DEVELOPMENTAL STUDIES FOR THERAPEUTIC APPROACHES USING ENDOTHELIAL CELLS DERIVED FROM MOUSE EMBRYONIC STEM CELLS

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Wszystkim, których kocham

Dedicated to whom I love

‘By dojść do źródła, trzeba płynąć pod prąd’

(To reach the source, one needs to go upstream)

(Um die Quelle zu erreichen, muss man stromaufwärts gehen)

Stanisław Jerzy Lec

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Dziękuje Wam z całego serca za Wasz trud i poświęcenie, abym mogła dojść do miejsca w którym teraz jestem.

Abbreviations

ΔCt - delta (threshold cycle)

AAV - **a**deno-**a**ssociated **v**irus

BMP4 - **b**one **m**orphogenetic **p**rotein 4

bp - **b**ase **p**air

BSA - **b**ovine **s**erum **a**lbumin

CAM - **c**ell **a**dhesion **m**olecule

CD - **c**luster of **d**ifferentiation

cDNA - **c**omplementary **d**eoxyribonucleic **a**cid

CF - **c**ystic **f**ibrosis

CO_2 - carbon dioxide

COPD - **c**hronic **o**bstructive **p**ulmonary **d**isease

CVD - **c**ardio-**v**ascular **d**isease

DAPI - 4'6-diamidino-2-phenylindole

ddH_2O - **d**ouble **d**istilled water

DNA - **d**eoxyribo**n**ucleic **a**cid

DPLD - **d**iffuse **p**arenchymal **l**ung **d**isease

EBs - **e**mbryoid **b**odies

EC - **e**ndothelial **c**ell

E-DNA - viral **e**xtrachromosomal DNA

EpiSC - post-implantation **epi**blast-derived **s**tem **c**ell

ESCs - **e**mbryonic **s**tem **c**ells

FACS - **f**luorescence-**a**ctivated **c**ell **s**orting

FCS - **f**etal **c**alf **s**erum

FGF - **f**ibroblast **g**rowth **f**actor

Flk1 - **f**etal **l**iver **k**inase

GAPDH - **g**lycer**a**ldehyde 3-**p**hosphate **de**-**h**ydrogenase

gDNA - **g**enomic **d**eoxyribo**n**ucleic **a**cid

GFP - **g**reen **f**luorescent **p**rotein

GSK3 - **g**lycogen **s**ynthase **k**inase 3

HEK cells - **h**uman **e**mbryonic **k**idney cells

hESCs - **h**uman ESCs

ICM - **i**nner **c**ell **m**ass

IDLV - **i**ntegration **d**eficient **l**entiviral **v**ector

iFCs – **i**nactivated **f**eeder **c**ells

IgG - **i**mmuno**g**lobulin **G**

ILD - **i**nterstitial **l**ung **d**isease

IN - protein **in**tegrase

IPF - **i**diopathic **p**ulmonary **f**ibrosis

iPSCs - **i**nduced **p**luripotent **s**tem **c**ells

KDR - **k**inase insert **d**omain **r**eceptor (a type III receptor tyrosine kinase)

Klf4 - **K**rüppel-**l**ike **f**actor 4

LIF - **l**eukemia **i**nhibitory **f**actor

LTRs - **l**ong **t**erminal **r**epeats

LV - **l**enti**v**irus

MP - **m**atrix **p**rotein

MEFs - **m**ouse e**m**bryonic **f**ibroblasts

MEK/ERK - **m**itogen-activated protein **k**inase/**e**xtracellular signal-**r**egulated **k**inase

mEndo cells - **m**ouse **end**othelioma cells

mRNA - **m**essenger ribonucleic acid

NCD - **n**on-**c**ommunicable **d**isease

Oct4 -**oct**amer-binding transcription factor **4** or POU5F1 - POU domain, class 5, transcription factor1

PAH - **p**ulmonary **a**rterial **h**ypertension

PCR - **p**olymerase **c**hain **r**eaction

PDGFRα - **p**latelet-**d**erived **g**rowth **f**actor **r**eceptor α

PECAM-1 - **p**latelet **e**ndothelial **c**ell **a**dhesion **m**olecule-1

PFA - **p**ara**f**orm**a**ldehyde

PI3K - **p**hosphoinositide-3 **k**inase

qPCR - **q**uantitative polymerase chain reaction

RNA - **r**ibo**n**ucleic **a**cid

rpm - **r**evolutions **p**er **m**inute

RRE - **R**ev-**r**esponsive **e**lement

RT - **r**everse **t**ranscriptase

SC1 - pluripotin

SIN - **s**elf-**i**nactivating

Sox2 - sex determining region Y-box 2

SSEA1 - **s**tage-**s**pecific **e**mbryonic **a**ntigen-1(CD15)

TBS/T - **t**ris **b**uffered **s**aline with **t**ween 20

TF - **t**ranscription **f**actor

TKR - **t**yrosine **k**inase **r**eceptor

VE-cadherin - **v**ascular **e**ndothelial-**c**adherin

VEGF - **v**ascular **e**ndothelial **g**rowth **f**actor

VEGFR - **r**eceptor for **v**ascular **e**ndothelial **g**rowth **f**actor

VSV-G - **v**esicular **s**tomatitis **v**irus **e**nvelope **g**lycoprotein G

WHO - **W**orld **H**ealth **O**rganization

Semantics

°C - degree Celsius

g - gram

M - molar

nm - nanometer

U - unit

µg - microgram

µl - microliter

µM - micromolar

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Abstract

The lungs together with the heart are the core of the human body. These organs maintain the homeostasis and provide substantial elements for proper functioning of the organism. Any kind of damage or dysfunction to these organs, results in serious disorders and general imbalance of the organism. Most critical are defects in the endothelial barrier, which consist of the endothelial cells (ECs). The vascular system is padded with a single layer of ECs. Endothelial cell structure and functional integrity are essential for the maintenance of the vessel wall and the circulation. Endothelial cell injury, activation or dysfunction is a feature of many pathologic states, just to mention few like inflammation or effects on vascular tone.

Regenerative medicine, including cell replacement therapy, is a promising alternative for disease treatments. The application of *in vitro*-generated (and if required) genetically amended cells could help to recover the function of damaged parts of a specific organ. One of the approaches is to establish or restore normal function of affected endothelial cells, by therapies based on embryonic stem cells (ESCs). However, generation of those cells is ethically debatable. Development of induced pluripotent stem cells (iPS) from somatic cells has emerged as a solution. This enables the generation of patient- and disease-specific iPS cells, which may produce therapeutic cell populations without immune rejection and moral dispute. Regenerative therapies seem to carry a great promise to treat endothelial dysfunction in respiratory and cardiovascular diseases.

To investigate the sites of integration of ES cell-derived endothelial (progenitor) cells, new cell lines from murine ES cells using lentiviral (LV) transduction were generated. In order to achieve the aim it had been decided to test different promoter-resistance-gene combinations. Therefore, a set of vectors containing murine promoters: VE-Cadherin (vascular endothelial) or VEGFR2 (vascular endothelial growth factor) in connection with GFP (green fluorescent protein) and antibiotic resistance genes hygromycin B, neomycin and puromycin, were produced and validated. It was feasible to generate and screen a cell line within a few weeks.

The newly established cell lines are suitable for monitoring endothelial differentiation and selection by means of antibiotic resistance. These experiments demonstrated that for an effective antibiotic selection of the desired cell type, further investigations are required. It was possible to produce murine iPS cell lines, containing the above-mentioned transgenes. Carefully selected and purified murine ECs subsets will be used for *in vivo* studies in tumor angiogenesis models in subsequent studies.

Zusammenfassung

Die Lunge, zusammen mit dem Herzen, bildet den Kern des menschlichen Körpers. Gemeinsam erhalten diese Organe die Homöostase und stellen wesentliche Substanzen für die einwandfreie Funktion des Organismus zur Verfügung. Störungen oder Dysfunktionen in Teilen dieser Organe oder deren Gesamtheit führen zu schwerwiegenden Erkrankungen sowie zu einem physiologischen Ungleichgewicht. Sehr kritisch sind dabei Defekte in der endothelialen Barriere, die aus Endothelzellen aufgebaut ist. Das weit verzweigte vaskuläre System ist mit einer einzelnen Schicht dieser Endothelzellen ausgekleidet. Die Endothelzellstruktur sowie die funktionale Integrität dieser Schicht sind essentiell für die Aufrechterhaltung der Blutgefäßwand und deren zirkulatorische Funktion. Verletzungen, Aktivierungen oder Dysfunktionen der Endothelzellen sind Charakteristika verschiedener pathologischer Zustände, z.B. im Zuge von inflammatorischen Prozessen oder bei krankhaften Veränderungen des vaskulären Tonus. Die Ansätze der regenerativen Medizin, die auch eine Zellaustausch-Therapie beinhalten, sind eine vielversprechende Möglichkeit, derartige pathologische Prozesse zu behandeln. Die Verwendung *in vitro*-generierter und falls notwendig gentechnisch veränderter Zellen könnte zur Regenerierung der Funktion der gestörten Teile des spezifischen Organs beitragen. Die Etablierung von Therapien, basierend auf embryonalen Stammzellen könnte eine Möglichkeit sein, die normale Funktion pathologisch betroffener Endothelzellen wiederherzustellen. Jedoch befindet sich die Generierung derartiger Zellen im Spannungsfeld ethischer Diskussion.

Daher ist die Entwicklung induzierter pluripotenter Stammzellen (iPS) aus somatischen Zellen eine Möglichkeit, diesen Debatten entgegenzutreten. Die Etablierung von iPS-Zellen ist individuell je nach Patient und Erkrankung und könnte zur Produktion von therapeutisch verwendbaren Zellpopulationen führen, die ohne Abstoßungsreaktion nutzbar sowie ethisch unbedenklich sind. Derartige regenerative Therapien sind eine vielversprechende Entwicklung zur Behandlung endothelialer Dysfunktionen in respiratorischen und kardiovaskulären Erkrankungen. Um die Funktionalität der aus Stammzellen entwickelten endothelialen (Vorläufer) Zellen zu analysieren und räumliche Verteilung zu verfolgen,

wurden unter Verwendung der lentiviralen Transduktion neue Zelllinien aus Mausstammzellen mit verschiedenen Promoter/Resistenzgen-Kombinationen generiert.

Die Vektoren besitzen einen spezifischen murinen Promotor, VE-Cadherin (Vaskulär-endotheliales) oder VEGFR2 (Vascular Endothelial Growth Factor Rezeptor), der die Expression von GFP (grün fluoreszierende Protein) reguliert, und ein Gen für antibiotische Resistenz, wie Hygromycin B, Neomycin oder Puromycin. Die verschiedenen hiermit generierten Zelllinien wurden etabliert und erwiesen sich als geeignet, eine Endothelzelldifferenzierung und Selektion auf Basis der spezifischen Antibiotikaresistenzen zu verfolgen. Die experimentellen Ergebnisse zeigen jedoch, dass weitere Untersuchungen notwendig sind, um eine Selektion des gewünschten Zelltyps vornehmen zu können. Die Generierung der murinen iPS Zelllinien, die die oben genannten Transgene beinhalten, befindet sich daher in einem derzeit voranschreitenden Entwicklungsprozess. Sorgfältig selektierte und aufgereinigte murine Endothelzellpopulationen sollen im weiteren Verlauf unter Inanspruchnahme eines Tumor-Angiogenese Modells in *in vivo* Studien eingesetzt werden.

1. Introduction

1.1. The respiratory and cardiovascular system

The lungs represent a very unique organ in the human body, not only do lungs have to endure contact with various particles and microorganisms during the entire life but also fulfill other functions. Among those tasks are, for example filtration of blood running to the systemic circulation, or absorption of metabolically active peptides within this circuit (Effros, 2006). The exceptional structure of the lungs helps to complete the most important role which is the distribution of the oxygen and elimination of carbon dioxide from the circulation (Tomashefski and Farver, 2008). The healthy human lung weighs around 1 kg, where 40% to 50% of the mass is blood (Effros, 2006). Lungs are embedded in a shiny visceral pleura and with time the pleura gathers black pigmentation. This pigmentation is the result from contact with the particles from the inspired air (Tomashefski and Farver, 2008). The development of the mammalian lungs is strictly correlated with the appearance of surfactant. Surfactant is a mix of lipids and proteins which reduces the surface fraction of the air-fluid interface of the lungs (Effros, 2006). The surfactant is secreted by type II alveolar cells, these cells have cubical shape and large basal nucleus. The lamellar inclusion bodies are characteristic for type II cells and are randomly distributed in the cell. The lamellar inclusion bodies are the precursors of surfactant. Type II cells establish 60% of the surface cells but account only for around 5% of the alveolar surface. The type II pneumocytes provide a reservoir that matures into type I alveolar cells. Type I cells stretch along the alveolar wall and have a flattened nucleus and a broad area of adjacent cytoplasm. In contrast to type II cells, type I cells cover 95% of the alveolar surface, but form just 40% of the alveolar lining cells. The type I pneumocytes are less resistant to injury then the type II cells (Tomashefski and Farver, 2008).

During human development, the first appearance of lungs falls on around 26th day. The lungs are generated from the primitive gut which accesses the surrounding mesenchyme. After the 26th week of embryonic development, the type I and II pneumocytes emerge from differentiated epithelial cells. The secretion of surfactant does not occur until the last weeks of gestation. The final formation of

the alveoli takes place after birth, approximately until a child reaches eight years of age (Harrison, 2005). In the Update in Pediatric Lung Disease 2012 a very interesting investigation conducted by Narayanan and colleagues is recalled. In that study, data were obtained from the non-invasive evaluation of alveolar size by helium-3 (^{3}He) magnetic resonance imaging. The results imply that there is almost a doubling in the number of alveoli during childhood and adolescence (age from 7 to 21) in humans. These data lead to the conclusion that late alveolarization is possible. Therefore, the lung could recuperate from damage that arose in the early stage of life (McColley and Morty, 2013). In total the number of alveoli reaches around 400 million with the entire surface area of 70 m^2. Nevertheless, at the age of 30 or 40 a gradual expansion of air space in the lungs follows.

During birth, the adaptation of lungs from a fluid-secreting organ to the structure, which takes in the liquids, is occurring. Prior to birth, the breathing movements exist and some amniotic fluid is swallowed. Between the thorax and the abdomen there is a musculo-fibrous separating pane, the diaphragm. The formation of the diaphragm, commences in the third week after fertilization. the diaphragm is the primary muscle of respiration (Harrison, 2005). The diaphragm is innervated by the phrenic nerve, which during embryogenesis originates from the cervical spinal cord (C3, 4, and 5) (Effros, 2006).

In higher vertebrates, the first organ which is structured during embryogenesis is the vasculature, encompassing the heart and blood vessels. Around the 3rd week of human embryogenesis, the cardiovascular system emerges. The vascular-alveolar network in the lungs is the principal place of gas exchange. After oxygenation in the lungs, the blood is distributed in the body by the pumping heart. The exchange of gases from tissues is accomplished through capillary beds (Schwarz and Cleaver, 2009).

Pulmonary and cardiac function are the most significant mechanisms to sustain human life (Tsai and Lee, 2011). These systems are to some extent autonomous and pursue individual patterns of functional organization. The integration of changes in respiratory movements and blood pressure occurring as an adaptation to changing conditions, indicates structural and functional interaction between respiratory and angiokinetic centers. The respiratory and circulatory systems are subject to independent neural pathways in response to signals originating from pulmonary, vascular and cardiac stretch receptors.

the most important function of the neurons in the cardiovascular system is the guarantee to sustain the gas exchange in the circumstances that threaten the normal functioning of the organism (Donina, 2011). The dynamic mechanical properties of the heart and lungs assure complementary response between lung and cardiac volumes and pressures.

1.2. Endothelium

The complete vascular structure is cushioned with endothelium, which is comprised of an endothelial cell (EC) monolayer (Sumpio et al., 2002). The endothelium embeds the vascular wall and the adventitia (Lerman and Zeiher, 2005). In a fully-grown human organism, the endothelium constitutes a structure of almost 1 kg. The endothelial cells, similar to hematopoietic cells, originate from precursor cells called hemangioblasts. The precursor cells emerge from differentiated mesenchymal cells. The hemangioblast becomes a pre-endothelial cell, which can convert into a committed hematopoietic cell or endothelial cell. It was proved that endothelial cells can moreover re-differentiate into mesenchymal cells and intimal smooth muscle cells. Endothelial cells are very flat, with a centrally located nucleus. The ECs shape uniform line on the inside of the vessels and at the junctions. Between cells, there are overlapping regions which help to seal the vessel. the intercellular junctions are very important, helping to maintain the integrity of the vessel (Alberts et al., 2002). The endothelium functions as a barrier, which is semi-accessible and regulates the relocation of small and large molecules (Sumpio, Riley, and Dardik 2002). Pinocytotic vesicles are very characteristic of endothelial cells. These structures are formed by small vesicles adjacent to the cell membrane, through which the exchange of particles from the bloodstream to the underlying tissues is carried out (Sumpio et al., 2002). Many molecules are located on the surface of endothelial cells. Those molecules serve as receptors and interaction sites for various essential particles. A molecule of high importance, which maintains homeostasis and that is synthesized in endothelial cells is Factor VIII (von Willibrand Factor). This molecule takes part in the process of blood clotting. Endothelial cells are also involved in the inflammatory response. The reaction of endothelial cells, to eliminate inflammation, is manifested by opening of intercellular junctions. This action permits

the release of large amounts of fluid from the blood plasma into the encompassing tissues, which develops swelling (Alberts et al., 2002).

A schematic drawing illustrating some of the processes in which the ECs are involved is depicted in Fig. 1.

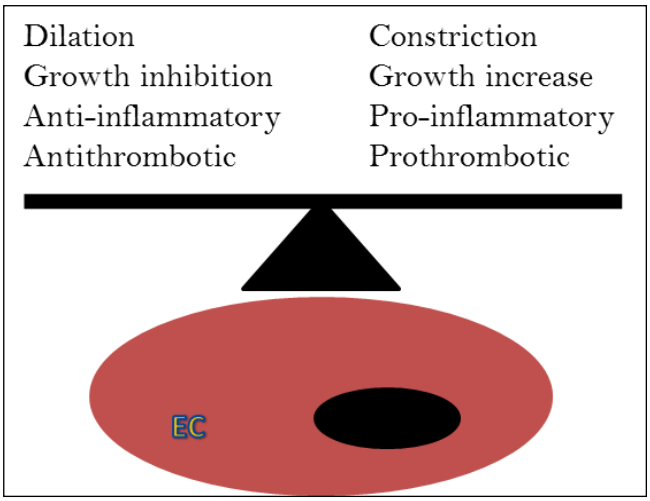


Figure 1: Scheme illustrating the balance of the endothelial cell functions. Abbreviation: EC - endothelial cell.

Various disruptions in endothelial integrity and function will lead to pathological conditions, including atherosclerosis, diabetes, or pulmonary hypertension (Sumpio, Riley, and Dardik 2002). It is crucial to maintain the right balance between the injury and repair of endothelium in order to diminish cardiovascular events as the regenerative potential of mature endothelial cells is very low (Shantsila, Watson, and Lip 2007).

There are two more terms inseparably connected with endothelial cells: vasculogenesis and angiogenesis (Fig. 2). Vasculogenesis is defined as a process of *de novo* formation of blood vessels from angioblasts. The angioblasts assemble and become organized to form a linear cluster within which a lumen will develop. In the process of vasculogenesis, the first embryonic vessels are assembled. Angiogenesis referrers to growth and remodeling of the pre-existing primitive vasculature. Angiogenic mechanisms occur as natural events in developing organs and tissues. In angiogenesis there are two different actions recognized: sprouting angiogenesis and angiogenic remodeling ((Fig. 2 image 2.) and 3.)). Sprouting angiogenesis describes the growth and elongation of new vessels from existing vessels. Angiogenic remodeling refers to various alterations to which the pre-existing

vessels are subjected. The already formed vessels can change shape and size, including narrowing of the vessel diameter. Those transformations are frequently the result of a response to changes in hemodynamic pressure. Vasculogenesis and angiogenesis often appear simultaneously, for example, in developing tissues and organs, when the vascular beds are being formed (Schwarz and Cleaver 2009) (Fig. 2 (4)).

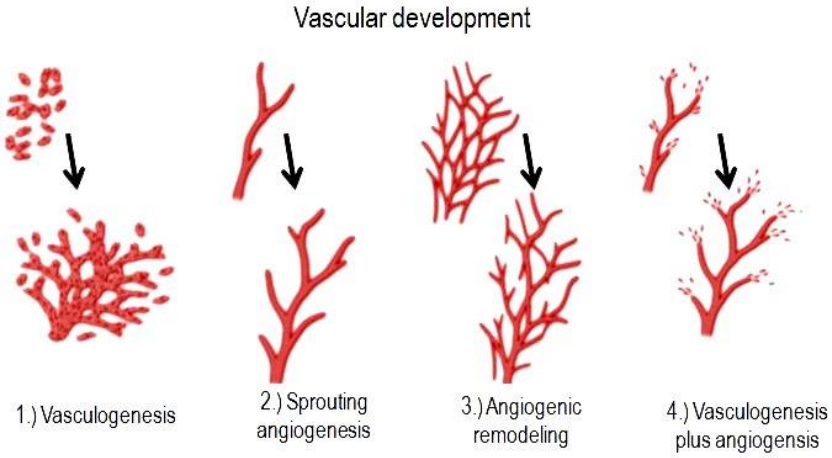


Figure 2: Formation of blood vessels. Adapted from: Schwarz and Cleaver 2009.

1.2.1. Endothelial dysfunction in respiratory and cardiovascular diseases

Endothelial dysfunction is associated with high rates of morbidity and death (Lerman and Zeiher 2005). In Fig. 3 the statistics concerning importance of endothelial dysfunction are presented. These data were obtained from the Medline (PubMed) trend browser. On this webpage numbers of entries concerning articles in PubMed (Medline) published each year are available. The search phrase was "endothelial dysfunction".

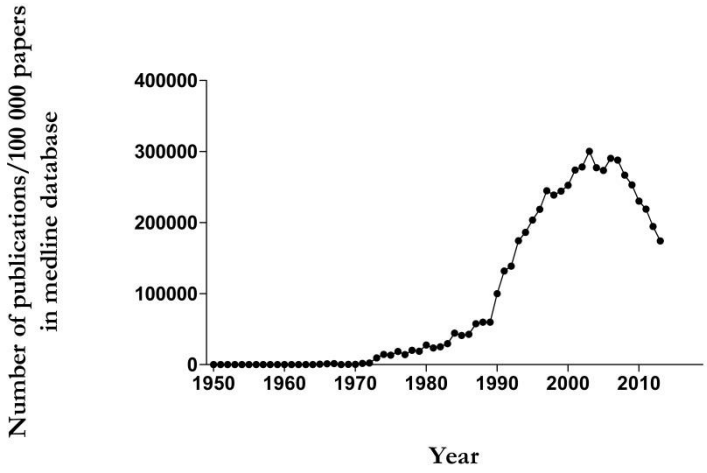


Figure 3: Changes in the numbers of publications concerning endothelial dysfunction over the past seven decades.

Endothelial dysfunction should be interpreted more as an endothelium activation, which ultimately may lead to arterial diseases. Activation of the endothelium means a shift from an inactive phenotype to a phenotype associated with defence responses of the organism. The molecular cascade that is activated in the endothelium, by elements that cause cardiovascular diseases (CVD), involves the release of chemokines, cytokines and adhesion molecules. These molecules interplay with elements in the blood such as leukocytes and aim inflammation in particular tissues. Repeatedly occurring cardiovascular risk factors may wear out the defensive capacity of the endothelial cells. The repercussions of that state may cause loss of endothelium functionality and stability. The damage to endothelial integrity is connected with the degree of injury and intrinsic ability to repair. The damage can be fixed by mature endothelial cells which can multiply and substitute missing cells. Alternatively, circulating endothelial progenitor cells (EPC) can be recruited to sites of injury. The EPC are found in the peripheral blood, where these cells originate from the bone marrow. The progenitor cells are able to transform into mature endothelial cells (Deanfield et al., 2007). Endothelial malfunction or injury are characteristic of pulmonary system illnesses like interstitial lung diseases (ILDs), which include idiopathic pulmonary fibrosis (IPF) (Cottin, 2013). Two other diseases in which endothelial dysfunction plays a crucial role are pulmonary arterial hypertension (PAH) and chronic obstructive pulmonary disease (COPD).

Once arteriolar remodeling has occurred, the endothelium is involved in the functional modifications of the pulmonary vasculature (Guazzi et al., 2014). Among CVD the most common disorders are coronary heart diseases (angina and heart attack), heart failure, congenital heart disease and stroke (Povsic and Goldschmidt-Clermont, 2008).

1.3. Therapeutic perspectives

According to the World Health Organization (WHO), 38 million (68%) out of 56 million of global deaths in 2012 were caused by noncommunicable diseases (NCDs). The NCDs are defined as chronic diseases which cannot be passed from person to person and are generally of long duration and slow progression. The WHO statistics from the year 2012, reveal that CVDs account for 46% of all deaths among NCDs. The major factors responsible for NCDs include rapid development and globalization, aging and an unhealthy way of life (http://www.who.int). The WHO figures concerning the mortality rate in cardiovascular and chronic respiratory diseases for 2012 are presented in Fig 4.

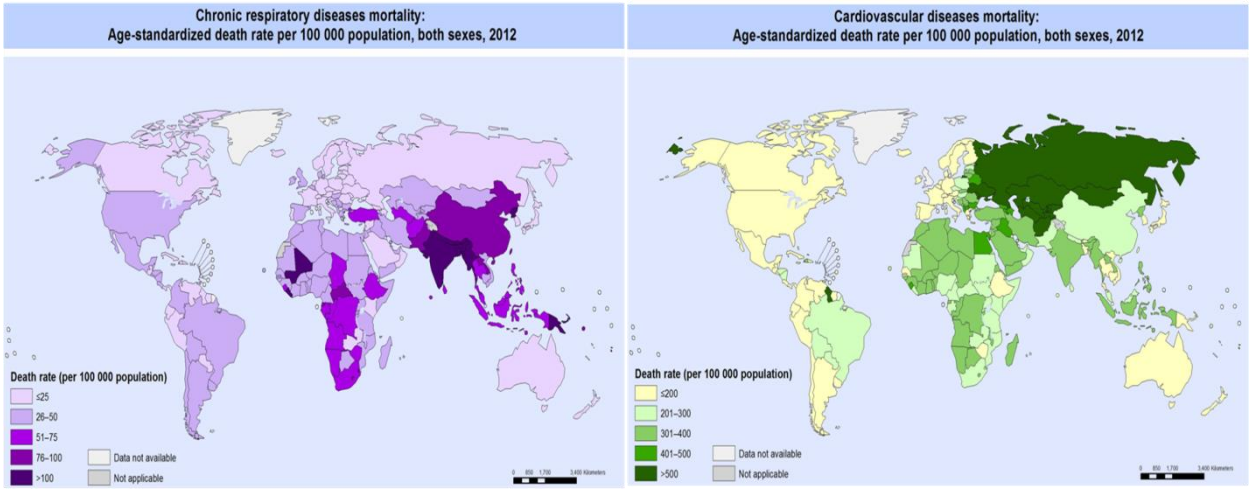


Figure 4: WHO data concerning death rate caused by chronic respiratory and cardiovascular diseases.
Source: http://www.who.int.

Given the trends reported above, the influence of NCDs on the socio-economic situation in countries all over the world is very high. In the group of NCDs are highlighted diseases which are inevitably linked to endothelial dysfunction, hence, it is even more challenging to find treatments for these disorders.

In many cases it is too late for prevention, thus other management steps need to be undertaken. Regenerative therapies seem to carry a great promise to treat endothelial dysfunction in respiratory and cardiovascular diseases.

1.3.1. Gene therapy

Gene therapy is a novel method that could be used for treating a sickness by introducing a gene into a patient's cells rather than using pharmacological medications or device therapies. This technique can be used to substitute a defective gene, or to insert a new gene to cure or to positively alter the medical progression of a disorder. In order to benefit from gene therapies, it is important that (after Misra, 2013):

- the inquired disorder is well understood,
- the deficient gene was recognized and a functioning copy needs to be obtainable,
- the defined cells in the body, which will undergo the treatment, need to be selected and approachable,
- an effective way to transfer the functional copies of the gene to right target cells need to be accessible.

Gene-based therapies depend on the introduction of a gene or small sequences of nucleic acids to the faulty cell or tissue. Sometimes the replaced gene is intended to modify the product of the defective gene and in this way cures the disease. The general classification of gene-based therapies includes two groups: germline and somatic gene therapies. In the germline method, the cells in focus are sperm or egg. Modifications made in those cells, prior to conception, will be transferable to the next generation. In the somatic cell technique the alterations are made to already mature cells. Gene-based therapies carry the possibility to carefully address diverse stages of a disorder and regenerative progress (Devaney et al., 2011). In order to deliver genes to desired tissues, many systems including viral and non-viral methods are available.

A short overlook of the existing methods for gene transfer is presented below (Kolb et al., 2006):

1. Liposomes - an artificial sphere-shaped vesicles consisting of a lamellar phase lipid bilayer. Liposomes seem to be non-immunogenic, but when utilized with plasmids, liposomes cause considerable immune response.

2. Adenovectors - the biggest asset is the exceptional efficiency of gene delivery. The gene expression is temporary and additionally the immunogenicity of the human adenovirus (of two serotypes: Ad2 and Ad5) hampers potent re-administration.

3. AAV (adeno-associated vectors) - demonstrated a great precision of infection and extended expression in the tissue. These vectors are assumed to generate a weaker inflammatory and immune response than adenoviruses.

4. Retro and lentivirus vectors - *in vivo* applications have been restricted due to the necessity of obtaining high titers to achieve a satisfactory degree of expression. Retroviral vectors can only transfect non-quiescent cells, whereas lentiviruses (LV) can also transfect non-dividing cells.

The "candidate" diseases for gene-based therapies include interstitial disorders or COPD, which are resultant from many endo- and exo-genous factors (Kolb et al., 2006). Concerning CVD, the area of restorative angiogenesis is in focus, preliminary data from clinical trials reveal encouraging results with only a few side-effects (Wolfram and Donahue, 2013).

1.3.2 Cell replacement therapy

Cell replacement therapies carry high expectations for patient-specific personalized treatments in the near future. In the cell replacement therapies, cells acts as a tool to mend dysfunction and deficits, where even nanoparticles or biomolecular methods fail. The most demanding task in the development of these strategies is to comprehend and manage the therapy itself and the reaction caused in the body of the patient (Dudek et al., 2014). There are two leading methods of reprogramming characterized (Thomas Graf, 2011):

- the induction of pluripotency in somatic cells leading to induced pluripotent stem cells (iPS),
- the transformation of somatic cells into already specialized cell type (transdifferentation).

The iPS cells, which can be converted into any kind of cell type, provide hope for the cure of various diseases. It is not yet possible to differentiate iPS cells into the cells which will possess the same attributes as the cells being replaced. Other issues that also need to be addressed are: (i) how to extend the cell production, (ii) finding ways to exclude tumorogenic factors and how to (iii) cutback the time required for growing, differentiation, selection and validation of the cells. At present, the available methods for cell-based therapies are lengthy and too expensive to be applied for severe organ failure. Currently, one issue can be dealt with, the immune rejection of implanted cells. The engrafted iPS cells can be derived from the patient and transplanted as autologous cells. This helps to avoid the immune response caused by histocompatibility mismatch (Fox et al., 2014). Increasing interest has also emerged in the lung field, where the focus is on recognition and application of endogenous progenitor lung cells. Precursor cell populations had been found in the mesenchyme, epithelium and endothelium in the distal lung. Insight into to these progenitor cells throughout development and in injury and regeneration will help to apply progenitors in therapies in which the capacity of endogenous cells in restoration, of an organ, is triggered (Collins and Thébaud, 2014).

1.4. Embryonic stem cells

The field of stem cells remains thrilling, undiscovered and a questionable sector of scientific endeavour. Though this research holds promise to transform the approach in which various human diseases can be cured. The phenomenal and unique abilities of stem cells make them suitable for use in replacement therapies and drug development (Alexander van Servellen & Ikuko Oba, 2014). The stem cells represent an unspecialized pluripotent cell population. To the exceptional features of ESCs are included ability to self-renew and the potential to differentiate into any cell type of the mature organism (Power and Rasko, 2011). Stem cells are often referred to as naïve cells, because the chromatin is folded lightly over the histone proteins. In mature and specialized cells, chromatin is

firmly attaching to the histones. In that manner the regions of DNA, in the differentiated cells, which are not in use are switched off (Takahashi et al., 2007).

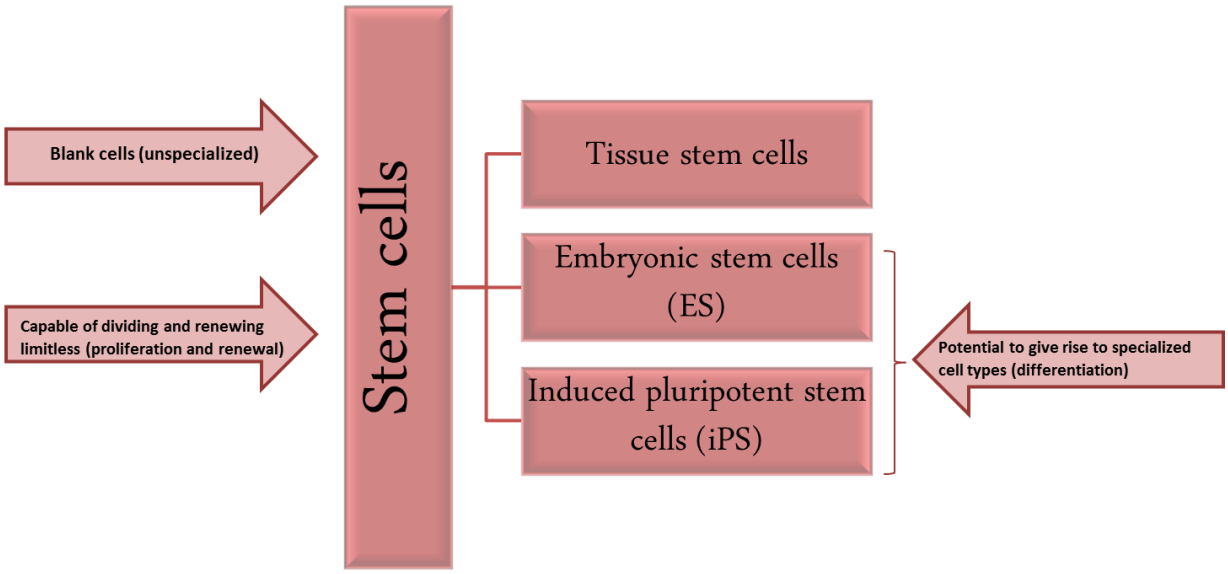


Figure 5: Classification of stem cells.

Stem cells can be divided into three categories:

1. Embryonic stem cells, obtained from embryo.
2. Tissue/adult stem cells.
3. Induced pluripotent stem cells.

The cells derived from the epiblast in culture, exhibit pluripotency and can differentiate into over 200 cell-types of the human body. However, these cells are not able to generate the cells which make extra-embryonic tissues. The ESCs can be maintained in the culture almost endlessly. The hES (human) cells are obtained from embryos in the blastocyst-stage and in the majority cases, those embryos are collected from *in vitro* fertility clinics, where the embryos were donated for use in scientific research. During embryonic development, almost all cells become specialized, but there remain some populations of undifferentiated cells which can be found in various parts of the organism. Those subsets of cells in case of sudden need for rapid aid, can be activated and start to divide. In this manner, the inner balance in the body can be maintained. These stem cells are named "somatic" or

"adult" stem cells. What distinguishes adult stem cells from ES cells is the limited ability to differentiate. The "adult" stem cells are multipotent, this means that can only transform into two or more mature lineages associated with the tissues of its origin. The last group of stem cells is constituted by iPS cells. Due to the "reverse engineering" it became possible to unlock already mature adult cells. The reprogramming of skin fibroblasts by Takahashi and Yamanaka in 2006 represented a breakthrough. Fibroblasts were driven back to a naïve state by expression of a cocktail of different transcription factors. The iPS cells turned out to be identical to ES cells in morphology and performance (Power and Rasko, 2011).

