoff et al., 2000). The hESC lines were routinely cultured on a feeder layer of irradiated primary mouse embryonic fibroblasts (MEFs) monolayer, obtained from bioproduct distributor ATCC-LGC Standards. MEF cells were seeded on two 0.1% gelatin coated 6-well cell culture plates (NUNC) in MEF medium (Table 1), 5 x 10^5 cells per well.

Table 1: MEF medium

MEF medium	Stock concentration	Final concentration
DMEM (11960-044) (Invitrogen)		
Fetal Calf Serum (Sigma-Aldrich)		10%
Penicillin (Invitrogen)	5000 U/ml	50 U/ml
Streptomycin (Invitrogen)	5000 µg/ml	50 µg/ml
GlutaMAX (Invitrogen)	200 nM	2 mM
Non Essential Amino Acids (Invitrogen)		100x dilution

hESC lines were passaged every three days and hESC growth medium (Table 2) replaced daily. To accelerate cell growth the growth medium was supplemented with 10 ng/ml bFGF and 10% conditioned medium. Conditioned medium (CM) was collected from MEF cells which secrete defined and undefined factors facilitating both cell growth and pluripotency. Approximately 4 ml of hESC growth medium without bFGF was applied to wells that contained only MEF cells. Every 24 hours the medium was collected and replaced with fresh hESC growth medium. CM was filter-sterilized using 0.22 µm filter before use.

Table 2: hESC growth medium

hESC medium	Stock concentration	Final concentration
DMEM/F-12 + GlutaMAX (31331-028) (Invitrogen)		
Conditioned medium (CM)		10%
KO Serum Replacement (Invitrogen)		20%
Non Essential Amino Acids (Invitrogen)		100x dilution
GlutaMAX (Invitrogen)	200 nM	2 mM
Penicillin (Invitrogen)	5000 U/ml	50 U/ml
Streptomycin (Invitrogen)	5000 µg/ml	50 µg/ml
2-Mercaptoethanol (Invitrogen)	55 mM	77 µmol/ml

3.1.1 Feeder-free hESC culture

To maintain hESCs undifferentiated without feeder layer, cells were feeder depleted and cultured on CELLstart (1:50 dilution in 1 x PBS) or Matrigel (1:50 dilution in DMEM). The Matrigel used was a Growth Factor Reduced BD Matrigel^{TM} Matrix (BD Biosciences). CELLstart (Invitrogen) is a xeno-free defined substrate that only contains components of human origin. It provides attachment for the cells and hESC promotes pluripotency.

When grown without feeders, cells were either cultured in 50% CM and 50% normal hESC growth medium combined with 10 ng/ml bFGF supplemented or in mTeSR^{TM}1 medium (STEMCELL Technologies). mTeSR1 medium is a feeder-independent maintenance medium for hESCs. It is a serum-free defined formulation and does not require addition of other growth factors.

3.1.2 Passage of human embryonic stem cells

1. Pre-warm hESC growth medium (Table 2) at 37°C.
2. Remove and discard culture medium and wash cells once with 1 ml 1 x PBS.
3. Dissociate cells using 0.5 ml TrypLE (Invitrogen) and incubate at 37°C for 1 min.
4. Inactivate TrypLE with 2.5 ml hESC growth medium and transfer to a 15 ml tube.
5. Pipette 6-10 times to get a single cell suspension.
6. Split cells in 1:10 ratio.

3.2 hESC differentiation

Three differentiation methods were used in this study; Embryoid bodies “hanging-drop” method, embryoid bodies in suspension and SpinEBs method. In all these methods growth factors were used to facilitate mesodermal differentiation.

3.2.1 Embryoid bodies "hanging-drop" method

1. Dissociate hESCs using TrypLE and incubate at 37°C for 1 min.
2. Inactivate TrypLE, count the number of cells and dilute in differentiation medium (Table 3) to give 60.000 cells/ml (=1.200 cells/20 µl).
3. Add 10 ml of 1 x PBS in a 10 cm cell culture dish to sustain humidity.
4. Pipette 20 µl cell suspension onto the lid of the cell culture dish. Turn the lid on top of the culture dish, the drops should be hanging vertically downwards.
5. Incubate at 37°C for 6 days.

   Note: After 3-5 days of culture each hanging drop contains an EB that is comprised of various differentiated cell types.

All experiments were performed with 10 ng/ml BMP4 supplemented or untreated as a control. After 6 days EBs were transferred to 0.1% gelatin coated 96-well plates in differentiation medium for further culturing. Culture medium was changed routinely and cells observed for beating areas around day 12.

3.2.2 Embryoid bodies in suspension

This protocol was merged from two published papers emphasizing on cardiomyocyte differentiation (Leschik et al., 2008; Yang et al., 2008). There, they describe the use of several growth factors facilitating mesodermal differentiation, preferably to cardiomyocytes around day 14. All growth factors were supplied by PeproTech.

1. hESC lines grown on MEFs in 6-well tissue culture plates.
2. Dissociate hESCs into clumps using 1 ml of 1 mg/ml dispase (Invitrogen) diluted in DMEM/F12 medium.
3. Incubate for 15 min at 37°C with mild shaking.
4. Inactivate the dissociative agent, scrape cells and place in 15 ml tubes.

5. Feeder-deplete hESCs by transferring onto 0.1% gelatin coated plates for 30 min at 37°C.
6. Harvest non-attached cells and spin down at 800 rpm for 4 min.
7. Resuspend in differentiation medium (mix very gently) and transfer clumps to 12-well low attachment plates (BD-Falcon) in differentiation medium containing 5 ng/ml BMP4.
8. 24 h later – add BMP4 resulting in 10 ng/ml BMP4 total, additionally add 5 ng/ml bFGF and 3 ng/ml Activin A.

   EBs form in 2-3 days. After 7-8 days in suspension, EBs can be transferred onto gelatin-coated plates at a density of 2 EBs per cm^2 and culture.

9. Each EB is examined for the presence of beating cardiomyocytes at day 12.

Table 3: hESC differentiation medium

hESC differentiation medium	Stock concentration	Final concentration
hESC growth medium (Table 2)		
α-Monothioglycerol (α-MTG)	1.3%	400 µM

α-Monothioglycerol (α-MTG; Sigma-Aldrich) is a 3-Mercaptopropane-1,2-diol used in cell culture to prevent free radicals in the medium.

3.3 Western blotting

Cells were lysed in lysis buffer (appendix B) and samples then sonicated. 2X sample buffer (appendix B) was added and samples boiled at 95°C for 10 minutes before running them on 10% or 12,5% SDS PAGE gels, depending on the size of the protein being blotted. Proteins were then transferred to a PDVF membrane followed by preincubation for 30 minutes in 5% milk diluted in TBS-T at RT. Primary antibodies (Table 4) were mixed with TBS-T containing 3% milk and incubated on a shaker over night at 4°C. Following incubation the membranes were washed in TBS-T and then incubated with secondary antibodies (Table 4) on a shaker for 1 hour at RT. Finally, the membranes were treated with ECL Western Blotting Detection System (GE Healthcare) and developed in a dark room with Dürr Med Medicine 260 developing device.

Table 4: Antibodies used for western blotting

Antibody against	Cat. No	Origin	Type	Dilution	Supplier
Actin	MAB1501R	Mouse	Primary	1:10000	Millipore
Phospho-Smad1	S463/465	Rabbit	Primary		Cell Signalling
Anti-Mouse	NA931	Sheep	Secondary		GE Healthcare
Anti-Rabbit	NA934	Donkey	Secondary		GE Healthcare

3.3.1 Spin EBs differentiation

This published protocol is relatively new, describing hESC differentiation using EBs in defined serum-free medium called BPEL (Table 5) (Ng et al., 2008). BPEL (Bovine Serum Albumin Polyvinylalchohol Essential Lipids) is neutral and permissive for the effects of exogenously added growth factors. Growth factors were supplemented in BPEL medium facilitating mesodermal differentiation.

Day 0:

1. Split hESCs ($2.3x10^6$) using normal passaging procedure (see 3.1.2) onto matrigel coated plates in 50% CM and 50% normal hESC growth medium with 10 ng/ml bFGF.
2. Store at 37°C overnight or until 70-90% confluent.

Day 1:

3. Use sterile 96-well V-shaped low-attachment plates.
4. Add 100 µl dH_2O to the 36 outer wells of each plate to humidify the EBs during the differentiation process.
5. Rinse cells with 1 x PBS.
6. Dissociate cells to generate single-cell suspension.
7. Inactivate, resuspend and dilute the total number of cells needed supplemented with growth factors.

    - Optimum cell number is 2000-3000 cells/well
    - Need 1.8 x 10^5 cells in 6 ml BPEL medium for each 96-well plate

8. Aliquot 100 µl of the hESC suspension in BPEL medium (Table 5) into each well.
9. Aggregate cells by centrifugation at 1100 rpm for 2 min at 4°C to induce the formation of spin EBs and incubate at 37°C.

Day 2:

10. Add 10 µl BPEL differentiation medium supplemented with 10 ng/ml BMP4, 5 ng/ml bFGF and 3 ng/ml Activin A to where required.

Day 8:

11. Transfer spin EBs to 0.1% gelatin coated flat-bottomed, tissue culture-treated 96-well plates (BD-Falcon) in fresh BPEL medium for further culturing and incubate at 37°C.

Examine spin EBs after day 12 for beating areas and change medium routinely.

Table 5: BPEL medium

Compound	Stock concentration	Amount	Final concentration
Iscove´s modified Dulbecco´s medium (IMDM)	1x	86.2 µl	
Ham´s F-12 nutrient mixture	1x	86.2 µl	
Deionized BSA	10%	5 ml	2.5 mg/ml
Polyvinylalcohol (PVA)	5%	5 ml	
Linoleic acid	10.000x	20 µl	100 ng/ml
Linolenic acid	10.000x	20 µl	100 ng/ml
SyntheChol	7.200x	28 µl	2.2 µg/ml
α-Monothioglycerol (α-MTG)	1,30%	600 µl	~ 400 µM
rh Insulin-transferrin-selenium-ethanolamine sol.	100x	2 ml	
Protein-free hybridoma mixture II (PFHMII)	1x	10 ml	5%
Ascorbic acid 2 phosphate	5 mg/ml	2 ml	50 µg/ml
Glutamaxl (L-alanyl-L-glutamine)	200 mM (100x)	2 ml	2 mM
Penicillin	5000 µg/ml	2 ml	50 µg/ml
Streptomycin	5000 U/ml	2 ml	50 U/ml
	Total	200 ml	

3.3.2 Immunofluorescent staining

Cell cultures were fixed with 2% paraformaldehyde in 1 x PBS for 30 min, followed by 8 min incubation in Triton X-100 in 1 x PBS to permeabilize the cell membrane. Both steps were performed at room temperature. Samples were then preincubated in PBS and 4% goat serum (GS). Primary antibody (Table 6) was mixed with PBS containing 4% GS and incubated on a shaker over night at 4°C. Then samples were washed in PBS/0.05% Tween and incubated with fluorescently labeled secondary antibody (Table 6) for 2 hours in dark. Samples were rinsed again with PBS/0.05% Tween and then mounted with cover slips using Fluoromount (Sigma-Aldrich) for preservation of the fluorescent signal. Immunofluoresence was visualized using a confocal Zeiss LSM 5 Pascal laser scanning microscope.

Table 6: Antibodies used for immunofluorescent staining

Antibody against	Cat.No	Origin	Type	Isotype	Dilution	Supplier
Islet-1	39.4D5-c	Mouse	Primary	IgG2b	1:50	DSHB
Cy3 anti-mouse	115-165-146	Goat	Secondary	IgG	1:250	Jackson ImmunoResearch

3.4 Lentiviral vector preparation

For lentiviral transfection, three plasmids were used: pLVTHM cloning vector, psPAX2 packaging plasmid and pMD2.G envelope plasmid for production of viral particles (Addgene, see maps in appendix A). All plasmids were provided in the form of *E.coli* bacterial stabs and spread on LB agar plates supplemented with 100 µg/ml ampicillin (AMP) and incubated overnight in 37°C (see appendix B). The next day colonies were put in liquid LB medium and incubated in 37°C shaker for 20 hours (see appendix B). After verifying plasmids using restriction site analysis, plasmids were amplified and frozen for storage.

pLVTHM plasmid has an additional ClaI site which is blocked by Dam methylation. In order to use ClaI for cloning the plasmid was grown in a Dam negative bacterial strain. After plasmid preparation, plasmids were analyzed using agarose gel electrophoresis and restriction enzymes (using Gene construction kit program, Texto biosoftware). After plasmid verification, plasmids were amplified and frozen in glycerol stock. pLVTHM was digested with ClaI and MluI ready to use.

3.5 MicroRNA-126 design

For pLVTHM vector, primers were designed using Primer3 PCR primer program online (Primer 3 v.0.4.0) and DNA amplified from human genomic DNA (provided by Bjarki Guðmundsson). MicroRNA-126 (miR-126) was amplified both with 100 bp flanking sites and the whole intron 7 spanning ~500 bp. For cloning into the pLVTHM vector, restriction sites (MluI and ClaI) were designed on the 5´ and 3´ ends of the primers (Table 7).

Table 7: Primer sequences for Intron 7 and miR-126 primers

Species	Primer	Primer sequence	Product	PCR conditions
Human	Intron 7 EGFL7 f	5´-gcg acg cgt gtg tgg ctg tta ggc atg gt-3´	483	AT = 65
	Intron 7 EGFL7 r	5´-gcc atc gat agg cag agc cag aag act ca-3´		
Human	mir126 EGFL7 f	5´-gcg acg cgt gag gga gga tag gtg ggt tc-3´	339	AT = 66
	mir126 EGFL7 r	5´-gcc atc gat cag agg tct cag ggc tat gc-3´		

Both the PCR reactions were performed in 50 µl reaction volumes (Table 8) in triplicates with minus controls. The PCR program was the same for both Intron 7 primers and miR-126 primers varying slightly in annealing temperature (AT) (Table 9).

Table 8: PCR reaction for intron 7 and miR-126 primers

	Volume (µl)
10x Pfu buffer (Fermentas)	5
Genomic DNA (150-230 ng/µl)	2
dNTP (10 mM)	1
Forward primer (5 µM)	0.8
Reverse primer (5 µM)	0.8
$MgSO_4$ (25 mM)	6
Pfu polymerase (Fermentas)	0.6
Deionized H_2O	33.8
Total	50

Table 9: PCR program for Intron 7 and miR-126 primers

	Intron 7		miR-126	
Step	Temperature (°C)	Time	Temperature (°C)	Time
1	95	5 min	95	5 min
2	95	30 sec	95	30 sec
3	65	30 sec	66	30 sec
4	72	2 min	72	2 min
	Cycle to step 2 for 35 more times			
5	72	10 min	72	10 min
6	4	∞	4	∞

PCR products were analyzed with agarose gel electrophoresis and DNA purified from the solution using GFX PCR DNA (see 3.5.1).

3.5.1 Purification of DNA from solution

1. Place one GFX column in a collection tube for each purification.
2. Add 500 µl of capture buffer to the GFX column.
3. Transfer the DNA solution to the GFX column and mix thoroughly.
4. Centrifuge in a microcentrifuge at full speed for 30 sec and discard the flow-through.
5. Add 500 µl of wash buffer to the column and centrifuge at full speed for 30 sec.
6. Transfer column to a fresh 1.5 ml tube, apply 10-50 ml of elution buffer (Type 4) and wait for 1 min at RT.
7. Centrifuge at full speed for 1 min and store at -20°C.