1.4.1. Murine embryonic stem cells

Embryonic development commences with fertilization, after meiotic and then mitotic divisions the fertilized oocyte becomes a diploid cell. When the structure consists of eight cells it receives the name morula and undergoes the first localization and is subjected to morphological processes. The blastocyst is composed of 16 to 32 cells and represents a further increase in size and next stage of embryo development. Some fluid starts to gather and the pressure forces the formation of a cavity within the blastocyst. A cluster of cells accumulates on one side of the cavity, those cells are the pluripotent inner cell mass (ICM). The external layer of epithelium constitutes the trophectoderm. After embryo implantation, the ICM has completed a second round of lineage differentiation and has transformed into a primitive endoderm (which coats the cavity) and into the pluripotent epiblast. The cells from the epiblast are exclusively able to become an embryo proper. The trophectoderm and the primitive endoderm establish the other embryonic tissues, like for example, the placenta (Wennekamp et al. 2013). In mammalian development, at the early stage of an embryo, cells are able to differentiate into all cell types of the adult organism and above all, into the gametes. This ability is a hallmark of the epiblast tissue, which lasts only for a very short time. The cells which will be obtained from the next phases of the developing embryo are named postimplantation epiblast stem cells (EpiSCs). Certainly those cell types need different maintenance conditions and execute diverse gene programs. It can be stated that the determinative force is unleashed during the development of the epiblast cells within the ICM of the blastocyst. In support of this statement are molecular studies

which demonstrated that the key transcription factors (Oct4, Sox2) are not expressed in the zygote (Nichols and Smith 2012).

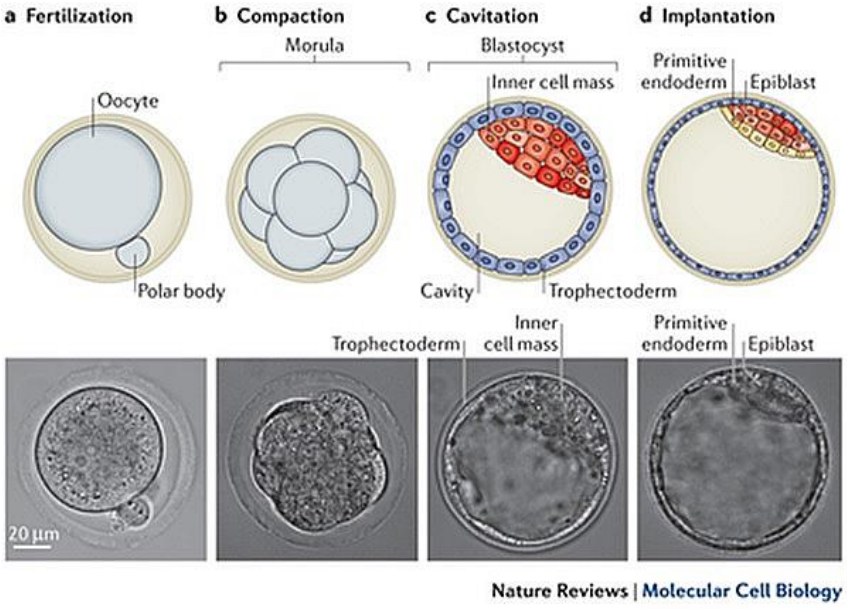


Figure 6: Mouse embryonic development. Mouse pre-implantation development: from fertilized oocyte to blastocy. Images courtesy of Sebastian Wennekamp, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany (Wennekamp et al., 2013).

The first mouse ESCs were derived from the cells obtained from the ICM of the blastocyst which was cultured on the layer of mitotically inactivated fibroblasts (iFC) with fetal calf serum-supplemented medium. For conducting those extremely important experiments in 1981, three scientists need to be acknowledged: M. Evans, M.H. Kaufman, and G.R. Martin. In order to establish the culture conditions, in the test phase pluripotent embryonal carcinoma cells were used by Martin and Evans in 1975. Similarly to carcinoma cells, ESCs form teratomas when injected into mice. The ultimate proof for the pluripotency was achieved by Bradley and colleagues (1984), by blastocyst injections which yielded chimeric mice. Typically, the ESC derivation is made by removal of the whole blastocyst or isolation of the entire ICMs and then culture of the isolated structure. In 1997, Brook and Gardner, after the single cell isolation succeeded in generating the ES cell lines from the microdissected epiblast. The above-mentioned experiment by Brook and Gardner confirmed that the murine epiblast at the prenatal day 4.5 (E4.5), is the source of ES cells (Nichols and Smith, 2012). The genes from ES cells in chimeric mice can be transferred to the next generation. Particular changes can be introduced

into the genes of ES cells and those cells can be placed back into the blastocyst. The new-born mice will possess the amended genes. Such mice can be used to model many human diseases (Blair, Wray, and Smith 2011).

The ESCs are very susceptible to pH or temperature oscillations and to overgrowth. Improper culture conditions may lead to unplanned differentiation of the ES cells, even though the cells are grown on iFCs and in the presence of leukemia inhibitory factor (LIF) (Turksen 2002). LIF is involved in the self-renewal process of ES cells. The key sources of this factor for ESCs culture are iFCs and exogenous LIF which is added to the medium (Tremml, Singer, and Malavarca 2008). The LIF action is initiated by the LIFR/gp130 receptor which leads to STAT3 stimulation. The transcription factor STAT3 plays a crucial role in controlling the self-renewal of stem cells. Differentiation and lineage commitment is blocked by STAT3 signaling. Simultaneously the MEK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) signaling pathway is initiated (Graf, Casanova, and Cinelli 2011a). The MEK/ERK pathway impacts many actions inside the cell, like for example propagation, differentiation and survival. The MEK/ERK signaling in ESCs has a negative impact on cell self-renewal by counteracting STAT3 action. The MEK/ERK pathway is not yet fully recognized in hESCs (Li et al. 2007). Numerous experiments have confirmed that LIF is important in managing self-renewal and maintaing the pluripotency in ES and iPS cells (Graf, Casanova, and Cinelli 2011).

1.4.2. *In vitro* differentiation of embryonic stem cells

Although much time has elapsed since the first ESCs isolation, many questions remain unanswered. The great ability of ESCs to determine three primary germ lineages, the capacity of stem cells to be used to generate transgenic animals was reported by Gossler and colleagues (Gossler et al., 1986). The possibility to amend the genome of stem cells by means of homologous recombination was presented by Thomas and Capecchi in 1987. Another milestone in ES cell research was reached by Smithies and colleagues, who demonstrated that the changes made to the genes which are then re-implanted into the blastocyst, are able to carry the modifications into the developing germline. So far, only mouse ES cells, among other mammalian stem cells, transmit the transformed genome to the germline (Turksen, 2002). Embryonic stem cells serve as a great experimental prototype of

mammalian embryogenesis. In mammals, the blastocyst (the preimplantation embryo) consists of the following sections: (i) the trophectoderm, (ii) the hypoblast and (iii) epiblast. The epiblast is responsible for generating the fetal tissues. The two first structures mentioned-above (i) and (ii) form organs and extraembryonic tissues. The blastocyst of rodents is very much like in other mammals in arrangement. There are dissimilarities in the time at which the three sections appear and the duration of preimplantation growth which in mice lasts four days and in primates seven to ten days (Medvedev et al., 2010).

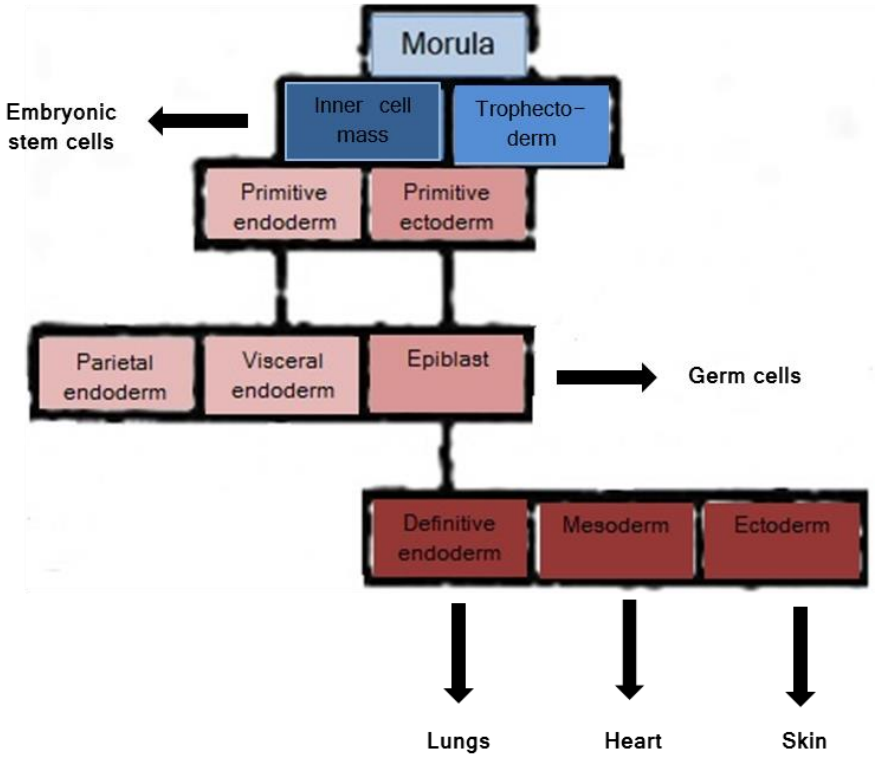


Figure 7: The embryonic development of the mouse illustrating correlations between early cell subsets and the three primary germ layers. Adapted from: (Keller, 2005).

Self-renewal and pluripotency make ES cells a perfect experimental system for studying the mechanisms behind cell differentiation. The capability of ES cells to differentiate into almost any kind of cell of the human body, make ES cells very desirable for use in regenerative medicine (Medvedev et al., 2010). In order to obtain clinically applicable cell subsets, ES cells are differentiated *in vitro* to form so called embryoid bodies (EBs). There are three common methods of differentiation:

- suspension culture,

- culture in methylcellulose semi-solid media,
- hanging drops culture.

The EB is described typically as an aggregate of ESCs in suspension culture, which is able to generate all three germ layers of an embryo (Bratt-Leal et al., 2009).

The formation of EBs is dependent on many factors: the quality of fetal calf serum (FCS), the presence of various growth factors and the ESC line, the time and amount of the ES cells at the starting point (Chen et al., 2011). Prior to triggering differentiation in ES cells, LIF and feeders need to be removed from the culture. The suspension (static or rotary-orbital) culture of EBs requires seeding of ES cell suspensions on a non-adherent Petri dish. The ESCs start to aggregate due to cell-cell adhesion forces. The EBs obtained through this method are usually asymmetric in shape but high in number (Bratt-Leal et al., 2009). Better quality EBs are achieved by placing the plates on a shaker or cells in the small bioreactors (Spinner flasks®), which provide continuous flow of medium. This condition offers improved access to nutrients and cytokines or growth factors and makes the production of EBs more scalable (Li et al., 2013). Single-cell suspensions or clusters of ES cells can also be put onto a hydrogel matrix. The hydrogels like methylcellulose or hyaluronic acid are thermoresponsive and provide cell aggregates of clonal derivation. This semi-solid culture allows for high reproducibility but the amount of EBs is significantly reduced. The most consistent embryoid bodies in known quantities are obtained by hanging drops method. The ES cells are placed in a very small amount of medium on a Petri dish and the plate then is inverted. The cells fuse due to the gravity forces and form EBs. The maintenance of hanging drops cultures is rather challenging and not easily scaled-up (Bratt-Leal et al., 2009). Each of the above-mentioned approaches has pros and cons. The 3-D arrangement of EBs provides an insight into cell-cell interplay, which is crucial for developmental mechanisms (G. Keller 2005, Murry and Keller, 2008).

Gordon Keller the "guru" in the ESC field, underlines three important factors which need to be obeyed if ESCs will be used as a standard for lineage commitment. Primarily, the establishment of a protocol which promotes a dynamic and repeatable way of collecting the desired cell types must be achieved.

A big advantage would be the possibility to integrate the differentiation process with the selection method. This kind of combination would increase the pool of cells for collection. The second issue concerns proper lineage development. The ESCs need to mimic the processes that occur when the lineage commitment appears in the embryo. The last objective implies that the generated cell subsets will possess characteristic functional abilities both *in vitro* and *in vivo* (Keller, 2005).

1.4.3. Differentiation towards endothelial cells

The origin of endothelial cells needs to be considered together with lineage commitment, more precisely, with the mesoderm. In the embryo the mesoderm is shaped between the outer layer, the ectoderm and the most inner layer, the endoderm (Turksen, 2002). Day 15 in human embryogenesis is considered as the hallmark of the initiation of gastrulation. On that day, a temporary formation the primitive streak is structured and the establishment of that transitory formation starts from the anterior epiblast. During the course of gastrulation, the epiblast undergoes transformation from a bilaminar into a trilaminar disc. Around day 16 of embryogenesis, part of the cells from the epiblast move along the primitive streak and settle in the gap between the epiblast and the embryonic definitive endoderm. In this way, the third germ layer is constituted the intraembryonic mesoderm (Larsen, 2008). That movement of the cells in and over the primitive streak is named epithelio-mesenchymal transition. The three germ layers after transformation in the embryo are referred to as: (i) ectoblast/derm dorsally located, (ii) mesoblast/derm situated in-between and (iii) ventrally based replacement of the hypoblast endoblast/derm (Smith, 2001).

In the 3rd week of human embryogenesis the blood vessels and blood islands appear inside the yolk sac. The blood islands mature beside the endoderm and divide into separate hemangioblasts, encircled by endothelial progenitor cells. The blood cells originate from the hemangioblasts and the progenitor ECs establishes the endothelium of blood vessels. The splanchnopleuric mesoderm converts into angioblasts under the influence of molecules excreted by the endoderm. Fusing, deflated mesodermal angioblasts are turning into endothelial cells which form vesicular structures. These complexes merge further, to finally constitute arterial, venous and lymphatic channels. Even though the early blood cells

come from the yolk sac, afterwards, the blood cells are produced by the bone marrow, liver, thymus and spleen. Two key theories have been proposed. The first assumes that the hemangioblasts, which are bipotential, generate endothelial precursor cells and the primitive erythroid. Alternatively, the hemogenic endothelium originates hematopoietic stem cells and endothelial progenitors (Atala and Lanza, 2012). During the transition from an epithelium to mesenchymal subset, cells cease to express E-cadherin, which is the epithelial cell adhesion molecule (CAM). That batch of mesodermal cells commences expressing the Flk1 receptor. The VEGFR2 (vascular endothelial growth factor receptor 2) in the mouse is also known as the fetal liver kinase (Flk1) and in humans is referred to as KDR (insert domain receptor). The group of Flk1 positive cells ($Flk1^+$) comprises endothelial progenitor cells. The $Flk1^+$ cells, during the further development of the embryo, start to produce blood islands. The conducted experiments revealed that mice which are deprived of Flk1 are unable to generate blood islands and die. Meanwhile, a distinct subset of cells emerges from the paraxial mesoderm, which is characterized by expression of PDGFRα (platelet-derived growth factor receptor α). Surprisingly, clusters of these cells are able to produce endothelial cells but fail to deliver hematopoietic cells. At the same time, the $Flk1^+$ lateral mesoderm gives rise to endothelial and hematopoietic cells. The endothelial cells, of which the sources were either the proximal or lateral mesoderm, establish the vasculature of the yolk sac and the body of the embryo. On the contrary to primitive erythroids, which are derived from lateral mesoderm, the definitive erythrocytes originate from endothelial cells. Those EC express VE-cadherin (vascular endothelial-cadherin, CD144), which is essential for proper vascular development. Investigations confirmed a close connection of the differentiation pathway of endothelial and hematopoietic cells. This fact is causing complications when trying to separate these two subsets of cells using surface markers. At the beginning of embryogenesis, both types of cells express the same markers PECAM-1 (platelet endothelial cell adhesion molecule-1/CD 31), CD 34, AA4 (CD93) and isolectin IB4. The above-mentioned cell-surface markers prove that there is a strong affiliation of endothelial cells with hematopoietic cells (Turksen, 2002). Fig. 8 depicts the heterogeneity of endothelial cell origin and the differentiation processes during embryogenesis. Comprehending the molecular basis of the diversity of endothelium would provide the necessary tools to use in the treatments of vascular network (Aird, 2012).

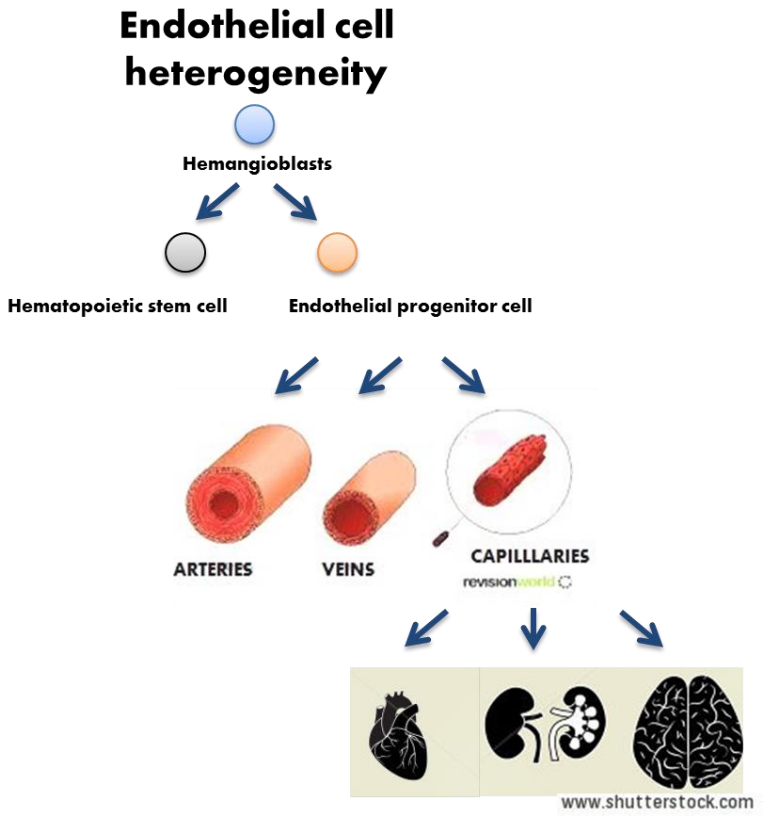


Figure 8: Endothelial differentiation. From hemangioblasts originate (i) blood cells and (ii) angioblasts, the endothelial progenitor cells, which mature to become the endothelial cells of arteries, veins and capillaries. Capillaries constitute the capillary bed, which is a linking system of capillaries, feeding organs and tissues. Adopted from: (Aird, 2012).

1.4.4. Serum- and feeder-free (2i) cell culture of mouse embryonic stem cells

The conventional ESC culture requires inactivated feeder cells, fetal calf serum and other extrinsic components. Serum derived from animals is the basic component which is applied to sustain and propagate cells. The fetal calf/bovine serum consists of diverse proteins, growth factors, hormones. These factors make the composition of the culture medium undefined. The presence of xenobiotics in the maintenance medium for human cells would put on risk the medical application of the therapeutical cells. Therefore, it becomes inevitable to detect small molecules which would sustain the self-renewal capacity of ESCs in the absence of iFC and animal serum (Li and Ding 2010, Van der Valk et al. 2010). A cell which is pluripotent is characterized as a naïve cell without a defined differentiation plan. Important questions are related to the state of pluripotency itself and how it is

preserved. Some answers were found by investigations on the ERK pathway, which appeared to be responsible for maintaining ESCs in the pluripotency phase. What is more, it was proved that by inhibition of GSK3 (glycogen synthase kinase 3) this effect was boosted. There is a view suggesting that embryonic stem cells should be perceived as cells which are fundamentally in a proliferative mode, which is independent of epigenetic regulation and does not require external stimulation. The balance of these conditions is confirmed by the uniformity of ESCs, which were maintained in the culture with the MEK/ERK and GSK3 inhibitors. Further surveys helped to reach the conclusion that LIF and BMP4 (bone morphogenetic protein 4) strengthen the capacity of self-renewal by inhibiting the lineage differentiation (Wray et al., 2010). Human ESCs display substantial dissimilarities in phenotype and signaling in contrast to mouse ESCs. The mES cells should be considered as cells that resemble pluripotent stem cells from ICM, while hES cells ought to be perceived as representing the late epiblast stage (Li and Ding 2010, Van der Valk et al. 2010). Joining of the PD0325901 inhibitor of MEK and the CHIR99021 inhibitor of GSK-3 and addition of LIF, maintain mESCs in the state of self-renewal and protects from spontaneous differentiation. However, these conditions are not sufficient for hESCs or hiPSCs maintenance. For this reason, an extra molecule needs to be included, TGF-β receptor inhibitor. The TGF-β/Activin A/Nodal signaling pathway turned out to be critical for hESCs or iPSCs to persist in an undifferentiated state. Blocking MAP kinase and GSK-3 signaling was named "dual inhibition" (2i) (Silva et al. 2008, Li and Ding 2010).

Determination of the small molecules responsible for self-renewal of mESCs in the defined culture conditions, was evaluated by many experiments. Pluripotin (SC1) is the discovered molecule, which controls self-renewal of ESCs. SC1 alone can maintain undifferentiated mES cells. This small molecule function through the double inhibition of ERK and Ras-GAP (Ras GTPase-activating protein) extracellular signals. The mESCs culture in medium supplemented with SC1 sustained ESCs capability to commit into three germ layer lineages *in vitro* and *in vivo* in chimeric mice (Chen et al., 2006). When Ras-GAP is inhibited the self-renewal of ES cells is boosted by strengthening the PI3K (phosphoinositide-3 kinase) signaling pathway. From the other side, the blockade of ERK1 hampers

the differentiation of embryonic stem cells. The discovery of pluripotin confirmed that the self-renewal of ESCs occurs aside from extrinsic deactivation of intrinsic proteins which promote differentiation. Two chemical blockers CHIR99021 and PD0325901 inhibit the GSK3 and MEK pathways by enhancing the durable generation of mESCs in the undifferentiated state, with omission of extrinsic proteins. However, the Wnt/β-catenin signaling pathway is stimulated by deactivation of GSK3. This action enhances ES cell differentiation towards the mesoderm, when additional small molecules or self-renewal proteins are not present. The application of the small molecules in the cell culture can improve the outcomes by scaling up the more homogeneous cell populations (Li and Ding, 2010a). In order to create fully defined media, without FSC and iFC support for the ESC culture, two more supplements are vital: B27 and N2. The N2 supplement is a chemically defined, 100(x) concentrate of Bottenstein's N2 formulation and is used as a substitute of general blood serum. This supplement helps to keep ES cells in the undifferentiated state. The B27 supplement consists of determined ingredients mainly antioxidants and free radical killers (Ying et al., 2008).

1.4.5. Controversy concerning embryonic stem cells

The ES cells allowed for handling the mouse genome in order to better encompass the embryonic development and get to know the potential of the undifferentiated cells and their fate commitment (Keller, 2005). Only a minority of people is denying the medical capabilities of stem cells investigation. Queries relates to efficacy and safeness of the ESCs use, derivation or donation. Also social concerns are being raised like, how costly the treatments would be and if they would be then available to all? The biggest controversy is connected with the origin of cells and use of the human embryos (J.Barfoot, D.Bruce, G.Laurie, and N.Bauer, J.Paterson and M.Bownes, 2014). Human ESCs are considered as never-ending pool of all the body’s cells. This phenomenon can be utilized in cell therapies of yet not curable disorders, for disease modelling and drugs testing. The pathways which are responsible for ensuring the genomic stability of hESCs have to be identified. These signalling pathways have crucial importance when it comes to continuous culture and lineage commitment. Furthermore, solutions to acquire pure cell subsets of only differentiated hESCs products, in a large number, are required (Fu and Xu, 2012).

1.5. Induced pluripotent stem cells

"Induced pluripotent stem cells - pluripotent cells that can be generated from many different types of somatic cells by expression of only a few pluripotency-related transcription factors, and that have properties of embryonic stem cells." (Plath and Lowry, 2011).

1.5.1. Characteristics of the pluripotency state

The retroviral vector was used to transduce mouse skin fibroblasts and this resulted in reversing the cells to the naïve-like stage (Power and Rasko, 2011). Following the work of the father of the iPS cells, Shinya Yamanaka, the induction of pluripotency needs to be regarded as bringing together three main scientific views:

1. Nuclear reprogramming in amphibians (J. Gurdon, 1962).
2. The ESCs discovery by: M.J. Evans and M.H. Kaufman in UK and G. Martin and A. Smith in USA (1981).
3. Detection of leading transcription factors (TF) in *Drosophila* (Schneuwly et al., 1987).

The iPS cell discovery sparked new areas of research and initiated discussion about whether iPS cells are truly identical to ESCs. The first iPS cells were obtained by Takahashi and Yamanaka in 2006 as a result of reprogramming of mouse fibroblasts. In order to induce the pluripotency, a mix of TFs was used. One year later the results of reprogramming were confirmed with the use of human fibroblast by Takahashi. A similar achievement was reported by a group of James Thomson at the University of California at Santa Barbara (Yamanaka, 2012). Adult somatic cells were reprogrammed to pluripotent stem cells due to the imposed expression of TFs. The ability to reverse cell fate raised a question concerning how the TFs impacted the differentiation and epigenetic pattern of the cell during the natural growth and in reprogramming (Stadtfeld and Hochedlinger, 2010). The reprogramming was achieved by applying only 4 TFs: Oct4 (POU5F1), Sox2 (sex determining region Y-box 2), Klf4 (Krüppel-like factor 4), and c-Myc (Myc). After around one to two weeks, first reprogrammed cell colonies are appearing. The reprogramming efficiency is on the level of 3-5% in

a time frame of two weeks. For this outcomes presumably responsible are epigenetic obstacles (Plath and Lowry, 2011). Furthermore, the transduction with retro- or lentiviruses may cause the insertional mutations. That is why new approaches had been applied in order to eliminate the possibility of integration of the extrinsic reprogramming factors to the genome (He et al., 2014).

The iPS cells are free from any moral objectives and immune rejection of an allograft. Nowadays, the reprogramming of somatic cells with the cocktail of TFs: Oct4, Sox2, Klf4 and c-Myc (OKMS) into pluripotent stem-like cells must be seen as a change in the perception of ultimate faith of the cell's planned development. The view concerning pluripotency need to be revised, it cannot be understood as being above epigenetic control, but rather as stabilized case of interacting differentiation signals. There is also an attempt, to prove that pluripotency markers can also act as conventional lineage specifiers, which lead ESCs to differentiate into a definitive lineage and at the same time to prohibit their specification to reciprocally unshared lineages (Wu et al., 2013, Shu et al., 2013).

The induced pluripotent stem cells had given a hope for generation of patient- and disease-specific cells in order to model the disorder or to use the obtained cells in therapies, without the risk of immune rejection (Stadtfeld and Hochedlinger, 2010). The more approachable solution would be to create universal cell bank with prefabricated immune-proved pluripotent stem cell lines and to with the most often presented haplotypes in a community (Power and Rasko, 2011).

1.6. Gene transfer

Gene transfer enables shift of a given gene from one DNA double stranded helix to another DNA molecule. The possibility to amend the DNA structure allows for generation of the organisms with improved chances for existence. Alterations made in the DNA sequence may be utilized in the medicine by modifying the faulty protein. Many systems of gene delivery exist: micro- or macroinjection, viral systems, liposome or calcium phosphate mediated gene transfer and gene transfer through peptide (Khan, 2010).

1.6.1. Lentiviral system of gene delivery

The lentiviruses belong to the family of retroviruses (*Retroviriade*) which utilizes viral reverse transcriptase (RT) and protein integrase (IN) in order to stably deliver viral genomes to the host (Sakuma et al., 2012). There are three main attributes of retroviral vectors, which make these viruses an alluring system of gene transfer:

1. ability to insert the transgene into the genome of the host,
2. possibility to carry nearly 10 kb of cDNA,
3. no transmission of the proteins determined in the packaging vector.

The viral RNA genome contains cis-acting sequences, which are crucial for: (i) packaging, (ii) reverse transcription, (iii) nuclear translocation and integration, (iv) essential proteins encrypted by gag and env genes and (v) the chemical products of the pol gene. All mentioned elements constitute the potential virion on the surface of the host cell membrane. In the lentiviruses, the activation of Gag and Pol requires a catalyst named Rev (Merten and Rubeai, 2011). The HIV virus genome contains single-stranded sense RNA of ~9kb which is responsible for encrypting main viral proteins. The *gag* gene is coding the core proteins, the *pol* enzymes which are needed for viral propagation and the *env* is coding glycoprotein from the viral surface. The regulatory proteins Rev and Tat are liable for transcription. LTRs (long terminal repeats) are at the ends of viral genome and are necessary for transcription, reverse transcription (RT) and incorporation. All transferred viral proteins, or genomes are being compiled at the surface of the plasma membrane. When the "undeveloped" virions are out of the cell, the Gag and Gag-Pol stimulate the viral protease (PR) which "maturates" the viral particles to become infectious. The commonly used lentiviral vectors went through a long process to reach the current state. Below is presented a structured order of the lentiviral constructs development:

1. The precursor vectors were divided into two plasmids: one for HIV-1 proviral DNA with the changes in the *env* gene, the second plasmid conveying Env. That separation of the Env protein created a virus which was able to conduct only one infection due to the lack of *env*.

2. Pseudotyping with VSV-G (vesicular stomatitis virus envelope glycoprotein G) broaden the viral tropism. The VSV-G possesses a phosphatidylserine, a membrane element, which makes the vector able to infect different type of cells.
3. The first generation HIV-1-based lentiviral vectors, this stage was reached by dividing the vector parts into three plasmids: (i) a packaging vector, (ii) an Env plasmid encrypting the viral glycoprotein, (iii) a transfer vector. The separation enabled the transfer of a transgene without the risk of activation of the viral proteins in the host cell.
4. The second-generation lentiviral vectors are lacking following proteins: Vif, Vpu, Vpr or Nef. That amendment of accessory proteins again elevated the biosafety degree.
5. The self-inactivating (SIN) vectors with a deletion in the U3 region of the 3'-LTR.
6. The third-generation lentiviral vectors Tat-independent vectors. Safety usage of that system is increased due to the removal of further six HIV genes, yet these vectors are showing low yield.

Nowadays, the vectors are being assembled from several plasmids, which lack HIV-1 accessory proteins and with self-inactivating alterations, still not affecting the transduction ability *in vitro* or *in vivo* (Sakuma et al., 2012).

1.6.2. Integrase deficient lentiviral vectors

The high efficiency of lentiviral vectors in transducing cells was praised, as it purely relies on viral integration with the genome of the targeted cell. This assumption had been questioned using integration deficient lentiviral vectors (IDLV). That kind of LV is generated with a mutation in the sequence of the protein called integrase (IN). This mutation inhibits the viral integration, at the same time there is an increase in the number of the vector episomes in the host cell. The mentioned circular vector episomes do not have replication signals and are progressively absent in the dividing cells, although are durable in non-dividing cells (Wanisch and Yáñez-Muñoz 2009).

Upon accessing the cell and integration with the viral DNA, the lentiviral vector generates transcriptionally active episomal forms of DNA. Episomes are considered to be the circular forms of viral extrachromosomal DNA (E-DNA). The E-DNA is transcriptionally active, thus synthesizing

RNA and proteins (Michelini et al., 2010). The integrase deficient LVs may be remodeled to become replicating episomes. This means that IDLVs could be applied for stable transduction of dividing cells. That type of a vector subsequently could be relevant for use in treating disease and dysfunction. The integration of the virus is a complex action involving: (i) vector 3'-end processing, (ii) strand transfer (insertion of the viral DNA to target cell), (iii) gap reparation and finally (iv) ligation (Wanisch and Yáñez-Muñoz, 2009). There had been various experimental results announced concerning the impact of mutations of different amino acids in the IN, in order to generate lentiviruses with disabled mechanism of integration. The protein integrase is considered to be pleiotropic and thus affecting viral activities not connected with the integration (class II mutation). The class I mutations are concerning strictly IN role in DNA cleavage and integration. For obtaining the class I mutant commonly substitutions in the catalytic triad of HIV-1, IN are implemented. These three amino acids are as follow: D64, D116, and E152 (Wanisch and Yáñez-Muñoz, 2009).

1.6.3. Endothelial specific promotors

The vascular endothelial growth factor (VEGF) together with the VEGF receptor (VEGFR) are the key players in normal and dysfunctional angiogenesis (Shibuya 2011). It would be beneficial to control the vasculature, both by enhancing the growth of vessels and by preventing the angiogenesis in tumor formation (Galas and Liu 2014). VEGF is a part of the PDGF supergene family and VEGF signaling protein acts as a homodimer structure. With the VEGF group so far seven representatives are affiliated, the VEGFR class of genes consists of three to four units and this is related with the vertebrate species. The first member of the VEGF family is VEGF-A which is responsible for angiogenesis and permeability of the vessels via controlling following receptors: VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk1 in mice). While the VEGF-C and the VEGF-D together with the VEGFR-3 (Flt-4) determines lymphangiogenesis (Shibuya 2011). The VEGF-E is encoded by the *Orf* virus, a zoonotic parapoxvirus (Wise et al. 2012). The VEGF receptors are mostly tyrosine kinase receptors (RTKs). In experiments described in this thesis the focus was put on Flk1 receptor which is assumed to be the marker for the earliest subsets of endothelial and blood cells. The encouraging studies were

conducted, amongst others, by Yamashita describing that Flk1 positive ESCs can establish endothelial cells *in vitro* (Yamashita et al. 2000).

Shear stress is inevitable to maintain the vascular homeostasis, control vascular remodelling or atherogenesis. Platelet endothelial cell adhesion molecule (PECAM-1) is responsible for direct transmission of mechanical forces. The vascular endothelial cell cadherin is acting as an adaptor and VEGFR2 stimulates phosphatidylinositol-3-OH kinase. All the specified elements constitute a mechanosensory complex (Tzima et al. 2005). In the light of these findings, the second promoter applied in the vectors used in the course of this doctorate was Ve-cadherin. The vascular endothelial cadherin (Ve, CD 144) is a defined endothelial specific adhesion molecule. The cadherin is settled in the intracellular junctions of endothelial cells. Apart from the role of the Ve-cadherin in adhesion, this molecule is also important for cell propagation and cell death and adjusts VEGFR activity. The CD 144 is leading the actions of endothelium but also guards the permeability of the blood vessel wall for various cells and substances. The cadherins belong to the large family of CAM and are characterized by the extracellular cadherin domain (EC-domain). Cadherins regulate adhesion through homophilic, Ca^{2+} dependent interplays. The CD 144 molecule had been reported to control contact restrictions of growing cells, thus adversely interacting with cell propagation activated by VEGFR-2 (Vestweber, 2007).

To sum up the used promoters sequences for generating the viral vectors were as follow:

1. Studies from Kappel et al. (1999) showed that a 939bp fragment between -640bp and +299bp in combination with a 510bp enhancer sequence located between +3437bp to +3947bp in the second intron, is enough for a specific expression.

2. According to analyzes of Gorry et al. (1999), where various lengths of the promotor region were tested, it appeared that the fragment between -2486bp and +64bp should be used. That specified promoter region shows the best specificity.

1.7. Hypothesis and aim

The underling hypothesis of all the experiments in the framework of this doctoral thesis, was the assumption that it is possible to isolate specific cell type from differentiated murine embryonic stem cells (mESCs). Consequently the aim of the investigations was to generate endothelial (progenitor) cells by means of lentiviral transgene delivery. The lentiviral vectors were also a subject of the investigation, in order to achieve the safest possible tool to impact the host genome (IDLVs). The results obtained from the experiments carried out on animal ES cells will be applied to studies concerning induced pluripotent stem cells (iPS). The studies on murine cells will serve as a starting point and "training" field, on which the mistakes might be made without serious repercussions. Inquiries concerning hES/hiPS cells, involve in addition, studies on specific culture conditions for these types of cells (xenobiotic-free culture media). Finally, the generated endothelial progenitor cells could serve as a source of therapeutically applicable cell subsets for treating vascular and parenchymal diseases of the lung.

2. Materials

2.1. Equipment

Table 1: Equipment used in the laboratory.

Equipment name	Producer
3D Sunflower Mini-Shaker	Kisker Biotech GmbH (Steinfurt, Germany)
Bacteria shaker (Innova 44)	New Brunswick Scientific (Hamburg, Germany)
Balance	A&D Weighing (San Jose, USA)
Cell culture incubator (DHD AutoflowCO_2 Air-Jacketed incubator)	Nuaire (Plymouth, USA)
Cell culture incubator (Galaxy 170S)	New Brunswick Scientific (Hamburg, Germany)
Cell culture incubator (Innova CO170)	New Brunswick Scientific (Hamburg, Germany)
Spinner flasks system (CELLSPIN)	Integra Biosciencee AG (Fernwald, Germany)
Confocal microscope (LSM 710)	Zeiss (Oberkochen, Germany)
Cooling-centrifuge (5430R)	Eppendorf (Wesseling-Berzdorf, Germany)
Cooling-centrifuge (Fresco 17)	Thermo Scientific (Waltham, USA)
Corning® bottle-top vacuum filter	Corning B.V. Life Sciences (Amsterdam, the Netherlands)
Electrophoresis chamber	PeqLab (Erlangen, Germany)
Fine scale	Pinacle, Denver instruments (Göttingen, Germany)
Flow cytometry machine (FACScalibur)	BD Bioscience (Heidelberg, Germany)
Gel imaging and documentation	Intas (Göttingen, Germany)
Gene Pulser Xcell™ Electroporation System	BioRad (Munich, Germany)
Heating block	HLC BioTech (Pforzheim, Germany)

High end fluorescent microscope Imager Z.1	Zeiss (Oberkochen, Germany)
Laminar flow cabinet (Labguard Class II Type B1)	Nuaire (Plymouth, USA)
Microwave (R93ST-AA)	Sharp (Hamburg, Germany)
Mini centrifuge (C130 1T)	Labnet International (Edison, USA)
Neubauer cell counting chamber	Marienfeld (Luda Königshofen, Germany)
Optical microscope (DMIL)	Leica (Nussloch, Germany)
PCR machine (Mastercycler ep gradientS)	Biometra/Eppendorf (Hamburg, Germany)
Pipetboy	Integra Biosciences (Fernwald, Germany)
Pipettes (0.5-10 µl, 10-100 µl, 100 -1000 µl)	Brand GMBH + CO KG (Wertheim, Germany)
Sigma 3_16P centrifuge	Thermo Scientific (Waltham, USA)
Spectrophotometer (NanoDrop ND-1000)	Peqlab (Erlangen, Germany)
Table centrifuge (Multifuge 1S)	Thermo Scientific (Waltham, USA)
Table centrifuge (Multifuge 35R)	Thermo Scientific (Waltham, USA)
Vacuum pump	KNF Lab (Freiburg, Germany)
ViiA 7 Real-time PCR system	ABI/Life Technologies (Darmstadt, Germany)
Water bath	Microm (Walldorf, Germany)

2.2. Chemicals and reagents

Table 2: Chemicals used in the experiments.

Name	Producer
Acetone	Roth (Karlsruhe, Germany)
Agarose NEEO Ultra Quality	Roth (Karlsruhe, Germany)
Calcium chloride ($CaCl_2$)	Roth (Karlsruhe, Germany)
Dimethylsulfoxide (DMSO)	Serva Feinbiochemica (Heidelberg, Germany)
Disodium hydrogen phosphate (Na_2HPO_4 x $2H_2O$)	Roth (Karlsruhe, Germany)
Ethanol	Roth (Karlsruhe, Germany)
Ethanol (absolute ≥99.8%)	Merck (Darmstadt, Germany)
Ethidiumbromide (10 mg/ml)	Roth (Karlsruhe, Germany)
Ethylendiamintetraacetate (EDTA)	Roth (Karlsruhe, Germany)
Glycerine 99.5%	Roth (Karlsruhe, Germany)
HEPES	PAA (Cölbe, Germany)
Isopropanol	Roth (Karlsruhe, Germany)
Liquid nitrogen	Linde AG (Pullach, Germany)
Magnesium chloride ($MgCl_2$)	Sigma Aldrich (Steinheim, Germany)
Methanol	Roth (Karlsruhe, Germany)
MOWIOL	Calbiochem/Merck (Darmstadt, Germany)
n-Butanol	Roth (Karlsruhe, Germany)
Paraformaldehyde (4% PFA)	VWR Syngene (Darmstadt, Germany)
Potassium chloride (KCl)	Roth (Karlsruhe, Germany)

Potassium dihydrogen phosphate (KH_2PO_4)	Roth (Karlsruhe, Germany)
Sodium chloride (NaCl)	Roth (Karlsruhe, Germany)
TRIS acetate salt	Roth (Karlsruhe, Germany)
Triton X-100	Roth (Karlsruhe, Germany)
Tween 20	Sigma Aldrich (Steinheim, Germany)

2.3. Antibodies and fluorescent dyes

Table 3: Antibodies and fluorescent dyes.

Name	**Producer**

Primary antibodies (dilution)

Anti_TurboGFP rabbit (1:500)	Evrogen (Heidelberg, Germany)
Anti-Human/Mouse Oct3/4 conjugated with PE (1:50)	eBioscience (San Diego, USA)
Anti-Human/Mouse Sox2 conjugated with Alexa Fluor®488 (1:50)	eBioscience (San Diego, USA)
Ve-Cadherin (CD 144) rat anti mouse (1:300)	BD Bioscience (Heidelberg, Germany)
PECAM-1 (CD31) rat anti mouse (1:300)	BD Bioscience (Heidelberg, Germany)
Flk1 rat anti mouse (1:100)	BD Bioscience (Heidelberg, Germany)
Oct3/4 (C10) mouse (1:100)	Santa Cruz Biotechnology, Inc (Dallas, USA)
SSEA1 (MC480) mouse (1:100)	Cell Signaling Technology (Boston, USA)

Secondary antibodies and IgG controls (dilution)

Alex 555 goat anti rat (1:1.000)	Invitrogen (Carlsbad, USA)
Alexa 488 goat anti mouse (1:1.000)	Invitrogen (Carlsbad, USA)
Alexa 488 goat anti rabbit (1:1.000)	Invitrogen (Carlsbad, USA)
Alexa 647 goat anti rabbit (1:1.000)	Invitrogen (Carlsbad, USA)
mouse IgG (1:50)	Invitrogen (Carlsbad, USA)
rabbit IgG (1:400)	Cell Signaling Biolabs (Boston, USA)
Rat IgG2 conjugated with PE (1:50)	Biolegend/Biozol (Eching, Germany)
Rat IgG2a K isotype control conjugated with FITC (1:50)	eBioscience (San Diego, USA)

Viability staining

7-aminoactinomycin D (7-AAD)	eBioscience (San Diego, USA)
Propidium iodide (PI)	BD Bioscience (Heidelberg, Germany)

Nuclear counterstain (dilution)

4'6-Diamidin-2-Phenylindol (DAPI) 1:1.000	Invitrogen (Carlsbad, USA)

Sera for blocking buffers

Goat serum	PAA (Pasching, Austria)
Rabbit serum	Chemicon (Limburg, Germany)

Buffers

Cytofix/Cytoperm™	BD Bioscience (Heidelberg, Germany)

2.4. Enzymes

Table 4: Restriction enzymes.

Name and sequence	Producer
AgeI (BshTI) a|ccggt	Thermo Scientific (Waltham, USA)
BglII a|gatct	Thermo Scientific (Waltham, USA)
BspTI (AflII) c|ttaag	Thermo Scientific (Waltham, USA)
ClaI (Bsu15I) at|cgat	Thermo Scientific (Waltham, USA)
Eam1105I (AhdI) gacn|nnnngtc	Thermo Scientific (Waltham, USA)
KpnI ggtac|c	Thermo Scientific (Waltham, USA)
MreI cg|ccggcg	Thermo Scientific (Waltham, USA)
NcoI c|catgg	Promega (Madison, USA)
NheI g|ctagc	Thermo Scientific (Waltham, USA)
SalI g|tcgac	Fermentas (Pittsburgh, USA)
SapI (LguI) gctcttc$(N)_1$|	Thermo Scientific (Waltham, USA)
ScaI agt|act	Thermo Scientific (Waltham, USA)
SdaI (SdfI) cctgca|gg	Thermo Scientific (Waltham, USA)
SmaI ccc|ggg	Thermo Scientific (Waltham, USA)
SwaI (SmiI) attt|aaat	Thermo Scientific (Waltham, USA)

2.5. Primers

Table 5: Sequences of the used primers.

Name	Sequence 5'>3' (number of nucleotides)
A_flk_enhancer	CTGCGTTGCCAACTTCAAGG (20)
AP_GE_scr	TGAAAGGGACGGGAGCCACTG (21)
AP_screen_WPRE	AGTGCACACCACGCCACGTT (20)
copGFP-R	TTCAGGGTGCCGGTGATGCG (20)
cPPT-scr-F	GGGGGTACAGTGCAGGGGAAA (21)
Flk1_FW	AAACCTCTTGGCCGCGGTGC (20)
Flk1_REV	AGGGCTCGATGCTCGCTGTG (20)
FP-Chd5-rt	CGGCCCGCCACTGTCTTGT (20)
FP-Flk-rt	GAGCGCTGTGAACGCTTGCC (20)
GAPDH murine F	CGAGACCCCACTAACATCAAA (21)
GAPDH murine Re	TGCATTGCTGACAATCTTGAG (21)
GATA4_FP	GACGTGGGAGCATCCTGGGC (20)
GATA4_RP	TCCCGTCCCATCTCGCCTCC (20)
HIV1_PSS_F	CGCAGGACTCGGCTTGCT (18)
HIV1_PSS_R	GACGCTCTCGCACCCAT (17)
hVE-Cad-F	CTATAATCGATGCCCCTCCAATCTGTCTTGTC TACC (36)
hVE-Cad-R	CATATGCTAGCGCCTGGCTGCCTCCCCTTC (30)
hVE-Cad-scr-R	GCAGAGGAGGAGGGCAGGGG (20)

Hygro-BshTI-F	GTATACCGGTCCGGGAGCTTGTATATCCATTTTCG (35)
Hygro-SalI-R	CATATGTCGACGCGGCGGTGGAATCGAAATCT (32)
IntD64V_forw_1	GCAGCTAGTTTGTACACATTTAGAAGGAAAAG (32)
IntD64V_rev_1	CATATTCCTGGGCTACAGTCTACTTGTC (28)
KDR-enh-F	TGCATGTATGTGTGGAATTGGGGAATG (27)
KDR-enh-R	ATGCTGAGCCTGGGCAGATCAAG (25)
KDR-enh-scr-R	TCTAGTGCGCTTCCCCTGGT (20)
KDR-prom-F	AGCTGGCCTCCTTCCCCTGG (20)
KDR-prom-R	TCCTGCACCTCGAGCCGGG (19)
KDR-prom-scr-R	CCAGTTCGCCAACATTCCCGC (21)
mKlf4-FP	TGTGACTATGCAGGCTGTGGC (21)
mKlf4-RP	GGCCCTGTCACACTTCTGGC (20)
mM-myc-FP	GCCCGCGATCAGCTCTCC (18)
mM-myc-RP	CTCGTCGCAGATGAAATAGGGC (21)
mNanog-FP	GAACGCCTCATCAATGCCTGC (21)
mNanog-RP	TGTTCTCCTCCTCCTCAGGGC (21)
mOct4-FP	CAAGTTGGCGTGGAGACTTTGC (22)
mOct4-RP	CCCCAAGGTGGATCCTCTTCTGC (22)
mSox2-FP	GGGCTCTGTGGTCAAGTCCG (20)
mSox2-RP	CGCTCTGGTAGTGCTGGGC (19)
Myc_fwd	GCGTGGGGAGCAAACAGG (18)

Myc_rev	GACACGAGCTGACGACAACC (20)
Neo-BshTI-F	GATAACCGGTCGCATGATTGAACAAGATGGATTGC (35)
Neo-SalI-R	CTATAGTCGACTTTCGAACCCCAGAGTCCCG (31)
Nes_FP	TCCAGGAGCGCAGAGAGGCG (20)
Nes_RP	GAGGTGTGCCAGTTGCTGCCC (21)
PGK_Hygro_screenF	GTTAATGTGGCTCTGGTTCTGG (22)
PGK_Hygro_seqR	GTCGTCCATCACAGTTTGCC (20)
pJet_forw	CGACTCACTATAGGGAGAGCGGC (23)
pJet_rev	AAGAACATCGATTTTCCATGGCAG (24)
psPax2_forw	GGGTGCCCACACTAATGATGTGAAA (25)
psPax2_rev	TCCCCTGCACTGTACCCCC (19)
Puro-BshTI-F	CTATAACCGGTACCATGACCGAGTACAAGCCCA (33)
Puro-SalI-R	GATATGTCGACTCAGGCACCGGGCTTGCG (29)
RP-Chd5-rt	CCAAGGGCTTGCCCACTCGG (20)
RP-Flk-rt	ACCATGAGAGGCCCTCCCGG (20)
Snail_FP	TCTGCACGACCTGTGGAAAGGC (22)
Snail_RP	TGGCACTGGTATCTCTTCACATCCG (25)
SP neo-IRES scre	AGGACATAGCGTTGGCTACCCG (22)
SP_neo_IRES_scr	AGGACATAGCGTTGGCTACCCG (22)
SP_PGK_Hyg#6	TGTGTAGAAGTACTCGCCGATAG (23)

2.6. Vectors

This subsection contains descriptions of the backbones of all the vectors which have been used in the experiments described in this thesis. Where indicated, vectors were bought from Addgene. The remaining vectors were modified *in situ*.

1. The pMD2.G vector was used for viral particles production in HEK cells. Plasmid was bought from Addgene (pMD2.G was a gift from Didier Trono to Addgene, plasmid # 12259).

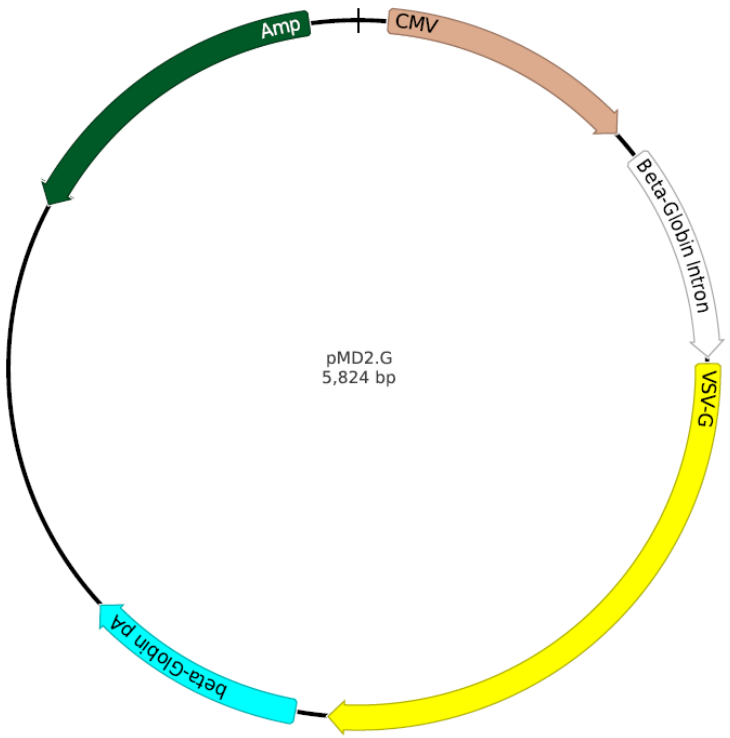


Figure 9: Backbone of the pMD2.G - envelope vector.

CMV - promoter/enhancer of the Cytomegalovirus, *VSV-G* - glycoprotein of the vesicular stomatitis virus, *pA* - polyadenylylation signal, *Amp* - ampicillin resistant gene for selection in *E. coli*.

2. The psPAX2 (2nd generation lentiviral packaging plasmid) was used for viral particles production in the HEK cells. Plasmid was bought from Addgene (psPAX2 was a gift from Didier Trono to Addgene, plasmid # 12260).

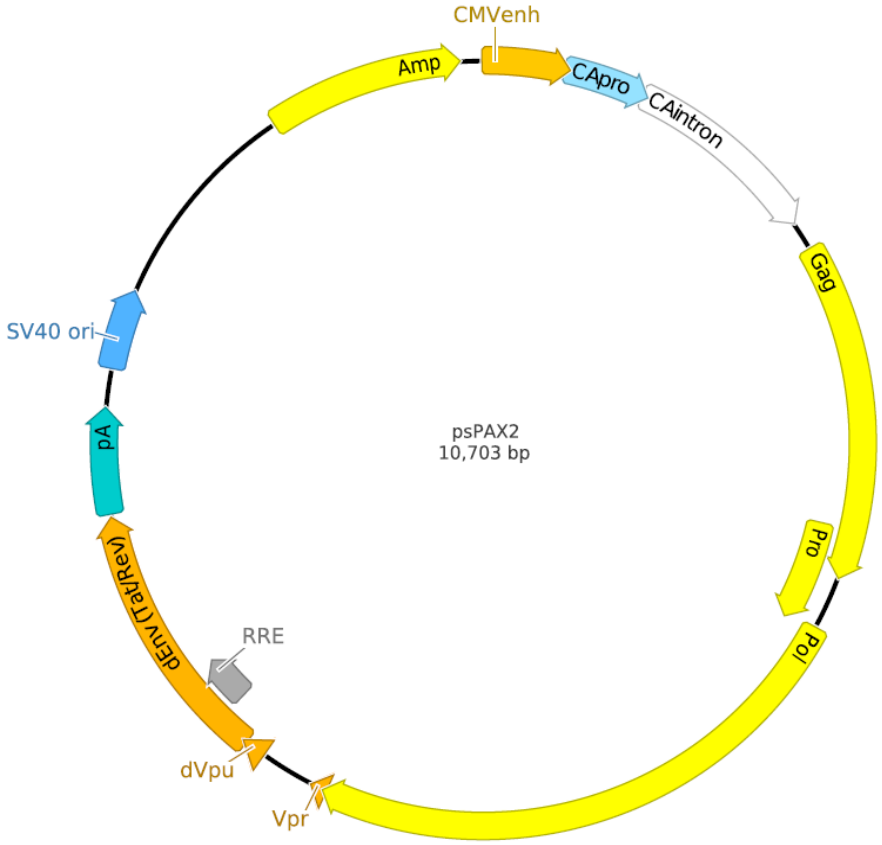


Figure 10: Backbone of the psPAX2 - packaging vector.

CMVenh - enhancer of the Cytomegalovirus, *CApro* - chicken beta actin promoter, *CAintron* - chicken beta actin intron, *Gag* - group antigen, *Pro* - protease, *Pol* - polymerase, *dEnv* - envelope proteins including the genes for Tat und Rev, *RRE* - reverse responsive element, *pA* - polyadenylation signal, *SV40 ori* - simian virus 40 origin, *Amp* - ampicillin resistant gene for selection in *E. coli*.

3. The psPAX2 IntD64V - integrase deficient vector. It was used for generation of integrase deficient viral particles for induction of pluripotency. Plasmid was modified by means of site-directed mutagenesis (SDM) in house.

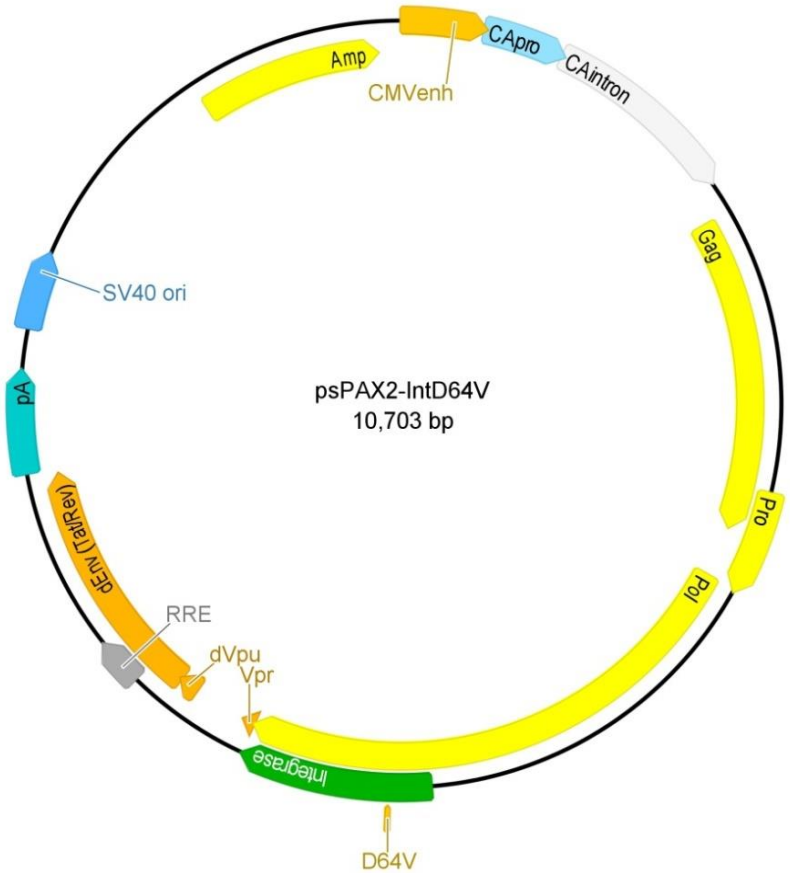


Figure 11: Backbone of the psPAX2-IntD64V - packaging vector.

CMVenh - enhancer of the Cytomegalovirus, *CApro* - chicken beta actin promoter, *CAintron* - chicken beta actin intron, *Gag* - group antigen, *Pro* - protease, *Pol* - Polymerase, *dEnv* - envelope proteins including the genes for Tat und Rev, *RRE* - reverse responsive element, *pA* - polyadenylation signal, *SV40 ori* - simian virus 40 origin, *Amp* - ampicillin resistant gene for selection in *E. coli*.

4. The pGZ_CMV vector is used for expression of transgenes under a CMV promoter. For research purposes stated in this thesis the pGZ_CMV vector had been modified as it is shown in the sections 5 to 8. This vector was available in house.

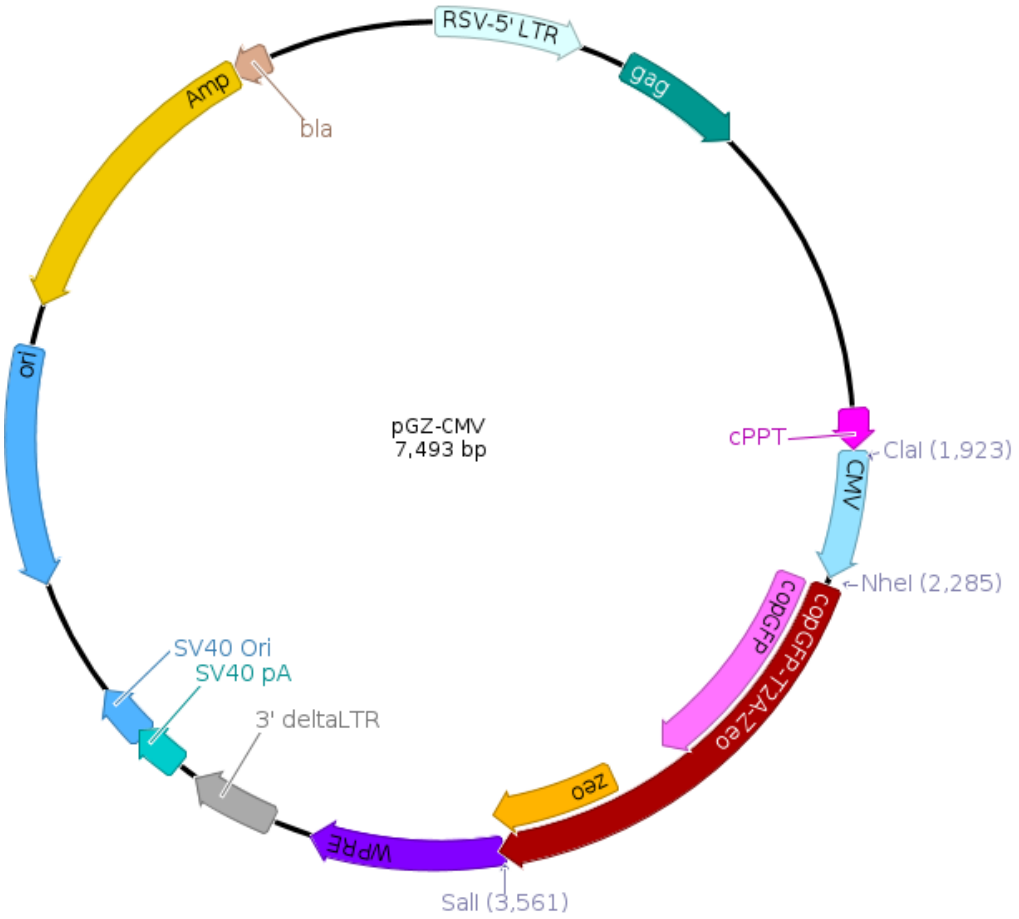


Figure 12: Backbone of the transfer vector pGZ-CMV.

RSV-5'LTR - long terminal repeat, required for viral packaging and transcription, *Gag* - packaging signal, *RRE* - rev responsive element, *cPPT* - central polypurine tract (includes DNA Flap region) involved in nuclear transcription and integration of transduced viral genome, *copGFP* - GFP-reporter gene from copepod *Pontellina plumata*, *Zeo* - zeocin resistance gene, *WPRE* - posttranscriptional regulatory element which enhances the stability of the viral transcripts, *ori* - allows high copy replication in *E. coli*, *pA* - transcription termination and polyadenylation, *Amp* - ampicillin resistant gene for selection in *E. coli*.

5. The pG*-mVE - all vectors contain murine Ve-cadherin promoter, but each of the vector has different resistant gene for antibiotic selection. Those are SIN (self-inactivating) vectors, which can be used for virus production by means of transfection and consequently for transduction of mESCs.

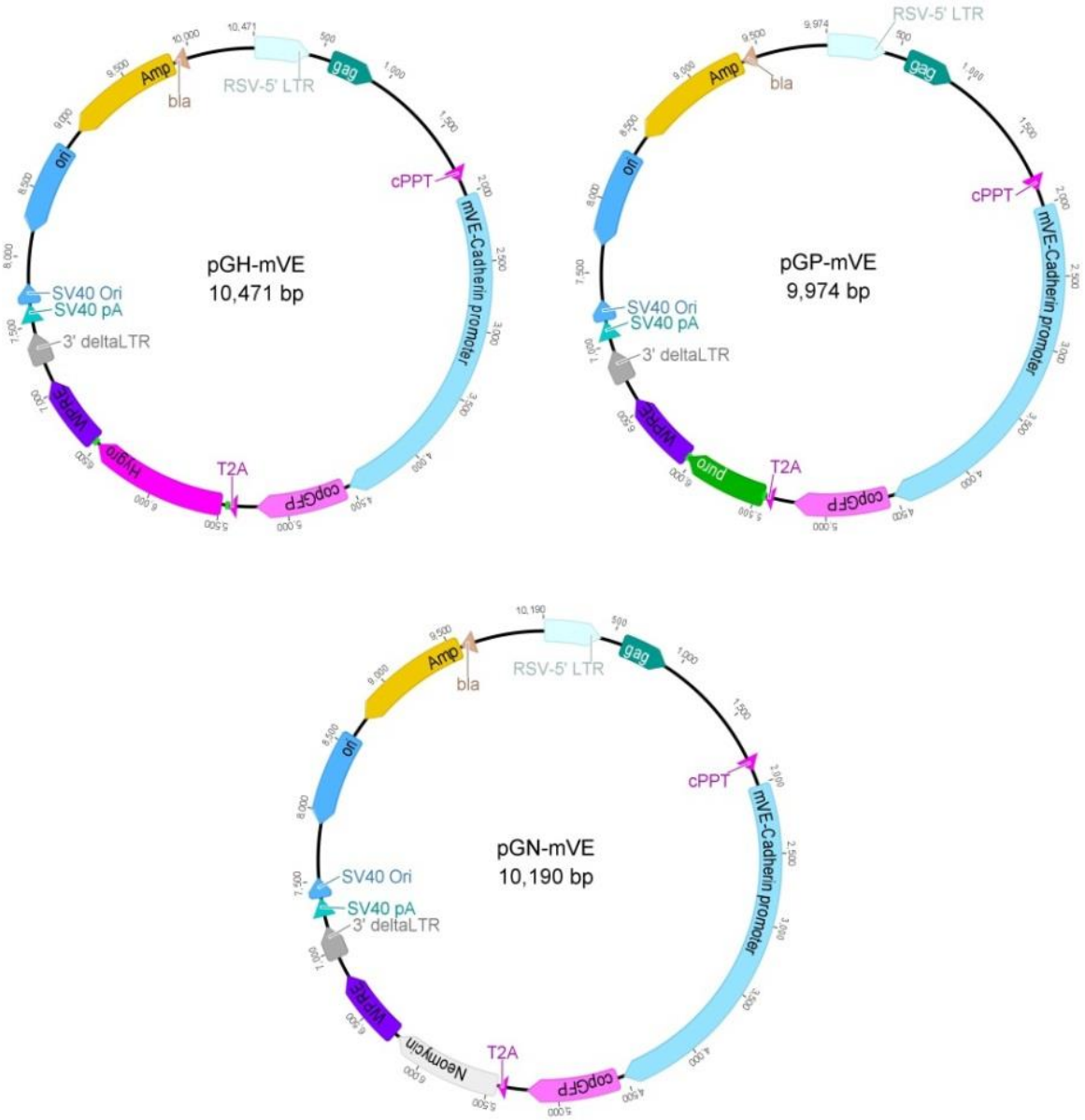


Figure 13: Backbones of the transfer vectors: pGH-mVE, pGN-mVE and pGP-mVE.

RSV 5' LTR - long terminal repeat, required for viral packaging and transcription, *Gag* - packaging signal, *cPPT* - central polypurine tract (includes DNA Flap region) involved in nuclear transcription and integration of transduced viral genome, *copGFP* - GFP-reporter gene from copepod *Pontellina plumata*, *T2A* - 2A peptide from *Thosehasigma* virus to mediate protein cleavage, *WPRE* - posttranscriptional regulatory element which enhances the stability of the viral transcripts, *3'LTR* - required for viral reverse transcription, self-inactivating 3'LTR with deletion in U3 region prevents formation of replication competent viral particles after integration into genomic DNA, *SV40 pA* - transcription termination and polyadenylation, *SV40 Ori* - allows episomal replication of plasmid in eukaryotic cells, *ori* - allows high copy replication in *E. coli*, *Amp* - ampicillin resistant gene for selection in *E. coli*, *H* - hygromycin, *N* - neomycin and *P* - puromycin, *SIN* - self-inactivating vectors lacking viral enhancers/promoters in the 3' long terminal repeat (LTR).

6. The pG*-Flk1- all vectors contain murine Flk1 promoter, but each of the vector has different resistant gene for antibiotic selection. Those are SIN vectors, which can be used for virus production by means of transfection and consequently for transduction of mESC.

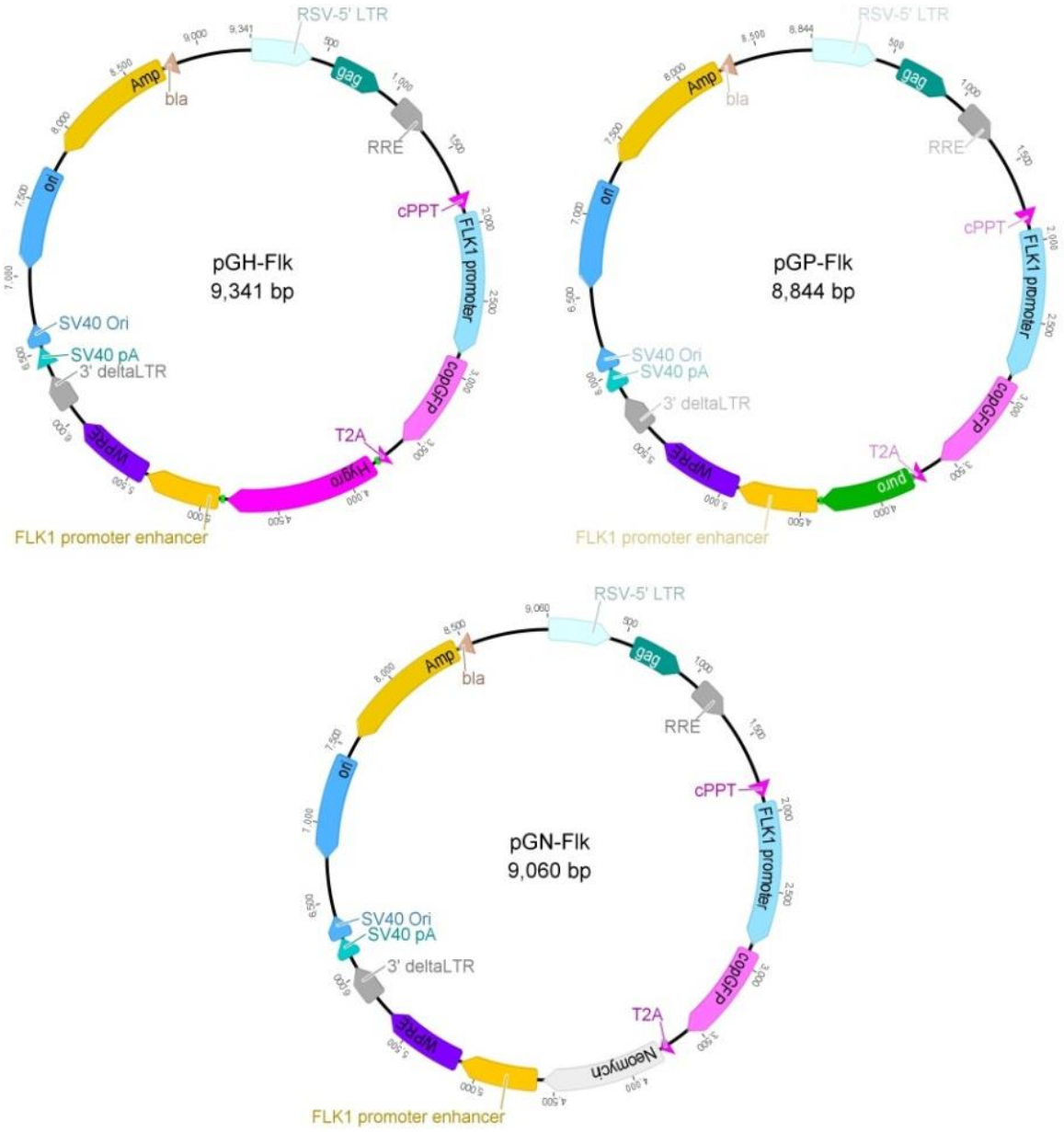


Figure 14: Backbones of the transfer vectors: pGH-Flk, pGN-Flk, and pGP-Flk.