After purification, DNA was digested with ClaI and MluI and DNA concentration measured with NanoDrop. The inserts were ligated into pLVTHM vector with T4 DNA ligase (Fermentas) as described by the manufacturer and transformed using electroporation. After electroporation the transformation mix was plated on LB agar with ampicillin (50 µg/ml) and incubated for 16-18 hours at 37°C.

3.5.2 Sequencing plasmid preps

Primers were designed to sequence pLVTHM plasmid on both sides of the inserts for verification (Table 10). This protocol was used for sequencing the plasmid preps with BigDye Terminator v.3.1 Cycle Sequencing Kit from Applied Biosystems.

Table 10: pLVTHM sequencing primers

Primer	Primer sequence	Length
pLVTHM seq f	5´-gca tgt cgc tat gtg ttc tg-3´	20
pLVTHM seq r	5´-aga gac cca gta caa gca-3´	18

1. Mix the sequencing reaction (Table 11), adding the BigDye v.3.1 last. If sequencing many samples, make a mastermix leaving out the DNA and primer unless they are the same for all samples. *Protect the BigDye from exposure to light.*

Table 11: Sequencing reaction

Compound	Solution concentration	Amount (µl)	Final concentration
DNA	200 ng/µl	1	~ 20 ng/µl
Sequencing Buffer	5 x	1.5	0.75 x
Primer	1.6 µM	1	0.16 µM
BigDye v.3.1.		1	
MQ H_2O		5.5	

2. Mix carefully and centrifuge before starting the sequencing program (Table 12). Allow the block to heat up before adding samples.

Table 12: Sequencing program

Step	Temperature (°C)	Time
1	96	10 sec
2	96	10 sec
3	50	5 sec
4	60	2 min
Cycle to step 2 for 25 more times		
5	60	7 min
6	12	∞

3. Remove samples from the PCR block and spin down carefully. *Protect samples from light.*

3.5.3 Precipitation of sequencing reaction

1. Transfer the sequence reactions (10 µl) to 1.5 ml tube.
2. Add 50 µl of a water-NaOAc-glycogen stock solution (Table 13).

Table 13: Water-NaOAc-Glycogen stock solution for 16 sequencing reactions

Compound	Solution concentration	Concentration in Reaction
MQ H_2O	900 µl	
3 M NaOAc (pH 5.2)	100 µl	81 mM
Glycogen (20 mg/ml)	5 µl	2.7 mM

3. Add 125 µl of ice-cold 96% ethanol and mix well by inverting.
4. Spin down at 10.500 RPM (11.710 RCF) for 15 min at 4°C.
5. Aspirate to remove supernatant. Do not touch the invisible pellet, located bottom side facing outwards.
6. Wash with 250 µl of 70% ethanol. Spin down at 10.500 RPM for 5-6 min at 4°C. Remove ethanol by suction and repeat.
7. Let the ethanol evaporate in the dark at RT or for 3-4 min at 55°C.
8. Add 10 µl of HiDi formamide, vortex twice and spin down at 10.500 RPM.
9. Load samples in the sequencing tray, spin down carefully.

3.6 Lentiviral work

3.6.1 Transfection

HEK-293T cells (human embryonic kidney) were transfected with pLVTHM containing either miR-126 insert or Intron 7 insert. Cells were also transfected with pLVTHM empty vector as a control. The amounts below are given for a T25-culture flask (60 mm).

1. Plate cells so they reach confluence of 60-90% at the time of transfection.
2. Add 5 µg of plasmid DNA into 2 ml of serum-free high glucose DMEM (OPTI-MEM, Invitrogen) in a microcentrifuge tube.
3. Gently mix TransPass D2 Transfection Reagent (New England BioLabs) tube before pipetting. Do not vortex.
4. Add 5 µl TransPass D2 Transfection Reagent per tube and mix well by flicking. Incubate at room temperature for 20-30 min to form the transfection complexes.
5. Wash cells once with serum-free medium.
6. Aspirate the culture medium and replace immediately with the transfection mixture. Rock the plate gently and place it in the incubator for 2-3 hours incubation in 37°C.

After incubation discard the transfection medium and replace with 5 ml of complete growth medium containing serum and incubate for 24 hours. Remove medium containing the lentiviral particles, centrifuged and filter-sterilized before use. Lentiviral particles can be collected for an additional 24 hours.

3.6.2 hESC infection

Coat 24-well culture dish with CELLstart (1:50) prior to seeding. Passage hESCs when 80% confluent using normal passaging procedures (3.1.2) and seed in 200 µl of medium made of 50% conditioned medium and 50% hESC growth medium. Immediately after passage, place 200 µl of fresh transfection medium containing 15 µg/ml polybrene (Hexadimethrine bromide). Incubate at 37°C for 18-24 hours. Remove medium and replace with fresh 50:50 medium without polybrene and check for GFP expression.

3.6.3 RNA Isolation

For RNA isolation, RNeasy Mini Kit from Qiagen was used which is designed to purify RNA from small amounts of starting material. With this RNeasy procedure, all RNA molecules longer than 200 nt are purified and ready to use. RNA isolation was mainly performed on EBs in suspension or spin EBs, in some cases cells from monolayer cultures were harvested and RNA isolated.

1. Harvest cells according to step 1a or 1b.
    a. **Cells grown in suspension**: Pellet cells by centrifuging for 5 min at 300 x *g*. carefully remove all supernatant by aspiration and proceed to step 2.
    b. **Cells grown in monolayer**: Harvest cells using normal passaging procedure (see 3.1.2). Pellet cells, remove supernatant and proceed to step 2.
2. Disrupt cells by adding Buffer RLT (see appropriate volume Table 14). Vortex or pipette to mix and proceed to step 3. *Addition of β-mercaptoethanol (β-ME) to Buffer RLT is recommended, 10 µl β-ME per 1 ml Buffer RLT.*

Table 14: Volumes of Buffer RLT

Number of cells	Volume of Buffer RLT
‹ 5 x 10^6	350 µl
5 x 10^6 - 1 x 10^7	600 µl

3. Homogenize the lysate by passing it at least five times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe.
4. *Continue according to RNeasy Mini Kit protocol*.

After isolation, RNA concentration was measured using NanoDrop spectrophotometer and RNA integrity was determined on Agilent 2100 Bioanalyzer RNA Nano chips. Samples were then stored in -80°C.

3.6.4 cDNA synthesis

For cDNA synthesis SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) was used. This system is optimized to synthesize first-strand cDNA from purified total RNA.

1. Use 1-5 µg of total RNA and add RNase-free water up to 10 µl final sample amount.
2. Mix 10 µl of total RNA with 10 µl of 2x Rxn Mix (RT Reaction Mix) and incubate at 65°C for 5 min.
3. Let stand on ice for 1 min and then add 2 µl of RT Enzyme Mix (1:10 of total sample volume). Incubate for 30-50 min at 50°C.
4. Terminate reaction by incubation at 85°C for 5 min and place on ice.
5. Add 1 µl RNase H (2 U/µl) and incubate at 37°C for 20 min.

After cDNA synthesis PCR reaction was performed on samples according to Table 15 with primers for hARP (Biomers) housekeeping gene (Table 17). DNA was amplified using standard PCR program (Table 16)

Table 15: PCR reaction solution

Reaction solution	Volume (µl)
10x DreamTaq Buffer (Invitrogen)	2.5
$MgCl_2$ (Invitrogen)	1.5
dNTP Mix, 2mM each (Invitrogen)	0.5
Forward primer (Biomers, 100 pmol/µl)	0.5
Reverse primer (Biomers, 100 pmol/µl)	0.5
Water, nuclease-free (Invitrogen)	18.5
cDNA	1
DreamTaq DNA Polymerase (Invitrogen)	0.06
Total	25

Table 16: Standard PCR program

Step	Temperature (°C)	Time
1	95	1 min
2	95	45 sec
3	60	1 min
4	72	1 min
Cycle to step 2 for 30 more times		
5	72	7 min
6	4	∞

3.6.5 Quantitative real-time PCR (qRT-PCR)

Real-time PCR was performed on diluted samples and 15 µl reactions carried out using Maxima SYBR green/ROX qPCR master mix (Fermentas). The control gene used was hARP. The procedure was carried out according to the manufactures protocol using the following primers (Biomers, Table 17). After the run, the 7500 Software v2.0 (Applied Biosystems) gives a Ct value for each sample. Ct values are then used to calculate relative quantification values, or fold difference, relative to the selected house-keeping gene.

Table 17: Primers used in this work

Species	Primer	Primer sequence	Product	PCR conditions
Human	hARP F 569 hARP R 684	caccattgaaatcctgagtgatgt tgaccagcccaaaggagaag	115 bp	AT = 60°C
Human	hISLETf-Q hISLETr-Q	ttgtacgggatcaaatgcgccaag aggccacacagcggaaaca	109 bp	AT = 60°C
Human	hNKX2.5f-Q hNKX2.5r-Q	acctcaacagctccctgactct ataatcgccgccacaaactctcc	155 bp	AT = 60°C
Human	hTf-Q hTr-Q	cagtggcagtctcaggttaagaagga cgctactgcaggtgtgagcaa	122 bp	AT = 60°C
Human	hCTNTf hCTNTr	ttcaccaaagatctgctcctcgct ttattactggtgtggagtgggtgtgg	166 bp	AT = 62°C
Human	hKDRf hKDRr	cctctactccagtaaacctgattggg tgttcccagcatttcacactatgg	219 bp	AT = 60°C
Human	hTBX5f hTBX5r	aaatgaaacccagcataggagctggc acactcagcctcacatcttaccct	200 bp	AT = 60°C
Human	hCD34f hCD34r	tgaagcctagcctgtcacct cgcacagctggaggtcttat	200 bp	AT = 57°C
Human	CD31f CD31r	atcatttctagcgcatggcctggt atttgtggagggcgaggtcataga	159 bp	AT = 60°C

3.7 Adenoviral infection of hESCs

hESCs were infected with adenoviruses expressing constitutively active (ca) ALK2, caALK3, caAlk6 and LacZ using a multiplicity of infection (MOI) of 100. Optimized infection efficiency of the adenovirus had previously been verified. All constructs are tagged with hemagglutinin protein, HA. Medium containing adenoviruses was removed from the cells after 24 hour infection and replaced with fresh hESC growth medium.

4 Results

Our knowledge about differentiation methods that direct hESCs into mesodermal differentiation, especially into cardiomyocytes, is rather limited and techniques generally used to initiate *in vitro* differentiation of hESCs result in a mixture of random cell types. (Ng et al., 2008; Passier and Mummery, 2003).

Recently described method for differentiation of hESCs addresses a number of shortcomings in previously discussed differentiation methods. In this method differentiation is initiated by aggregating a known number of undifferentiated hESCs in a 96-well low-attachment plate resulting in the formation of EBs of uniform size in each well. With this method a new defined serum-free medium was introduced that is 'neutral' and permissive for the effects of exogenously added growth factors (Ng et al., 2008).

In order to achieve success in stem cell therapy, pathogen-free differentiated cells need to be generated with a high ratio and quality of a desired cell type (Ng et al., 2008; Passier and Mummery, 2003). For that to become a reality, methods for directed differentiation need to be more efficient. To promote effective cardiovascular differentiation it is necessary to clarify and control signaling pathways that regulate the establishment of this lineage during embryonic development (Zhu et al., 2009).

4.1.1 Role of growth factors in cardiac differentiation

The cardiac mesoderm is one of the earliest stages of differentiation towards cardiomyocytes, monitored by the temporal expression of Flk-1 (KDR) (Kattman et al., 2011; Yang et al., 2008). Cardioinductive activity can be at least partially mimicked by a number of growth factors, including Activin A, TGFβ, BMPs and FGF (Kattman et al., 2011; Zhu et al., 2009). In previous reports it has been documented that BMP2/4, both alone and in combination with Activin A and FGF, induces cardiovascular development (Laflamme et al., 2007; Leschik et al., 2008; Takei et al., 2009; Yang et al., 2008).

Because of these published reports it was interesting to examine the role of BMP4 alone or in combination with Activin A and bFGF in the differentiation of HUES9 and HES2 stem cell lines. EB induction was facilitated both in suspension and by using the spin EB method described earlier, in combination with serum-free medium to get only the effects of added growth factors on the differentiation.

4.1.2 Treatment with growth factors gives rise to more compact EBs

To trigger spontaneous differentiation, undifferentiated hESCs colonies were dissociated into clumps and suspended in serum-free medium on non-adherent surfaces. Within three days of cell culture, compact EBs had formed. HUES9 was cultured in serum-free medium alone, supplemented with 10 ng/ml BMP4 or a growth factor (GF) cocktail of 10 ng/ml BMP4, 5 ng/ml bFGF and 3 ng/ml Activin A. These different culturing conditions resulted in a morphology change where untreated EBs were small and loose whereas EBs treated with growth factors had a more compact morphology as aimed for when forming EBs in suspension. Difference between EBs cultured with BMP4 alone or a GF cocktail could not be detected (Figure 9).

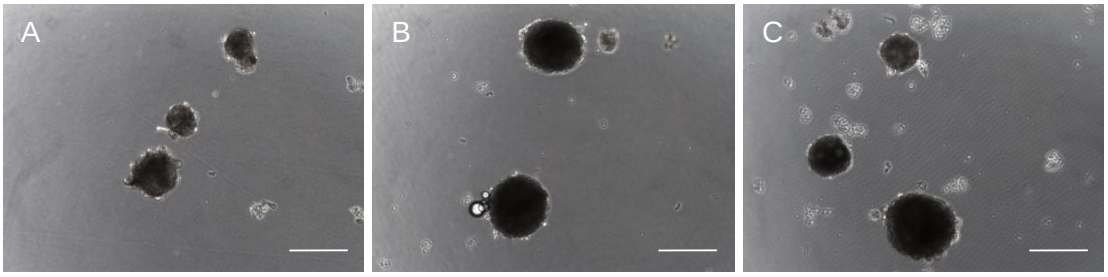


Figure 9: Growth factor treated hESCs form more compact EBs than untreated hESCs.
HUES9 cell line cultured in suspension. Differentiation at day 9. (A) Untreated. (B) 10 ng/ml BMP4 treated hESCs. (C) 10 ng/ml BMP4, 5 ng/ml bFGF and 3 ng/ml Activin A treated. Bars represent 200 µm.

4.1.3 Formation of beating areas

To maintain pluripotency, HES2 cells were cultured on MEF feeder-layer forming round colonies in a monolayer with defined borders (Figure 10; A). One day prior to start of experiment, cells were passaged onto matrigel for feeder depletion and grown to 70% confluency (Figure 10; B). Then they were treated with TrypLE to generate single-cell suspension and cells diluted to 3.000 cells per well in BPEL differentiation medium supplemented with the desired growth factors and centrifuged to promote the formation of spin EBs (Figure 10; C). After 24 hours in BPEL medium, single cells had generated single EBs with rough borders (Figure 10; D) but compact spin EBs had formed two days later (Figure 10; E). After 8 days of culturing in low-attachment plates (Figure 10; F-G) cells were transferred to gelatin coated wells for further differentiation. EB´s compact morphology was still visible after 9 days of differentiation (Figure 10; H) and after that cells started to migrate. The expected time of visible beating cardiomyocytes is around day 12 and cultures were kept going up to day 30 reaching their plateau (Figure 10; I).