RSV 5'LTR - long terminal repeat, required for viral packaging and transcription, *Gag* - packaging signal, *cPPT* - central polypurine tract (includes DNA Flap region) involved in nuclear transcription and integration of transduced viral genome, *copGFP* - GFP-reporter gene from copepod *Pontellina plumata*, *T2A* - 2A peptide from *Thosehasigma* virus to mediate protein cleavage, *WPRE* - posttranscriptional regulatory element which enhances the stability of the viral transcripts, *3'LTR* - required for viral reverse transcription, self-inactivating 3'LTR with deletion in U3 region prevents formation of replication competent viral particles after integration into genomic DNA, *SV40 pA* - transcription termination and polyadenylation, *SV40 Ori* - allows episomal replication of plasmid in eukaryotic cells, *ori* - allows high copy replication in *E. coli*, *Amp* - ampicillin resistant gene for selection in *E. coli*, *H* - hygromycin, *N* - neomycin and *P* - puromycin, *SIN* - self-inactivating vectors lacking viral enhancers/promoters in the 3' long terminal repeat (LTR).

7. The pG*-hVE – all vectors contain human Ve-cadherin promoter, but each of the vector has different resistant gene for antibiotic selection. Those are SIN vectors, which can be used for virus production by means of transfection and consequently for transduction of mESC.

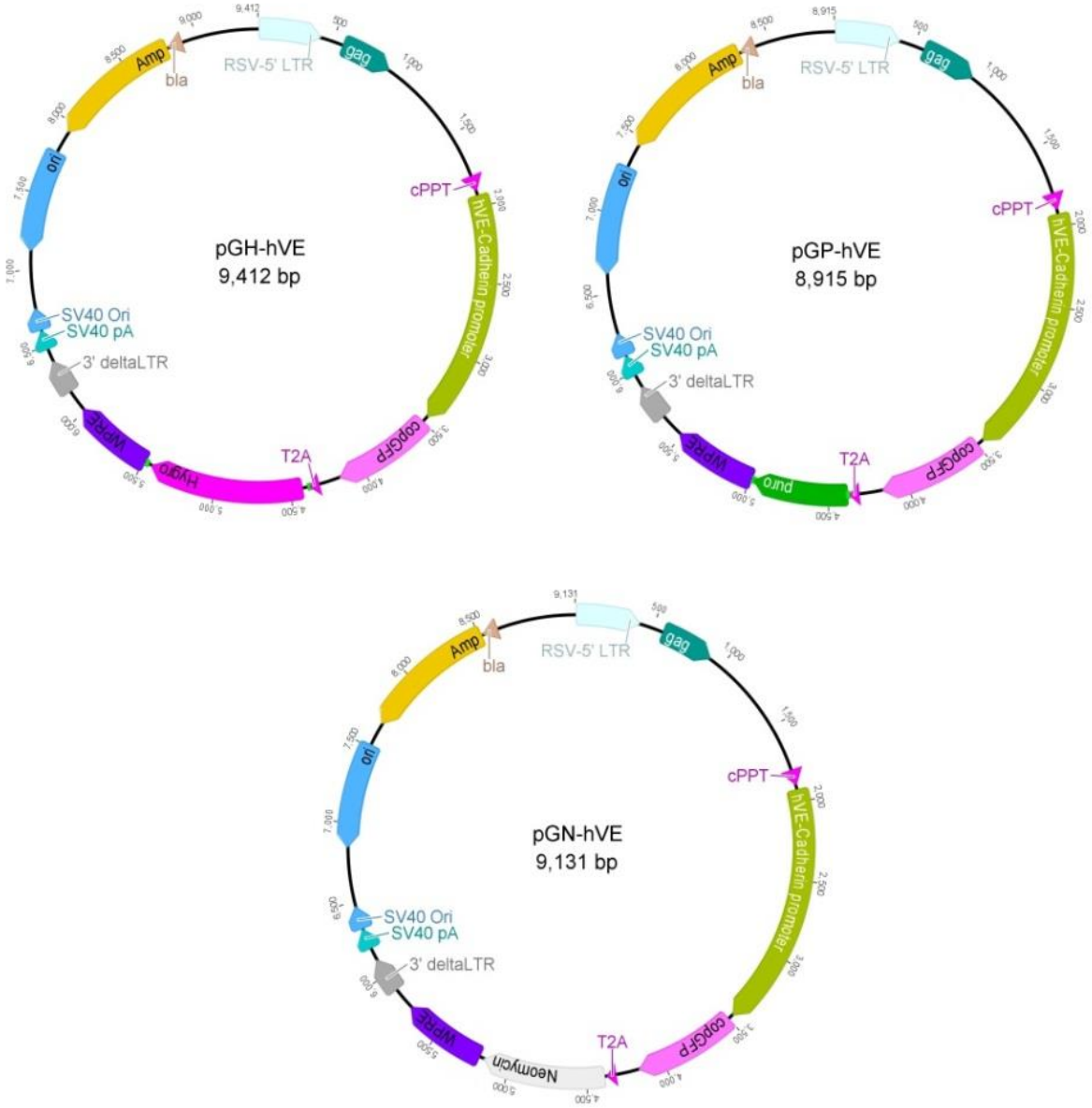


Figure 15: Backbones of transfer vectors with human promoters: pGH-hVE, pGN-hVE and pGP-hVE.

RSV 5'LTR - long terminal repeat, required for viral packaging and transcription, *Gag* - packaging signal, *cPPT* - central polypurine tract (includes DNA Flap region) involved in nuclear transcription and integration of transduced viral genome, *copGFP* - GFP-reporter gene from copepod *Pontellina plumata*, *T2A* - 2A peptide from *Thosehasigma* virus to mediate protein cleavage, *WPRE* - posttranscriptional regulatory element which enhances the stability of the viral transcripts, *3'LTR* - required for viral reverse transcription, self-inactivating 3'LTR with deletion in U3 region prevents formation of replication competent viral particles after integration into genomic DNA, *SV40 pA* - transcription termination and polyadenylation, *SV40 Ori* - allows episomal replication of plasmid in eukaryotic cells, *ori* - allows high copy replication in *E. coli*, *Amp* - ampicillin resistant gene for selection in *E. coli*, *H* - hygromycin, *N* - neomycin and P - puromycin, *SIN* - self-inactivating vectors lacking viral enhancers/promoters in the 3' long terminal repeat (LTR).

8. The pG*-KDR- all vectors contain human KDR promoter, but each of the vector has different resistant gene for antibiotic selection. Those are SIN vectors, which can be used for virus production by means of transfection and consequently for transduction of mESC.

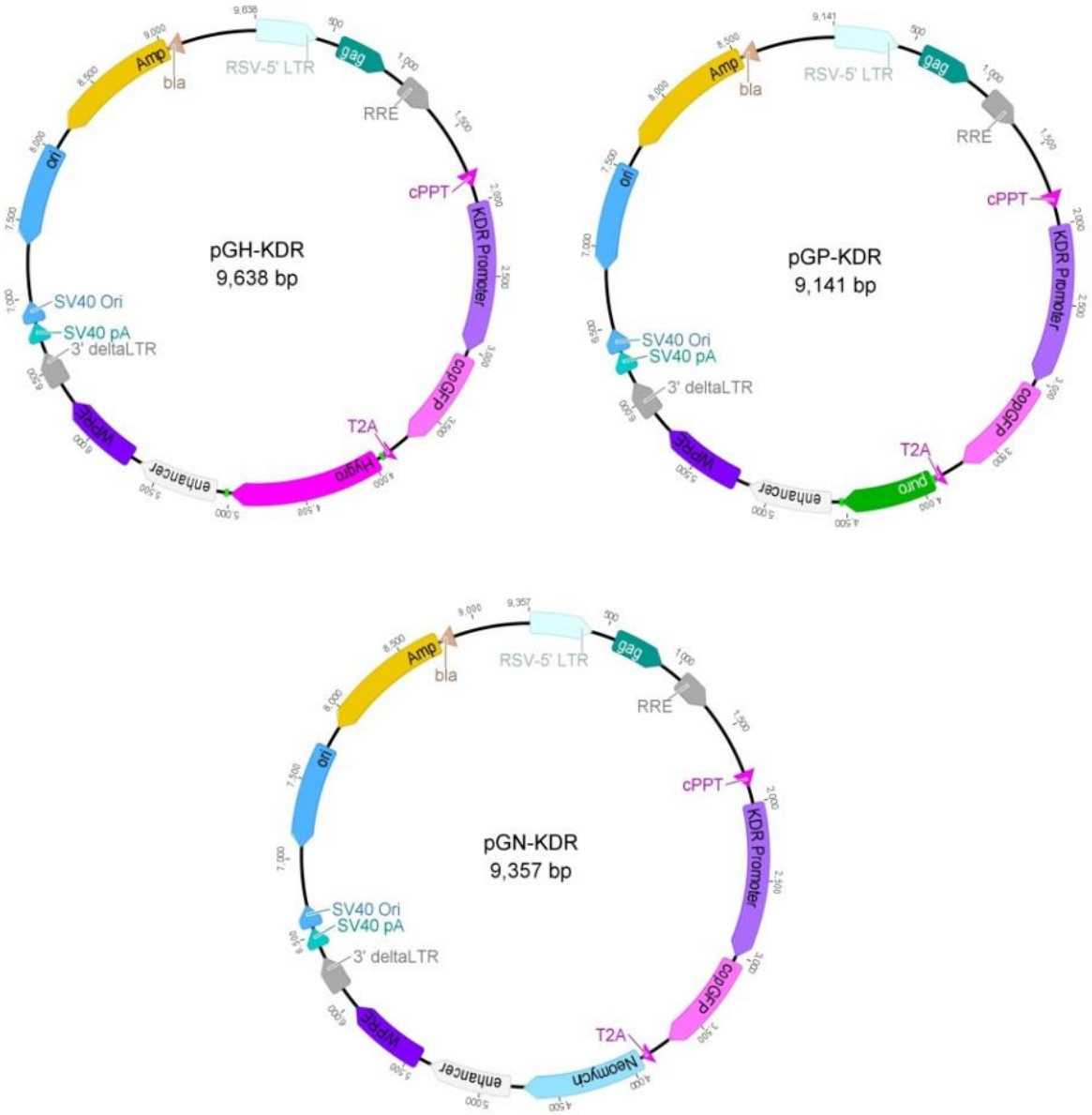


Figure 16: Backbones of transfer vectors with human promoters: pGH-KDR, pGN-KDR and pGP-KDR.

RSV 5'LTR - long terminal repeat, required for viral packaging and transcription, *Gag* - packaging signal, *cPPT* - central polypurine tract (includes DNA Flap region) involved in nuclear transcription and integration of transduced viral genome, *copGFP* - GFP-reporter gene, from copepod *Pontellina plumata*, *T2A* - 2A peptide from Thosehasigma Virus to mediate protein cleavage, *WPRE* - posttranscriptional regulatory element which enhances the stability of the viral transcripts, *3'LTR* - required for viral reverse transcription, self-inactivating 3' LTR with deletion in U3 region prevents formation of replication competent viral particles after integration into genomic DNA, *SV40 pA* - Transcription termination and polyadenylation, *SV40 Ori* - allows episomal replication of plasmid in eukaryotic cells, *ori* - allows high copy replication in *E. coli*, *Amp* - ampicillin resistant gene for selection in *E. coli*, *H* - hygromycin, *N* - neomycin and *P* - puromycin, *SIN* - self-inactivating vectors lacking viral enhancers/promoters in the 3' long terminal repeat (LTR).

9. The pKP332 (Lenti-OSK) vector was used for induction of pluripotency. The plasmid was bought from Addgene (plasmid # 21627) and was primarily used in the experiments published by Chang (Chang et al., 2009).

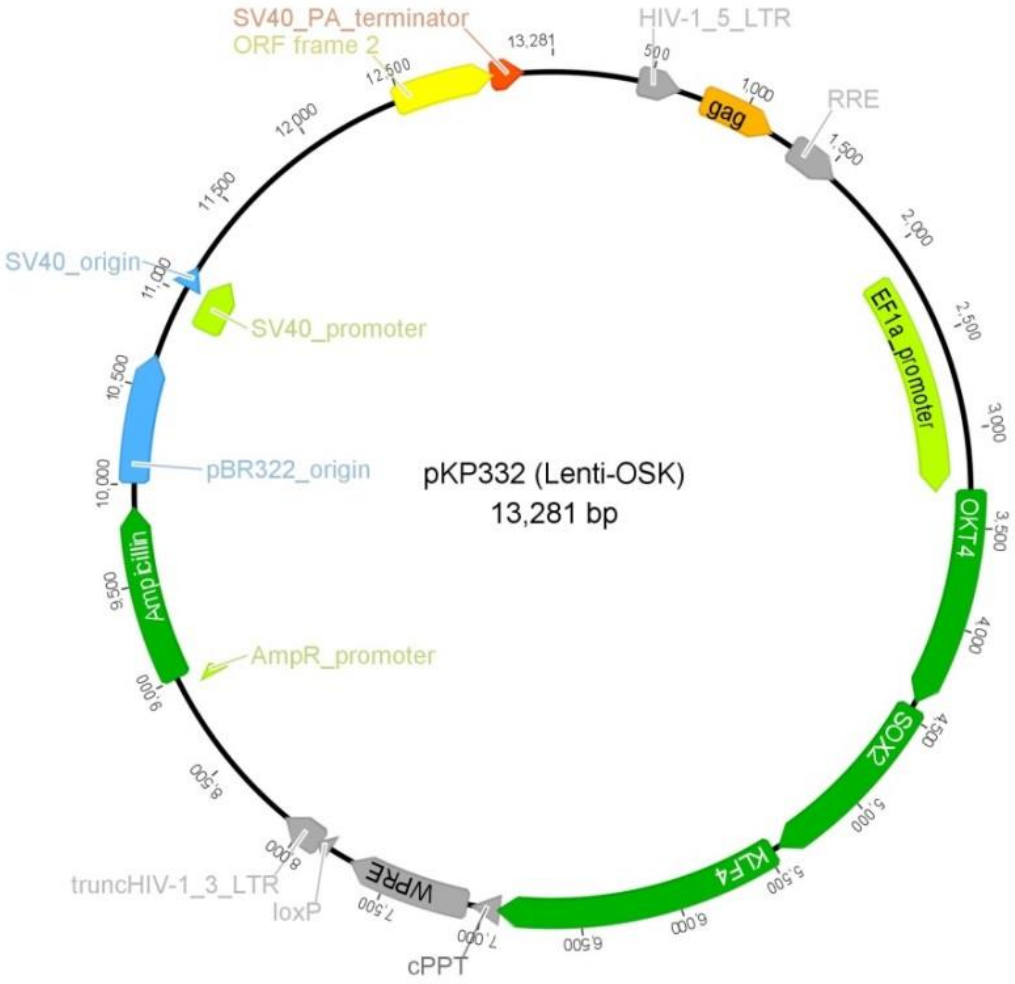


Figure 17: Backbone of pKP332 (Lenti-OSK) vector.

1(PTV1) - porcine *Teschovirus* 2A sequences that function as cis-acting hydrolase elements (CHYSELs) to trigger "cleavage" and ribosome skipping linked with human Oct4, Sox2 and Klf4 cDNAs, *SV40 Ori* - allows episomal replication of plasmid in eukaryotic cells, *ori* - allows high copy replication in *E. coli*; *Amp* - ampicillin resistant gene for selection in *E. coli*, *WPRE* - posttranscriptional regulatory element which enhances the stability of the viral transcripts, *cPPT* - central polypurine tract (includes DNA Flap region) involved in nuclear transcription and integration of transduced viral genome, *RRE* - rev responsive element, *pBR322* - an *E. coli* plasmid cloning vector containing the origin of replication from pMB1 (a plasmid in the ColE1 compatibility group), *EF1a_promotor* - human elongation factor 1α subunit promotor for high level expression, transcription factors: *Oct4* - POU5F1 a homeodomain, *Sox2* - (sex determining region Y)-box 2, *Klf4* - Kruppel-like factor 4.

10. The pLM-fSV2A (Lenti-OKMS) vector was used for induction of pluripotency. This vector was bought from Addgene (plasmid # 27512) and was primarily used in the experiments published by Papapetrou (Papapetrou et al., 2011).

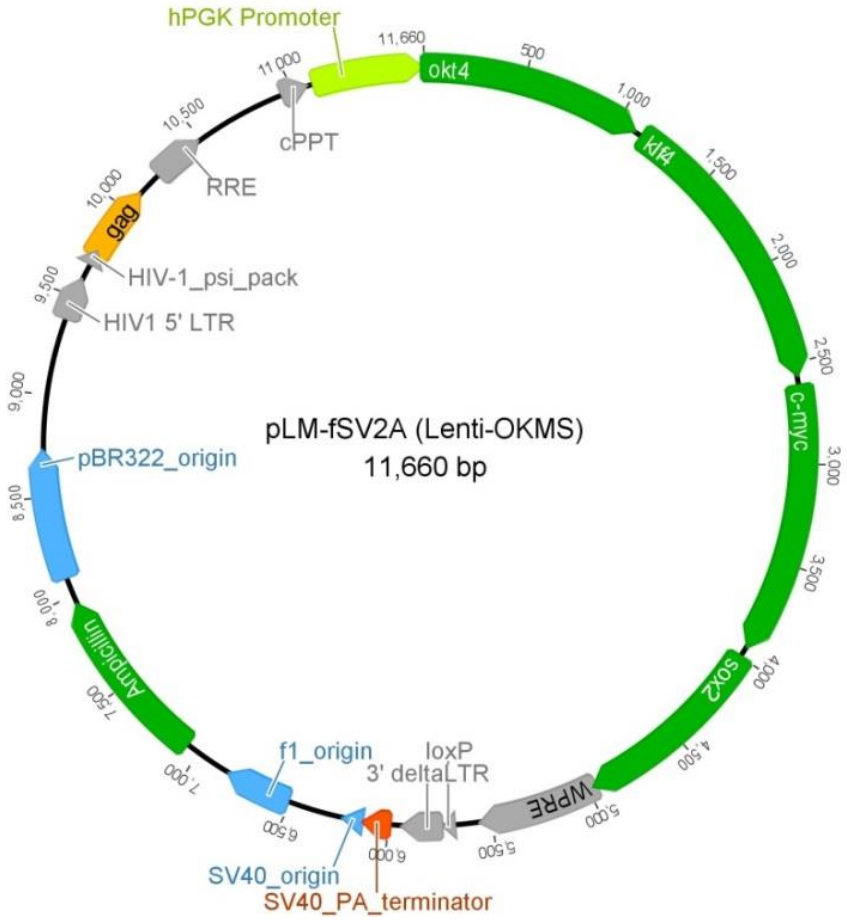


Figure 18: Backbone of pLM-fSV2A (Lenti-OKMS) vector.

hPGK_promoter - phosphoglycerate kinase promoter drives transgene expression subcloned within the multicloning sites, *pBR322* - an *E. coli* plasmid cloning vector containing the origin of replication from pMB1 (a plasmid in the ColE1 compatibility group), *Amp* - ampicillin resistant gene for selection in *E. coli*, *WPRE* - posttranscriptional regulatory element which enhances the stability of the viral transcripts, *cPPT* - central polypurine tract (includes DNA Flap region) involved in nuclear transcription and integration of transduced viral genome, *RRE* - rev responsive element, transcription factors: *Oct4* - POU5F1 a homeodomain, *Klf4* - Kruppel-like factor 4, *c-Myc* - a regulator gene, *Sox2* - (sex determining region Y)-box 2.

2.7. Kits and standards

Table 6: Kits and standards used in the experiments.

Kit or standard	Producer
Agarose Gel Extraction Kit	Jena Bioscience (Jena, Germany)
DNA marker (Roti®-Load DNA)	Roth (Karlsruhe, Germany)
DNase treatment Kit	Promega (Madison, USA)
DNeasy Blood and Tissue Kit	Qiagen (Hilden, Germany)
dNTPs	Thermo Scientific (Waltham, USA)
Fast-n-Easy Plasmid Mini-Prep Kit	Jena Bioscience (Jena, Germany)
GeneJet™RNA Purification Kit	Thermo Scientific (Waltham, USA)
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems/Life Technologies (Darmstadt, Germany)
ImmoMix™Red	Bioline GmbH (Luckenwalde, Germany)
MangoMix™	Bioline GmbH (Luckenwalde, Germany)
NuceloBond Xtra Maxi Kit	Macherey-Nagel GmbH (Düren, Germany)
PCR Purification Kit	Jena Bioscience (Jena, Germany)
Pfu Polymerase	Fermentas (Pittsburgh, USA)
Phusion® High Fidelity DNA Polymerase	New England Biolabs (Ipswich, USA)
QIAamp® Viral RNA Mini Kit	Qiagen (Hilden, Germany)
RNeasy® Mini Kit	Qiagen (Hilden, Germany)
Shrimp Alkaline Phosphatase (SAP)	Fermentas (Pittsburgh, USA)
SYBER® Select Master Mix	Applied Biosystems/Life Technologies (Darmstadt, Germany)
T4 Ligase	Fermentas (Pittsburgh, USA)

T4 Polynucleotidkinase (PNK)	Fermentas (Pittsburgh, USA)
Taq Polymerase	Bioline GmbH (Luckenwalde, Germany)
TurboFect	Fermentas (Pittsburgh, USA)

2.8. Media

2.8.1. Media, buffers and solutions used in the cell culture

Table 7: Cell culture media.

Name	Producer
0.05% Trypsin-EDTA	Gibco Invitrogen (Karlsruhe, Germany)
1-Thioglycerol (3-Mercapto-1,2-diol)	Sigma Aldrich (Steinheim, Germany)
2.5% Trypsin	Gibco Invitrogen (Karlsruhe, Germany)
Ampicillin	Serva Feinbiochemica (Heidelberg, Germany)
Ascorbic Acid (vitamin C)	STEMCELL Technologies (Köln, Germany)
B-27 supplement	Gibco Invitrogen (Karlsruhe, Germany)
BD SMC4 (small molecule cocktail of inhibitors)	BD Bioscience (Heidelberg, Germany)
Fibroblast Growth Factor-basic (bFGF)	Life Technologies (Darmstadt, Germany)
Bovine Albumin Fraction V Solution (BSA)	Gibco Invitrogen (Karlsruhe, Germany)
Bovine Pituritary Extract (BPE)	Sigma Aldrich (Steinheim, Germany)
GSK-3 inhibitor CHIR99021 [3 mM]	Reagents Direct (Encinitas, USA)
Collagenase type IV	Gibco Invitrogen (Karlsruhe, Germany)
Deoxyribonuclease I from bovine pancreas (DNaseI)	Roche (Indianapolis, USA)
Dispase	VWR Syngene (Darmstadt, Germany)

Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM-F12)	PAA (Cölbe, Germany)
Dulbecco's Modified Eagle Medium with Glucose (DMEM)	Gibco Invitrogen (Karlsruhe, Germany)
Dulbecco's Phosphate Buffer Saline (PBS) without Ca^{2+} & Mg^{2+}	PAA (Cölbe, Germany)
Fetal calf serum (FCS)	PAA (Cölbe, Germany)
Fibronectin	BD Bioscience (Heidelberg, Germany)
Gelatin from porcine skin, type A	Sigma Aldrich (Steinheim, Germany)
Geltrex™ LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix	Life Technologies (Darmstadt, Germany)
Geneticin disulfate (G418) powder	Sigma Aldrich (Steinheim, Germany)
Ham's F 12 Nutrient Mixture	Gibco Invitrogen (Karlsruhe, Germany)
Heparin	Sigma Aldrich (Steinheim, Germany)
Hygromycin B	Sigma Aldrich (Steinheim, Germany)
Iscove's liquid medium with stable glutamine (IMDM)	Biochrom AG (Berlin, Germany)
Knockout DMEM (KO DMEM)	Gibco Invitrogen (Karlsruhe, Germany)
Knockout Serum Replacement (KO replacement)	Gibco Invitrogen (Karlsruhe, Germany)
Leukemia inhibitory factor (LIF)	Millipore (Darmstadt, Germany)
L-Glutamine	PAA (Cölbe, Germany)
MCDB 131 Medium	PAN Biotech (Aidenbach, Germany)
Mitomycin C	Sigma Aldrich (Steinheim, Germany)
N-2 supplement	Gibco Invitrogen (Karlsruhe, Germany)
Neomycin	Sigma Aldrich (Steinheim, Germany)
Neurobasal® Medium	Gibco Invitrogen (Karlsruhe, Germany)
Non-essential amino acids (NEAA)	PAA (Cölbe, Germany)

MEK inhibitor PD0325901 [1 mM]	Reagents Direct (Encinitas, USA)
Penicillin/Streptomycin (Pen/Strep)	PAA (Cölbe, Germany)
Poly (2-hydroxyethyl methacrylate) (polyHEMA)	Sigma Aldrich (Steinheim, Germany)
Polybrene (Hexadimethrin bromide) [10 mg/ml]	Millipore (Darmstadt, Germany)
Sodium pyruvate (Pyruvat)	PAA (Cölbe, Germany)
β-Mercaptoethanol [2 mM]	Sigma Aldrich (Steinheim, Germany)
VitronectinXF™	STEMCELL Technologies (Köln, Germany)

2.8.2 Cell culture media composition

Each medium containing all ingredients after preparation was filter sterilized and kept in 4 °C up to six weeks.

- **Complete ES-medium for ESCs culture:**

  Iscove's modified DMEM (IMDM) containing 25 mM HEPES and it is optimal for the cell culture conducted when the concentration of CO_2 is at the level of 5%.

  IMDM with 15% (v/v) FCS, 1% (v/v) NEAA, 1% (v/v) Pen/Strep, 1% (v/v) Pyruvate, 4.4 µl β-Mercaptoethanol, 10^6 U/ml LIF (stock concentration 10^7 Units in 1 ml of phosphate buffered saline)

- **Differentiation medium:**

  ES-medium without LIF

- **MEF-medium for culturing murine embryonic fibroblasts:**

  DMEM with 15% (v/v) FCS, 1% (v/v) Pen/Strep, 1% (v/v) NEAA

- **Medium for mitotic inactivation of MEFs:**

  DMEM with 5% (v/v) FCS, 1% (v/v) Pen/Strep, 1% (v/v) NEAA and 10 µg/ml (w/v) mitomycin C (stock 50 mg powder of which 2 mg mitomycin C and 48 mg of NaCl)

- **mEndo medium - medium for culturing mouse endothelioma cells (mEndo):**

  MCDB 131 medium with 15% (v/v) FCS, 50 µg/ml (w/v) L-glutamine, 50 µg/ml (w/v) BPE, 100 µg/ml (w/v) heparin, 1% (v/v) Pen/Strep

- **Medium for culturing HEK 293T cells:**

  DMEM with 10% (v/v) FCS and 1% (v/v) Pen/Strep

- **Freezing medium:**

  DMEM with 50% (v/v) FCS and 10% (v/v) DMSO

- **HEPES buffer:**

  Composition for 3 L stock: 21.21 g of NaCl, 1.2 g of KCl, 0.18 g of KH_2HPO_4, 0.24 g of Na_2HPO_4 (x) $7H_2O$, 3 g of glucose, 0.03 g of phenol red, 14.3 g of HEPES. Dissolve all chemicals in 2.5 L of cell culture water, adjust pH to 7.3 (with NaOH or HCl accordingly), fill up to 3 L. Distribute into 500 ml bottles, autoclave and store in 4 °C for several weeks.

- **0.2% (w/v) gelatin solution for coating the cell culture dishes:**

  Dissolve 1 g of gelatin type A in 500 ml of cell culture water and autoclave at 121 °C for 15 min.

2.8.3. Bacterial culture media composition

- **LB medium (Roth, Karlsruhe, Germany):**

  The formulation of this broth is based on the LB broth described by E.S. Lennox (Lennox, 1955). LB medium was used for the growth and maintenance of *E.coli* strains, this broth is nutrient rich and specifically developed for the isolation of pure recombinant strains.

  Preparation: dissolve 20 g of the LB medium in 1 L of purified water and autoclave. Afterwards add 800 µl of ampicillin (stock 100 mg/ml).

- **LB agar (Roth, Karlsruhe, Germany):**

  The LB agar was established by E.S. Lennox for growth and maintenance of pure cultures of recombinant strains of *E. coli* (Lennox, 1955).

  Preparation: suspend 16 g of the LB agar in 500 ml of purified water, then heat with frequent agitation and boiled for 1 min to completely dissolve the medium. Autoclave at 121 °C for 15 min. Before pouring the agar on the Petri dishes, add 400 µl of ampicillin (stock concentration 100 mg/ml).

- **SOC medium:**

  SOC medium is appropriate for use in the final step of cell transformation to obtain maximal transformation efficiency of *E. coli* (Hanahan, 1983). Use of SOC medium increases the molecular uptake, while stabilizing the cells and at the same time maximizing the transformation efficiency.

  Preparation:

  tryptone 2 g, yeast extract 0.5 g, NaCl 0.2 ml [15 M], KCl 0.25 ml [1M], $MgCl_2$ 1 ml [1 M], glucose 2 ml [1 M] constitutes the SOB medium (Super Optimal Broth).

  To the ready SOB solution containing the first four reagents, after autoclaving at 121 °C, add sterile $MgCl_2$ and glucose.

2.8.4. Xenobiotic-free media composition (2i media)

- **Serum free ES medium (SFES-basal media), composition for 500 ml:**

  50% (v/v) Neurobasal medium, 50% (v/v) DMEM/F12, 0.5% (v/v) N-2 supplement, 1% (v/v) B-27 supplement, 0.6% (v/v) BSA, 1% (v/v) Pen/Strep

  Aliquot 10(x) into 50 ml conicals and wrap each in tin foil, store at 4 °C.

- **Complete 2i medium:**

  Take one aliquot of SFES medium and add:

  50 µl MEK inhibitor PD0325901 [1 mM], 50 µl GSK-3 inhibitor CHIR99021 [3 mM], 500 µl glutamine [200 mM], 0.63 µl monothioglycerol (MTG) [11.9 M], 50 µl LIF [10^6 U/ml]

- **Serum free differentiation medium (SFD), composition for 150 ml:**

  75% (v/v) IMDM, 25% (v/v) Ham's F12, 0.5% (v/v) N-2 supplement, 1% (v/v) B-27 supplement, 1% (v/v) Pen/Strep, 0.6% (v/v) BSA (0.05%), 1% (w/v) glutamine (2 mM) Directly before use supplement with 1% (v/v) ascorbic acid and 0.002% (v/v) MTG ($1.5(x)10^4$M).

- **"STOP" buffer to terminate the trypsin activity:**

  50% (v/v) IMDM, 50% (v/v) serum, DNAse (10 mg/ml)

- **Freezing medium:**

  50% (v/v) IMDM, 40% (v/v) serum, 10% (v/v) DMSO

- **VitronectinXF™ solution for coating the cell culture dishes:**

  Thaw Vitronectin XF™ at room temperature and then dilute in PBS to reach a final concentration of 10 µg/ml (i.e. use 40 µl of Vitronectin XF™ per 1 ml of buffer). Prepare the dilution in 50 ml conical tube and mix gently. Coated plates, after incubation, should be washed once with PBS before use.

2.9. Cell lines

- **Embryonic stem cell line - E14Tga2a.4**

  This is a derivative of one of several embryonic stem cell lines developed by M. Hooper in 1987 (Hooper et al., 1987). Cells were obtained from the 129P2/OlaHsd mouse strain.

- **Mouse embryonic fibroblasts (MEFs)**

  MEFs are obtained from d13.5 to d14.5 postcoitum (p.c.) mouse embryos. After propagation cells can be aliquoted and frozen indefinitely in liquid nitrogen.

In order to use MEFs as feeders, cells first need to be mitotically inactivated (see paragraph 3.2.1.).

- **Mouse endothelioma cells (mEndo)**

  It is an immortalized cell line which possesses endothelial characteristic. This cell line was used as a positive control to validate the vectors created for establishing the reporter cell lines.

- **Human Embryonic Kidney cells (HEK 293T)**

  Human embryonic kidney cells have been transformed by exposing cells to sheared fragments of adenovirus type 5 DNA (Graham et al., 1977). The 293T cells are derivative of 293 cells but are stably expressing the SV40 large T antigen. That antigen can bind to SV40 enhancers of expression vectors increasing in that manner protein production. The HEK cell line was used for viral particle (VP) production.

3. Methods

3.1. Molecular methods

3.1.1. Isolation of total RNA and DNase treatment

The isolation of total RNA from cell culture samples was performed by using GeneJET™ RNA Purification Kit or RNeasy® Mini Kit according to the manufacturers' protocol. Each sample was eluted in 50 µl of nuclease free water. Next, the quality and concentration of RNA were measured on NanoDrop. After determination of RNA concentration, samples were normalized to an end concentration of 100 ng/µl. Next, DNase treatment was applied and samples were incubated for 30 min at 37 ºC.

Set up of the DNA digestion reaction:

Component	Volume
10(x)DNase Reaction Buffer	2 µl
DNase	2 µl
RNA sample	15.5 µl
RNase inhibitor	0.5 µl
Total volume per reaction:	**20 µl**

3.1.2. Determination of nucleic acid concentration

The DNA yield can be estimated by absorbance (optical density), agarose gel electrophoresis, or by use of fluorescent DNA-binding dyes. Nucleic acids and proteins have absorbances maxima at 260 and 280 nm, respectively. The ratio of absorbance at these wavelengths has been used to estimate the purity of proteins and nucleic acids. The ratio of ~1.8 is generally accepted for DNA and for RNA ~2.0.

3.1.3. Synthesis of cDNA from total RNA (reverse transcription)

The synthesis of DNA from an RNA template, by means of reverse transcription, creates a complementary DNA (cDNA). The reverse transcriptases utilize an RNA template and a short primer complementary to the 3' end of the RNA to drive the synthesis of the first strand of cDNA. That strand will be used immediately as a template for the polymerase chain reaction (PCR). A mixture of reverse transcription and PCR reactions allow for the detection of low amounts of RNA in a sample and production of the corresponding cDNA. Applications of engineered reverse transcriptases boost the efficiency of full-length product arrangement, at the same time providing that the copying of the 5' end of the mRNA transcript is complete. In the Promega Kit a Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) is used as an RNA-dependent DNA polymerase.

RT-PCR reaction mix:

Component	**Volume per reaction**	**End concentration**
ddH_2O	4.2 µl	N/A
10(x)RT Buffer	2 µl	1(x)
10(x)Random Primers (hexamers)	2 µl	1(x)
25(x)dNTP Mix [100 mM]	0.8 µl	1(x)
MultiScribeTM-RT [50 U/µl]	1 µl	50 U
RNA	10 µl	100 ng/µl
	Total volume:	**20 µl**

The PCR was run in the Mastercycler Ep Gradient S machine, with the following set up:

Time	Temperature	Process
10 min	25 ºC	Primer binding to an RNA
2 h	37 ºC	Reverse Transcription
5 min	85 ºC	Inactivation of MultiScribe™ reverse transcriptase
>5 min	4 ºC	∞

3.1.4. DNA amplification by polymerase chain reaction (PCR)

To be able to investigate single genes or particular DNA regions of interest, large quantity of nucleic acid are required for analysis. To avoid isolation of single copy of desired DNA, it is better to have multiple copies of a target. PCR makes it possible to produce millions of copies of a specific DNA sequence in a fairly short time. This technique was developed by Kary Mullis and associates in 1984.

In experiments described in this thesis, PCR was used for both analytical and preparative purposes.