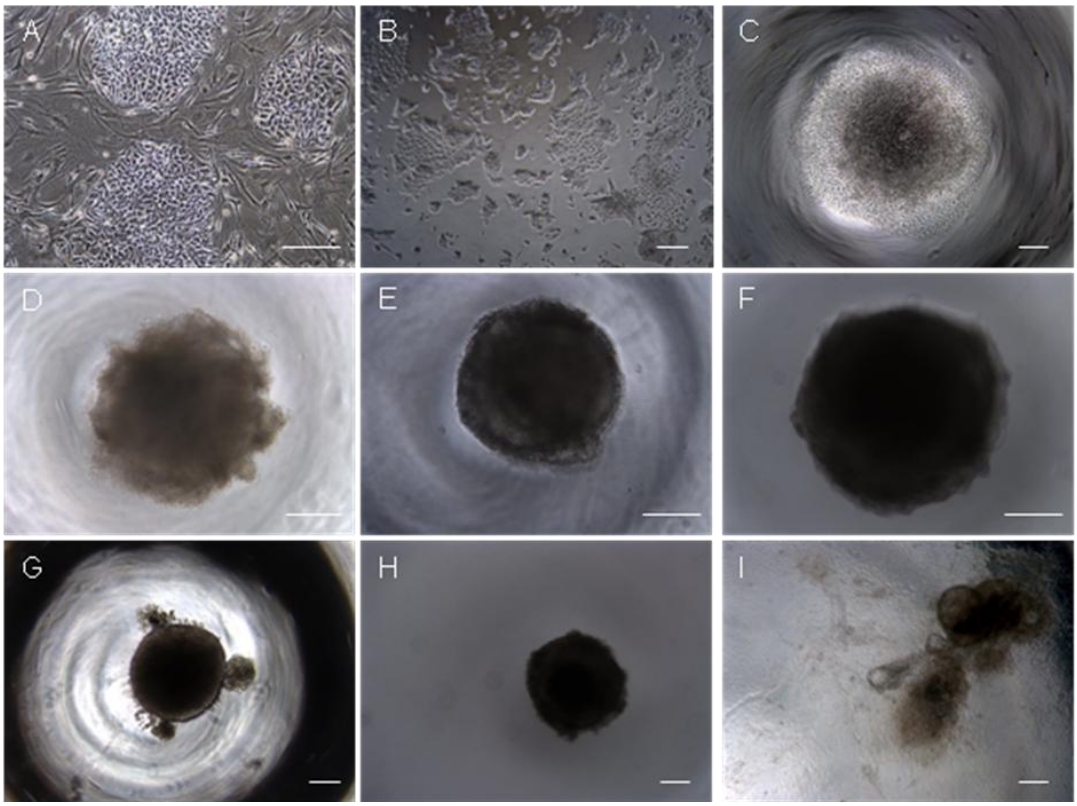


Figure 10: Morphology of spin embryoid bodies generated in BPEL medium.
(A) Undifferentiated HES2 on MEF feeder layer. (B) Undifferentiated HES2 on matrigel. (C) Undifferentiated HES2 cells after centrifugation on day 0. (D) EBs with rough borders generated after 24 hours. (E) EBs at day 2 of differentiation. (F) Day 7 of differentiation. (G) Day 8 of differentiation. (H) Further differentiation on gelatin, two days after transfer. (I) Beating cardiomyocytes on gelatin at day 13 of differentiation. Bars represent 200 µm.

Spin EBs were initiated in either untreated BPEL medium or supplemented with 10 ng/ml BMP4 and cultured for 8 days. EBs were then transferred to gelatin coated wells for further differentiation where cultures were split and half of the untreated EBs were put in medium supplemented with 10 ng/ml BMP4 and half of the treated EBs were placed in medium supplemented with 25 ng/ml BMP4. This addition of higher BMP4 concentration has been shown to affect the induction of beating cardiomyocytes (Takei et al., 2009).

Beating areas were first observed at day 13 of differentiation and to our surprise most were detected in untreated EBs or in around 24% of total wells (Table 18). Cultures were routinely checked, medium changed every 3 days and number of beating areas counted. After 27 days of differentiation beating areas had reached a plateau and a final counting revealed that untreated EBs produced the highest number of beating areas (~38%). EBs cultured for 8 days with 10 ng/ml BMP4 and then further differentiated with 25 ng/ml BMP4 produced around 29% beating areas of total wells (Table 18).

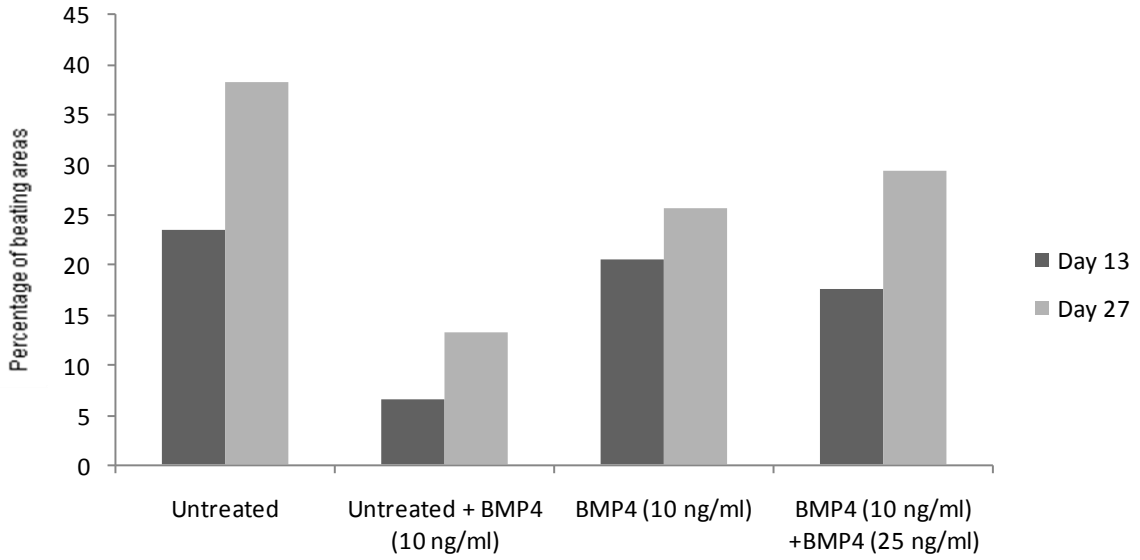


Table 18: Untreated EBs produce the highest percentage of beating areas.
Spin EBs where cultured for 8 days untreated or with 10 ng/ml BMP4 supplemented. Cultures were split after transfer; half of untreated EBs were cultured in 10 ng/ml BMP and half of the BMP4 treated EBs were further subjected to 25 ng/ml BMP4.

The beating areas observed were of different sizes, shapes and had different "beating" rhythms. In some wells the beating area comprised the whole cell aggregate whereas in others it was only a small part of it, irrelevant of different treatments and culture duration. In Figure 11 beating areas are shown taken as snapshots from videos.

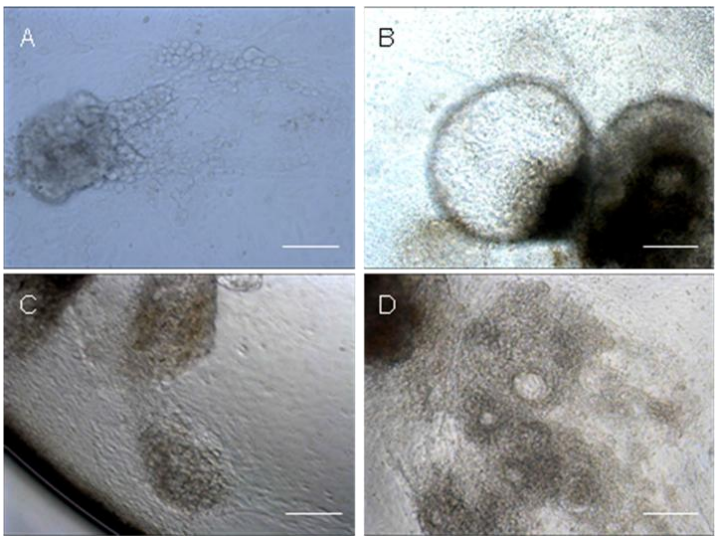


Figure 11: hESC derived cardiomyocytes have different morphology and beating rhythms.
HES2 cultures were kept on 0.1% gelatin after day 8. (A) Beating area at day 15 of differentiation. (B) Beating area at day 21. (C-D) Beating areas at day 24. Bars represent 200 µm.

In Figure 12 three pictures are lumped together of the same beating area at two different time points of the differentiation taken as snapshots from videos. Figure A and B are taken at day 17 of differentiation showing the area contracting (B) and resting (A). The same area is shown resting in figure C at day 24. More videos are included on a CD supplemented with the thesis.

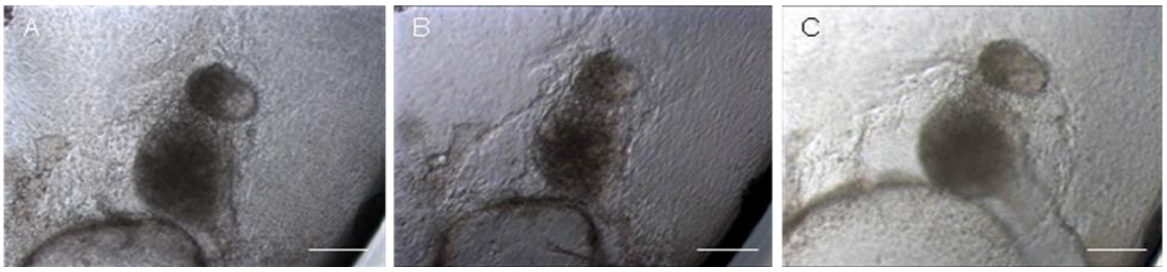


Figure 12: Beating area at two different time points.
(A) Beating area in resting mode at day 17 of differentiation. (B) Beating area contracting. (C) Beating area at day 24. Bars represent 200 µm.

4.1.4 Infection with caALK2 receptor accelerates the amount of beating areas

BMP4 is a member of the TGFβ superfamily that binds to its receptors located in the cellular membrane. This binding causes phosphorylation of transcription factors known as Smad proteins. Members of the BMP subfamily activate type I receptors called ALK2, ALK3 and ALK6 resulting in phosphorylation of Smad1/5/8 (Miyazawa et al., 2002; Valdimarsdottir and Mummery, 2005). My previous results showed that stimulation with BMP4 did not result in induction of beating areas contrary to published reports (Kattman et al., 2011; Takei et al., 2009; Yang et al., 2008). Thus, we found it interesting to investigate the kinetics of phosphorylated Smad1/5/8 (pSmad1/5/8) in hESCs. In the regulation of specificity of cellular biological responses the duration of Smad signaling is a critical determinant and important to cell fate decisions. This is well demonstrated during *Xenopus* embryogenesis where differences in the duration of Smad signaling are carefully controlled (Grimm and Gurdon, 2002). A difference in the duration of Smad signaling has been shown in mouse endothelial cells where Smad2 phosphorylation is stable for at least 6 hours whereas Smad1/5 phosphorylation is transient and absent 3 hours after stimulation (Goumans et al., 2002). HES2 cells were cultured on CELLstart and stimulated with 10 ng/ml BMP4 in serum-free medium. Samples were collected at indicated time points and phosphorylation levels of Smad1/5/8 examined. The levels of pSmad1/5/8 increased the first hour of treatment, reaching the highest levels one hour after stimulation. After 6 hours,

phosphorylation levels started to diminish and at 20 hours the levels were similar to untreated HES2 and cells treated with Noggin, an inhibitor of the BMP pathway (Figure 13).

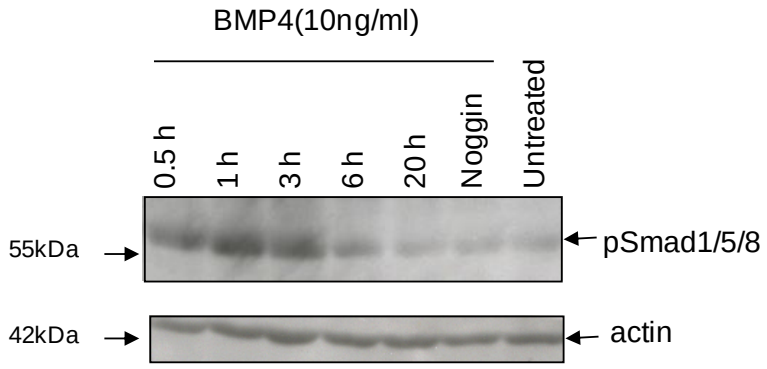


Figure 13: Phosphorylation of Smad1/5/8 diminishes after 6 hours in culture.
HES2 cells were cultured with or without 10 ng/ml BMP4 and lysed for western blot analysis harvested at indicated time points. Actin was used as a loading control.

In order to get more insight into the role of BMP4 in induction of beating cardiomyocytes, hESCs were infected with constitutively active ALK2, ALK3 or ALK6 receptors (caALK2/3/6). hESCs on matrigel were infected with adenoviral constructs containing inserts of constitutively active BMP type I receptors tagged with hemagglutinin (HA). hESCs were also infected with adenoviral constructs containing LacZ as a negative control for comparison. When cells reached 70% confluency they were differentiated using the spin EB method in BPEL medium. EBs were transferred to gelatin coated plates after 7 days for further culturing. Beating cardiomyocytes can be expected in culture around day 12 of differentiation but interestingly, beating areas were first observed at day 8 in EBs infected with caALK2. Beating areas were observed in all cultures but noticeably much more areas were beating in EBs with caALK2 (Figure 14; A). Numbers of beating areas are taken together.

When examined on a protein level by western blot analysis it was evident that infection with caALK2/3/6 resulted in phosphorylation of Smad1 protein, demonstrating that the BMP pathway was active. caALK2 infection resulted in the most prominent phosphorylation levels (Figure 14; B) which correlates with the high number of beating areas seen in Figure 14; A. hESCs infected with LacZ served as a negative control where phosphorylation was barely detectable. Stimulation of LacZ infected cells stimulated with 10 ng/ml BMP4 resulted in an increase of phosphorylation compared to LacZ alone (Figure 14; B). The value of phosphorylation resulting from caALK3 and caALK6 infection also correlates to the numbers of beating areas (Figure 14; A).

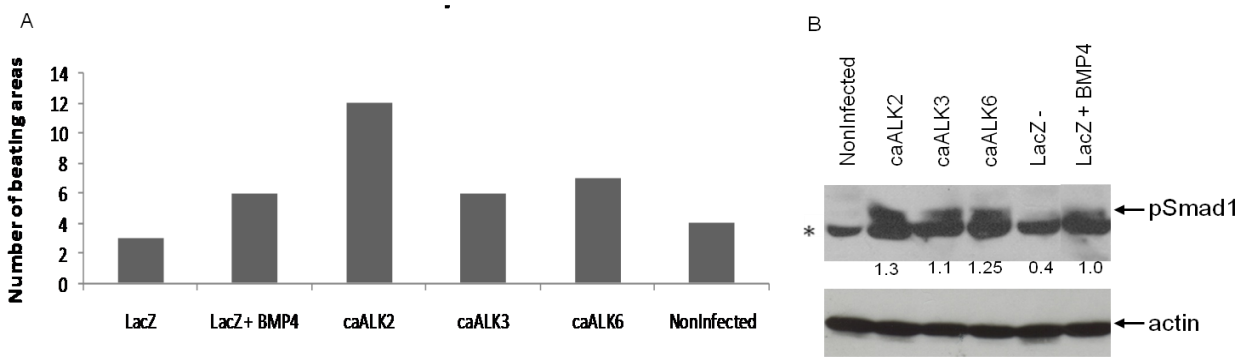


Figure 14: caALK2/3/6 increases Smad1 phosphorylation correlating to number of beating areas.
hESCs were infected with adenoviral constructs of constitutively active (ca) ALK2/3/6. (A) After 8 days in culture, beating areas were visible. (B) Number of beating areas correlate to phosphorylation levels of Smad1 with the highest levels in caALK2. LacZ infected cells were stimulated with 10 ng/ml BMP4 for 1 hour before harvesting. Asteriks indicate non-specific bands appearing in all samples. Actin was used as a loading control.