For the detection of positive bacterial clones or selection of ESC clones, MangoMix containing Taq Polymerase was used. The Taq Polymerase is a highly thermostable DNA polymerase from the thermophilic bacterium *Thermus aquaticus.*

Preparative PCR was utilized for amplification of DNA fragments which were used as inserts for production of plasmid DNA (pDNA) constructs. In preparative PCR Pfu polymerase instead of Taq polymerase was applied. The Pfu polymerase possesses proof reading ability, which makes it superior in comparison to the other polymerase.

3.1.5. Real Time PCR

For the purposes of the experiments presented in the thesis, changes in gene expression during cell differentiation were explored. To conduct tests with real time PCR, a florescent dye called SYBR® Green was used. This is a universal probe, which attaches to all double-stranded DNA. This allows the detection and monitoring by measuring the increase in fluorescence throughout the cycle. SYBR®

Green has an excitation maximum of 494 nm and emission of 521 nm. The threshold cycle (Ct) is the quantitative endpoint for real-time PCR. The Ct is defined as the PCR cycle, at which the fluorescent signal of the reporter dye, exceeds an arbitrarily set up threshold. Presentation of the generated data as the Ct values guarantees that the PCR is in the exponential phase of amplification. The numerical value of the Ct is inversely linked to the amount of amplicon in the reaction.

3.1.6. Gel electrophoresis

To confirm the specificity of the primers, verify the DNA fragments after digestion, ligation, or other PCR products, the gel electrophoresis method was used. It is a common method for separating DNA by size (i.e. length in base pairs) for visualization and purification. The molecules to be separated are driven by an electrical field through a small porous gel. The speed at which the molecules migrate through the gel is inversely connected with the lengths of the molecule. Bands can be detected by staining and visualized by illumination with 300 nm UV light. Ethidium bromide, the added dye, is a DNA intercalator which inserts itself between the base pairs in the double helix.

3.1.7. DNA restriction

Restriction enzymes are DNA-cutting enzymes found in bacteria. Those enzymes cleave the sugar-phosphate backbone of the DNA. In order to prevent self-ligation of the vector, shrimp alkaline phosphatase (SAP from *Pandalus borealis*), which dephosphorylates the vector was added.

Over the course of experiments carried out in this PhD project, the restrictive digestion of DNA was used for cloning purposes, as well as for the purification of desired plasmid fragments. Those fragments were obtained by means of gel electrophoresis followed by gel extraction, which is necessary to obtain sufficient amount of the product. For the digestion reaction, commonly 5 µg of the vector DNA and 1µg of the insert(s), originating from the PCR, were applied.

Example setup of the digestion reaction:

Component	Volume
10(x)buffer (adequate for the particular enzyme)	2 µl
Restriction enzyme	1-2 U
DNA	~ 1 µg
H_2O	Sufficient amount to bring the **total** reaction **volume** to **20 or 50 µl**

Reaction mix was separated by gel electrophoresis and the band exhibiting the expected size was extracted and purified with Agarose Gel Extraction Kit.

3.1.8. DNA ligation

Ligation was catalyzed by T4 DNA ligase, this enzyme is obtained from bacteriophage T4. The ligase mediates formation of phosphodiester bonds between the free 3'-hydroxyl terminus with the 5'-phospate group of adjacent DNA. T4 DNA ligase will link cohesive end termini as well as repair single stranded nicks in double helix of DNA.

Ligation mix for 20µl reaction:

Component	Amount
10(x)T4 DNA Ligase Buffer	2 µl
Vector DNA	50-100 ng
Insert DNA	in molar ratio of 1:3 over vector
Nuclease-free H_2O	up to 20 µl
T4 DNA Ligase	1 µl

The mixture was incubated for 30 min to 1 h at room temperature (22 °C). Depending on need the mixture was enriched with polyethylene glycol (5% PEG per reaction), which "packs" the aqueous solution of the ligation mix, thus increasing the concentration of the DNA and ligase, making the interaction more efficient.

3.1.9. Electroporation

Transformation of bacteria is a method in which the naked DNA molecules will be taken up by bacterial cells, by means of electric field the permeability of the bacteria cell membrane is increased. When the extrinsic DNA has an origin of replication recognized by the polymerase of the host cell, then the bacteria will replicate the incorporated DNA as its own.

In order to remove salts from the sample, the DNA was precipitated. This procedure is crucial since in the subsequent electroporation step, the salt content could cause interference.

The protocol for DNA precipitation was as follows:

1. Fill up the reaction mix with water up to 100 µl.
2. Mix with 1 ml of *n*-butanol and vortex thoroughly.
3. Centrifuge for 20 min at maximum speed at room temperature.
4. Pellet should be well visible. Discard supernatant and add 500 µl of 70% ethanol to wash the pellet. Centrifuge for 5 min at the highest speed.
5. Pellet should be now white, dry it for about 10-15 min. Then suspend the precipitated DNA in 10 µl of ddH_2O.

An aliquot (100 µl) of electrocompetent bacteria was thawed on ice and 50 µl of the bacterial suspension was mixed with 10 µl of the precipitated DNA. The remaining bacteria were used for the ligation control, where instead of insert water was added (colonies on the plate will indicate undigested or self-ligated vector). Mix was kept on ice and pipetted into a 1 mm plastic cuvette. The dried-off cuvette was placed in the electroporator and standard for bacteria current pulse was applied (1.8 kV and capacitance of 25 µF with a resistance of 1 Ω), bacteria were mixed with 800 µl of pre-warmed SOC medium, to stimulate the recovery of the microorganisms.

The mixture was transferred to a 1.5 ml reaction tube and maintained in a heating block (37 °C) for 40 min with shaking at 500 rpm. After the incubation, bacteria were distributed on agar plates containing ampicillin selective medium and incubated at 37 °C overnight.

3.1.9.1. Bacterial strain

An *Escherichia coli* XL1-Blue strain was used for the plasmid propagation. That type of bacteria has the following genotype:

recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´proAB lacI^{q}ZΔM15 Tn10 (Tet^{r})]

This strain of *E. coli* is the most common strain used in cloning. The XL-1-Blue strain is tetracycline-resistant and also has endonuclease deficiency (*endA*). The *endA* mutation deletes endonuclease and this has a significant impact on the quality of plasmid preparations. This strain is recombination-deficient (*recA*$^{-}$) and that results in better insert stability. Another characteristic of this specific *E. coli* strain is the *hsdR* mutation, which prevents the cleavage of cloned DNA by the *EcoK* endonuclease system. While *E. coli* DNA is protected from degradation by an equivalent methyl-transferase, foreign DNA will be cut at the specific sites. The deletion of *hsdS* eliminates both the endonuclease and methyl-ltransferase activities of *EcoK*. The gene (F' Δ(*lacZ*)*M15*) constitutes the omega-fragment of β-gal; Δ(*lac-proAB*) deletes the β-gal gene on the chromosome. Several plasmids code for the α-peptide of β-galactosidase (*lacZ*). The α-peptide is able to connect with the omega-fragment of β-galactosidase, which is carried on the *F'* (α-complementation). When β-galactosidase is built again in this way it can cleave X-gal and then the blue colonies on an X-gal plate will emerge. Inserts cloned into the plasmid polylinker (multiple cloning sites) disrupt the α-peptide gene and accordingly the growing colonies will be white (www.neb.com, NewEngland Biolabs).

3.1.10. Ligation control after bacterial transformation

There are two methods to ensure that the grown bacteria had been properly transformed. The first utilizes digestion enzymes to screen the clones. As it was mentioned previously (3.7.1.), the restriction endonucleases will create unique band patterns of the digested vectors.

In order to isolate enough of plasmid DNA for experiment the liquid culture was used.

Protocol for liquid bacteria culture:

1. Add 3 ml of liquid LB to a culture tube (liquid medium should be supplemented with 800 µl of ampicillin).
2. Pick a single colony from given LB agar plate using a sterile pipette tip (it is important to pick one single colony only). Drop the tip into the liquid LB and swirl.
3. Loosely cover the tube with a sterile aluminium foil or a cap that is not air tight.
4. Incubate bacterial culture at 37 °C overnight in a shaking incubator at 250 rpm.
5. Next day a cloudy haze in the media should be observed (evidence of bacterial growth).
6. Remove tubes from the incubator and pick out the pipette tip with forceps and spin down the liquid cultures in 15 ml tube at 4.000 rpm at 4 ºC for 5 min.
7. Discard the supernatant and let excess liquid to drain by inverting tube on a paper towel.
8. Proceed with a mini prep following the kit instruction or freeze the pellet in -20 °C.

After the mini prep, vector in the amount of 1 µg was digested with the adequate restriction enzyme.

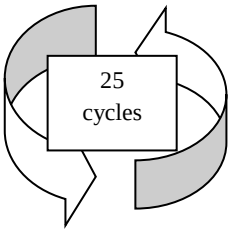
The second possibility for detection of bacterial colonies, with inserted desired cloning product, was performed using PCR. Primers used for the screen, were designed in a way to include a part from the vector and a piece from the insert. This approach guarantees that the vector and insert are correctly oriented. Taking into consideration colony density on the plate which served as a control (ligation without insert), colonies from the ligation plate were picked in the appropriate ratio. The picked clones were suspended in 10 µl of liquid medium and incubated in a shaking heating block (37 °C) for 1-2 h. To the reaction tube containing 5 µl of the incubated sample 100 µl of water was added. Tubes were centrifuged for about 1 min at maximum speed and 95 µl of the supernatant was discarded. The remaining volume with the pellet was incubated for 5 min at 95 °C to lyse bacteria. The last step required brief centrifugation at the highest speed. For PCR mix 1 µl of the supernatant was used.

Reaction mix:

Component	Volume
H_2O	2.6 µl
Buffer Top Taq Mix	5 µl
Forward primer for screen	0.2 µl
Reverse primer for screen	0.2 µl
Loading dye	1 µl
Template	1 µl
Total volume	**10 µl**

PCR-program set up:

Time	Temperature
2 min	95 ºC
15 s	95 ºC
15 s	58 ºC
10 s	72 ºC
1 min 40 s	72 ºC
∞	4 ºC



The bacterial colonies showing a band at the accurate height, after the gel electrophoresis, were used for liquid culture. After the overnight incubation the mini prep was performed.

3.1.11. Plasmid preparation

A plasmid preparation is a technique by which plasmid DNA is extracted and purified. The general concept behind the function of the kit is the preparation of plasmid DNA from chromosomal DNA.

The available kits are named after the size of the bacterial culture and corresponding plasmid yield: miniprep, midiprep or maxiprep. The plasmid DNA yield will be subjected to the plasmid copy number, type and size, the strain of bacteria, the growing conditions and the kit applied.

For the purpose of the experiments carried out in the course of this research two approaches had been utilized:

- to extract small amounts of plasmid DNA (10-50 µg) the Fast-n-Easy Plasmid Mini-Prep Kit was used,
- to obtain bigger quantities of plasmid DNA (up to 1 mg) NuceloBond Xtra Maxi Kit was applied.

In the first step, bacteria are lysed in the alkaline conditions and the lysate is applied, under defined salt settings, on the specially design columns. The plasmid DNA is selectively bound and purified from RNA, proteins and other cellular contaminants. The cells should not be exposed to alkaline conditions for too long, because this may lead to the plasmid denaturation, resulting in faster migration in the agarose gel or causing resistance to digestion by restriction endonucleases.

3.1.12. Viral vectors production

Vectors for establishing a reporter cell line

Vectors with murine promotors (Fig. 13 and 14)

In the first step of the pFlk vector creation, the α-mMHC promotor was replaced by Flk1 promoter in the pMHC plasmid. Later to replace the promoter the following restriction sides were used: XhoI and BspTI. The sequences of the Flk1 promoter and its enhancer were provided by Stefanie Bachmann from Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH).

The promoter was subcloned using indicated primers:

forward: SP Flk promoter: 5'catat**ctcgag**cgacccagccaggaagttc3'

reverse: AS Flk promoter: 5'gtata**cttaag**cctgcacctcgcgctgg3'

(Bolded nucleotides specify the recognition sequences of restriction enzymes).

The enhancer was amplified by use of specified primers:

forward: SP Flk enhancer: 5'tc**gagctc**taaatgtgctgtctttagaagcc3'

reverse: AP Flk enhancer: 5'ta**cccggg**gtccaataggaaagcccttt3'

To the plasmid containing the desired promoter and enhancer was cloned on the SacI and SmaI restriction sites. In the original vector, there was no restriction site between the neomycin resistance gene cassette (Neo) and polyadenylation (pA) signal. Due to that fact it was possible to cut out Neo from the primary vector without pA signal. Amplification was made by using the showed primers:

forward: SP_neo_BspTI: 5'tgcaggc**cttaag**gcggc3'

reverse: AP_neo_SalI_hTm: 5'gtata**gtcgac**gccgatcccctcagaagaactc3'

Next, the Neo cassette was ligated to the interfaces of BspTI and SalI into the plasmid already comprising Flk1 promoter and the enhancer.

In the case of building up the pVe-Cad construct also pMHC plasmid was used. Similarly, the α-mMHC promoter was replaced by Ve-cadherin promoter. In 1998 Gorry described and analyzed the activity of this promoter (Gorry et al., 1998). The template for subcloning was prepared from genomic DNA and the promoter region was placed between -2486 to +24 base pairs. In that step the subsequent primers were used:

forward: SP_VE-Cad: 5'gtata**ctcgag**catgcagtgcaggagggagccagaa3'

reverse: ASP_VE-Cad: 5'ctata**cttaag**agtctgtccagggccgagctttgtg3'

From already completed pFlk vector, the whole neo-IRES-EGFP cassette was removed and subcloned to pVe-Cad construct at the interface of BspTI and SacI.

It is important to mention, that at the very beginning of the project Dr. Sven Becker from MPI Bad Nauheim, created also vectors containing zeocine as a resistance gene pGZ_Flk and pGZ_Ve-Cadherin. These constructs were used in the transitional stages of cloning procedures presented in the thesis.

The next step covered replacement of CMV promoter from pcDNA™5/TO plasmid. This promoter has a tendency to be silenced in the ES cells. That is why it had been replaced by PGK promoter. The new promoter was amplified from pMHC vector with the following primers:

forward: SP-PGK-MluI: 5'gtata**acgcgt**taccgggtaggggaggcgctt3'

revers: AP-PGK-BspTI: 5'ctata**cttaag**ggtggcgggatgcaggtcga3'

and then ligated to the generated plasmid at the subsequent restriction sites: MluI and BsptI.

After sequencing of the already assembled plasmids, it appeared that a mistake had been made. While cutting out the neo-IRES-EGFP cassette at the site of BsptI, the T2A sequence had been also removed. T2A gene flanked by multiple cloning sites should facilitate the simultaneous expression of multiple genes. In order to rescue the situation, the T2A sequence was ligated again to the vectors. The synthetic linkers were applied, which are single-stranded pieces of DNA of usually 8-12 base pairs in length. Linkers self-associate in solution to create regular helixes, which are even at both ends. Upon attachment of a linker to the cDNA, each of the strands is cleaved with the suitable restriction enzymes and new sticky-ends are generated. These ends will be complementary to the one created by similar handling of the vector (Greene, 1998).

Scheme of actions undertaken to recover the T2A sequence:

1. Linker synthesis with T4 Polynucleotide Kinase (T4 PNK) which catalyzes the transfer of the γ-phosphate from ATP to the 5'-OH group of oligonucleotides.
2. Digestion of the vectors with AgeI.
3. Ligation of the T2A linker to the vectors.
4. Exchange of the T2A-Zeo sequence, from PGZ-VE by the T2A-Neo sequence from pGN-Flk by using MreI and SalI restriction enzymes (generation of pGN-VE).

In order to add the puromycin cassette, the pLKO.1cloning vector was used. This vector was obtained from a shRNA library. The sequence encoding that resistance gene was cut out at the interface of MreI and SalI. After amplification the puromycin cassette was subcloned to constructs containing Flk1

promoters. For attaching the hygromycin to the backbones, its coding sequence was obtained from pGK_GFP vector. This vector was received from Dr. Marten Szibor from MPI Bad Nuaheim.

Vectors with human promoters (Fig. 15 and 16)

The sequences for KDR and hVe- Cadherin promoters were derived from the human blood received from the blood bank (University Hospital of Giessen). The blood was delivered as a buffy coat. The genomic DNA was extracted with the DNeasy Blood and Tissue Kit accordingly to the manufacturers' manual. In the PCR reaction the human endothelial promoters were amplified with specifically designed primers. In case of the KDR promoter it was also important to amplify the enhancer. This short sequence of DNA binds with the proteins in order to promote the gene transcription. The role of the KDR enhancer was described, among others, in the work conducted by Patterson (Patterson et al., 1995).

Following the PCR amplification, the human endothelial promoters were sub-cloned to already generated vectors:

1. hVe-Cadherin promoter with the use of following primers:

forward: hVE-Cad-F: 5'ctataatcgatgcccctccaatctgtcttgtctacc3'
revers: hVE-Cad-R: 5'catatgctagcgcctggctgcctccccttc3'

2. for the KDR promoter subsequent primers were applied:

forward: KDR-prom2-F: 5'gtataatcgattccactgaagcacgctggca3'
revers: KDR-prom-R: 5'gatatgctagctcctgcacctcgagccggg3',

and the oligos for the KDR enhancer:

forward: KDR-enh-F: 5'gtatgtcgactgcatgtatgtgtggaattggggaatg3'
revers: KDR-enh-R: 5'gatacccgggatgctgagcctgggcagatcaag3'

The T2A sequence was now completed and the Neo sequence had been cut out of the Flk-pGN vector by using the MreI and SalI restriction enzymes. The neomycin cassette was ligated into the pGN_KDR

construct, to confirm if the vector was assembled correctly screen with copGFP_F and AP-T2A-screen primers was performed.

The pGN-hVE vector din not have SalI restriction site. This site was required for the exchange of resistance genes. To solve that, vectors PGZ-CMV and pGN-hVE had been digested with ClaI and NheI. The hVE-Cadherin promoter was then ligated instead of CMV in the PGZ-vector and the exchange of T2A-zeo cassette was accomplished. The next two resistance genes hygromicin B and puromycin were added in the same way it was described for the vectors containing murine endothelial promoters.

Integrase deficient lentiviral vector (Fig. 11)

The described in this subparagraph, psPAX2-IntD64V lentiviral vector, contains a point mutation in the amino acids sequence (D64 residue) of a protein called integrase (Shaw and Cornetta, 2014).
To obtain the IDLV, the psPAX 2 vector was used in the process of site-directed mutagenesis (SDM). The psPAX2 due to its size (over 10.000 bp) and the risk of mutations in the fragments that are important for its functioning, could not be used in the SDM. Only relevant part of the vector was amplified by using the subsequent primers:

forward: psPax2_forw: 5'gggtgcccacactaatgatgtgaaa3'

reverse: psPAX2_rev: 5'tcccctgcactgtaccccc3'

Fragment for amplification was removed from the psPAX2 plasmid at the unique interface of AlfII and SwaI. The PCR product was then ligated into the cloning vector pJet1.2 (Fermentas), which contains the same restriction sites. The size of the cloning vector after ligation was 4 kb and was suitable for introducing the point mutation in the sequence of the chosen protein. The amino acid residue aspartic acid (D) was substituted by valine (V). The primers for SDM were designed with mismatched base pairs and used for the PCR of the entire Pjet1.2 vector. In this reaction the Phusion® High-Fidelity DNA Polymerase was applied.

The following primers were utilized:

forward: IntD64V_forw_1: 5'gcagctagtttgtacacatttagaaggaaaag3'

reverse: IntD64V_rev_1: 5'catattcctgggctacagtctacttgtc3'

The sequence coding the integrase protein with the introduced point mutation was cut out from pJet1.2 vector with the restriction endonucleases AflII and SwaI. The two mentioned restriction enzymes were applied in a double digest reaction, where the modified protein sequence was pasted back to the psPAX2 vector.

3.1.13. Sequencing

The sequencing was conducted in order to ensure, that the positive clones after ligation, or the prepared vector constructs do not contain any mutations. This check was performed by an external laboratory StarSEQ®GmbH.

Each sample for sequencing, prior to sending, was prepared in the following manner:

1. 400-700 ng DNA,
2. 1 µl of forward or reverse primer,
3. filled up with dd H_2O to the final volume of 7 µl.

3.1.14. Viral test

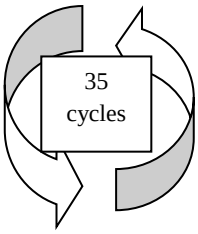
In order to transfer the transduced ESCs from S2 area to the S1 laboratory it is crucial to exclude any content of viral particles in the medium. The mRNA was isolated from the supernatant with the QIAamp® Viral RNA Mini Kit. The aliquots of supernatant (150 µl) were prepared from medium which was exchanged to HEPES buffer in a clone picking procedure (subparagraph 3.2.6.). The cDNA was synthesized and used for the PCR screen to check possible content of viral particles.

Reaction mix:

Component	Volume
H_2O	3 µl
2(x)Immo Mix	5 µl
HIV1_PSS_F	0.5 µl
HIV1_PSS_R	0.5 µl
cDNA	1 µl
Total volume	**10 µl**

PCR set up:

Time	Temperature
10 min	95 °C
10 s	95 °C
10 s	53 °C
10 s	72 °C
1 min	72 °C
∞	4 °C



3.2. Cell culture methods

The cell culture experiments were performed under sterile conditions in the clean laminar flow hoods and using sterile materials and aseptic techniques. All cell lines were kept at 37 ºC with 5% of CO_2 concentration and in 95% of relative air humidity incubators. Thawing, splitting and freezing of the cells were conducted under the general rules of the cell culture.

When it was important to know the exact number of passaged cells, cells were counted. Cell counting was performed using the Neubauer counting chamber. Cells were counted within four large squares, next the cell concentration in the suspension was calculated according to the following equation: cell number/ml = number of counted cells / 4(x)10^4 (x) dilution factor.

Mycoplasma test

None of the cell culture is free from risk of mycoplasma contamination, which are the smallest and simplest self-replicating prokaryotic organisms. In the course of all cell culture experiments, cell batches were checked routinely for mycoplasma presence.

Mycoplasma testing was carried out by use of PCR method. Samples for mycoplasma check were prepared in two ways. In the first the aspirated medium, from the plate of tested cells, was centrifuged and then the pellets were resuspended in 20 µl of water. Samples were heated in the 97 °C for 5 min. Alternatively, the samples for testing were obtained from purification of genomic DNA from cells. In this case the cell suspension was spun down for 5 min at 200 (x) g and then washed once with 1 ml of PBS. After the last centrifugation the genomic DNA was isolated by use of DNeasy Blood and Tissue Kit. Following controls were included in the experimental set up:

- positive control, a sample know to be contaminated
- internal control consisting of a DNA fragment with the same primer sequences for amplification, the internal control is of a different size than the amplicon of mycoplasma-contaminated samples

For each sample (including controls) to be tested PCR reaction mixes were prepared in two configurations:

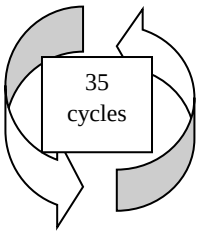
- one containing the internal control,
- second without the internal control.

Reaction mix:

Component	Amount
H_2O	7 µl
2(x)MangoMix	10 µl
myc-fwd primer	0.5 µl
myc-rev primer	0.5 µl
Template	1 µl
Internal control (or without)	1 µl
Total volume per reaction	**20 µl**

PCR set up:

Time	Temperature
3 min	95 °C
30 s	95 °C
30 s	56 °C
10 s	72 °C
2 min	72 °C
∞	4 °C



The size of DNA fragments amplified, depending on the mycoplasma species, would appear at around 270 bp, indicating a mycoplasma contamination. The specific primers used for this PCR reaction are specified in the paragraph 2.5.

3.2.1. Isolation and inactivation of mouse embryonic fibroblasts

Embryonic stem cells need feeder cells (FCs) for the optimal growth. The cells which fulfill this task are mitotically inactivated mouse embryonic fibroblasts (iFCs). The MEFs are the primary cells which were isolated from mouse embryo at E13.5 to E14.5 of pregnancy. The mice were sacrificed by cervical dislocation. The animals were sprayed with alcohol and the abdomen was opened with flamed surgical utensils and the uterine horns containing embryos were dissected out when placed in a culture dish containing 10 ml of PBS. The uterus was transferred to the dish containing fresh PBS and swirled few times to remove blood. The uterine wall was opened with sharp forceps and the embryos were removed from uterus and amniotic sac and washed 3(x) times in PBS buffer. The amniotic sac, main vessels, mice heads and other tissues were discarded. The remains of the embryo were once again washed 2-3(x) in PBS and kept on ice. Each embryo was minced with small scissors or a new scalpel blade for 10-15 min. Then 2 ml of trypsin/EDTA (x) 2 solution (made from 10 (x) stock solution) was added and tissues were further minced. Additional 5 ml of trypsin/EDTA solution were added and pipetted up and down several times to break up tissue chunks and generate a cell suspension. The plate was incubated in 37 °C for 15 min with gentle agitation. To stop the enzymatic digestion 20 ml of MEF medium was added and mixed well to resuspend the cells. The suspension was centrifuged at 200(x)g for 5 min. The pellet was suspended in 30 ml of fresh MEF medium and distributed between 3(x) 10-cm cell culture plates, constituting passage 0. Cells were incubated overnight in 37 °C. The following day medium was exchanged. After reaching a confluence of 70-80%, MEFs were frozen for long term storage or passaged for further expansion.

MEFs as a primary cells have a limited lifespan in culture. In order to make use of these cells, as cells which provide the matrix and nutrients for ESCs, fibroblasts need to be mitotically inactivated. The interference with the ability to divide was completed with mitomycin C, which is isolated from *Streptomyces caespitosus* and inhibits DNA synthesis and nuclear division.

Protocol for preparation of feeder cells:

1. Defrost two vials of primary fibroblasts and plate cells on two 15-cm cell culture plates (MEFs medium).
2. Propagate the cells for two consecutive passages to reach the number of 10(x) 15-cm cell culture dishes.
3. When cells are confluent replace the medium for the one containing re-suspended mitomycin C (10 µg/ml final concentration). Incubate for 2 h but not longer than 4 h.
4. Aspirate the inactivation medium and wash cells 3(x) with PBS.
5. Trypsinize the cells, spin down and re-suspend in freezing medium. Distribute between 50 pre-labeled cryotubes.

Each time fresh cell culture dishes were coated with the 0.2% (w/v) gelatin solution and incubated for 40-60 min in the incubator (or left overnight for the next day). Shortly before using the plate, gelatin solution was aspirated. Cell from one cryovial after defrosting are enough to cover 6-8(x) 35-mm plates. These are the standard culture plates on which ESCs will be grown.

3.2.2. Murine embryonic stem cells

3.2.2.1. Cultivation of murine embryonic stem cells

The routine cell culture of ES cells starts from preparation of feeders. Mitotically inactivated MEFs are plated one day earlier on gelatinized plates. One hour before seeding mES cells, the complete medium is exchanged to the one containing LIF. The medium was exchanged every day and cells were split every second day at the ratio of 1:20 or 1:50.

3.2.2.2. Differentiation of mESCs

Upon removal of factors which maintain ES cells in unspecialized state, cells will start to differentiate. When cells are allowed to aggregate will form three-dimensional structures embryoid bodies (EBs), which give rise to derivatives of the three embryonic germ layers (Keller, 2005).

In order to trigger the differentiation processes ES cells need to be pre-differentiated. At that step, feeder depletion and adaptation to the medium without LIF was accomplished. Embryonic stem cells after passage were seeded on freshly gelatinized plate and after around 45 min the medium containing ES cells, which did not settled yet, was transferred on to a new gelatin coated plate. Cells were kept in ESC complete medium without LIF, for the next two days. After trypsinization, mESCs were counted and in a number of one million transferred on the 10-cm Petri dishes, which did not have an adherent surface or neither were coated with gelatin. The mES cells now were kept in 15 ml of ESC complete medium in a constant movement. To provide such conditions, Petri dishes were placed on the plate shaker and continuously gently agitated.

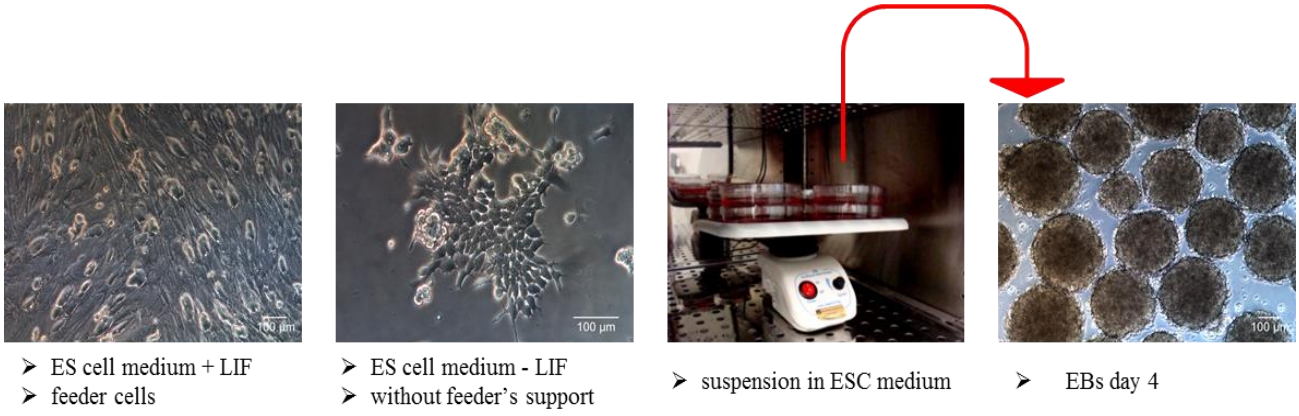


Figure 19: Stages of ES cells differentiation: standard cell culture, pre-differentiation and EBs formation.

3.2.3. Xenobiotic-free media

Protocol for serum- and feeder-free adaptation of murine ES cells:

1. Thaw one vial of feeder cells and plate them on gelatinized 6-well plate in IMDM containing 20% (v/v) FCS.
2. Defrost one vial of ES cells and seed them in one well of the 6-well culture dish in SFES medium (paragraph: 2.8.4.)
3. When the cells become confluent, passage mESCs in the 1:5 ratio onto remaining wells with iFCs.

4. Prepare two new gelatin coated 6-well plates without feeders. Trypsinize all five wells with ES cells. Wash cells with IMDM and spin down. Split cells in a 1:2 ratio. Cells from now on are maintained in the 2i medium (paragraph: 2.8.4.)

After feeder depletion cells can be now passaged on the nine new gelatinized 6-well plates. The splitting ratio was 1:5. To inhibit the enzymatic activity of trypsin the "STOP" solution was used (paragraph: 2.8.4.). Cells were washed once with IMDM after trypsinization.

5. Pre-label 100 cryovials and make sufficient amount of freezing medium (paragraph: 2.8.4.).
6. To simplify and speed up the whole procedure, one can add trypsin to all 50 wells at the same time and pull content of 5 wells into one 10 ml conical tube (step repeated then (x) 10). After washing re-suspend the pellet with 10 ml of freezing medium and distribute quickly between 10 cryovials. Repeat the steps with the remaining nine conical tubes.
7. Place all cryovials in the -80 °C freezer and after 24 h move them to the liquid nitrogen storage tank.
8. To start the new 2i culture, thaw one cryovial of feeder-free mESCs into concial tube containing 10 ml of IMDM. Spin down and plate in the 2i medium on the 6-cm culture plate. Plate should be coated in advance with gelatin or VitronectinXF™.

3.2.4. HEK 293T cells

The human embryonic kidney cells were used for production of viral particles according to the work presented by Naldini (Naldini et al., 1996). The maintenance of HEK cells required the use of 10-cm dishes and culture in HEK medium (paragraph 2.8.2.).

3.2.4.1. Viral particle production

In order to produce viral particles, HEK cells were seeded at the amount of 5(x) 10^5 cells per well on 6-well plate. The transfection mix was freshly prepared just before the procedure. The mix consisted of five components:

1. DMEM medium.

2. Transfer vector (from the vectors bank/accordingly to requirements of the planned experiment).
3. Packaging vector (psPAX2).
4. Enveloping vector (pMD2.G).
5. Transfection reagent (Turbofect).

To prepare the mix (per one well) 3 µg of total DNA (vector ratio 3:2:1 in the order used above) were diluted in 300 µl of DMEM. Turbofect was briefly vortexed and added in an amount of 5 µl. All the ingredients were gently mixed and incubated in the laminar flow hood for ~20 min. The transfection mixture was added on each well in drop-wise manner. Plate was gently rocked to achieve equal distribution of the mix. The expression of the transgene was observed after 48 h. To obtain the viral particles from the supernatant, the medium was collected and passed through the 0.20 µm cell strainer. The stocks of viral particles were used directly for the transduction or kept at -80 °C.

3.2.5. Mouse endothelioma cells

The mouse endothelioma cells (mEndo) were routinely cultivated on 10-cm gelatin coated plates and maintained in mEndo medium (paragraph 2.8.2.). Before the splitting, after medium removal, cells were incubated with PBS for around 20 min, to facilitate the cell detachment, the passage ratio was usually 1:5. The mouse endothelioma cells were used to validate the vector constructs applied for generation of reporter cell lines.

3.2.6. Stable transduction of mouse embryonic stem cells

Since the procedure is extensive, the most convenient way to present the method is to divide the protocol description into the particular days.

Day 1

Plate 5(x)10^6 HEK cells on 10-cm dish.

Day 2 Transfection of HEK cells with specific vectors using TurboFect

1. Replace the medium on the dish with 8 ml of fresh medium.

Note: Prepare transfection reagent immediately before transfection like described in the subsection 3.2.4.1.

2. Incubate the transfected cells and analyze the transgene expression after 24 to 48 h.

Day 5 Transduction of ES cells

1. Seed $3(x)10^3$ (per well) of ES cells in 6-wells of 24-well plate.

Note: The rest of the procedure from now on must be conducted in a S2 laboratory.

2. Prepare the required number of 0.5 ml reaction tubes containing 0.8 µl of polybrene.

Note: To obtain the right amount of cells per well and to double the amount of LIF in the medium, collect 18.000 cell in a conical tube and resuspend them in 3 ml of complete medium and add 6 µl of LIF (1.000 U of LIF per 1 ml of the medium). Distribute between the six wells, giving 500 µl of the cell suspension per well.

3. Take the supernatant from each well of the 6-well plate and pass through syringe filter.
4. Mix 0.5 ml of the viral particles with the content of the 0.5 ml reaction tube and add to the ES cells.
5. Freeze the remaining supernatant.
6. Prepare 10-cm dish(s) with iFCs one cryovial per dish is sufficient.

Day 6 Maintenance

1. Change the medium on the plates with iFC for the medium containing LIF.
2. Wash the 24-well plate containing ES cells with HEPES buffer.
3. Add few drops of trypsin/EDTA.
4. Take 1 ml of medium from a 10-cm plate and add to the well to stop the enzymatic activity, suspend cells well to avoid clumps and distribute evenly on 10-cm dish with feeder layer.

Day 8 Maintenance

Wash the 10-cm plates 2(x) with HEPES buffer and exchange the medium.

Day 9 Maintenance

1. Wash the 10-cm plates 2(x) with HEPES buffer and exchange the medium.
2. Prepare 96-well plate(s) with iFCs, using one cryovial per plate.
   Each well should contain 100 µl of medium.

Day 10 Clone picking

Note: Before picking clones collect the culture supernatant for virus testing.

1. Remove the medium from a 96-well plate and replace with the ESC complete medium (100 µl per well).
2. Prepare V-bottom 96-well plate(s) with 50 µl of trypsin/EDTA solution.
3. Take the first 10-cm plate and aspirate the medium and wash the cells once with HEPES buffer. Leave the cells in 15 ml of new buffer.
4. Pick carefully all non-differentiated colonies of ES cells with a yellow tip (200 µl) and put on the 96-well plate with trypsin/EDTA.

   Note: The 20 min time frame, while picking the clones, should not be exceed.