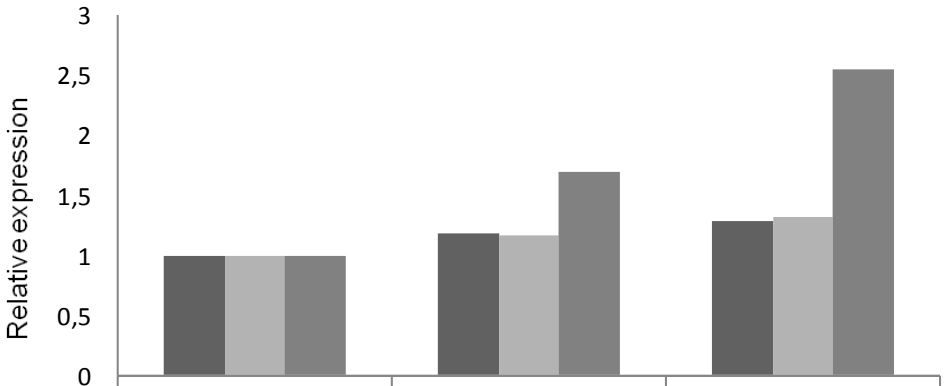
4.1.5 Stimulation with BMP4, Activin A and bFGF results in higher expression of mesodermal and cardiac markers

Because of previously published reports indicating a role for BMP4, Activin A and bFGF in the cardiomyocyte differentiation of hESCs we were interested to explore whether stimulation with BMP4 alone or in combination with Activin A and bFGF would have an effect on the relative gene expression of known early cardiac mesoderm markers and beating cardiomyocytes markers. To check for relative gene expression, spin EBs experiment was set up using combinations of BMP4, Activin A and bFGF. EBs were stimulated for 7 days with either 10 ng/ml BMP4 or a growth factor cocktail containing 10 ng/ml BMP4, 5 ng/ml bFGF and 3 ng/ml Activin A. After 7 days of differentiation samples were taken for real-time PCR analysis (qRT-PCR).

One of the earliest markers defing the mesoderm lineage is the T-box transcription factor brachyury (Kispert and Herrmann, 1994). Brachyury or T is expressed in the developing mesoderm and is downregulated upon patterning and specification (Pal, 2009). KDR is expressed in undifferentiated ESCs but the levels increase following brachyury expression in cardiovascular progenitors, representing one of the earliest cardiac mesoderm specifications. Islet-1 (Isl1) is a LIM homeodomain transcription factor which labels cardiac progenitors of the secondary heart field in the early embryo (Pal, 2009; Yang et al., 2008).

Therefore, I studied the molecular analysis of developing EBs which revealed that the relative expression of T was similarly expressed as KDR in 7 day old EBs irrelevant of different treatment procedures. The expression of ISL1 however was upregulated in EBs stimulated with BMP4 and more so with EBs stimulated with the growth factor cocktail (Table 19).

Table 19: Growth factor cocktail upregulates the cardiac progenitor marker Islet-1.
EBs were stimulated with BMP4 alone or in a growth factor cocktail containing BMP4, Activin A and bFGF for 7 days. Untreated EBs serve as a control.



	7d EBs untreated	7d EBs BMP4	7d EBs cocktail
T	1	1,185736798	1,29062309
KDR	1	1,170819015	1,324409929
ISL1	1	1,692777948	2,548478316

Previously, it has been shown that the expression of T is downregulated around day 7 in EB differentiation when stimulated with the growth factors used. Upon downregulation of T, KDR and ISL1 become upregulated with greater upregulation of ISL1 expression (Yang et al., 2008). To further confirm the upregulation on a RNA level, protein expression was assessed by immunofluorescently labeling the cells using antibody raised against Isl1. Importantly, Isl1 expression was highly increased in BMP4 treated cells compared to untreated and was strongest in cells that had been stimulated with the growth factor (Figure 15).

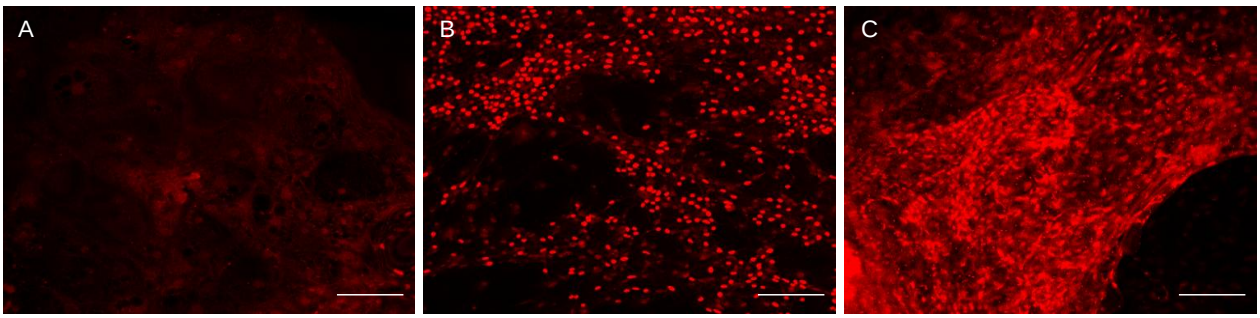
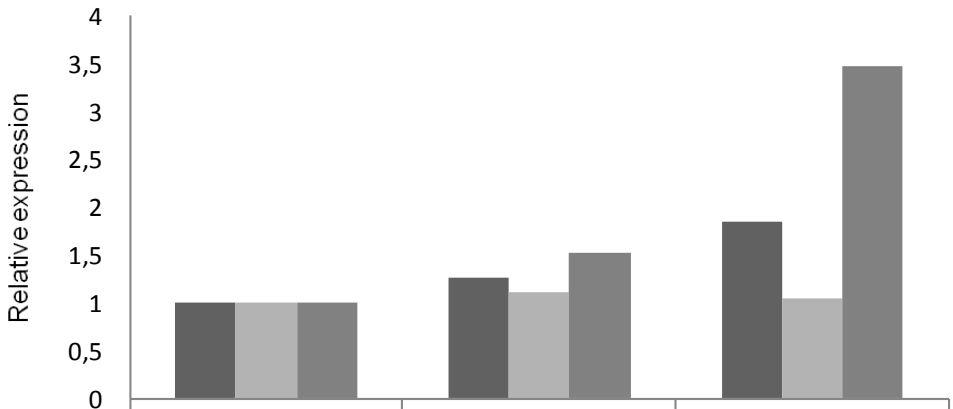


Figure 15: BMP4 alone and growth factor cocktail stimulates Islet-1 expression.
Immunofluorescent staining of 7 day old hESC EBs. Primary antibody used was mouse anti-Islet-1. Secondary antibody used was anti-mouse Cy3 (red). (A) Unstimulated hESCs. (B) hESCs stimulated with 10 ng/ml BMP4. (C) hESCs stimulated with a cocktail containing 10 ng/ml BMP4, 5 ng/ml bFGF and 3 ng/ml Activin A. Bars represent 200 µm.

Members of the T-box family of transcription factors are crucial for cardiac specification. Tbx5 is expressed in the primary heart field, hence Tbx5 is a well established cardiac marker (Zhu et al., 2009). The expression of cardiac troponin T (cTNT) marks the emergence of contracting cardiomyocytes. Further analysis of the 7 day old EBs revealed that expression of TBX5 was upregulated in EBs stimulated with BMP4. More upregulation was detected in EBs stimulated with the growth factor cocktail. However, only minor upregulation in cTNT expression was detected compared to untreated EBs. Until recently, $CD34^+$ cells have been thought to be hematopoietic stem cells. Studies have shown that human peripheral blood $CD34^+$ cells can differentiate into cardiomyocytes, endothelial cells and smooth muscle cells *in vivo* (Iwasaki et al., 2006; Yeh et al., 2003). Thus, I examined the expression of CD34 in this hESC population. CD34 was highly upregulated specifically with the growth factor cocktail stimulation (Table 20).

Table 20: Stimulation with growth factor cocktail highly upregulates CD34 hematopoietic marker.
HES2 EBs were stimulated with BMP4 alone or with a growth factor cocktail containing BMP4, Activin A and bFGF for 7 days.



	7d EBs untreated	7d EBs BMP4	7d EBs cocktail
TBX5	1	1,265727443	1,856355759
cTNT	1	1,102527607	1,035124779
CD34	1	1,517258742	3,476721691

It has been previously shown that the expression of Tbx-5 appears shortly after brachyury expression and it has also been demonstrated that cTNT expression is highly upregulated between days 8 and 10 of EB development (Yang et al., 2008; Zhu et al., 2009). The expression of the hematopoietic marker CD34 is not known to be apparent in cardiomyocytes but it was upregulated after stimulation with the growth factor cocktail to our surprise. However, it is known to be expressed in mesodermal precursors and mature endothelial and hematopoietic cells (Segev et al., 2005).

4.2 Function of miR-126 in hESCs

Studies of the mouse embryo and mouse embryonic stem cell (mESC) differentiation have provided clues indicating that mesoderm-derived lineages come from a common KDR positive cardiovascular progenitor representing one of the earliest stages in mesoderm specification to the cardiovascular lineages (Kattman et al., 2006). Cardiovascular research has been a prime focus for the last decades, where studies on cardiovascular progenitors in mESCs have implicated miR-126 in mouse cardiovascular development. The biologically active miRNAs miR-126 and miR-126* are encoded by *EGFL7* gene in vertebrates and both miRNAs are relevant for cardiovascular development. They have also been implicated in cardiovascular diseases as well as the formation of cancer as previously discussed (Fish et al., 2008; Nikolic et al., 2010; Wang et al., 2008). With qRT-PCR studies on cardiovascular progenitors expressing KDR in mESCs, miR-126 was found to be highly enriched indicating a role in cardiovascular cells (Fish et al., 2008; Wang et al., 2008). Because of its importance in cardiovascular cells in mice we decided to check for the role of miR-126 in hESC cardiomyocyte differentiation.

4.2.1 Establisment of pLVTHM viral vector

Viral vectors are used for delivering genetic materials into cells whether it is performed inside a living organism (*in vivo*) or in cell culture (*in vitro*). Lentiviruses are a common research tool used for this purpose because of their high-efficiency of infecting both dividing and non-dividing cells, long term stable expression and low immunogenicity (Campeau et al., 2009). pLVTHM lentiviral vector is an established 2nd generation transfer vector that uses the Histone H1 promoter to drive shRNA expression. It has an ampicillin (AMP) resistance gene and GFP (green fluorescent protein) for selection purposes (Addgene). pLVTHM has an additional ClaI site which allows for direct cloning of inserts. However, this site is blocked by overlapping DAM methylation. Thus, transformation of pLVTHM into a Dam negative strain was performed followed by digestion with ClaI and MluI (Figure 16; A). Both miR-126 with 100 bp flanking sites and the whole Intron 7 were amplified from human genomic DNA (Figure 16; B). We confirmed by sequencing that pLVTHM clones of interest had been successfully generated containing either miR-126 insert or intron 7.

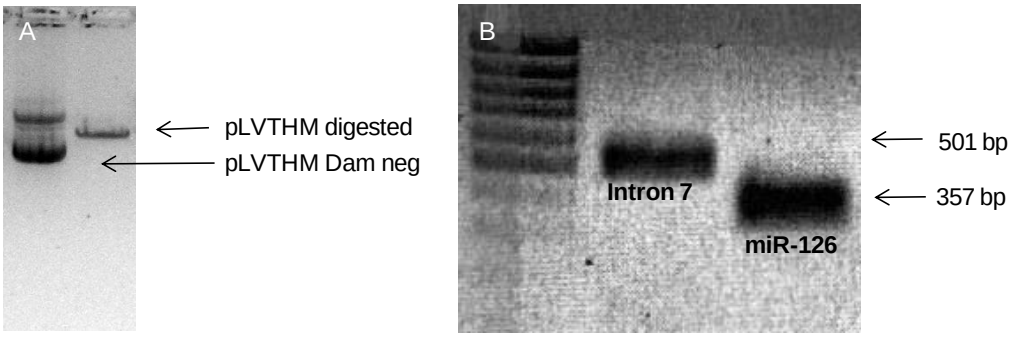


Figure 16: microRNA-126 inserts and pLVTHM vector.
(A) pLVTHM vector after transformation to a Dam negative strain and after digestion with ClaI and MluI. (B) Intron 7 and miR-126 inserts purified and digested with ClaI and MluI. Intron 7 is 501 bp and miR-126 is 357 bp.

4.2.2 HES2 morphology is independent of miR-126 expression

Transfection of lentiviral vectors is used to introduce foreign DNA into cells *in vitro*. With transfection the foreign DNA becomes stably introduced into the host cell genome. Recommended with pLVTHM for transfection are the packaging vector psPAX2 and envelope plasmid pMD2.G (see maps in Appendix A).

HEK-293T cells were transfected with pLVTHM, psPAX2 and pMD2.G in OPTI-MEM medium. After 24 hours, cells were examined for GFP expression, medium containing viral particles removed and replaced with fresh medium for another 24 hour incubation. Transfection was performed with pLVTHM containing Intron 7 insert, pLVTHM containing miR-126 insert, pLVTHM empty vector and a negative control (Figure 17).

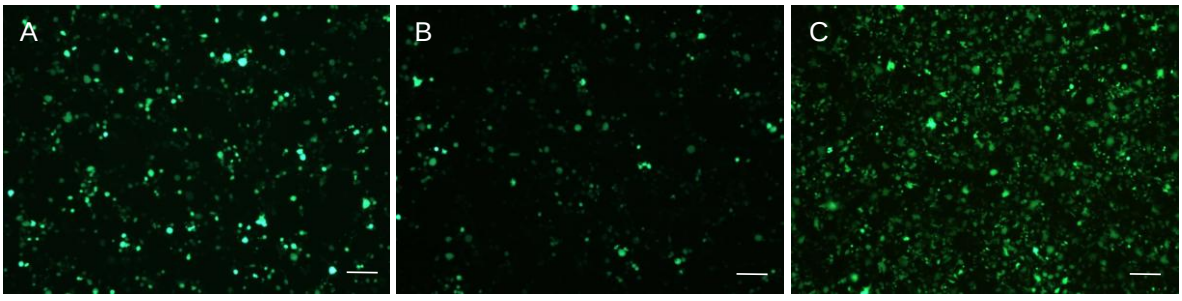


Figure 17: HEK-293T cells transfected with pLVTHM containing Intron 7, miR-126 or an empty vector.
GFP expression was assessed one day post infection (p.i.). (A) pLVTHM Intron 7, (B) pLVTHM miR-126 and (C) pLVTHM empty vector. No GFP expression was visible in the negative control. Bars represent 200 µm.

After removal from HEK-293T cells, medium containing lentiviral particles was filter-sterilized and immediately used for HES2 cell infection. HES2 cells were infected in medium containing no serum for 4-6 hours and then fresh medium containing serum supplemented to

cultures. GFP expression was usually apparent 24-48 hours after infection, whereas no GFP expression was detected in the negative control. No apparent culturing differences were seen between miR-126 and Intron 7 infected HES2 cells which could be maintained over ten passages with no apparent morphological changes. pLVTHM empty vector infection however, produced smaller cells that could only be maintained for five passages before contamination. Culture and morphology of infected HES2 cells was independent of miR-126 expression (Figure 18).

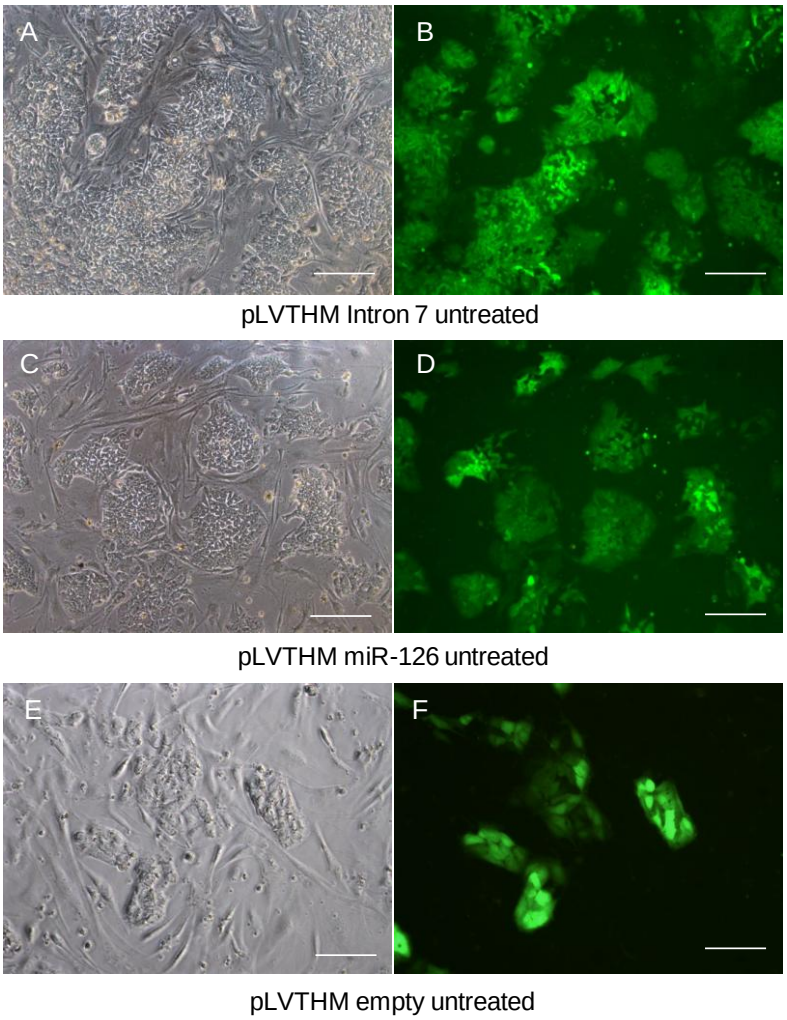


Figure 18: Morphology and GFP expression of miR-126 infected HES2 cells.
(A-B) pLVTHM Intron 7 untreated on MEF feeders. (C-D) pLVTHM miR-126 untreated on MEF feeders. (E-F) pLVTHM empty vector on MEF feeders. (A, C & E) Morphology. (B, D & F) GFP expression. Bars represent 200 µm.

4.2.3 hESCs produce extracellular matrix when differentiated in the presence of BMP4

Both spin EBs and EBs in suspension differentiation methods were performed on infected HES2 cells to see if miR-126 expression had any effect on the differentiation process. EBs in suspension proved to be more suitable in forming spherical and compact EBs. All experiments were performed with treatment of BMP4 (10 ng/ml) compared to untreated HES2 cells. Due to culturing difficulties, differentiation of HES2 cells infected with pLVTHM empty vector could not be performed.

Three days after differentiation initiation, spherical and compact EBs had formed. No apparent difference was seen between the two differently infected HES2 cells with or without BMP4 treatment (Figure 19).

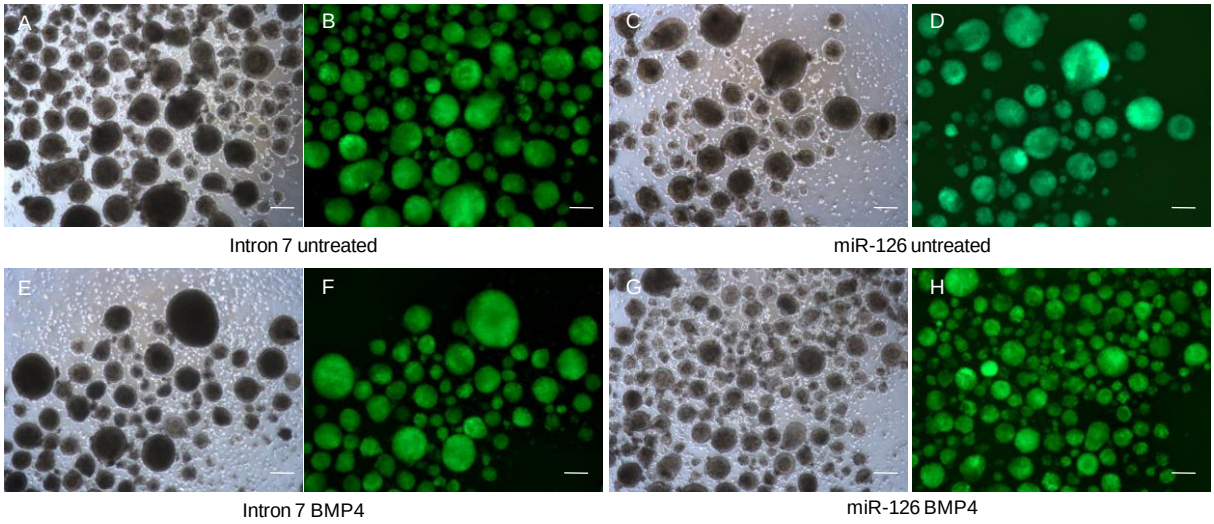


Figure 19: Cells infected with Intron 7 or miR-126 show no morphology differences.
HES2 infected cells differentiated on passage four (p.i.) and visulized at day 3 of differentiation. (A-B) Untreated pLVTHM HES2 Intron 7. (C-D) Untreated HES2 pLVTHM miR-126. (E-F) pLVTHM Intron 7 treated with BMP4. (G-H) pLVTHM miR-126 treated with BMP4. (A, C, E & G) Morphology. (B, D, F & H) GFP expression. Bars represent 200 µm.

When visualized after eight days of differentiation in the low-attachment plates it was apparent that unstimulated HES2 transduced cells had loosened up producing single cells compared to BMP4 stimulated cells. More interestingly, HES2 infected cells stimulated with 10 ng/ml BMP4 had produced extracellular matrix attaching them to the culture plates surface (Figure 20).

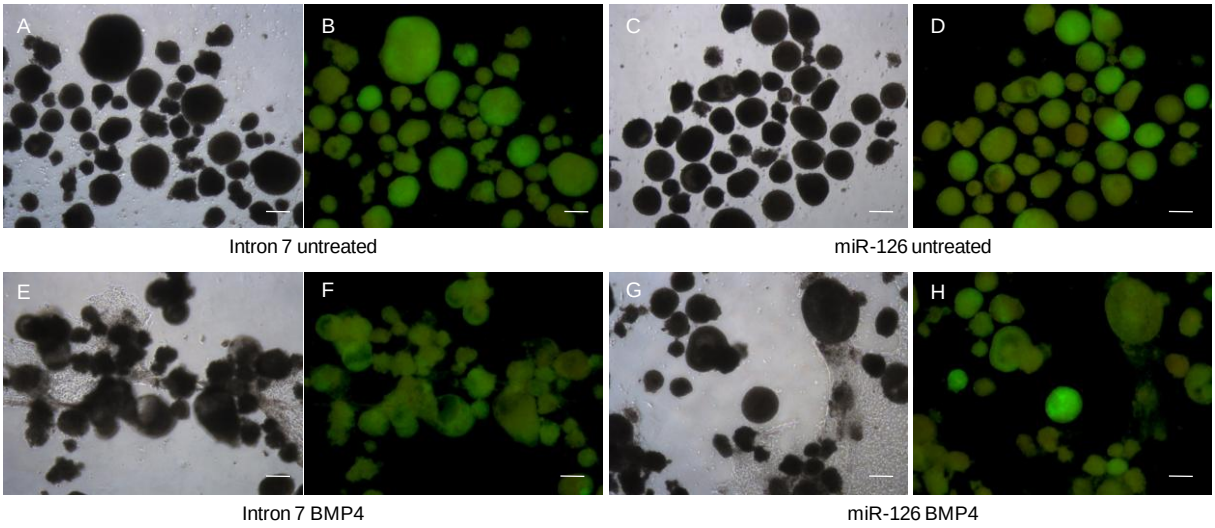


Figure 20: Treatment with BMP4 leads to ECM production facilitating cell attachment.
Differentiation performed on infected HES2 on passage four p.i. and visualized at day 8 of differentiation. (A-B) pLVTHM Intron 7 untreated. (C-D) pLVTHM miR-126 untreated. (E-F) pLVTHM Intron 7 treated with BMP4. (G-H) pLVTHM miR-126 treated with BMP4. (A, C, E & G) Morphology. (B, D, F & H) GFP expression. Bars represent 200 µm.

Cultures were examined again after 11 days of differentiation where GFP expression was still present in every well. EBs treated with BMP4 were attached to the plate's surface starting to migrate on a ECM layer, some of them forming some sort of tube-like structures (Figure 21).

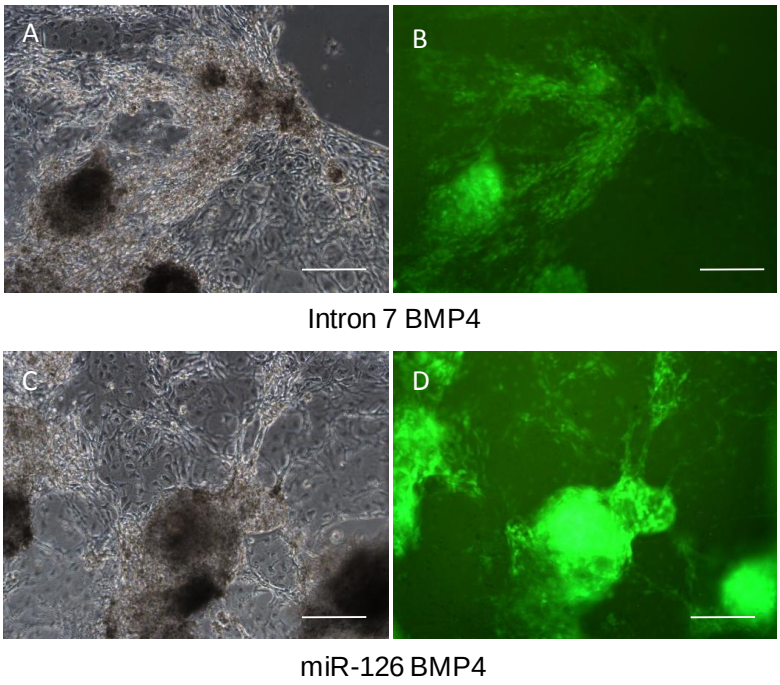


Figure 21: Infected hESCs migrate on ECM layer forming tube-like structures.
EBs visualized after 11 days of differentiation in suspension. (A-B) pLVTHM intron 7 BMP4 stimulated. (C-D) pLVTHM miR-126 BMP4 stimulated. (A & C) Morphology. (B & D) GFP expression. Bars represent 200 µm.

Members of the BMP family have been identified as potent inducers of ECM components. When overexpressed in joint cartilage in mice, BMP2 induces elevated expression of the ECM molecules collagen type II showing the highest expression on day 7. It has also been shown that upon stimulation with BMP2 elevated proteoglycan (ECM component) synthesis is clear at day 7 in mice (Blaney Davidson et al., 2007). Untreated pLVTHM Intron 7 infected cells had not attached to the surface after 15 days of differentiation becoming smaller and more irregular in shape. Untreated pLVTHM miR-126 infected cells had on the other hand started attaching to the surface but they were also smaller and more irregular then on day 11. BMP4 stimulated cells had migrated further, forming more tube-like structures on the surface of the plate (Figure 22). No beating areas were present after 15 days of differentiation.

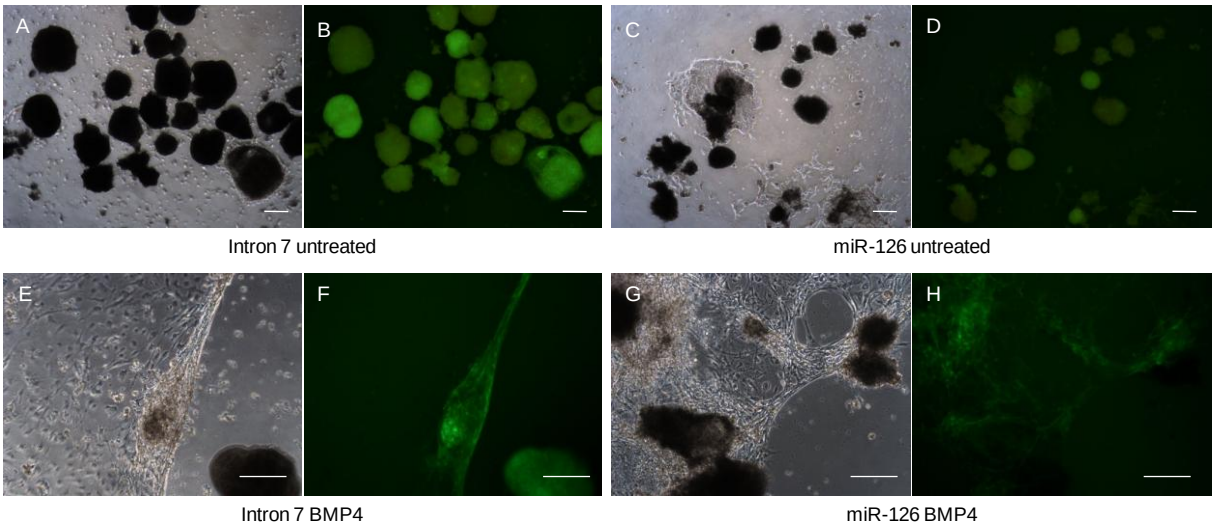


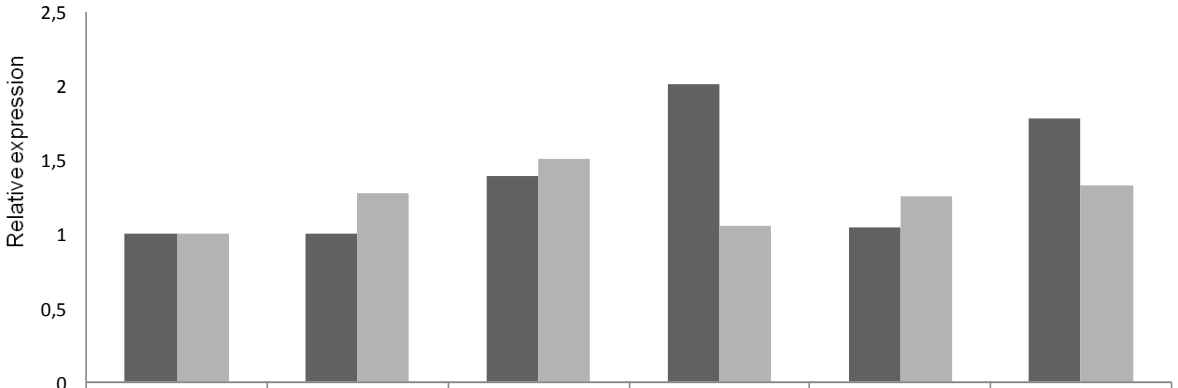
Figure 22: miR-126 expression is preferentially in tube-like structures.
EBs visualized after 15 days of differentiation in suspension. (A-B) Untreated pLVTHM Intron 7 infected cells. (C-D) Untreated pLVTHM miR-126 infected cells. (E-F) BMP4 treated pLVTHM Intron 7 infected cells. (G-H) BMP4 treated pLVTHM miR-126 infected cells. (A, C, E & G) Morphology. (B, D, F & H) GFP expression. Bars represent 200 µm.