5. Transfer the colonies on the 96-well plate with seeded iFCs (containing medium supplemented with LIF) and mix well.
6. Repeat with remaining plates and incubate overnight.

Day 11

Exchange the medium on the 96-well plate(s).

Day 12 Duplicating plates, pre-differentiation.

1. Gelatin coat 96-well plates, 2(x) per plate with clones.
2. Prepare freezing medium: 10% (v/v) DMSO with 45% (v/v) FCS and 45% (v/v) DMEM.

3. Put 150 µl of ESC complete medium in each well of one of the gelatinized plates.
4. Wash cells with 100 µl of HEPES buffer and add 50 µl of trypsin/EDTA.
5. Transfer 10 µl from trypsinized cells on the plate containing ESC complete medium.
6. Stop the trypsin activity with 40 µl of ESC medium.
7. Add 80 µl of freezing medium and mix well. Overlay each well with 50 µl of mineral oil, cover with parafilm and freeze (first plate).
8. After approx. 1 h transfer the entire medium, from the second plate, onto a new gelatinized 96-well plate and placed in the incubator.

Note: This step helps to remove feeders.

Day 14 Differentiation - EB formation

1. Coat 96-well plate with polyhema and leave for drying, next wash once with PBS.
2. Wash cells with HEPES buffer then add 20 µl of trypsin/EDTA and put for several minutes into the incubator.
3. Add 180 µl of complete medium to each well, mix and move on the earlier prepared 96-well plate.
4. Leave the plate in the incubator for four days to observe EB formation.

Note: When the EBs start to express GFP, plate the EBs and wait for formation of vessel-like structures. Then according to the number of the clone (well), mark those which are positive.

3.2.7 Cultivation of iPS cells

The iPS cells were grown either on a mitotically inactivated feeder cell layer or on Matrigel (Geltrex®).

Preparation of Matrigel plates:

Matrigel is a matrix, which is made of soluble basement membrane extract of the Engelbreth-Holm-Swarm (EHS) mouse tumor. This matrix gels at room temperature to form a genuine reconstituted basement membrane.

The Geltrex® should be aliquoted and frozen. All the preparation should be made on ice to prevent the matrix from solidifying.

Geltrex was diluted 1:1 with DMEM/F-12 medium and frozen in 250 µl aliquots:

- for 1:50 dilution add 6 ml of DMEM/F-12 to one aliquot
- for 1:25 dilution add 2.9 ml of DMEM/F-12 to one aliquot

1. Thaw one aliquot of Matrigel on ice.

2. Dispense 24 ml of cold DMEM/F 12 into a 50 ml conical tube and keep on ice.

3. Add thawed matrix to the cold DMEM/F 12 and mix well.

4. Immediately use the diluted Geltrex® solution to coat tissue culture plate.

5. It is very important to swirl the cultureware to spread the solution evenly across the surface.

6. Incubate at room temperature for at least 1 h, before use, remove the excess of Geltrex® solution (do not scratch the coated surface).

3.2.7.1. Murine iPS cells

Mouse induced pluripotent stem cells (miPS) were generated by transduction of genetically unmodified mouse embryonic fibroblasts with viral particles produced in HEK cells. To harvest the viral particles first the HEK cells were co-transfected with: psPax2, pMD2.G and OSK.

Maintenance of mouse iPS cells

1. Aspirate the medium and wash the cells twice with 2 ml of PBS. Remove PBS and add 0.5 ml of trypsin/EDTA solution and incubate at 37 °C for 10 min.
2. In the meantime, remove medium from 6-well plate with seeded feeder cells and add 2 ml per well of fresh complete ES medium.
3. Swirl the plate containing mouse iPS cells to remove the cells from the bottom of the plate. Add 1 ml of ES medium to the plate and re-suspend the cells by pipetting up and down to obtain a single cell suspension.
4. Distribute 0.2 ml of the mouse iPS cell suspension to each well of the 6-well plate.
5. After plating iPS cells, gently swirl the plate back-and-forth to evenly distribute the cells.

6. The ES medium must be changed every day and miPS cells need to be sub-cultured (ratio ~1:10) every two days.

3.2.8. Fluorescence and confocal microscopy imagining

In the course of the studies, apart from a conventional widefield optical microscope, the confocal microscope was used. Confocal microscopy gives noticeable advantages over a conventional optical microscopy through controlled depth of field and elimination or reduction of background information from the focal plane (Olympus corporation, www.olympus-global.com). The immune fluorescent (IF) methods are equally suitable for fresh as for fixed samples. The IF techniques are based on antibodies which are chemically conjugated to fluorescent dyes. The labeled antibodies bind (directly or indirectly) to the antigen of interest, which allows for its detection, by for example flow cytometry or visualization by fluorescence or confocal microscopy (Sawant et al., 2014).

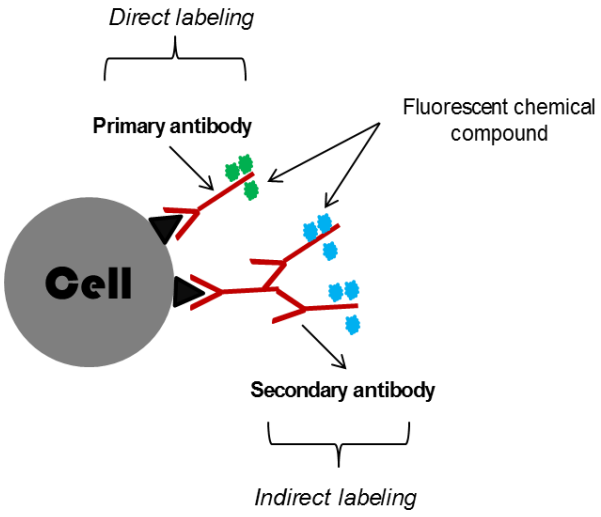


Figure 20: Immunofluorescence techniques: direct and indirect labeling. Adapted from: Sawant, Priyanka, et al. (2014).

3.2.8.1. Immunofluorescent staining of cells

The ES cells were stained to inspect the expression of pluripotency markers. In this subparagraph the general protocol for cell staining is provided. The ES cells for staining were grown in a 6-well plate. The medium in which cells were grown depended on the experiment setting. In each well four small, round cover slips (Ø13 mm) were placed. The cover slips were first immersed for 10 min in

70% (v/v) ethanol and coated with gelatin solution. After letting the cells grow and expand for two days, the medium was aspirated and the following steps were performed:

1. Wash cells once with PBS.
2. Add 1% (n/v) PFA and incubate for 20 min.
3. Aspirate PFA and add 0.1% (w/v) Triton X-100 and leave for 20 min.
4. Block with blocking solution for 30 to 45 min. The blocking solution: 5% serum (from the same species that the secondary antibody was raised) in PBS.
5. Remove blocking solution and wash once with 0.01% (v/v) PBST. Apply adequately diluted primary antibody in 100 µl (per cover slip) of blocking solution. Incubate for 1 h in room temperature.
6. Aspirate the primary antibody solution and wash once with 0.01% (v/v) PBST.
7. Apply secondary antibody at the required concentration in 100 µl of blocking solution. Transfer cover slips to a black box to protect from photobleaching. Incubate slides for 1 h at 22 °C.
8. Remove the secondary antibody solution and wash extensively with 0.01% (v/v) PBST.
9. Apply solution of counter staining dye and incubate for 5 min, do not extend the incubation time as this may result in a high background.
10. After washing carefully transfer cover slips (with a scalpel blade) on to the microscope slide and mount cover slips with MOWIOL (turn the cover slips upside down before mounting). Store the specimens at 4 °C in a microscope slide holder.

When conjugated antibodies were used step 7 was omitted. With each staining IgG controls were applied or the specimen was stained with secondary antibody only in order to verify its specificity.

For all washing steps a phosphate-buffered saline with Tween 20 (PBST) was used. PBST is a water-based salt solution, perfect as a wash buffer because the buffer reduces nonspecific binding, protein:protein interactions and background.

Preparation for 1(x): 10 mM phosphate, 150 mM NaCl, and 0.05% (v/v) Tween 20, pH adjusted to 7.4, stable for 1 year at room temperature.

3.2.8.2. Immunofluorescent staining of embryoid bodies

The embryoid bodies were stained to observe the differentiation, monitor the formation of vessel-like structures and expression of endothelial specific markers. In order to stain these three-dimensional structures, the EBs needed to be re-plated. The EBs at a given time point were collected in the middle of the plate by gentle swirling the dish. The medium was carefully aspirated and replaced with PBS. All EBs were then again collected in the middle of the culture plate. With use of a 1ml pipette tip, set at the smallest possible amount (~80 µl), the embryoid bodies were transferred into the wells of a 6-well plate. The 6-well plates with cover slips were prepared as in the protocol in subsection 3.2.8.1. The EBs were cultured in ESC complete medium until EBs expanded on the cover slips and developed vessel-like structures. On the day of staining the following stages were implemented:

1. Remove the medium and wash cells once with PBS.
2. Fixing (30 min) and permeabilization (10 min) steps depend on the antibody used. Therefore, the below provided adjustments were carried out:
    - for Ve-Cadherin staining 2% (v/v) PFA then 1% (v/v) Triton X-100,
    - for CD 31staining 2% (v/v) PFA then ice cold methanol,
    - for Flk1staining only ice cold methanol.
3. Block with blocking solution for 45 min.
4. Apply the primary antibody, diluted accordingly in 100 µl of blocking buffer for 1 h at room temperature.
5. Wash once with 0.01% PBST. Add secondary antibody solution, leave protected from light for 1 h at 22 ºC.
6. Wash 3(x) times with 0.01% (v/v) PBST and incubate for 5 min with nuclear staining solution.
7. Wash extensively and carefully transfer cover slips to the microscope slide (invert cover slips) and mount with MOWIOL.
8. Store the specimens in a microscope slide holder at 4 ºC.

The same procedure was applied to the GFP expressing cells. The used controls were unchanged, according to the subparagraph 3.2.8.1. If the cells had a transgene and were expressing GPF the antibody against GFP was applied simultaneously with primary antibody solution.

3.2.9 Flow cytometry analysis

Flow cytometry technology is used to study the physical and chemical features of particles suspended in a fluid as it passes through a laser beam. The cell components which are fluorescently labelled become excited by the laser and emit light at variable wavelengths. The estimation of number, shape and size of the sampled cells is measured on the basis of the fluorescence. Even up to thousands of events per second can be evaluated as they pass through the liquid stream.

This technique was utilized to:

1. Validate the functionality of produced vectors (transfection on mEndo cells).
2. Estimate the quality and amount of produced viral particles.
3. Investigate the expression of GFP under the endothelial specific promoters.
4. Survey the "peak" point of GFP expression in the developing EBs.
5. Compare expression levels of Oct4 and Sox2 in ES cells cultured in different conditions.

Protocol for preparation of cells expressing GPF for flow cytometry analysis (applicable to points from above 1 to 4):

1. Aspirate medium and wash cells once with PBS
2. Trypsinization:
    a. Add appropriate amount of trypsin/EDTA and incubate plate(s) for 10 min to speed up the enzymatic activity.
    b. In case of EBs preparation, collect them in the middle of the Petri dish and transfer sufficient amount of the cell aggregates to the 15 ml tube. Wash once with PBS and centrifuge at 200 (x) g for 5 min. To dissociate the EBs use 200 µl of dissociation solution (see below) and incubate for ~30 min. Stop the enzymatic activity with 500 µl of FCS. Wash once with IMDM or PBS, continue with point 4.

3. Detach cells from the plate and re-suspend cells with adequate amount of medium to stop the trypsinization.
4. Transfer cells to a 15 ml tube containing 5 ml of FACS buffer. Spin down for 5 min (x) 200g. Aspirate the liquid and repeat the washing step.
5. Re-suspend the pellet in 500 µl of FACS buffer and transfer to FACS tubes.
6. Approximately 5 -10 min before conducting the experiment add 5 µl of 7-Aminoactinomycin D (7-AAD) ready to use staining solution. Mix well and proceed with analysis.

   Samples ready to investigate generally should be kept on ice in the dark.

Used controls: samples with wild-type (no GFP expression) or un-transfected cells or transfected cells of non-endothelial origin like MEFs.

Dissociation solution (for 10 ml): mix 8 ml of trypsin EDTA with 1 ml of IMDM and 1 ml of DNase [stock 1 mg/ml]. Filter through 0.20 µm syringe strainer, keep at 4 ºC for up to one week.

Protocol for preparation of cells for flow cytometry analysis with antibody staining (point 5, p 105):

1. Points 1, 2a, 3, 4 applied as in paragraph 3.2.9..
2. Distribute appropriately cells between tubes, then fix and permeabilize with Cytofix/Cytoperm™ for 10 min on ice. After incubation add 1 ml of 0.1% (v/v) saponin solution to wash the cells. Spin down at 200 (x) g for 5 min.
3. At this stage cells were counted and in given amounts used for the experiments: $1(x)10^6$ cells for unstained control sample, $1(x)10^5$ for IgG controls and relevant samples.
4. Block cells with appropriate serum for 30 min, next wash with 0.1% (w/v) saponin solution.
5. Re-suspend the cell pellet in 100 µl (for $1(x)10^5$ cells) or in 200 µl (for $1(x)10^6$ cells) of saponin solution containing adequately diluted antibodies. Incubate for 30 min.

   Note: In all flow cytometry experiments with antibodies conducted in the thesis, conjugated antibodies were used.
6. Wash cells once with FACS buffer. The stained cells were suspended in 300 µl of the buffer, the unstained sample and controls in 500 µl.

7. Approximately 5-10 min before conducting the experiment add 5 µl of 7-AAD ready to use staining solution. Mix well and proceed with analysis.

Samples ready to investigate, generally should be kept on ice covered with tin foil. Used controls: unstained cells, IgG controls.

Flow cytometry buffers used for maintenance of the machine:

1. FACS Flow [FACS buffer] (Becton Dickinson, USA) and for re-suspending the samples,
2. FACS Clean (Becton Dickinson, USA),
3. FACS Rinse (Becton Dickinson, USA).

5% (w/v) Saponin solution: add 1 g of saponin to 20 ml of ddH_2O in a 50 ml conical tube to dissolve, mix gently as this is a detergent and may foam. Store at 4 °C for up to two weeks.

3.2.10. Software

For analyzing the data acquired during the course of experiments, following software were used:

- MacBiophotonics ImageJ - image processing program,
- Geneious R7 - DNA alignment, assembly and analysis ,
- GraphPad Prism 5 - statistics,
- Microsoft Office 2010,
- Microsoft Excell 2010,
- Microsoft PowerPoint 2010,
- Flowing Software 2.5.1. - flow cytometry analysis.

4. Results

4.1. Analysis of ESC differentiation

According, to what was mentioned at the beginning of this dissertation, the aim was to generate pure subsets of clinically-applicable endothelial cells. In order to achieve that goal, a fast, easily accessible and high-yield method to produce endothelial cells had to be established.

As it was described in the subparagraph 2.8.2., mES cells are co-cultured on a layer of mitotically inactivated feeder cells. Below are presented pictures illustrating the culture of iFCs and ES cells grown on fibroblasts.

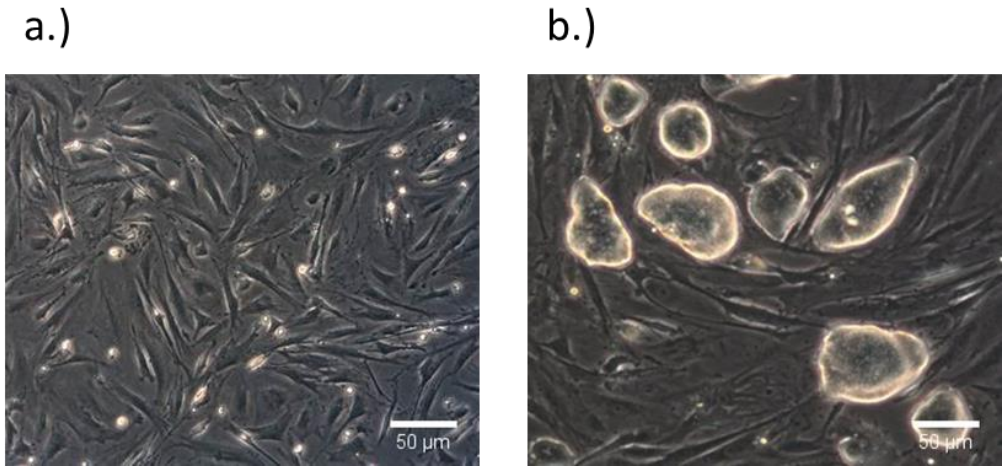


Figure 21: Cells in the culture: a.) inactivated mouse embryonic fibroblasts on a gelatin coated culture dish, b.) mouse embryonic stem cells co-cultured on the single layer of iFCs in ESC medium containing LIF.

It is important to maintain mESCs in the correct culture conditions as the cells are sensitive to changes in the environment. In the Fig. 21 picture (a) is showing inactivated fibroblasts which created a monolayer of feeder cells, in picture (b) healthy mESC colonies with shiny borders, can be observed. In the paragraph 3.2., was mentioned the importance of the mycoplasma test for the proper culture maintenance. Mycoplasma tests were carried out routinely prior to freezing the new batch of iFCs. After the PCR reaction the results excluding or confirming the mycoplasma presence were evident on the electrophoresed agarose gel (Fig. 22).

In the presented image (Fig. 22), mycoplasma-positive samples display a band at the height of ~270-300bp.

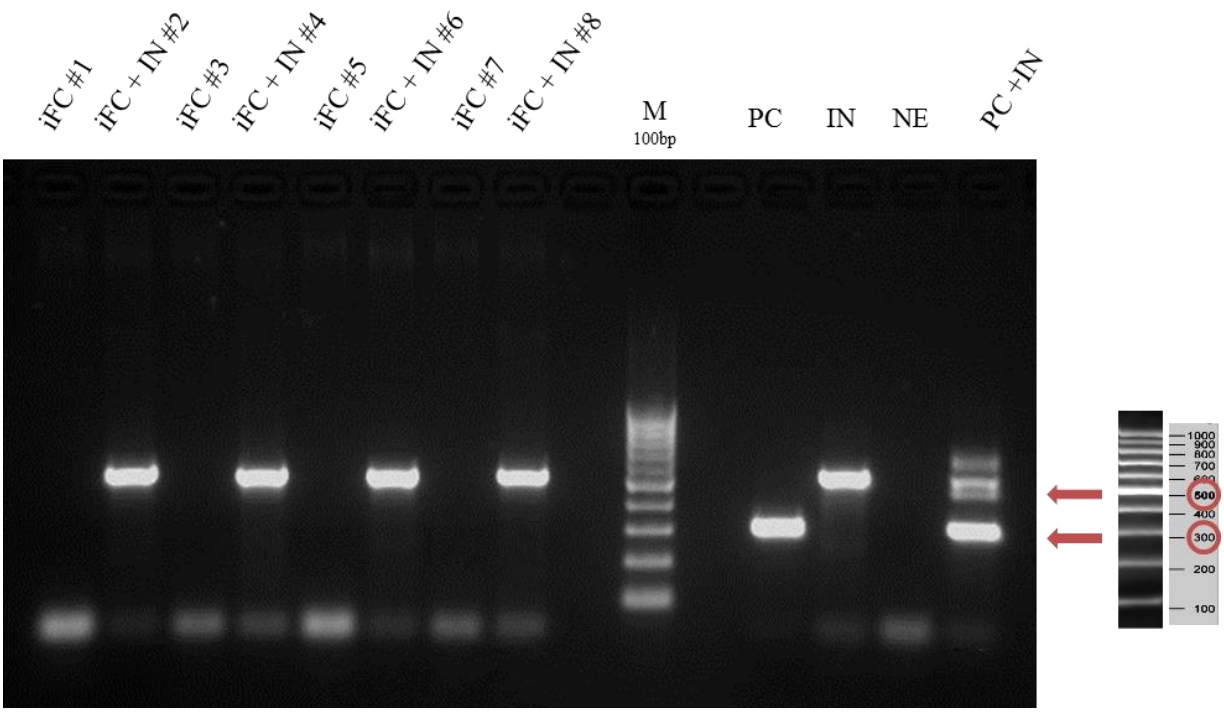


Figure 22: Test for possible mycoplasma contamination. PCR products obtained by agarose gel electrophoresis. In the samples: #1, 3, 5 and 7 as well in the one with negative control (NE) only. Samples: #2, 4, 6, 8 and internal control (IN) only. NE - ddH_2O instead of the template, PC - positive control, M - PCR marker ladder (JenaBioscence), iFC - inactivated feeder cells.

4.1.1. Generation of reporter cell line

The production of the viral vectors was described in the subparagraph 3.1.12.1. After many steps of cloning and sub-cloning the presented in Fig. 23 vector bank was created. The vectors contain combination of endothelial specific promotors and the resistance gene for antibiotic selection and the reporter gene GFP. In the designed vectors, two different, definite endothelial promoters were used. A pair of murine and a pair of human specific promoters. One of the chosen endothelial markers is Flk1 present in more progenitor cells. The second promoter used is Ve-Cadherin, which is a marker expressed in more mature endothelial cells. In case of human promoters the equivalent for Flk1 was KDR and the murine Ve-Cadherin was replaced by human gene coding that cadherin. With that pairs of promoters, the endothelial cells could have been captured in the two different stages of development.

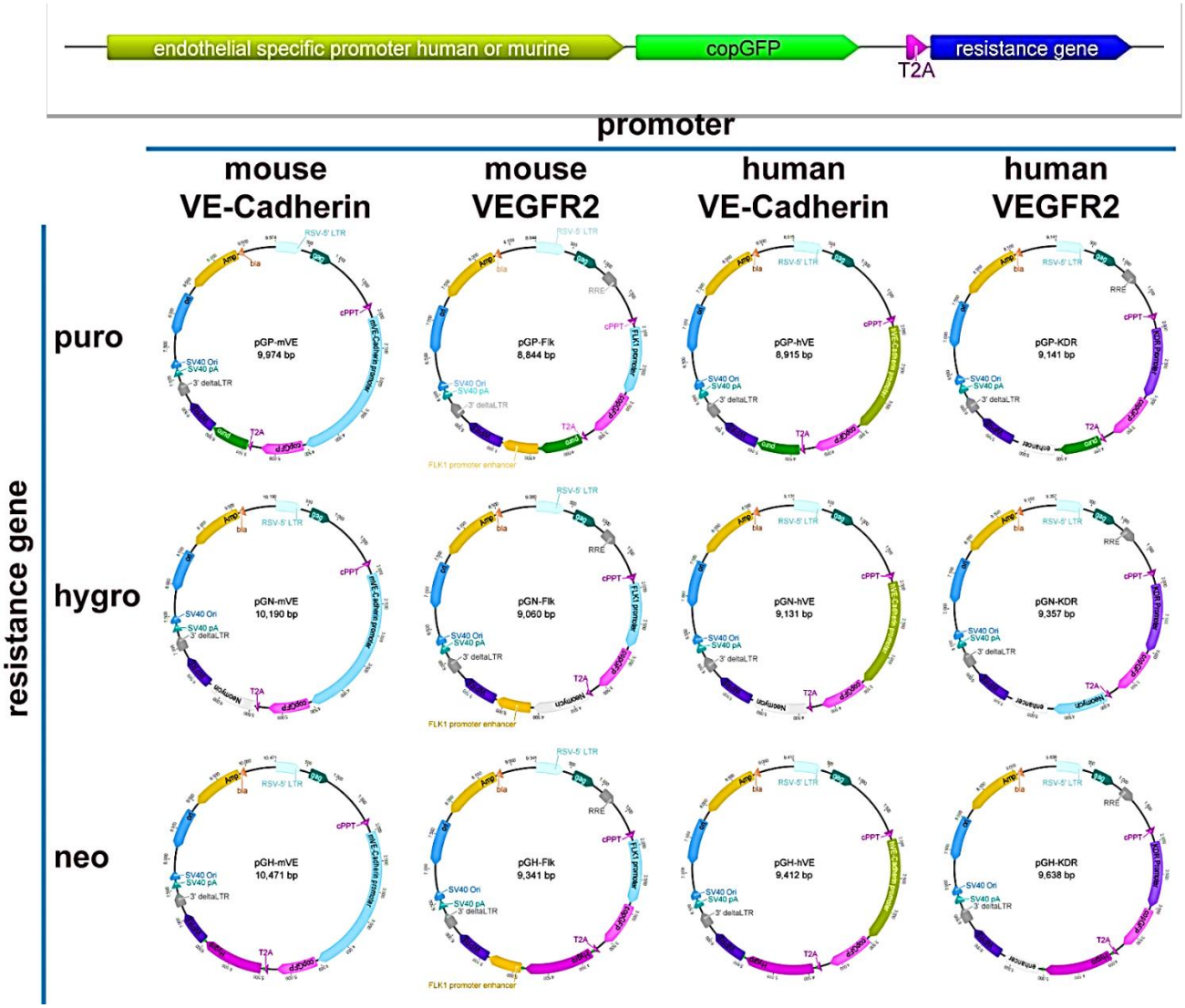


Figure 23: Vector bank. The depicted vectors present the combination of endothelial specific promoters: first two columns are showing mouse: Ve-cadherin and Flk1 promoters, the third and fourth columns are showing human: Ve-cadherin and KDR promoters. The three rows are indicating used antibiotic resistance gene: puromycin, hygromicin B and neomycin. Each vector also contains fluorescent protein GFP, which serves as an expression marker.

4.1.1.1. Optimization of the production process and validation of viral particles

To produce viral particles the HEK cells were used, next validation of the transfection efficiency was performed. The HEK cells are fairly easy to transfect and show a high transfection yield. Consistent and feasible system had been created to test the correlation between the expression and copy number. Below is presented a setup, of one from many, experiments applied to establishment the right concentration of the transfection "mixture". In regard to the subparagraph 3.2.4.1, into the transfection

mix were added helper vectors (psPAX and pMD2.G) plus pGZ_CMV vector containing GFP marker, transfection reagent Turbofect (TF) and basal not supplemented medium (DMEM) (Table 8). The ratio of the used three vectors was: 3:2:1, starting from the pGZ_CMV vector, next psPAX and lastly pMD2.G vector. The amount of the plated HEK cells also varied from $5(x)10^4$ to $5(x)10^6$, finally the chosen number of cells was $5(x)10^5$. In the Fig.24 are presented pictures which display the insensitivity of the GFP expression in the HEK cells from the tested combinations of the transfection mixtures.

Table 8: Test concentrations for the high efficiency transfections in HEK cells.

pGZ_CMV	psPAX	pMD2.G	End concentration
1µg	0.7 µg	0.3 µg	2 µg
1.5 µg	1 µg	0.5 µg	3 µg
2 µg	1.4 µg	0.7 µg	4 µg

DNA mix	Transfection reagent (TF)	DMEM
2 µg	5 µl	600 µl
3 µg	5 µl	600 µl
4 µg	5 µl	600 µl

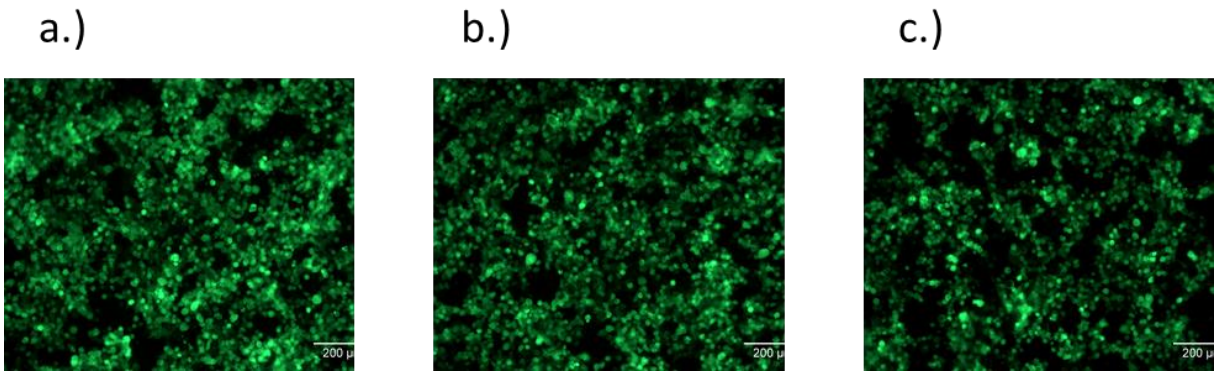
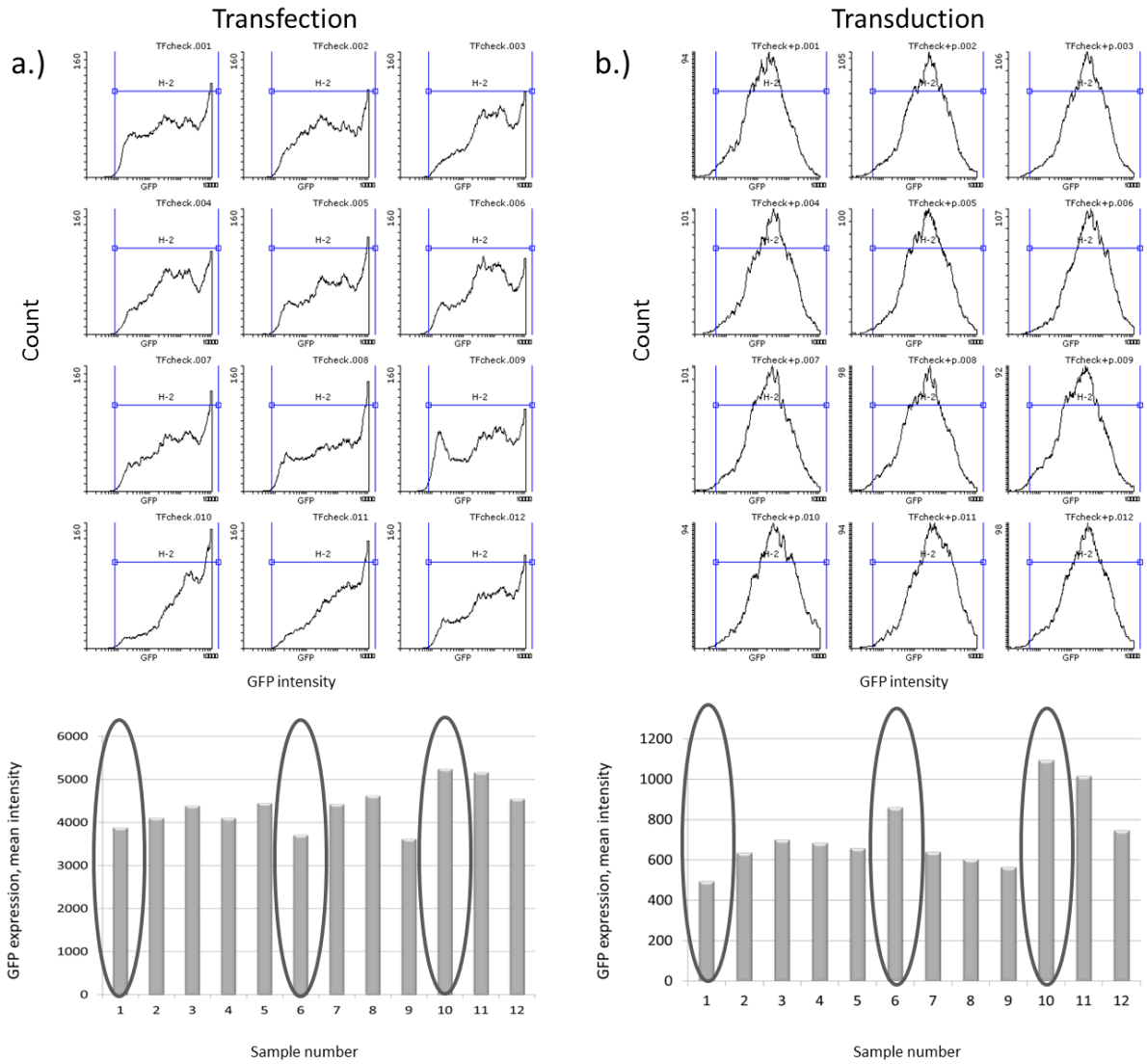


Figure 24: HEK cells expressing GFP after co-transfection. For each transfection $5(x)10^5$ of HEK cells were seeded on a plate. The amount of DNA from three plasmids (pGZ_CMV, psPAX, pMD2.G): a.) 2 µg, b.) 3 µg, c.) 4 µg used in each transfection. The transfection reagent TurboFect used in amount of 5 µl per reaction.

The task was to achieve a good quality of the viral particles and to create a stock of VP for further transductions. To achieve sufficient amount of the viral particles, after each transfection, the correlation between the transfection and transduction was verified. The presented graphs in Fig. 25 (a) and (b) show experiments, which were conducted in order to verify if there is analogy between the favourable results obtained after production of viral particles and efficiency of transduction. The data presented in the Fig. 25 panel (b) are showing experiments in which the substance called polybrene was used. Polybrene (Hexadimethrine bromide) is a cationic polymer helping to increase the efficacy of infection of certain cells with viral vectors. These experiments presumed to establish the impact from high expression of the transfected cells on the increase in expression after transduction. This interrelationship was confirmed, but this time with smaller amount of samples. The combinations of the transfection reaction to conduct the experiments presented in the Fig. 26, were taken from the specimens indicated by the ellipses in the Fig. 25.



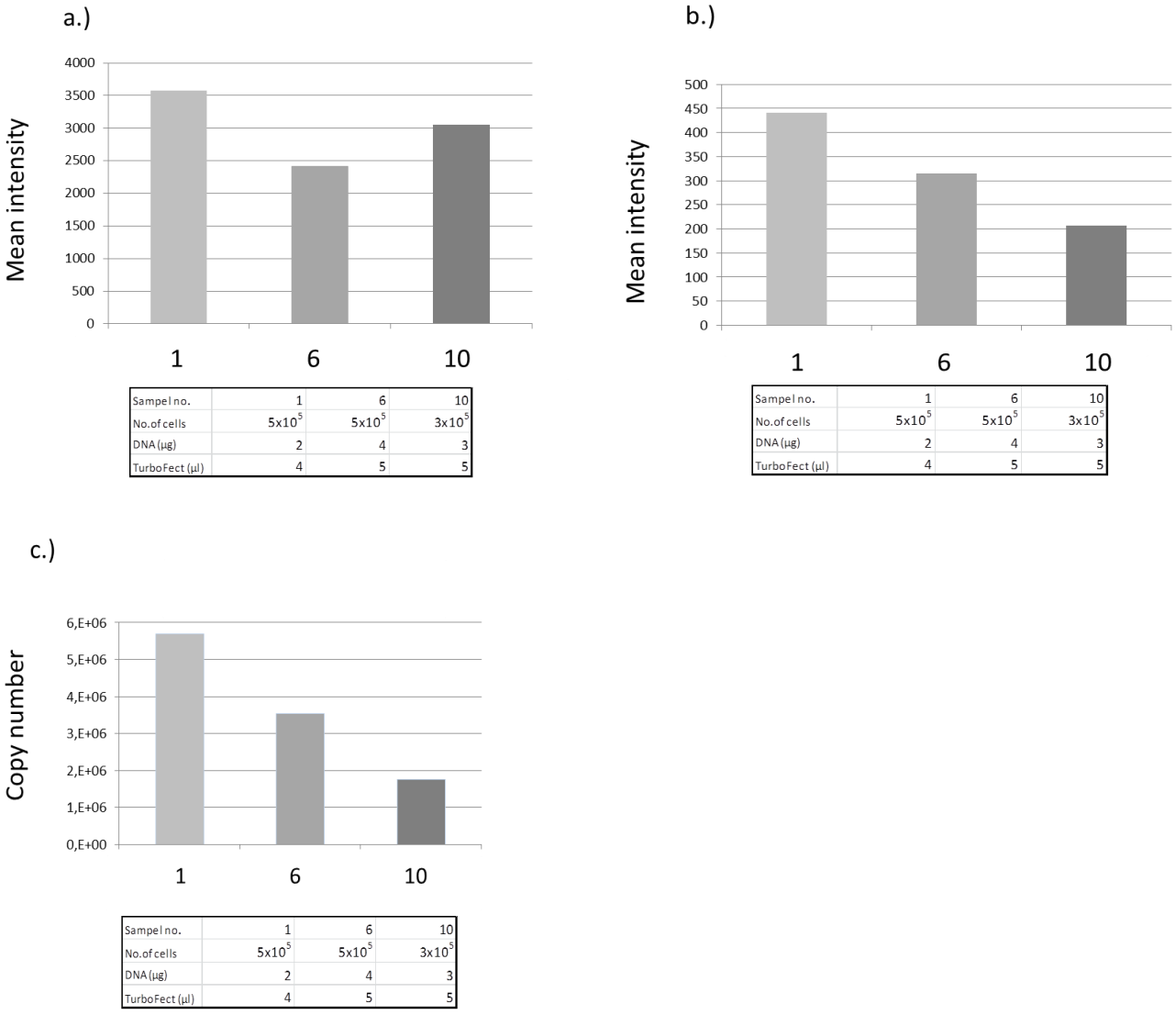
Sample no.	1	2	3	4	5	6
No.of cells	2	$5x10^5$	$5x10^5$	$5x10^5$	$5x10^5$	$5x10^5$
DNA (µg)	2	3	4	2	3	4
TurboFect (µl)	4	5	6	3	4	5

Sample no.	7	8	9	10	11	12
No.of cells	$5x10^5$	$5x10^5$	$5x10^5$	$3x10^5$	$4x10^5$	$6x10^5$
DNA (µg)	2	3	4	3	3	3
TurboFect (µl)	5	6	7	5	5	5

Figure 25: Flow cytometry analysis of GFP expression. Panel: a.) presenting GFP intensity of transfected HEK cells, b.) presenting GFP intensity of transduced HEK cells. Histograms are depicting the cell subsets with active GFP protein. Charts are showing the efficiency of those processes. The table below the chart in panel a.) is conveying information related to the experimental setup of the transfection. The sample numbers in both panels (under the both charts) are indicating the particular samples obtained from the transfection (panel a.)). The viral particles produced in that process were later used for transduction, the analyzed samples in this experiment are presented in the panel b.).