4.2.4 miR-126 overexpression upregulates cardiovascular markers in hESCs

Next, we were interested to look in more detail whether miR-126 infection of hESCs was facilitating cardiomyocyte differentiation by upregulating known mesodermal and cardiac markers. miR-126 and Intron 7 infected HES2 cells were differentiated in suspension with 10 ng/ml BMP4 supplemented for 8 days with untreated EBs as a control. The relative gene expression of the mesodermal marker brachyury (T) revealed that miR-126 and Intron 7 infected EBs stimulated with BMP4 were upregulated compared to untreated EBs. In all cases the brachyury expression was higher in HES2 cells infected with pLVTHM vector containing the inserts compared to cells

infected with an empty vector. The expression of KDR was also upregulated in EBs stimulated with BMP4, except in the case of EBs infected with miR-126 (Table 21).

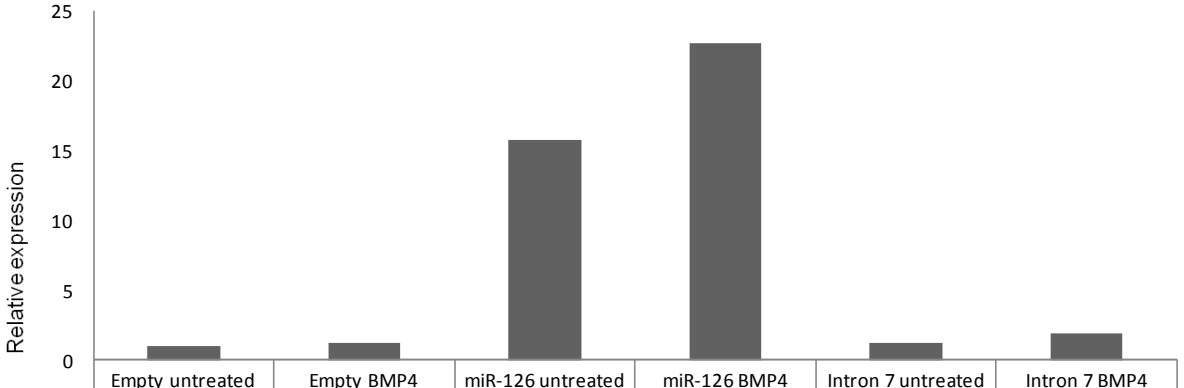
Table 21: Expression of brachyury and KDR is upregulated in miR-126 infected hESCs.
Relative expression of brachyury (T) and KDR in 8 day old EBs. EBs were formed from HES2 cells infected with pLVTHM empty vector, pLVTHM miR-126 and pLVTHM Intron 7. Cells were treated with BMP4 for 8 days or left untreated for comparison.



	Empty untreated	Empty BMP4	miR-126 untreated	miR-126 BMP4	Intron 7 untreated	Intron 7 BMP4
T	1	1,000331234	1,389930532	2,016690363	1,043474717	1,787317838
KDR	1	1,277370529	1,510169358	1,052735796	1,254571082	1,324805706

The relative expression of ISLET-1 was assessed. Surprisingly its expression was 15-fold upregulated in untreated miR-126 infected HES2 cells and upon stimulation with BMP4 the upregulation was over 22-fold compared to untreated pLVTHM empty vector (Table 22).

Table 22: ISLET-1 expression higly upregulates in HES2 cells infected with miR-126.
Relative expression of ISLET-1 in 8 day old EBs. EBs were formed from HES2 cells infected with pLVTHM empty vector, pLVTHM miR-126 and pLVTHM Intron 7. Cells were treated with BMP4 or not for 8 days. Results seen are taken as an average from two experiments.

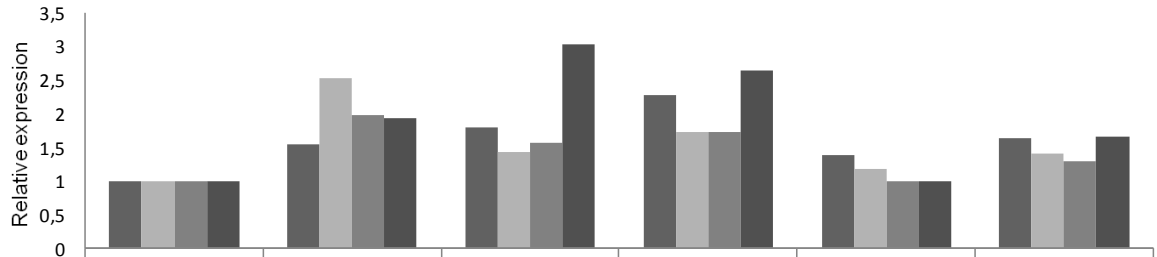


	Empty untreated	Empty BMP4	miR-126 untreated	miR-126 BMP4	Intron 7 untreated	Intron 7 BMP4
ISL1	1	1,171174292	15,79137172	22,72822854	1,262616219	1,91723829

Next, I looked at the expression of known cardiac precursor markers, contractile marker and endothelial marker in infected HES2. Infection with Intron 7 did not affect marker expression unless stimulated with BMP4 and then only slight upregulation was seen. miR-126 infection did however upregulate Nkx2.5 with further enhancement upon BMP4 stimulation.

Due to tube-like formation observed in our studies with miR-126 infected HES2, expression of a known endothelial marker, CD31 was examined. The expression of CD31 was highly upregulated in HES2 cells infected with pLVTHM vector containing miR-126 insert. In HES2 cells infected with pLVTHM empty vector and stimulated with BMP4, upregulation was detected in both cTNT and CD31. Furthermore, strongest upregulation of cardiac marker Tbx5 was observed in this population also (Table 23).

Table 23: miR-126 highly upregulates CD31 expression in hESCs.
Relative expression in 8 day old EBs. EBs were formed from HES2 cells infected with pLVTHM empty vector, pLVTHM miR-126 and pLVTHM Intron 7. Cells were treated with BMP4 or not for 8 days.



	Empty untreated	Empty BMP4	miR-126 untreated	miR-126 BMP4	Intron 7 untreated	Intron 7 BMP4
NKX2.5	1	1,542580933	1,796512074	2,288284321	1,39847576	1,641391162
TBX5	1	2,524386357	1,427336693	1,736189216	1,177097855	1,419821581
cTNT	1	1,974804011	1,563811109	1,745001623	1,001771824	1,294755521
CD31	1	1,933501313	3,027592911	2,63930298	1,003197246	1,666387474

In conclusion, the infection with pLVTHM containing miR-126 resulted in the upregulation of known mesodermal markers and cardiac precursor marker Isl-1. However, to my disappointment no upregulation was seen in cardiac markers. As previously demonstrated in recently published reports, the infection of HES2 cells with pLVTHM bearing miR-126 insert resulted in 3-fold upregulation of CD31, compared to empty vector. My results show some resemblance between the effect of miR-126 and BMP4 stimulation on gene expression, ECM deposition and cell attachment. Hence, we hypothesize that a connection resides between miR-126 expression and BMP4. miR-126 might inhibit BMP inhibitors (such a s Noggin, Gremlin) creating a feed-forward loop.

5 Discussion

5.1 Cardiomyocyte differentiation

The first aim of this study was to establish a successful differentiation protocol of hESCs to beating cardiomyocytes which had not been done in our lab. In the beginning of my research I used mainly two different differentiation methods, EBs in “hanging-drop” and EBs in suspension. Using these methods, EBs were cultured and maintained in either medium with serum or in a serum-free medium and treated with BMP4 alone or in combination with Activin A and bFGF, based on previous reports on cardiomyocyte induction (Laflamme et al., 2007; Leschik et al., 2008; Takei et al., 2009; Yang et al., 2008). When culturing EBs in suspension I found that they produced more compact morphology when stimulated with BMP4 alone or in combination with bFGF and Activin A compared to untreated EBs. Unfortunately, I was unable to induce beating cardiomyocytes using this method. Recently, an article was published describing a new differentiation method using a serum-free medium (BPEL) that is objective, i.e. the effects of added growth factors are not influenced by substances in the medium (Ng et al., 2008). I set up this method in our lab using HES2 cells where the morphology of spin EBs produced was as expected from previous reports.

Various differentiation experiments using spin EB method were conducted in this project with mixed results. I successfully produced beating areas from spin EBs cultured with four different conditions. Untreated spin EBs produced the highest percentage of beating areas or about 38%. Spin EBs cultured for 8 days treated with 10 ng/ml BMP4 and then further cultured in medium supplemented with 25 ng/ml BMP4 produced the 2nd best yield by producing around 29% beating areas. The lowest yield of beating areas resulted from spin EBs that were untreated for 8 days and then further cultured with 10 ng/ml BMP4 which supports the findings of other researchers suggesting that BMP4 is crucial the first 4 days for cardiac induction (Yang et al., 2008). Moreover, the time frame, in which BMP affects cardiomyocyte differentiation, is essential (Yuasa et al., 2005). Every experiment spanned a long culturing process, taking up to three weeks for final results. For this reason, experiments could not be repeated in all conditions.

5.2 BMP4 kinetics play a key role in differentiation

It is important to manipulate the key signaling pathways to optimize an essential first step in generating a specific lineage, the germ layer induction. It is known that different cell lines require different concentrations of growth factors to generate cardiovascular mesoderm population. This is due to the fact that kinetics of each cell line play a crucial role in differentiation (Kattman et al., 2011).

It has been shown that endogenous signaling can promote the development of specific cell populations. Furthermore, it has been demonstrated that endogenous signaling of the HES2 cell line, together with BMP4 stimulation, is sufficient in promoting a cardiac population. Successful induction of beating cardiomyocytes has also been accomplished with BMP4 and Activin A stimulation (Kattman et al., 2011; Yang et al., 2008). Although not strong, a difference in the upregulation of known mesodermal and cardiac precursor markers, KDR and Islet-1, was evident with stimulation where more upregulation was detected in 7 day EBs stimulated with the growth factor cocktail. In the case of cardiac markers, Tbx5 and cTNT, the expression of Tbx5 did increase with growth factor stimulation indicative of the presence of cardiac precursors, whereas the expression of cTNT did not. However, reports vary substantially regarding expression profile of specific lineage markers. Yang et al. demonstrate that cTNT expression becomes visible at day 8 in EBs stimulated with the growth factor cocktail hence, cTNT was possibly not yet expressed in my study.

Levels of endogenous signaling are known to vary between different cell lines, underlining the importance of cytokine screening as a tool for cardiac output optimization (Kattman et al., 2011). With this observation as a guideline we looked closer on the levels of pSmad1/5/8 in normal HES2 cultures stimulated with 10 ng/ml BMP4. One hour after stimulation the levels of pSmad1/5/8 peaked sustaining their level for at least 3 hours. After 6 hours phosphorylation levels started to diminish and after 20 hours levels were the same as for untreated HES2 cells and cells treated with Noggin, a BMP pathway inhibitor. Furthermore, we set up an experiment using constitutively active BMP type I receptors ALK2/3/6. We found that having these receptors constantly active greatly enhanced the induction of beating areas and especially with caALK2 receptor, compared to the LacZ control. The same was apparent on a protein level as western blot analysis showed that the phosphorylation of Smad1 protein was enhanced with the presence of caALK3/6 and more so with caALK2. These results clearly show that in HES2 cells stimulation with 10 ng/ml BMP4 is only sustained for less than 20 hours demonstrating the need for re-stimulation for the BMP pathway to be active at least the first 4 days of differentiation. Moreover, by constitutively activating one of BMPs receptors the BMP pathway itself becomes more active resulting in higher levels of Smad1 phosphorylation facilitating in a sufficient way the induction of beating cardiomyocytes. This data might explain the confusing results of the amount of beating cardiomyocytes observed when inducing hESCs with growth factors, without re-stimulation.

5.3 miR-126 leads to hESCs differentiation towards the endothelial lineage

The importance of microRNA-126 (miR-126) has clearly been revealed with studies in mESCs. In the beginning of this study reports had recently been published demonstrating the role of miR-126 in development of the cardiovascular system of mice. There, both EGFL7 gene, where miR-126 is located within intron 7, and miR-126 itself were found to be upregulated in cardiac precursors and upregulated in lung and heart tissues (Fish et al., 2008; Wang et al., 2008). Therefore we became interested in the role of miR-126 in cardiovascular differentiation in hESCs, with special focus on cardiomyocyte differentiation.

When designing the primers for miR-126 DNA amplification we were uncertain of the best suitable way to produce the right folding of the miRNA stem-loop. That is why we decided to design primers both for miR-126 with 100 bp flanking sites and for the whole Intron 7 wherein miR-126 resides. I accountered many problems along the way establishing the viral vectors, making it a longer task than first thought. The same was the case with the lentiviral transfection where I tried three procedures and two transfection reagents several times before accomplishing the production of GFP expressing HEK-293T cells. It has been reported that transducing hESCs can be problematic (Cao et al., 2010) which was also the case in my research. Nevertheless, when achieving successful infection the HES2 cells produced well defined and discrete colonies in culture. Both HES2 cells infected with miR-126 insert and Intron 7 insert could be maintained over 10 passages without any morphology changes. HES2 cells infected with pLVTHM empty vector were however only maintained for five passages before they got contaminated, leaving out this important control in most differentiation experiments.

In order to explore the effects of miR-126 infection on cardiomyocyte differentiation known mesodermal and cardiac markers were examined by qRT-PCR. HES2 cells infected with pLVTHM empty vector, miR-126 insert and Intron 7 insert were differentiated using spin EBs for 8 days, treated with 10 ng/ml BMP4 or left untreated as a control. In the empty control, small upregulation was only visible in KDR expression upon BMP4 stimulation. However, in both miR-126 and Intron 7 infected cells the expression of mesodermal marker brachyury (T) and KDR was upregulated in untreated cells but surprisingly, upon BMP4 stimulation expression of KDR was downregulated especially in cells infected with miR-126 insert. It has been previously described that stimulation with 50 ng/ml BMP4 results in an upregulation of KDR expression in hESC EBs compared to untreated at day 7. However, two days later this has completely rotated resulting in upregulation of KDR in untreated EBs and downregulation in BMP4 stimulated EBs (Goldman et al., 2009).