The obtained results (Fig. 25) were not evidently showing the inter-play between the transfection efficiency and the results of transduction. The next set of experiments was conducted only with specimens indicated by the ellipses in the Fig. 25.

The results in the Fig. 26 (a) are displaying the GFP expression of the virus-producing transfected HEK cells. Panel (b) in the Fig. 26 is depicting the results from flow cytometry analysis of harvested HEK cells after transduction. In case of samples 1 and 6, the correlation between the amount of viral particles and its impact on the transduction can be observed. In agreement with these observations are also data from the real time PCR test presented in panel (c) Fig. 26, which confirmed the results obtained from the flow cytometry analysis (Fig.26 (b)). The outcomes attained from the flow cytometry are reliable and delivered solid information concerning the titer of the produced VPs and the efficiency of the transduction.



Sampel no.	1	6	10
No.of cells	$5x10^5$	$5x10^5$	$3x10^5$
DNA (µg)	2	4	3
TurboFect (µl)	4	5	5

Figure 26: Validation of the titer of the viral particles. Panel: a.) is presenting flow cytometry analysis of GFP expression of transfected HEK cells, b.) is showing flow cytometry analysis of GFP expression in transduced HEK cells. In panel c.) the real time PCR data are displayed. The mRNA was extracted from the transduced HEK cells used in the experiment b.). The tables below each of the chart are indicating the conditions set up for the transfection as the viral particles production was the starting point prior to transduction.

4.1.1.2. Validation of the created vectors for establishing the stable mESC cell line

In order to verify, if the created vectors are functional, the assessment of their performance was performed with the use of mouse endothelioma cells. The mentioned immortalized cell line, described in subsection 3.2.5., was the correct tool to validate how well the vectors are working. The examinations were carried out on the basis of the GFP expression. Beneath in Fig. 27 are presented images displaying the cells expressing GFP gene under the control of specific endothelial promoter, accordingly to the transfer vector used for the transduction. The presented pictures are depicting, the expression of the GFP in the transduced cells confirming, that the specific endothelial promoters are working properly. As a negative control, MEFs were transduced with the same set of vectors and no GFP expression was observed (data not shown).

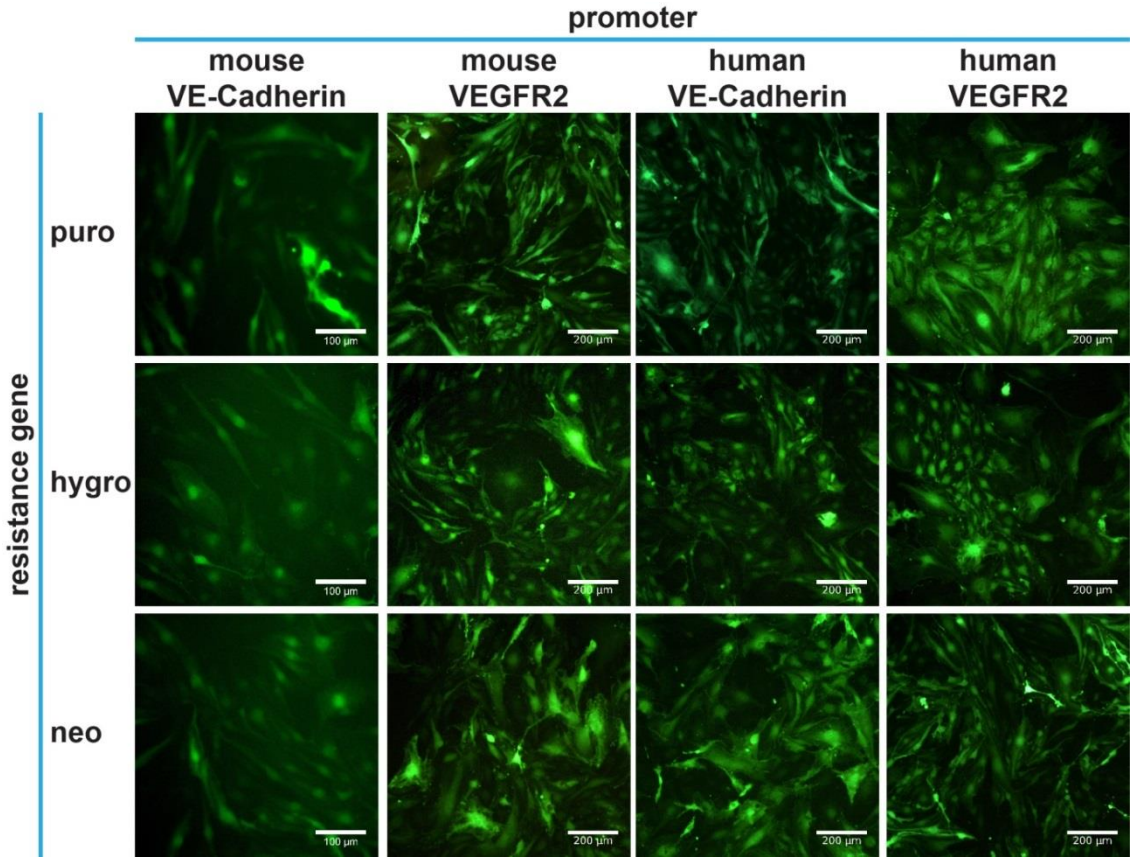


Figure 27: Functionality test of vector constructs with specific endothelial promoters. First two columns are presenting vectors containing murine promoters: Ve-cadherin and Flk1, the third and fourth columns are showing vectors with human promoters: Ve-cadherin and KDR. The three rows are indicating used resistance gene, antibiotic are as follows: puromycin, hygromicin B and neomycin. The tests were carried with the use of mouse endothelioma cell line.

The following histogram (Fig. 28) is reporting data from the flow cytometry analysis of GFP expression in transduced mEndo cells. In the graph are observed high GFP expression levels (lines: blue and red), confirming that the transduction with the generated vectors was achieved. Peaks of analyzed cell subsets were broad, reflecting the versatile states of the transduced mEndo cells. These data confirmed the heterogeneity of GFP expression levels, in different transduced cell groups.

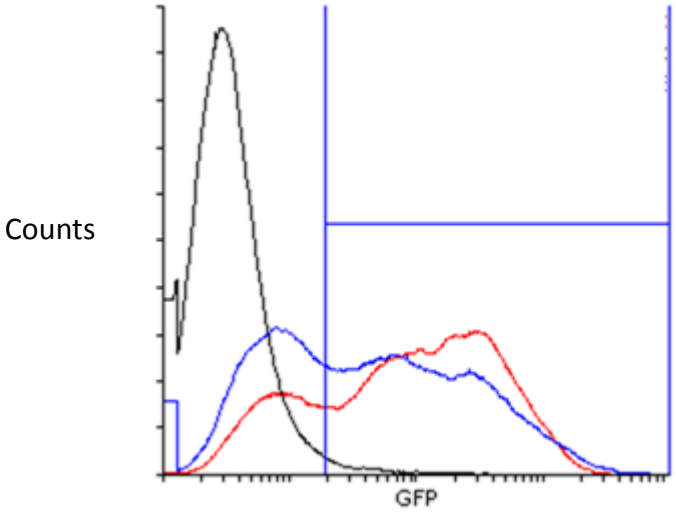


Figure 28: Flow cytometry analysis of GFP expression in transduced mEndo cells. The histogram is presenting the GFP expression, under the control of the different promoters used in the vectors. The different color lines are corresponding to: black - negative control (not transduced cells), blue - the pGP_mVe vector, red - the pGP_Flk1vector.

The experimental setup for the subsequent tests (Fig. 29) was designed in a following manner: the mEndo cells from one day after proceeding transduction were harvested and analyzed with the flow cytometry. The mEndo cells were transduced with three vectors combinations. The graphs (Fig. 29) depict the mean intensity of GFP fluorescence under the control of the specific promoters, from the transduced mouse Endothelioma cells. In the Fig. 29 (panel a) it is apparent, that the constructs containing hygromicin B as a resistance gene, displayed the lowest values of the GFP mean intensity. The second graph in Fig. 29 (panel b), exhibits results from the repeated experiments with only constructs containing hygromicin B as a selective antibiotic. That repetition aimed to achieve good efficiency of the vectors carrying hygromicin B gene. The experiment had identified best batches of viral particles for the transduction of mESCs from the best performing vectors.

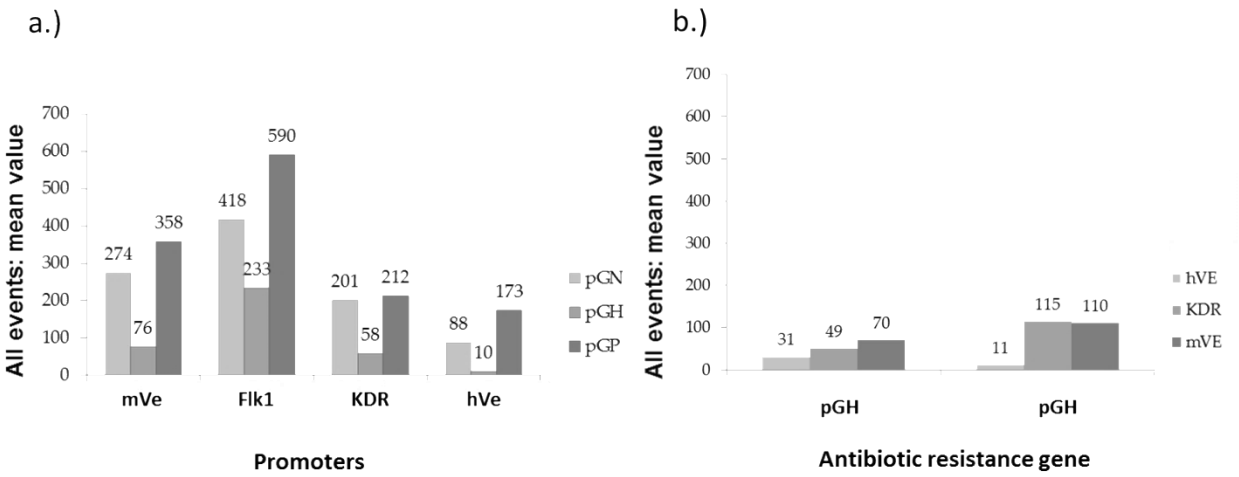


Figure 29: Quality control by flow cytometry analysis, of viral particle production. Graphs are showing mean intensity of GFP fluorescence of transduced mEndo cells under the control of the specific promoters. The used abbreviations are as follows: pGN - vector containing neomycin as a resistance gene, pGH - vector containing hygromicin B as a resistance gene, pGP - vector containing puromycin as a resistance gene, hVe - vector with human Ve-Cadherin promoter, KDR - vector with human VEGFR2 promoter, mVE - vector with mouse Ve-Cadherin promoter.

The above-presented experiment postulated to optimize different batches of produced viral particles. The result of this experiment enabled to prepare dilutions, according to the lowest achieved number from the produced VPs. The dilutions were used to transduce mEndo cells. The transduced mEndo cells were expressing the GFP at the same level (on all the plates), proving that the quality of VPs in each dilution were equal.

4.1.1.3. Transduction of mouse embryonic stem cells

The next step in the mESC differentiation protocol was the transduction of mouse ESCs with the produced and validated viral particles. After around one week, the emerging ESC clones (Fig. 30), could have been observed.

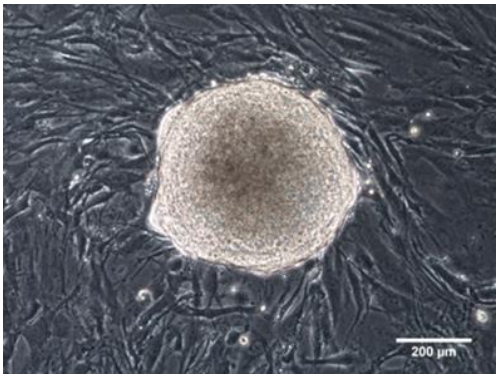


Figure 30: Clone of mouse ES cell emerging after viral transduction with the validated viral particles.

The developed clones were carefully picked under the microscope and transferred to the 96-well plates. The 96-well plates were duplicated, cells were passaged and one plate from the pair was frozen. The ESCs clones were used for the formation of embryoid bodies as it is shown in the picture (a) from Fig. 31. This step allowed for the preliminary screening and then by re-plating the EBs on gelatinized plates, for observation of the first signs of GFP expression restricted to vessel-like structures (Fig. 31 (b)). After the initial screen, in search for GFP expression under the control of the endothelial specific promoter, the main screening for vessel-like structures could begin Fig. 31 (c).

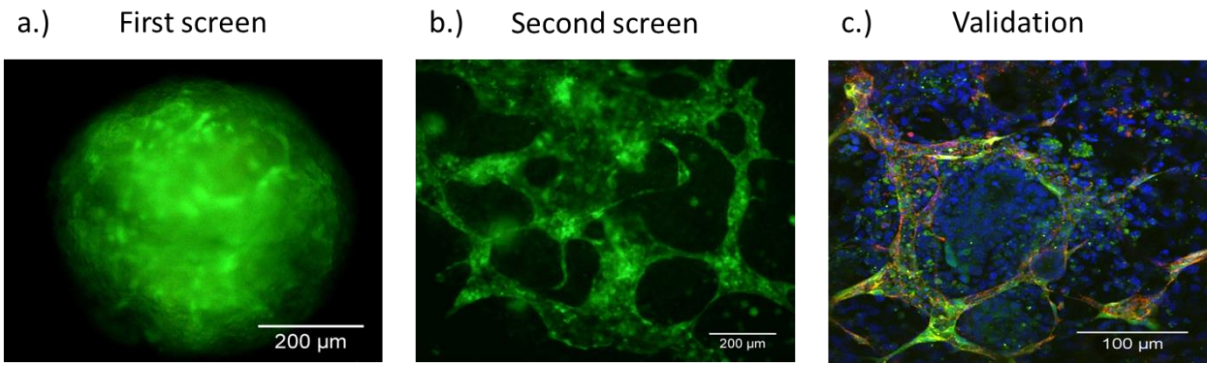


Figure 31: GFP expression under the control of the Flk1 promoter during the differentiation of a newly established murine ES cell line. Picture: a.) embryoid body in the suspension culture, showing dispersed GFP expression, b.) re-plated embryoid body with forming vessel-like structures expressing GPF, c.) re-plated embryoid body expressing GPF restricted to vessel-like structures, co-stained with the Flk1 antibody (GFP/Flk1/DAPI).

The first picture (a) displayed in the Fig. 31 demonstrates the EB expressing GFP maintained in the suspension. The GFP expression was a sign of the forming vessel-like structures, though the expression was not fully localized. Picture (b) in Fig. 31 presents more developed vessel-like structures formed in the re-attached to the surface embryoid body, here the GFP expression is more defined. The last image (c) in Fig. 31, displays co-staining with the Flk1 specific antibody. The Flk1 antibody is co-localized with GFP restricted to vessel-like structures.

4.1.1.4. Dissociation of embryoid body

Formation of EBs is commonly used as a method for initiating spontaneous differentiation toward endothelium, it is a "default" function triggered by FSC present in the medium. Tissue-like structures are often exhibited within EBs, including the appearance of blood islands, reminiscent of early blood vessel structures. The picture (a) in the Fig. 32 is presenting the standard image of the 3-D structures in the suspension culture. The panel (b) of the Fig. 32 is displaying the EB with the blood islets forming within the EB structure.

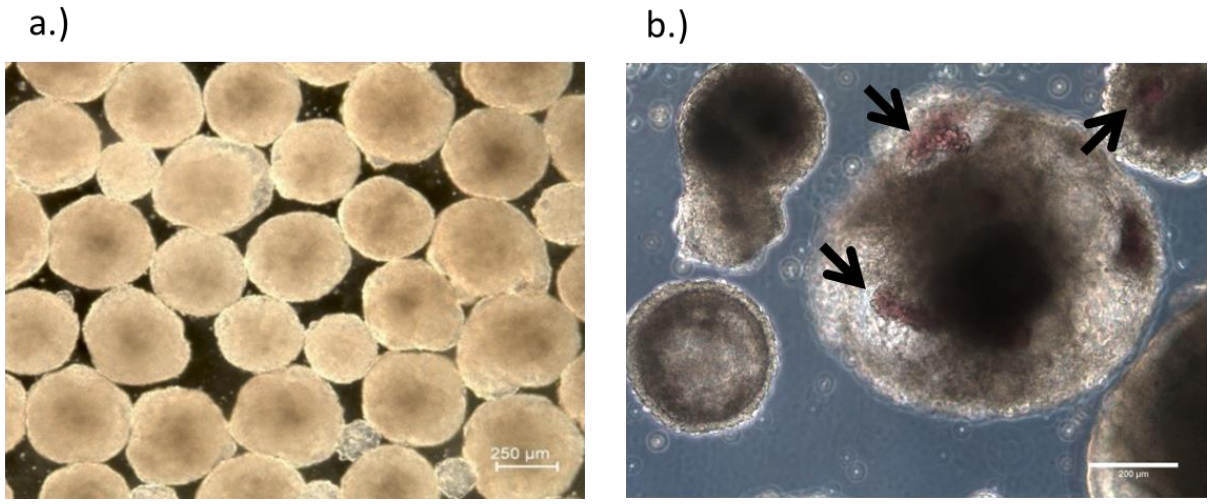


Figure 32: Three dimensional aggregates of the mES cells. Panel: a.) is showing routinely obtained EBs in suspension culture, b.) EBs in suspension with developing blood islands indicated by arrows.

The proper dissociation of EBs is crucial as it gives the possibility to obtain viable and numerous cells from the aggregates. That allows for acquisition of endothelial-like cells from differentiated mESCs and to execute necessary functionality assays. In order to achieve big number of viable cells, various methods of dissociation were tested. The viability counts were performed on the automated cell counter Countess®, with the use of Trypan Blue stain. Table 9 presents data from three consecutive experiments with different methods of EBs dissociation: trypsin/EDTA, Dispase, Collagenase, 2 mM EDTA, Dissociation solution.

Table 9: Combination of diffrent dissociation methods of EBs with regard to the cell viability.

Name	Experiment I	Experiment II	Experiment III
Tryp/EDTA	Total:$4.0x10^5$/ml Viability:82%	Total:$4.7x10^5$/ml Viability:90%	Total:$9.7x10^5$/ml Viability:95%
Dispase	Total:$6.0x10^4$/ml Viability:92%	Total:$4.3x10^5$/ml Viability:80%	Total:$7.2x10^5$/ml Viability:80%
Collagenase	Total:$2.9x10^5$/ml Viability:66%		
2 mM EDTA	Total:$2.0x10^5$/ml Viability:69%	Total:$3.8x10^5$/ml Viability:55%	
Diss.solution		Total:$2.1x10^5$/ml Viability:52%	Total:$1.1x10^6$/ml Viability:59%
Dispase/DNase			Total:$8.1x10^5$/ml Viability:94%
Diss.sol./Tryp			Total:$1.0x10^6$/ml Viability:88%

The bolded rows in Table 9 indicate the top results. In consideration to these results for the subsequent experiments, the in house made dissociation solution was used: the mix of trypsin EDTA with IMDM and DNase (refer to subparagraph 3.2.9.).

4.1.1.5. Embryoid bodies differentiation and antibiotic selection

EBs differentiation

In order to confirm, if the pre-selected clones from the preliminary screen were truly functional, the duplicated plates were defrosted and then clones from indicated wells of 96-well plate cultured again. The clones were kept in culture for some time in order to expand the cells and to create a frozen stock, in case if particular clones will be needed for future experiments. During the process of embryoid bodies formation (differentiation of mESCs), variations in the GFP expression were observed. These distinctions were visible in images from the fluorescent microscope, without need of additional staining. Fig. 33 presents progress or deterioration of the GFP expression in the EBs on day seven and day nine in suspension (Fig. 33 column 1 and 3). The EBs on day seven were re-plated and maintained in culture for nine days (Fig. 33 column 2). The seen in column 4 in Fig .33 EBs from day five, were seeded on gelatin coated plate and cultured for three days.

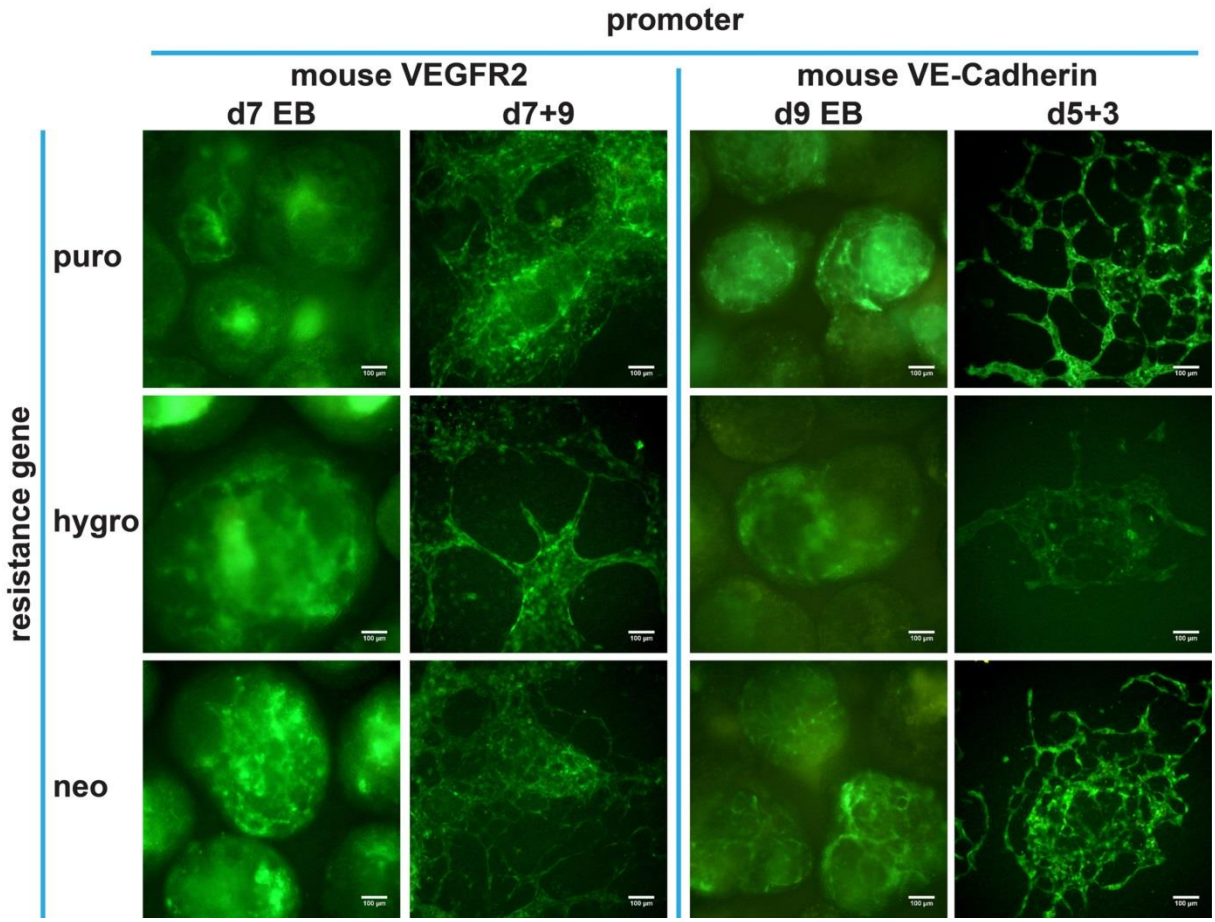


Figure 33: The established murine ES cell lines showing GFP expressing vessel-like structures. The first and third columns are showing EBs in suspension culture, columns second and fourth are presenting re-plated EBs. Above each column are indicated the age (d) of presented EB and in case of re-attached EBs also the time (+) of the maintenance on the plate. (Magnification 20 x)

To even more precisely validate the kinetics of GFP expression, another experiment was planned to obtain statistic data. The experiments were arranged in the particular manner in order to ensure a sufficient amount of EBs for at least a period of one week, from one batch of the ESCs every second day the ES cells were passaged and new plate for pre-differentiation was setup and again two days later the cells were put into suspension culture. With this routine, every day the EBs could have been analyzed by flow cytometry method for the GFP intensity.

Results for the vectors containing mouse Flk1 endothelial specific promoter, with combination of three different antibiotic resistance genes are presented in the Fig. 34. The outcomes for the Ve-cadherin promoter, were collected only from the vector containing neomycin (Fig. 35). On the basis of the experiments with the Flk1 promoter, it was unnecessary to conduct a GFP kinetics analysis, with

all the constructs, as the differences between each construct were not significant. The vector containing neomycin as a resistance antibiotic presented the best performance in the course of subsequent trials. In case of Flk1 promoter, the highest point of GFP expression was estimated for the day three and four. Thereafter the GFP expression was decreasing to pick up again around day eight or nine. The GPF kinetics for the Ve-cadherin promoter generated the peak of the expression on day six. To exclude variations in the GFP expression, resulting from the heterogeneity of the different cells population, the data were collected from the same start-up culture, for numerous parallel experiments.

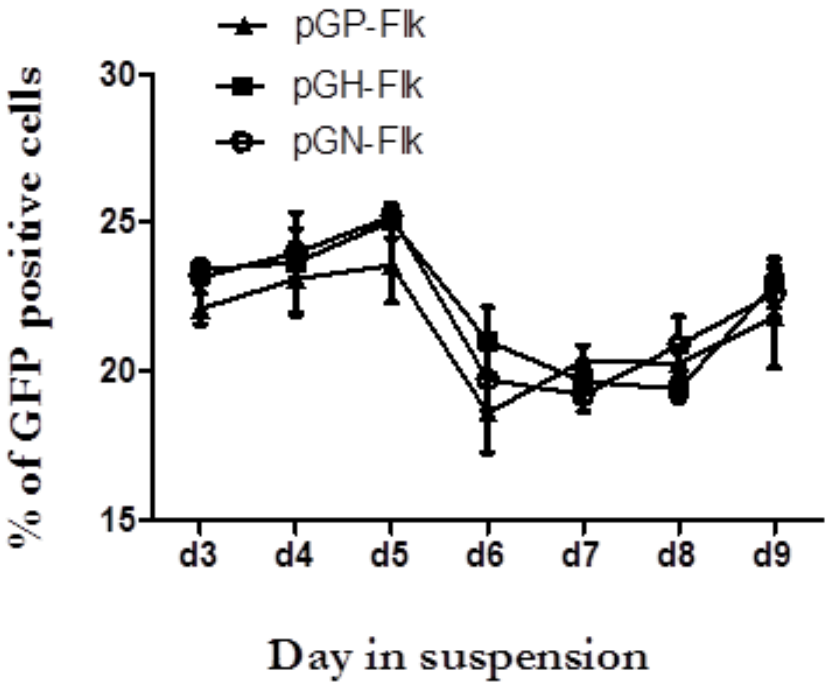


Figure 34: Time-course of GFP expression during differentiation of established cell lines under the control of the Flk1 promoter. Three different vectors containing various resistance genes for the antibiotic selection were used: pGN - vector with neomycin, pGH - vector with hygromicin B, pGP - vector with puromycin.

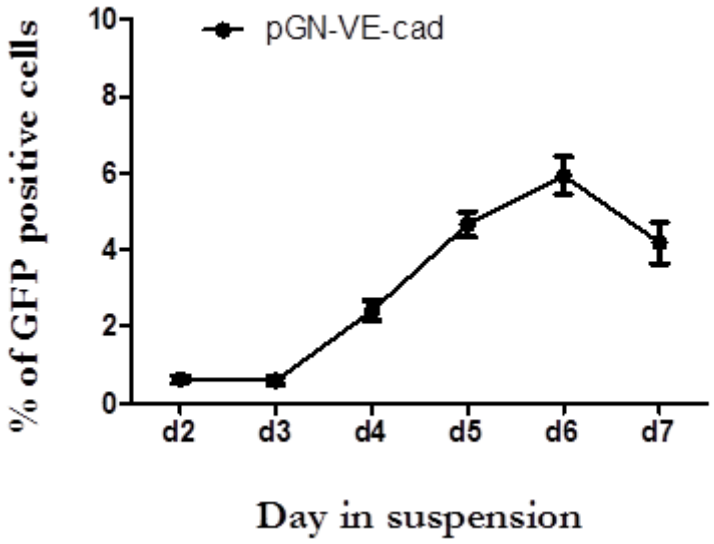


Figure 35: Time-course of GFP expression during differentiation of established cell line under the control of the Ve-Cadherin promoter, this vector contained neomycin, as a resistance gene for the antibiotic selection.

Determination of the peak of the GFP expression led to the prediction of a more accurate time point for the antibiotics application. In consequence antibiotic selection will enable collection of the pure subsets of endothelial cells.

Antibiotic selection

Diverse combinations of maintenance media were tested, in order to achieve higher amounts of mESCs differentiated towards endothelial cells. EBs were cultured in the following media: mEndo medium with altered concentration of FSC and also in the medium supplemented with VEGF or ESCs complete medium or serum free differentiation medium (SFD). Part of the results is presented in the Fig. 36. The introduced imagess are showing the same EBs in the bright field and in the fluorescent light. The various culture conditions impacted not only the GFP expression, but also the morphology of the EBs. In case of the maintenance of the differentiated mESC in SFD medium, the EBs were starting to spontaneously beat and the blood islets were observed (Fig. 36 (d)), arrows indicating the spots of blood islands.

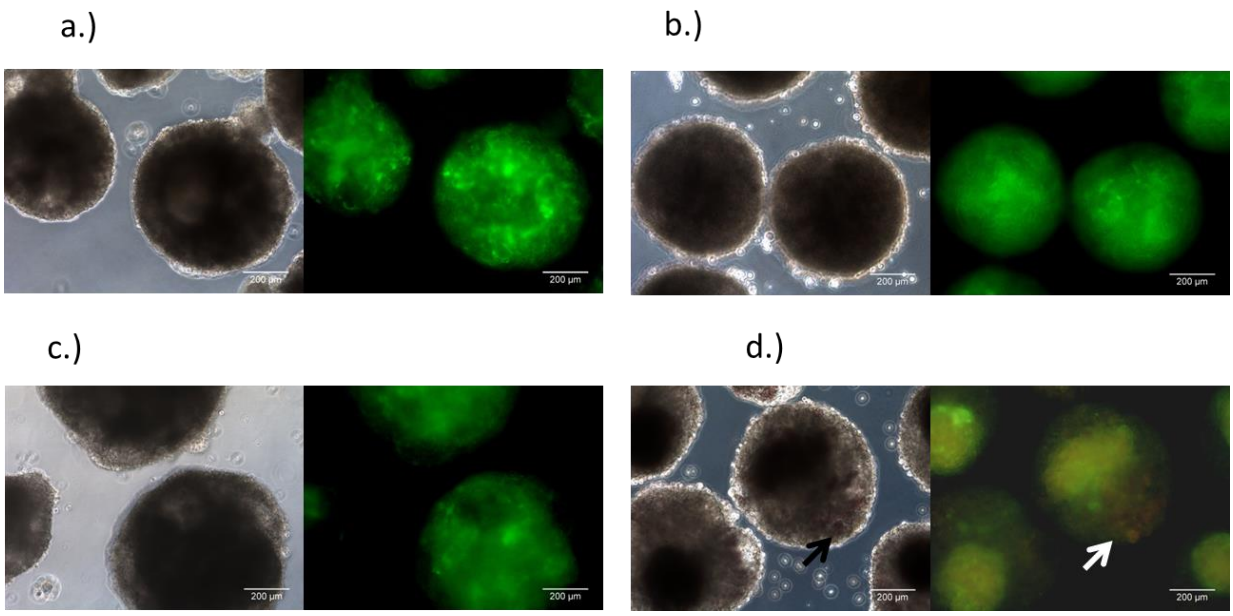


Figure 36: Light microscope and fluorescent images of the EBs maintained in different suspension culture conditions, arrows - indicating emerging blood islands. Panel: a.) EBs day five in 2% (v/v) mEndo medium, b.) EBs day five in 15% (v/v) mEndo medium, c.) EBs day five in the complete ESC medium, d.) EBs day seven in SFD medium.

Initially, just few reporter cell lines were selected and the focus was set on the GFP expression, which was quantified by flow cytometry. The GFP kinetics tests implied, that the antibiotics should be applied at the indicated by experimental outcomes time points. The expected enrichment of GFP did not lead to higher expression of endothelial specific markers. In the Fig. 37 are presented bright field and fluorescent images of EBs kept in suspension with the ESC complete medium, supplemented with antibiotic neomycin in the concentration of 500 µg per ml. The displayed EBs (Fig. 37) are the differentiated mESCs from the cell line containing as an endothelial specific promoter Ve-Cadherin and neomycin as a selection marker. It can be observed that these EBs are not having regular shapes and additionally in the panel (b) in the Fig. 37 are noticeable necrotic cores, which are the spots of probable hypoxia conditions. In addition, the GFP expression is dispersed and no vessel-like structures can be noted. In the Fig. 38, the same clone is shown, but re-plated on the gelatinized culture dish. The clone was first maintained in the suspension with complete ESC medium, until the EBs were formed. After culturing the EB for three days, the antibiotic containing medium was added and then the formation of vessel-like structure was observed and the GFP expression was revealed. The concentration of antibiotic was tested in the range from 500 to 1.500 µl per ml.

The vessel-like structures and the co-localizing GFP expression could not be observed in any conditions.