In miR-126 infected HES2 cells expression of Islet-1 was upregulated 15-fold compared to empty vector cells and upon BMP4 stimulation, expression was even more enhanced resulting in 22-fold upregulation. However, Intron 7 infected HES2 cells did not show upregulation in Isl1

unless treated with BMP4 and even then the upregulation was only slight. When examining cardiac markers no drastic upregulation was seen in HES2 miR-126 or Intron7 cells. However, upon BMP4 stimulation upregulation of cardiac precursor markers Nkx2.5 and Tbx5 and the contractile marker cTNT was detected in HES2 empty vector. In HES2 miR-126 cells the expression of a known endothelial marker, CD31, was highly upregulated at day 8 of differentiation, whereas upregulation of CD31 was only apparent in other cell types upon BMP4 stimulation. This correlates perfectly with the formation of tube-like structures observed in the differentiation of miR-126 infected cells.

It has been reported that in EB differentiation of sorted cells of the cardiovascular lineage the expression of Islet-1 is enhanced at day 10. In the same population CD31 was similarly expressed indicative of Islet-1 as a marker for both cardiomyocytes and endothelial cells (Yang et al., 2008). Similar reports have been published regarding cells positive of Islet-1 expression having the potential of developing into smooth muscle cells, cardiomyocytes and endothelial cells (Barzelay et al., 2010).

When looking at the overall results of the infection experiments, we speculate that more reliable results are achieved with miR-126 infection versus Intron 7. A recently published article describing the role of miR-126 in erythropoeisis uses lentiviral constructs designed with DNA fragments coding miR-126 with 185 bp upstream and 179 bp downstream from the precursor (Huang et al., 2011), similar to my design. Most likely, more efficient processing of miR-126 is achieved when HES2 are infected with miR-126 than with the whole Intron 7. We speculated that Intron 7 was maybe bearing more miRNAs obstructing the processing procedure, but that did not seem to be the case with further examination using *RNA*fold WebServer software online (data not shown).

5.4 BMP and/or miR-126 promote extracellular matrix deposition that supports vascular development

The first three days of differentiation no morphological differences were seen between the two differently infected HES2 cells, irrelevant of BMP4 stimulation. Around day 8 of culturing, cells stimulated with BMP4 had produced extracellular matrix (ECM) on the surface of the non-attachment plates, enabling them to attach. After 15 days in suspension more ECM had formed and HES2 had migrated on the layer forming tube-like structures, reminiscent of what endothelial cells can form. Furthermore, around day 15, untreated miR-126 infected HES2 cells had produced ECM and attached. Recently, a review article described ECM as one of the key components of the vascular system supporting endothelial cell proliferation, migration, survival and morphogenesis during blood vessel formation (Nikolic et al., 2010). EGFL7 has been suggested to be mainly located on the cell surface, attaching through interactions with ECM

molecules. Furthermore, the presence of EGFL7 in the ECM is facilitated by certain types of matrix proteins, such as fibronectin and collagen type I in mice (Schmidt et al., 2007). Microarray analysis on hESCs was previously conducted in the lab. Differential gene expression was assessed on hESCs stimulated for 4 days with BMP4 compared to untreated cells. I used this data to focus on the upregulation of ECM components upon BMP4 treatment. The vast majority thereof (such as fibronectin and collagen) was indeed upregulated but only slightly. Collagen type V and VI was ~2-fold upregulated in BMP4 induced hESCs (data not shown). Of note, EGFL7 was not upregulated at this time point in the differentiation when cells were harvested. We therefore speculate that whilst the expression of EGFL7 is linked to ECM, miR-126 must be linked to that expression and have a role in upregulating ECM components supporting vascular maturation. Nevertheless, we cannot exclude the possibility that the effect may be due to BMP4 alone. ECM deposition was however only detected in cells expressing miR-126 and not in empty vector when harvested for assessment of cardiovascular markers (data not shown).

5.5 Concluding remarks

The kinetics of a particular cell line needs to be evaluated so the optimal stage of germ layer induction can be established. Optimization of endogenous and exogenous signaling is crucial for determining the efficiency of cardiac differentiation. By constitutively activating ALK2/3/6 BMP type I receptors I have shown that when the BMP pathway is constantly active a more prominent induction of beating cardiomyocytes is achieved. Since first published reports of miR-126 importance in mice, a number of articles describing the role of miR-126 in endothelial and vascular cell development have been reported similar to my findings in hESCs. With miR-126 infection mesodermal and cardiac precursor markers become upregulated during hESC differentiation. However, cardiac markers were not upregulated upon infection, only in the case of HES2 empty vector stimulated with BMP4. This suggests that miR-126 steers cells further from the cardiac lineage. This statement was further verified with the upregulation of CD31 in miR-126 HES2 which indicates that miR-126 hESCs do preferably differentiate towards the endothelial lineage. Here, I show that with miR-126 infection hESCs are directed to the cardiovascular lineage with a more prominent role in the vascular lineage. In continued research it will be interesting to use Illumina´s sequencing technology to explore differentially expressed miRNAs at different time points of cardiovascular differentiation. This could give us information about novel microRNAs having role in hESC cardiovascular commitment.

Bibliography

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281-297.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215-233.

Barzelay, A., Ben-Shoshan, J., Entin-Meer, M., Maysel-Auslender, S., Afek, A., Barshack, I., Keren, G., and George, J. (2010). A potential role for islet-1 in post-natal angiogenesis and vasculogenesis. Thromb Haemost *103*, 188-197.

Blaney Davidson, E.N., Vitters, E.L., van Lent, P.L., van de Loo, F.A., van den Berg, W.B., and van der Kraan, P.M. (2007). Elevated extracellular matrix production and degradation upon bone morphogenetic protein-2 (BMP-2) stimulation point toward a role for BMP-2 in cartilage repair and remodeling. Arthritis Res Ther *9*, R102.

Bondue, A., Tannler, S., Chiapparo, G., Chabab, S., Ramialison, M., Paulissen, C., Beck, B., Harvey, R., and Blanpain, C. (2011). Defining the earliest step of cardiovascular progenitor specification during embryonic stem cell differentiation. J Cell Biol *192*, 751-765.

Campeau, E., Ruhl, V.E., Rodier, F., Smith, C.L., Rahmberg, B.L., Fuss, J.O., Campisi, J., Yaswen, P., Cooper, P.K., and Kaufman, P.D. (2009). A versatile viral system for expression and depletion of proteins in mammalian cells. PLoS One *4*, e6529.

Cao, F., Xie, X., Gollan, T., Zhao, L., Narsinh, K., Lee, R.J., and Wu, J.C. (2010). Comparison of gene-transfer efficiency in human embryonic stem cells. Mol Imaging Biol *12*, 15-24.

Chambers, I., and Smith, A. (2004). Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene *23*, 7150-7160.

Chen, J.F., Mandel, E.M., Thomson, J.M., Wu, Q., Callis, T.E., Hammond, S.M., Conlon, F.L., and Wang, D.Z. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet *38*, 228-233.

Cordes, K.R., and Srivastava, D. (2009). MicroRNA regulation of cardiovascular development. Circ Res *104*, 724-732.

Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., Wang, S., Morton, C.C., McMahon, A.P., Powers, D*., et al.* (2004). Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med *350*, 1353-1356.

Dambrot, C., Passier, R., Atsma, D., and Mummery, C.L. (2011). Cardiomyocyte differentiation of pluripotent stem cells and their use as cardiac disease models. Biochem J *434*, 25-35.

Dang, S.M., Kyba, M., Perlingeiro, R., Daley, G.Q., and Zandstra, P.W. (2002). Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. Biotechnol Bioeng *78*, 442-453.

Deb, K.D., Jayaprakash, A.D., Sharma, V. and Totey, S. (2007). Embryonic Stem Cells: From Markers to Market. Rejuvenation Research *11*, 19-37.

Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154-156.

Filipowicz, W., Bhattacharyya, S.N., and Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet *9*, 102-114.

Fish, J.E., Santoro, M.M., Morton, S.U., Yu, S., Yeh, R.F., Wythe, J.D., Ivey, K.N., Bruneau, B.G., Stainier, D.Y., and Srivastava, D. (2008). miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell *15*, 272-284.

Gan, Q., Yoshida, T., McDonald, O.G., and Owens, G.K. (2007). Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells. Stem Cells *25*, 2-9.

Gangaraju, V.K., and Lin, H. (2009). MicroRNAs: key regulators of stem cells. Nat Rev Mol Cell Biol *10*, 116-125.

Goldman, O., Feraud, O., Boyer-Di Ponio, J., Driancourt, C., Clay, D., Le Bousse-Kerdiles, M.C., Bennaceur-Griscelli, A., and Uzan, G. (2009). A boost of BMP4 accelerates the commitment of human embryonic stem cells to the endothelial lineage. Stem Cells *27*, 1750-1759.

Goumans, M.J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P., and ten Dijke, P. (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J *21*, 1743-1753.

Griffiths-Jones, S., Saini, H.K., van Dongen, S., and Enright, A.J. (2008). miRBase: tools for microRNA genomics. Nucleic Acids Res *36*, D154-158.

Grimm, O.H., and Gurdon, J.B. (2002). Nuclear exclusion of Smad2 is a mechanism leading to loss of competence. Nat Cell Biol *4*, 519-522.

He, L., and Hannon, G.J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet *5*, 522-531.

Heo, J., Lee, J.S., Chu, I.S., Takahama, Y., and Thorgeirsson, S.S. (2005). Spontaneous differentiation of mouse embryonic stem cells in vitro: characterization by global gene expression profiles. Biochem Biophys Res Commun *332*, 1061-1069.

Huang, X., Gschweng, E., Van Handel, B., Cheng, D., Mikkola, H.K., and Witte, O.N. (2011). Regulated expression of microRNAs-126/126* inhibits erythropoiesis from human embryonic stem cells. Blood *117*, 2157-2165.

Ivey, K.N., and Srivastava, D. (2010). MicroRNAs as regulators of differentiation and cell fate decisions. Cell Stem Cell *7*, 36-41.

Iwasaki, H., Kawamoto, A., Ishikawa, M., Oyamada, A., Nakamori, S., Nishimura, H., Sadamoto, K., Horii, M., Matsumoto, T., Murasawa, S.*, et al.* (2006). Dose-dependent contribution of CD34-positive cell transplantation to concurrent vasculogenesis and cardiomyogenesis for functional regenerative recovery after myocardial infarction. Circulation *113*, 1311-1325.

Jaenisch, R., and Young, R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell *132*, 567-582.

Jovanovic, M., and Hengartner, M.O. (2006). miRNAs and apoptosis: RNAs to die for. Oncogene *25*, 6176-6187.

Kattman, S.J., Adler, E.D., and Keller, G.M. (2007). Specification of multipotential cardiovascular progenitor cells during embryonic stem cell differentiation and embryonic development. Trends Cardiovasc Med *17*, 240-246.

Kattman, S.J., Huber, T.L., and Keller, G.M. (2006). Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. Dev Cell *11*, 723-732.

Kattman, S.J., Witty, A.D., Gagliardi, M., Dubois, N.C., Niapour, M., Hotta, A., Ellis, J., and Keller, G. (2011). Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell *8*, 228-240.

Keller, G.M. (1995). In vitro differentiation of embryonic stem cells. Curr Opin Cell Biol *7*, 862-869.

Khoo, M.L., McQuade, L.R., Smith, M.S., Lees, J.G., Sidhu, K.S., and Tuch, B.E. (2005). Growth and differentiation of embryoid bodies derived from human embryonic stem cells: effect of glucose and basic fibroblast growth factor. Biol Reprod *73*, 1147-1156.

Kispert, A., and Herrmann, B.G. (1994). Immunohistochemical analysis of the Brachyury protein in wild-type and mutant mouse embryos. Dev Biol *161*, 179-193.

Klimanskaya, I., Chung, Y., Meisner, L., Johnson, J., West, M.D., and Lanza, R. (2005). Human embryonic stem cells derived without feeder cells. Lancet *365*, 1636-1641.

Kondo, M., Wagers, A.J., Manz, M.G., Prohaska, S.S., Scherer, D.C., Beilhack, G.E., Shizuru, J.A., and Weissman, I.L. (2003). Biology of hematopoietic stem cells and progenitors: Implications for clinical application. Annu Rev Immunol *21*, 759-806.

Kozak, M. (2008). Faulty old ideas about translational regulation paved the way for current confusion about how microRNAs function. Gene *423*, 108-115.

Laflamme, M.A., Chen, K.Y., Naumova, A.V., Muskheli, V., Fugate, J.A., Dupras, S.K., Reinecke, H., Xu, C., Hassanipour, M., Police, S.*, et al.* (2007). Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol *25*, 1015-1024.

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science *294*, 858-862.

Lee, R.C., and Ambros, V. (2001). An extensive class of small RNAs in Caenorhabditis elegans. Science *294*, 862-864.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843-854.

Leschik, J., Stefanovic, S., Brinon, B., and Puceat, M. (2008). Cardiac commitment of primate embryonic stem cells. Nat Protoc *3*, 1381-1387.

Liu, N., Lu, M., Tian, X., and Han, Z. (2007). Molecular mechanisms involved in self-renewal and pluripotency of embryonic stem cells. J Cell Physiol *211*, 279-286.

Liu, N., and Olson, E.N. (2010). MicroRNA regulatory networks in cardiovascular development. Dev Cell *18*, 510-525.

Ludwig, T.E., Levenstein, M.E., Jones, J.M., Berggren, W.T., Mitchen, E.R., Frane, J.L., Crandall, L.J., Daigh, C.A., Conard, K.R., Piekarczyk, M.S.*, et al.* (2006). Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol *24*, 185-187.

Mannello, F., and Tonti, G.A. (2007). Concise review: no breakthroughs for human mesenchymal and embryonic stem cell culture: conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or ad hoc formula? All that glitters is not gold! Stem Cells *25*, 1603-1609.

Miyazawa, K., Shinozaki, M., Hara, T., Furuya, T., and Miyazono, K. (2002). Two major Smad pathways in TGF-beta superfamily signalling. Genes Cells *7*, 1191-1204.

Mogi, A., Ichikawa, H., Matsumoto, C., Hieda, T., Tomotsune, D., Sakaki, S., Yamada, S., and Sasaki, K. (2009). The method of mouse embryoid body establishment affects structure and developmental gene expression. Tissue Cell *41*, 79-84.

Mummery, C., Ward-van Oostwaard, D., Doevendans, P., Spijker, R., van den Brink, S., Hassink, R., van der Heyden, M., Opthof, T., Pera, M., de la Riviere, A.B.*, et al.* (2003). Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation *107*, 2733-2740.

Mummery, C.L., and van den Eijnden-van Raaij, A.J. (1999). Developmental tumours, early differentiation and the transforming growth factor beta superfamily. Int J Dev Biol *43*, 693-709.

Ng, E.S., Davis, R., Stanley, E.G., and Elefanty, A.G. (2008). A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nat Protoc *3*, 768-776.

Nikolic, I., Plate, K.H., and Schmidt, M.H. (2010). EGFL7 meets miRNA-126: an angiogenesis alliance. J Angiogenes Res *2*, 9.

Pal, R. (2009). Embryonic stem (ES) cell-derived cardiomyocytes: a good candidate for cell therapy applications. Cell Biol Int *33*, 325-336.

Passier, R., and Mummery, C. (2003). Origin and use of embryonic and adult stem cells in differentiation and tissue repair. Cardiovasc Res *58*, 324-335.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol *18*, 399-404.

Rosler, E.S., Fisk, G.J., Ares, X., Irving, J., Miura, T., Rao, M.S., and Carpenter, M.K. (2004). Long-term culture of human embryonic stem cells in feeder-free conditions. Dev Dyn *229*, 259-274.

Schmidt, M., Paes, K., De Maziere, A., Smyczek, T., Yang, S., Gray, A., French, D., Kasman, I., Klumperman, J., Rice, D.S.*, et al.* (2007). EGFL7 regulates the collective migration of endothelial cells by restricting their spatial distribution. Development *134*, 2913-2923.

Segev, H., Kenyagin-Karsenti, D., Fishman, B., Gerecht-Nir, S., Ziskind, A., Amit, M., Coleman, R., and Itskovitz-Eldor, J. (2005). Molecular analysis of cardiomyocytes derived from human embryonic stem cells. Dev Growth Differ *47*, 295-306.

Small, E.M., and Olson, E.N. (2011). Pervasive roles of microRNAs in cardiovascular biology. Nature *469*, 336-342.

Steele, A., and Steele, P. (2006). Stem cells for repair of the heart. Curr Opin Pediatr *18*, 518-523.

Stojkovic, M., Lako, M., Stojkovic, P., Stewart, R., Przyborski, S., Armstrong, L., Evans, J., Herbert, M., Hyslop, L., Ahmad, S.*, et al.* (2004a). Derivation of human embryonic stem cells from day-8 blastocysts recovered after three-step in vitro culture. Stem Cells *22*, 790-797.

Stojkovic, M., Lako, M., Strachan, T., and Murdoch, A. (2004b). Derivation, growth and applications of human embryonic stem cells. Reproduction *128*, 259-267.

Takei, S., Ichikawa, H., Johkura, K., Mogi, A., No, H., Yoshie, S., Tomotsune, D., and Sasaki, K. (2009). Bone morphogenetic protein-4 promotes induction of cardiomyocytes from human embryonic stem cells in serum-based embryoid body development. Am J Physiol Heart Circ Physiol *296*, H1793-1803.

Terskikh, A.V., Bryant, P.J., and Schwartz, P.H. (2006). Mammalian stem cells. Pediatr Res *59*, 13R-20R.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., and Hearn, J.P. (1995). Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A *92*, 7844-7848.

Thum, T., Catalucci, D., and Bauersachs, J. (2008). MicroRNAs: novel regulators in cardiac development and disease. Cardiovasc Res *79*, 562-570.

Valdimarsdottir, G., and Mummery, C. (2005). Functions of the TGFbeta superfamily in human embryonic stem cells. APMIS *113*, 773-789.

Vasudevan, S., Tong, Y., and Steitz, J.A. (2007). Switching from repression to activation: microRNAs can up-regulate translation. Science *318*, 1931-1934.

Vazin, T., and Freed, W.J. (2010). Human embryonic stem cells: derivation, culture, and differentiation: a review. Restor Neurol Neurosci *28*, 589-603.

Wang, S., Aurora, A.B., Johnson, B.A., Qi, X., McAnally, J., Hill, J.A., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2008). The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell *15*, 261-271.

Wang, Y., Keys, D.N., Au-Young, J.K., and Chen, C. (2009). MicroRNAs in embryonic stem cells. J Cell Physiol *218*, 251-255.

Wienholds, E., and Plasterk, R.H. (2005). MicroRNA function in animal development. FEBS Lett *579*, 5911-5922.

Xu, C., Inokuma, M.S., Denham, J., Golds, K., Kundu, P., Gold, J.D., and Carpenter, M.K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol *19*, 971-974.

Xu, R.H., Peck, R.M., Li, D.S., Feng, X., Ludwig, T., and Thomson, J.A. (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods *2*, 185-190.

Xu, R.H., Sampsell-Barron, T.L., Gu, F., Root, S., Peck, R.M., Pan, G., Yu, J., Antosiewicz-Bourget, J., Tian, S., Stewart, R.*, et al.* (2008). NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. Cell Stem Cell *3*, 196-206.

Yang, L., Soonpaa, M.H., Adler, E.D., Roepke, T.K., Kattman, S.J., Kennedy, M., Henckaerts, E., Bonham, K., Abbott, G.W., Linden, R.M.*, et al.* (2008). Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature *453*, 524-528.

Yeh, E.T., Zhang, S., Wu, H.D., Korbling, M., Willerson, J.T., and Estrov, Z. (2003). Transdifferentiation of human peripheral blood CD34+-enriched cell population into cardiomyocytes, endothelial cells, and smooth muscle cells in vivo. Circulation *108*, 2070-2073.

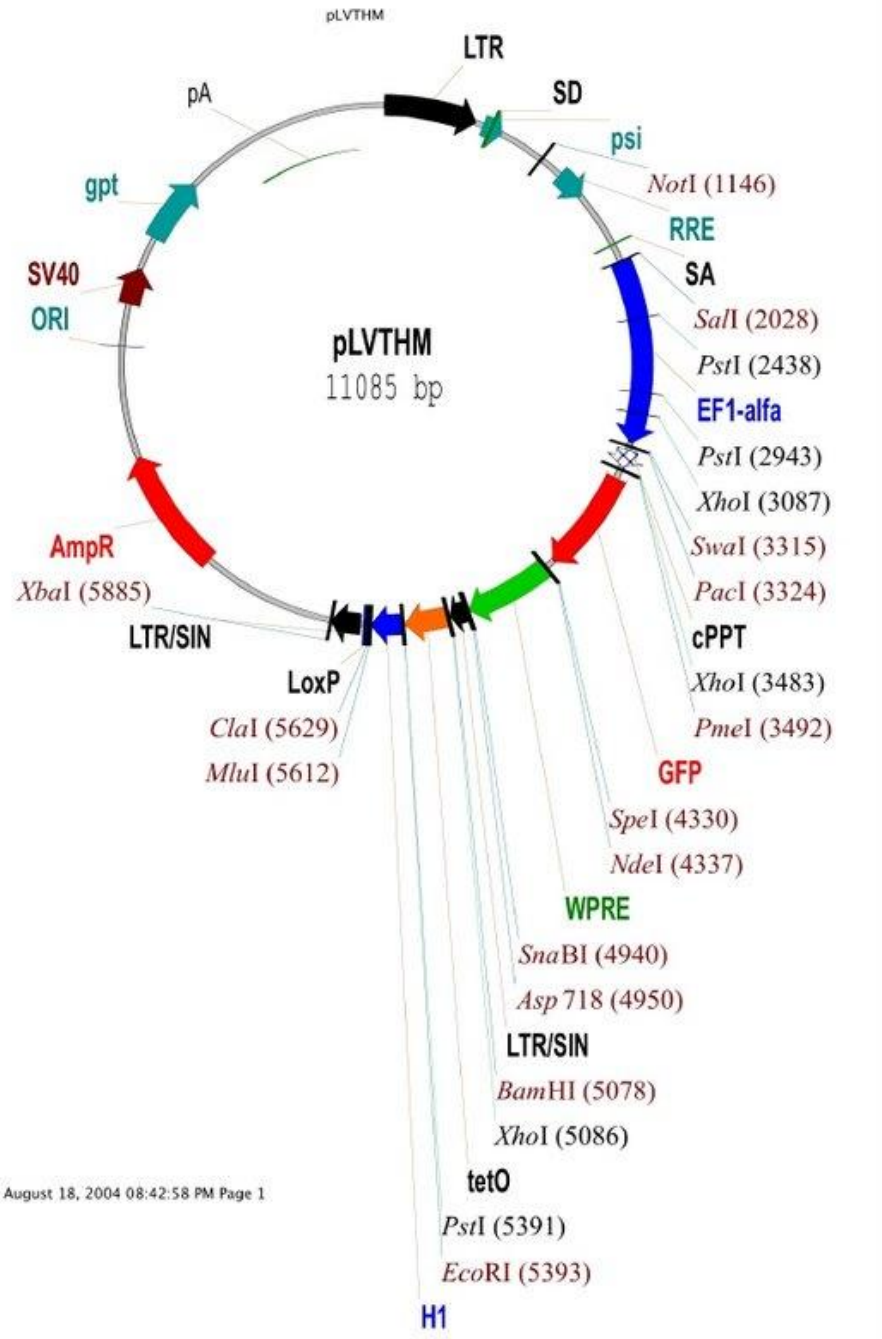
Yuasa, S., Itabashi, Y., Koshimizu, U., Tanaka, T., Sugimura, K., Kinoshita, M., Hattori, F., Fukami, S., Shimazaki, T., Ogawa, S.*, et al.* (2005). Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. Nat Biotechnol *23*, 607-611.

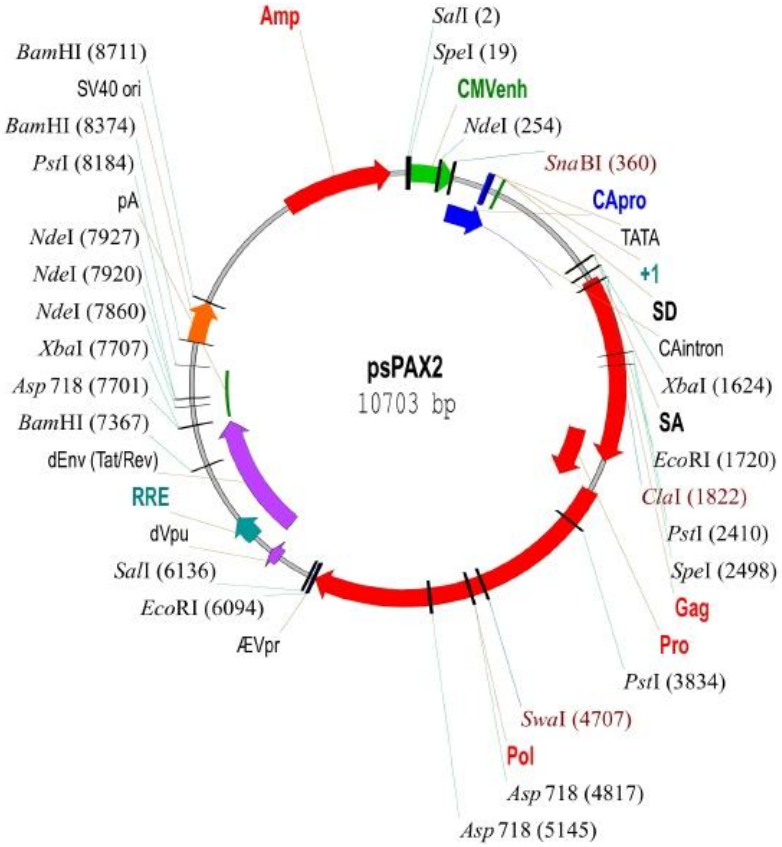
Zhao, Y., Samal, E., and Srivastava, D. (2005). Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature *436*, 214-220.

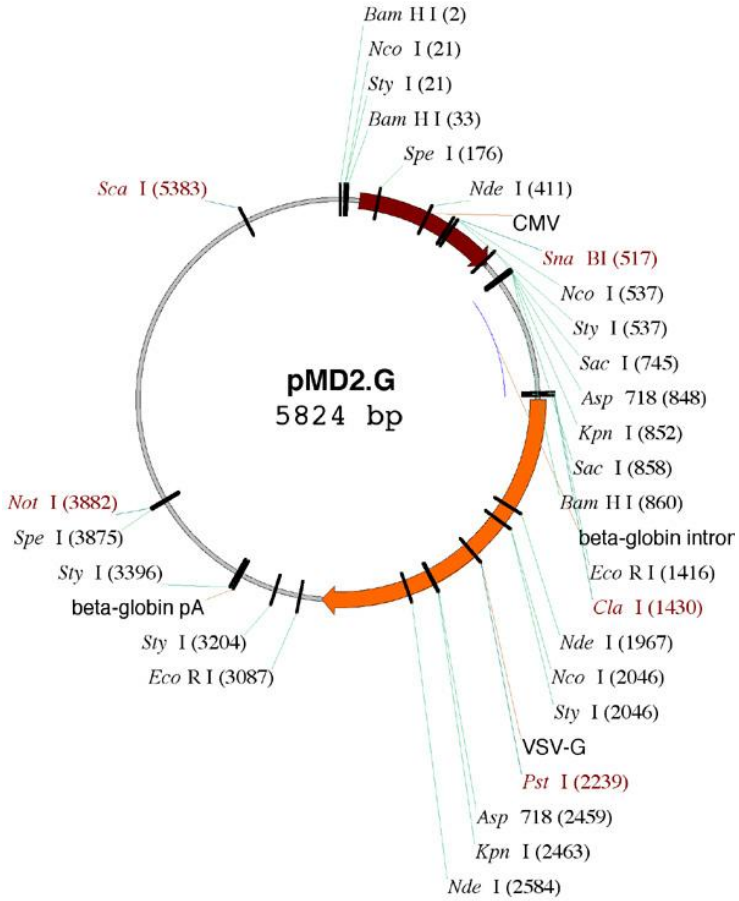
Zhu, W.Z., Hauch, K.D., Xu, C., and Laflamme, M.A. (2009). Human embryonic stem cells and cardiac repair. Transplant Rev (Orlando) *23*, 53-68.

Zwaka, T.P., and Thomson, J.A. (2003). Homologous recombination in human embryonic stem cells. Nat Biotechnol *21*, 319-321.

Appendix A: Lentiviral vector maps




psPAX2
10703 bp
Amp
SalI (2)
SpeI (19)
CMVenh
NdeI (254)
SnaBI (360)
CApro
TATA
+1
SD
CAintron
XbaI (1624)
SA
EcoRI (1720)
ClaI (1822)
PstI (2410)
SpeI (2498)
Gag
Pro
PstI (3834)
SwaI (4707)
Pol
Asp718 (4817)
Asp718 (5145)
ÆVpr
EcoRI (6094)
SalI (6136)
dVpu
RRE
dEnv (Tat/Rev)
BamHI (7367)
Asp718 (7701)
XbaI (7707)
NdeI (7860)
NdeI (7920)
NdeI (7927)
pA
PstI (8184)
BamHI (8374)
SV40 ori
BamHI (8711)


pMD2.G
5824 bp
Bam H I (2)
Nco I (21)
Sty I (21)
Bam H I (33)
Spe I (176)
Nde I (411)
CMV
Sna BI (517)
Nco I (537)
Sty I (537)
Sac I (745)
Asp 718 (848)
Kpn I (852)
Sac I (858)
Bam H I (860)
beta-globin intror
Eco R I (1416)
Cla I (1430)
Nde I (1967)
Nco I (2046)
Sty I (2046)
VSV-G
Pst I (2239)
Asp 718 (2459)
Kpn I (2463)
Nde I (2584)
Eco R I (3087)
Sty I (3204)
beta-globin pA
Sty I (3396)
Spe I (3875)
Not I (3882)
Sca I (5383)

Appendix B: Buffers and solutions

LB medium

1% Tryptone, 0.5% Yeast extract, 1% NaCl

LB agar

1% Tryptone, 0.5% Yeast extract, 1% NaCl, 1.5% Bacto agar

SOC medium

2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM Glucose

Lysis buffer

20 mM Tris (pH 7.4), 150 mM NaCl, 1% Trin X-100, 10% Glycerol, 1 mM PMSF, 10 µg/ml Aprotinin

2x Sample buffer

10% SDS, 10% 2-Mercaptoethanol, 20% Glycerol, 130 mM Tris, 0.1% Bromophenol blue,