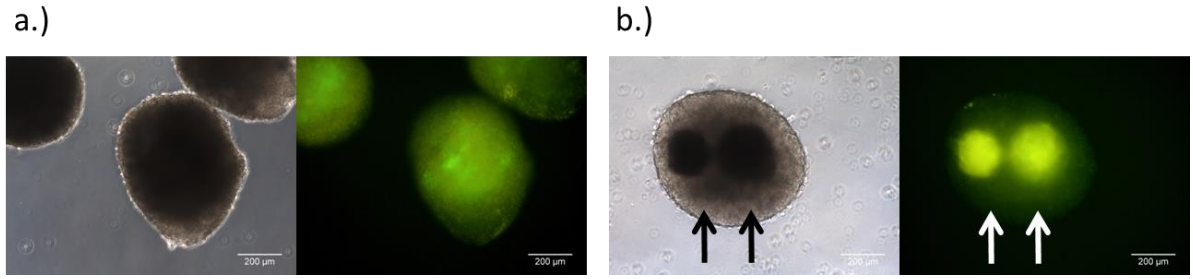


Figure 37: Embryoid bodies cultured in the ESC complete medium supplemented with the neomycin. Panel: a.) EB day seven cultured in the medium containing 500 µg/ml of antibiotic added at day five, b.) EBs day eleven cultured in the medium containing 500 µg/ml of antibiotic added at day three.

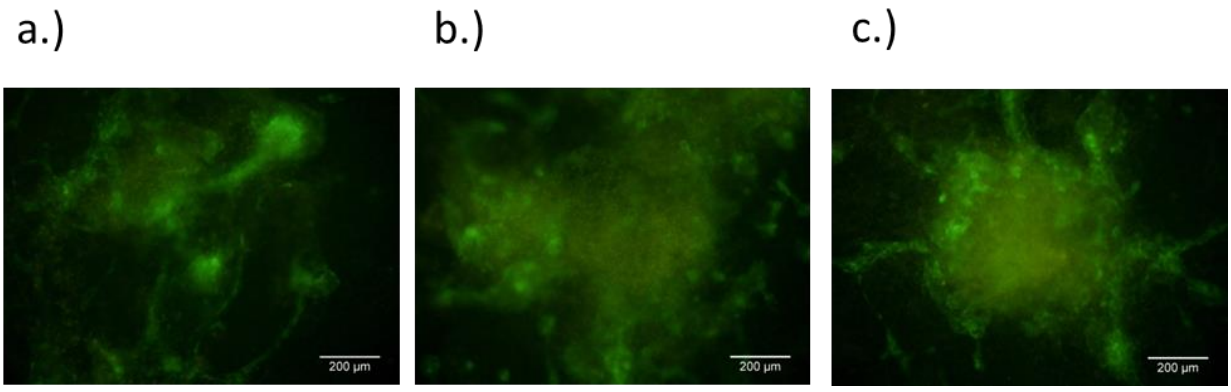


Figure 38: Re-plated embryoid bodies cultured in the ESC complete medium containing neomycin in different concentrations. Panel: a.) EBs day three medium containing 500 µg/ml of antibiotic was added at the day five, b.) EBs day three medium containing 1.000 µg/ml of antibiotic was added at the day five, c.) EBs day three, medium containing 1.500 µg/ml of antibiotic was added at the day five.

The observed enrichment of the GFP expression could not be confirmed because no endothelial markers were visible after staining with specific antibodies. Further experiments had been applied and the time-point of the antibiotic selection was moved earlier in the procedure. The antibiotic supplemented medium was introduced allready at the stage of the preliminary screening for vessel-like structures. From the picked clones, which apperead after transduction of mESCs, the EBs were formed and the one expressing the GFP were selected. These chosen EBs were re-plated and exactly at that moment the antibiotic selection had been started. The graphs in the Fig. 39 and Fig. 40 present data concerning EBs expressing GFP. The differentiated mESCs were maintained in the two conditions in

parallel, in medium with or without antibiotic. The selected clones contained as a resistance gene puromycin and were selected on the basis of the preliminary screen of the GFP expression during the differentiation. In Fig. 39 the presented data were collected from twelve clones, the following figure Fig. 40 displays data only from four out of the twelve clones. The records were collected from the flow cytometry analysis. The nominated clones were selected on the basis of visible GFP expression in the fluorescent light, during the preliminary screening. These clones were cultured in the medium supplemented with neomycin.

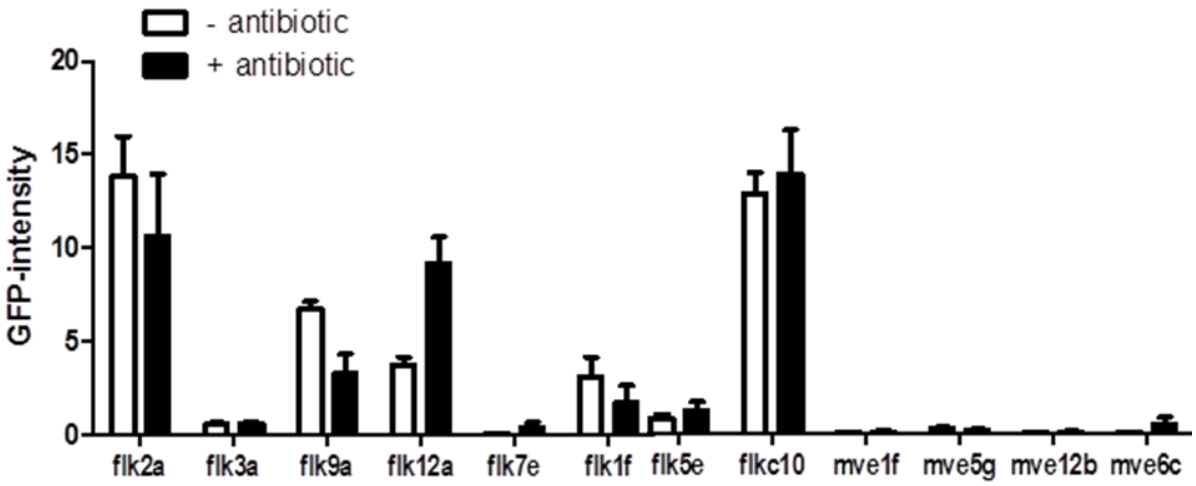


Figure 39: Antibiotic selection based on the GPF intensity. Graph is presenting data obtained from twelve clones, following number and letter abbreviations e.g. 2a are indicating the label of the clone taken for the purposes of the experiment. The GFP expression was driven by the Flk1 (flk) endothelial specific promoter or Ve-Cadherin (mve) endothelial specific promoter present in the vector.

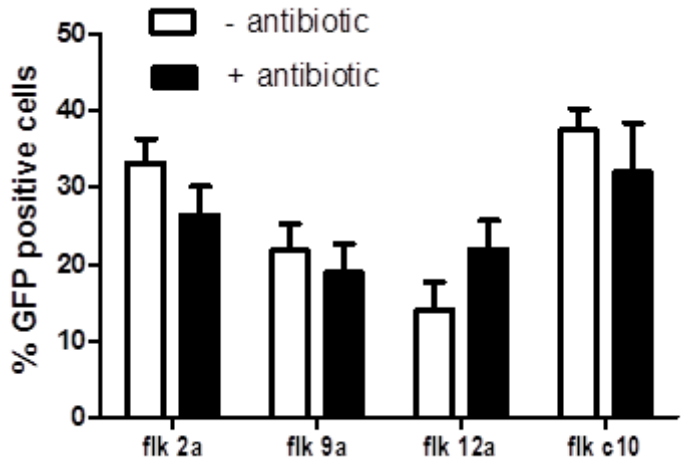


Figure 40: Antibiotic selection based on the GPF intensity. Graph is presenting data obtained from four clones, following number and letter abbreviations e.g. 2a are indicating the label of the clone taken for the purposes of the experiment. The GFP expression was driven by the Flk1 (flk) endothelial specific promoter present in the vector.

After the first flow cytometry analysis (Fig. 39), the experiment was continued with only four best-performing clones (Fig. 40). Clone 12a displayed the highest consistency in the performance and could have been differentiated into the desired cell type but the assumption was not confirmed by the immunocytostaining (Fig. 42). To further support the selection of the twelve and then four clones for the experiments, one of the chosen clones was observed in the process of pre-differentiation and during this process the expression of GFP was already observed (Fig. 41). The visible GFP expression was a sign of unspecific GFP activity, which at that stage should not appear. This fact was a reason to discriminate this clone from further screening. In the Fig. 42 the unspecific expression coming from the fluorescent marker of the clone pGN_flk_4a can be observed. The construct for establishing this clone contained Flk1 promoter and neomycin as a resistance gene.

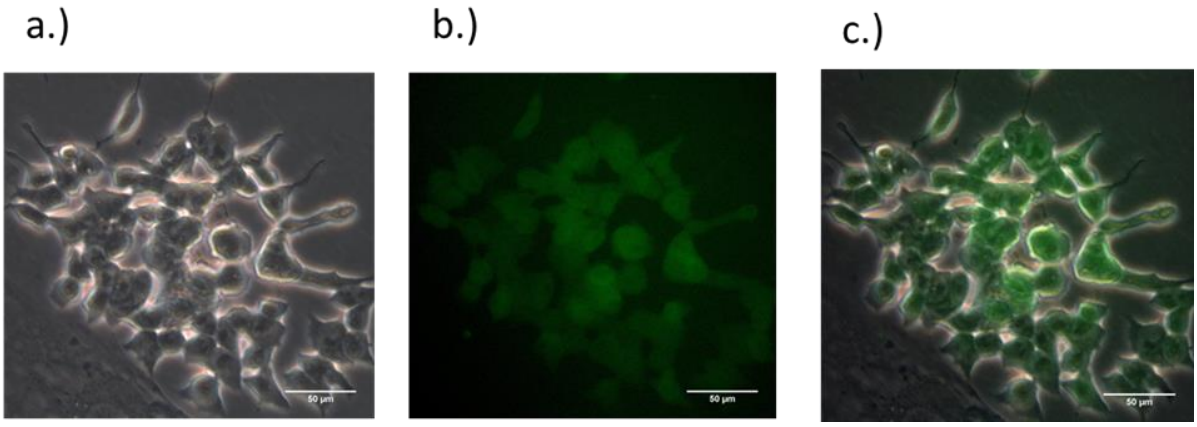


Figure 41: Newly established clone pGN_flk_4a showing signs of GFP expression in the pre-differentiation stage. Panel: a.) picture taken in the bright field, b.) picture taken in the fluorescent light, c.) merged images.

The selected four clones were used for immunocytostaining. The EBs generated from these clones were re-plated on gelatinized plates and after the re-attachment, started to form vessel-like structures. The medium was exchanged for the one containing antibiotic. The EBs were stained and the results are presented in the Fig. 42. images (a), (b) and (c) display re-plated EBs maintained in the medium supplemented with antibiotic, which was applied six days after seeding the EBs. The EBs were cultured to day eleven, until the staining was performed. The image (d) in the Fig. 42 is presenting four day-old EB which was maintained in antibiotic medium for five days. The clone presented in the image (d) from Fig. 42 does not display any co-localization with endothelial specific marker,

contrary to the staining with the Ve-Cadherin specific antibody. The observed GFP expression in Fig. 42 image (d) was concluded to be unspecific, because the GFP was not restricted only to vessel-like structures. The used antibodies were Ve-cadherin as a marker for vascular endothelium cadherin and DAPI as a nuclear dye.

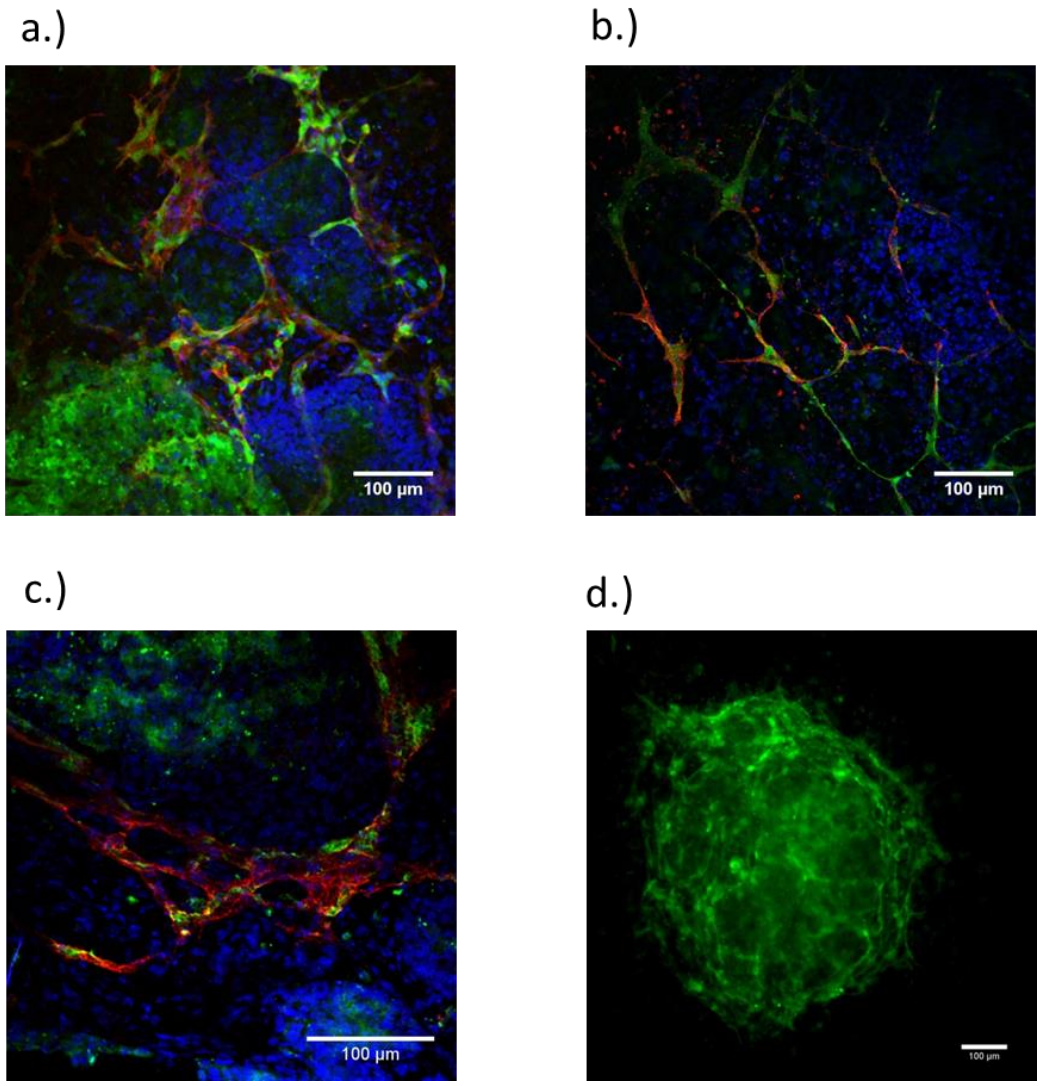
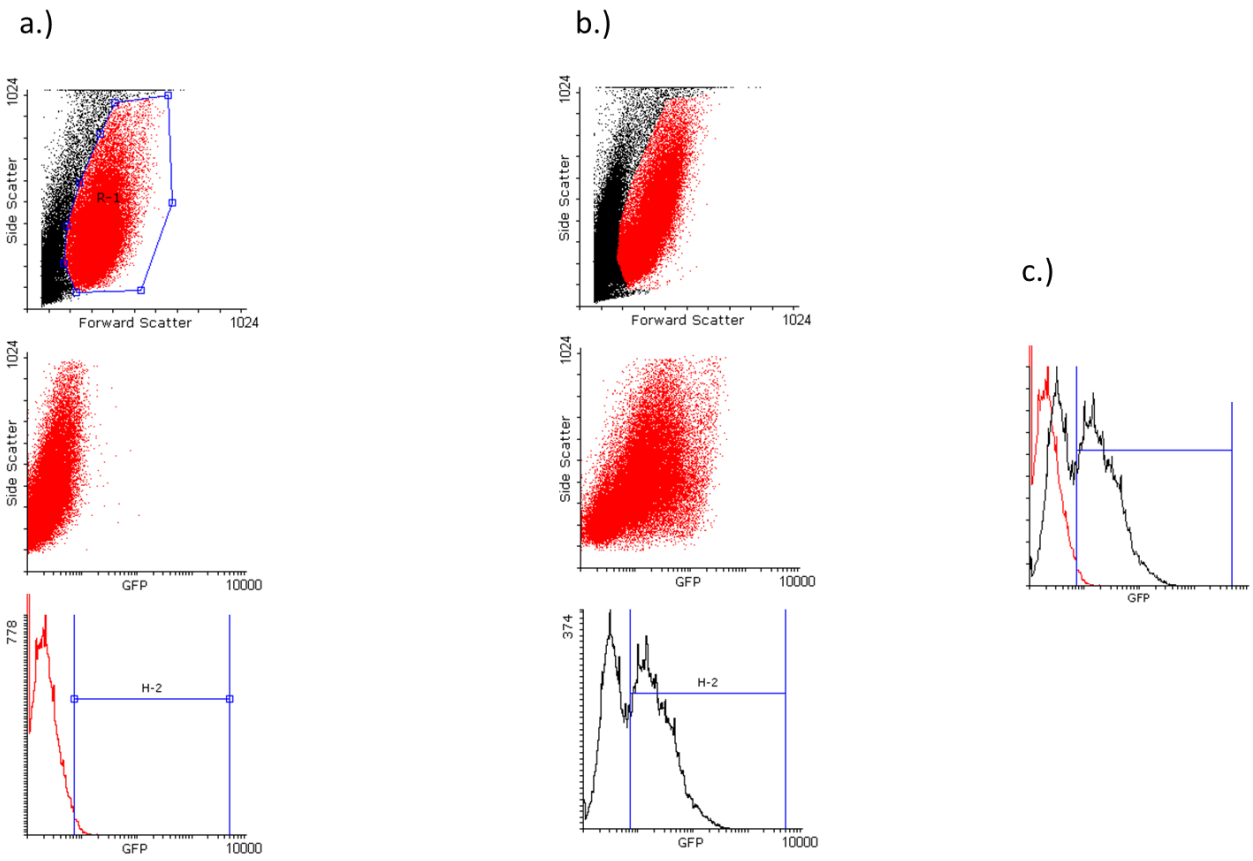


Figure 42: ES cell clones: antibiotic selection, staining with specific markers and co-localization of GPF expression driven by the specific endothelial promoters. Panel: a.) the presented clone was labeled 2a, the expression was driven by Flk1 promoter, construct had puromycin as a selection marker, the stained EB is four day-old, b.) the presented clone was labeled 9a, the expression was driven by Flk1 promoter, construct had puromycin as a selection marker, the stained EB is four day-old, c.) the presented clone was labeled 10c, the expression was driven by Flk1 promoter, construct had puromycin as a selection marker, the stained EB is four day-old, d.) the presented clone was labeled 12a, the expression was driven by Flk1 promoter, construct had puromycin as a selection marker, the stained EB is four day-old. The used antibodies are as follows: Ve-Cadherin/GFP/DAPI.

In order to exclude any coincidence of GFP expression occurring in the experiments, tests with the one of the established clones were conducted. The selected clone contained neomycin as a resistance gene and the GFP expression was driven by the Flk1 promoter (the clone not connected with the experiments presented in the Fig. 39 or Fig. 40). The clone generated from this construct was tested versus already well-known ES cell line E14, referred here as a wild-type and served as a negative control. The flow cytometry analysis of the GFP expression from the cell line and the clone were compared. The E14 ESCs were not displaying significant GFP expression. The small percentage of the GFP expressing cells, might occurred from the autofluorescence (Fig. 43). The GFP expression of the established clone was confirmed by the staining with the endothelial specific markers Flk1 and CD31, GFP antibody and DAPI which was used as a counter stain (Fig. 44).



	Events	% of Vis	Mean	GeoMean	Median	CV
All events	50.527	100,00	2,50	2,16	2,05	68,37
H-2	998	1,98	9,23	8,80	8,28	54,70

	Events	% of Vis	Mean	GeoMean	Median	CV
All events	49.554	100,00	20,34	10,14	10,00	154,97
H-2	29.147	58,82	32,09	22,45	19,63	114,57

Figure 43: Comparison of the GFP expression in the cell lines. Panel: a.) ESC E14 cell line (wild-type), b.) newly established cell line expressing GFP driven by the Flk1 endothelial specific promoter, c.) histogram with overlapping data from panel a.) and b.). The analyses were conducted with the flow cytometer. The tables below each graph are indicating the percentage of GPF positive cells in the analyzed cell subsets, the red circle in the table (panel b.)) indicates the significant increase in the level of GFP expression.

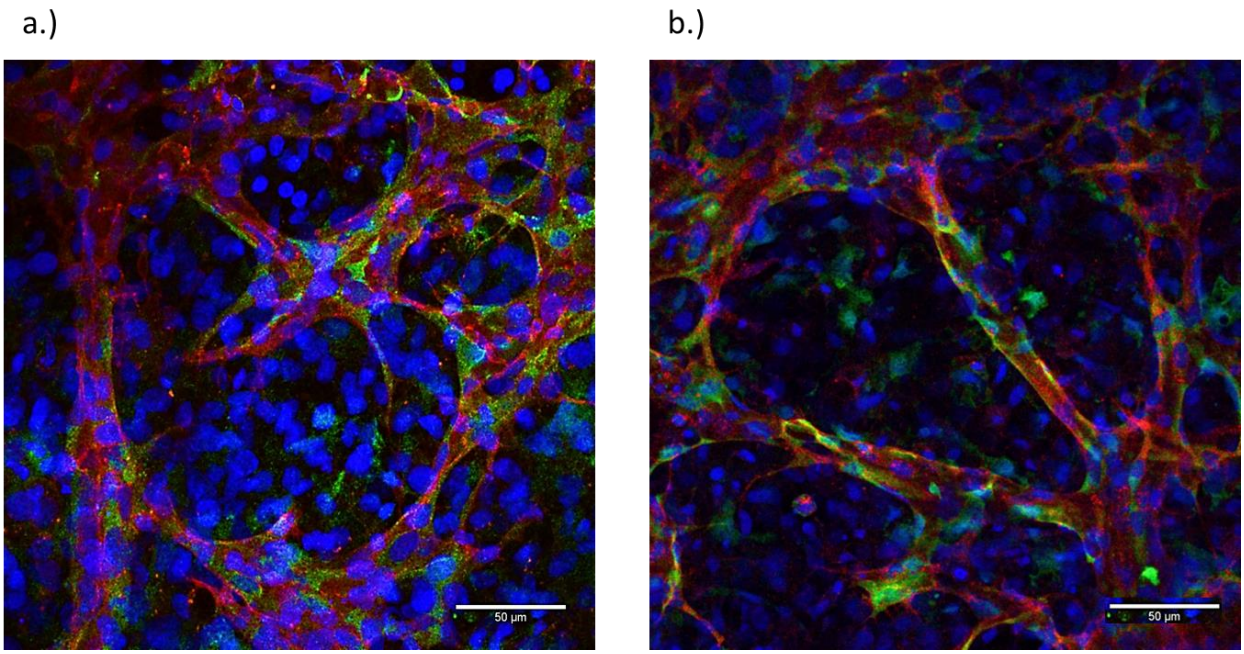


Figure 44: the newly established cell line expressing GPF under the control of the Flk1 promoter. Panel: a.) co-staining with the endothelial specific marker CD31 (PECAM - platelet endothelial cell adhesion molecule) and GFP antibody restricted to vessel-like structures, nuclei stained with DAPI (CD31/GFP/DAPI), b.) co-staining with the endothelial specific marker Flk1 and GFP antibody restricted to vessel-like structures, nuclei stained with DAPI (Flk1/GFP/DAPI).

4.2. Induction of the pluripotency

In the presented thesis the approach with the use of lentiviral system of transgene delivery was explored. In order to solve the remaining issue, the integration of the viral DNA into the host genome, the attempts of creating the non-integrating lentivirus (IDLV) were undertaken. Due to the introduction of the point mutation, into the integrase gene of the packaging plasmid, the integration of the transgene into the host genome could have been prevented. By means of the site-directed mutagenesis, the specific part of the packaging vector (psPAX2) was amplified then ligated into the cloning vector (pJet). The point mutation, mismatch of amino acids in the sequence of the protein integrase was introduced. After SDM the desired part, with the altered base pairs was re-ligated into the psPAX2 vector. In the Fig. 45 are visible vectors which were used in the SDM (detailed description in the subparagraph 3.1.12.2).

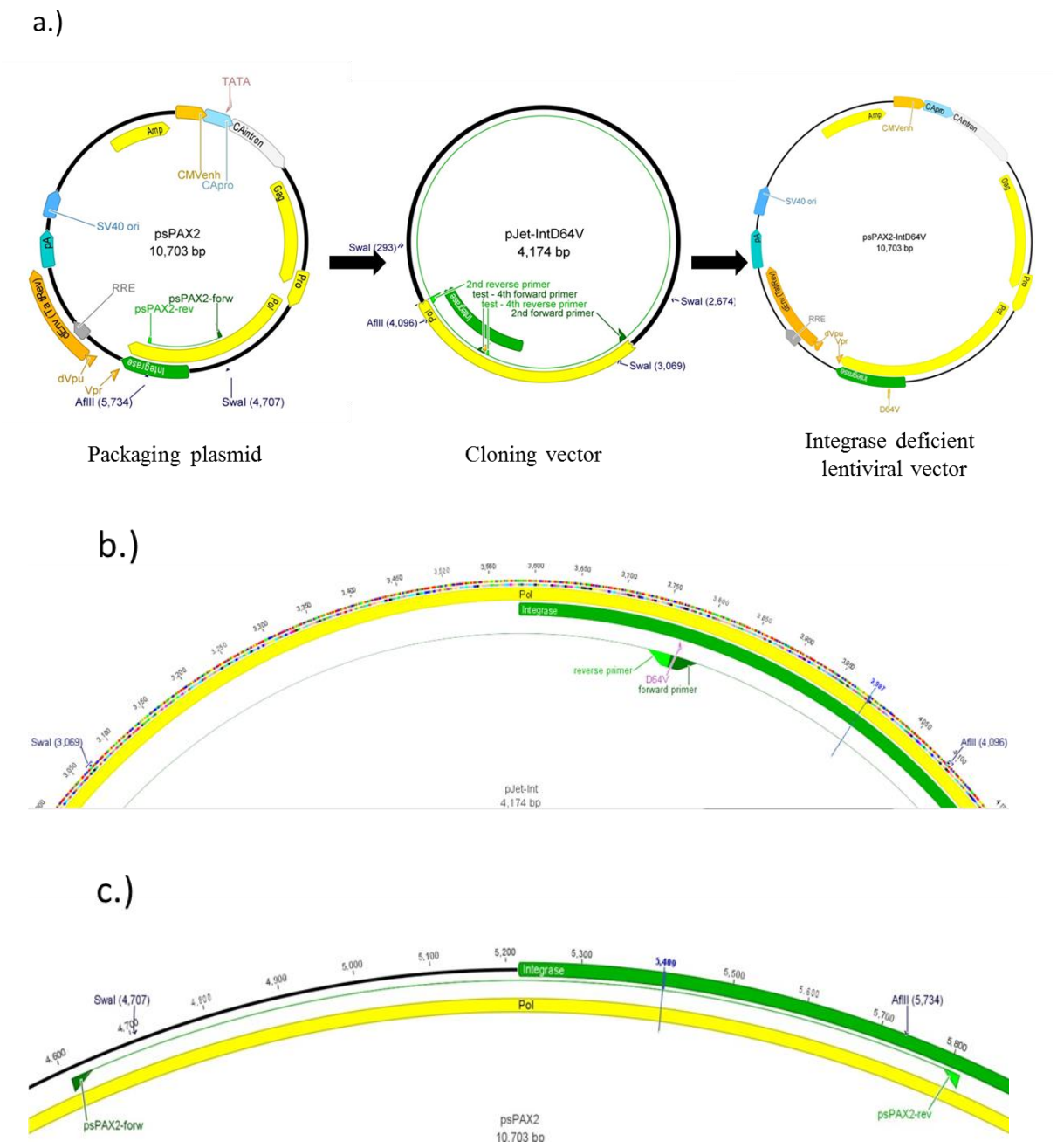


Figure 45: Site-directed mutagenesis strategy applied to the psPAX2 vector. Panel: a.) three vectors used in the SDM, b.) the PCR product ligated into pJet vector (pJet-Int vector), introduction of the desired point mutation by SDM was performed to achieve final product pJet-IntD64V, c.) psPAX2 vector with the unique primers which are flanking the SwaI and AflII fragment.

The functionality of the IDLV vector was estimated by the level of GFP expression in transduced HEK cells (Fig. 46). The experiment was run in parallel with the standard lentiviral vector (wild-type). In the Fig. 46 it can be observed that the GFP expression in the HEK cells transduced with the IDLV vector is diminishing after some time, due to the dilution-loss of episomes in the dividing cells.

These data are confirming that the introduced mutation, in the sequence of the protein integrase, is preventing the integration of the vector to the host cell.

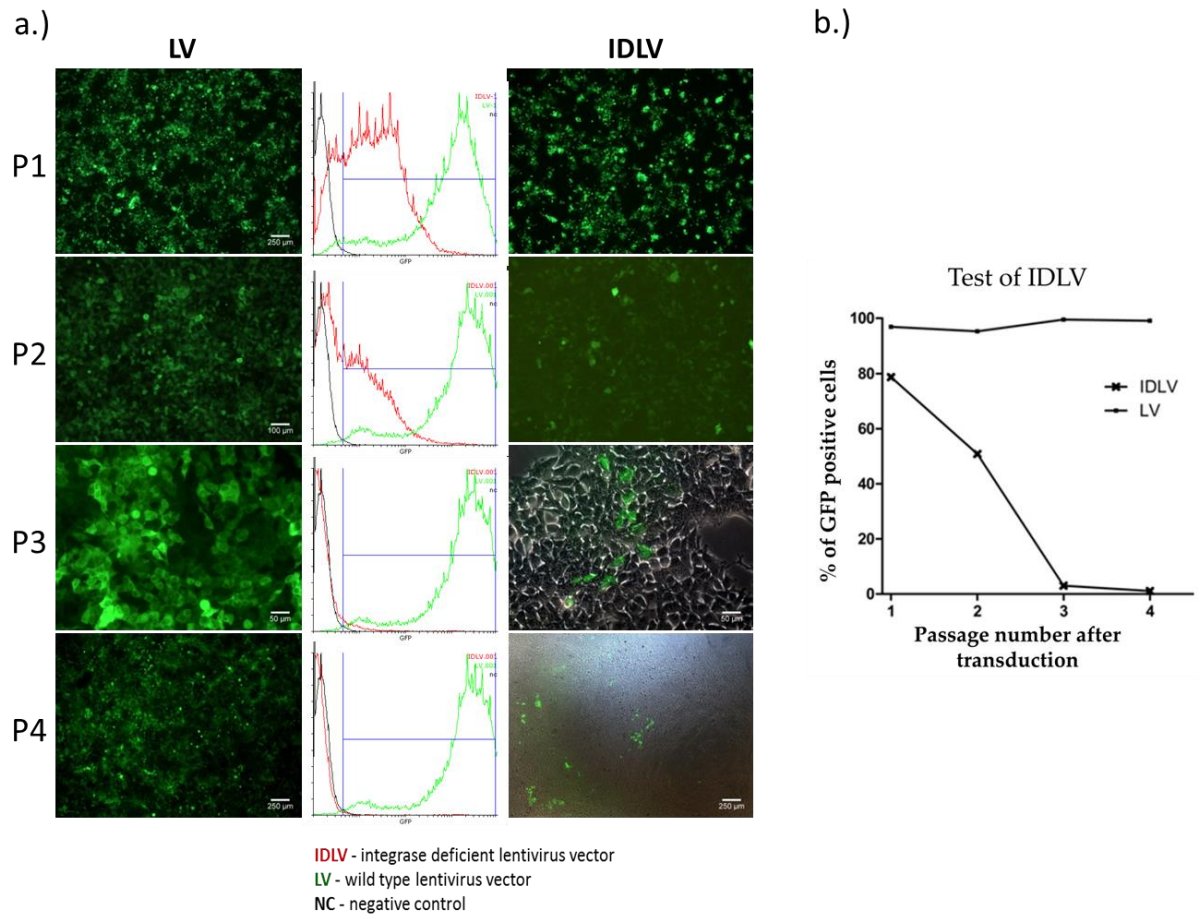


Figure 46: Comparison of the GFP expression in HEK cells after transduction with IDLV or LV (wild-type) construct, during the course of successive passages (P1-P4). The data were obtained through flow cytometry analysis (histograms in between the pictures). The presented fluorescent pictures of transduced HEK cells, panel a.) demonstrate that the expression of GFP was weakening in case of the cells transduced with the IDLV vector. The graph beside the pictures, (panel b.)), presents statistic values of the GFP expression levels.

The next step in reprogramming of MEFs was the transduction of mouse embryonic fibroblast. This action should reverse the status of the adult cell to the naïve stem cell. The experiment with IDLV construct was run side by side with the LV vector (wild type). In the experiment transfer vector Lenti-OKMS or Lenti-OSK were utilized (subsection 3.2.7.1). The induction of pluripotency was observed in the cells for generation of which the LV and OSK vectors were used. Additional tests were carried out in order to verify the endogenous expression of the transcription factors. To screen the iPS cells, the definitive primers were applied.

These primers could indicate the endo- and exogenous expression of the genes coding the particular transcription factor (Fig. 47). Figures 48 and 50 are displaying data confirming that there is no exogenous expression depending on the transgene. The concept of these specific primers was underlined by the fact that the exogenous mRNA contains only the coding sequences in contrast to the endogenous mRNA which encompasses also UTR regions (untranslated regions). The primers were designed in the way to also include the untranslated regions. Fig. 47 is depicting those distinct regions in one of the designed primers.

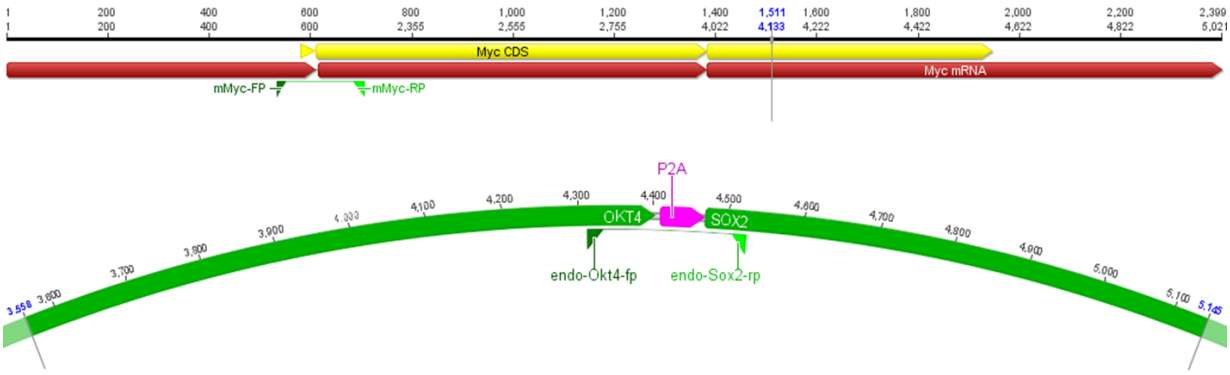


Figure 47: Example of the strategy implemented to design specific primers for detection of endo- or exogenous expression of the transcription factors.

To confirm that the designed primers were functioning properly, the PCR was run with the undifferentiated iPS cells (positive control). The results from that experiment are presented in the Fig. 48, picture (c) presents outcomes from the mRNA extraction. The PCR with the exogenous primers proved, that the undifferentiated iPS cells do not express the lineage markers, though the visible signal might be resulting from the present fibroblast. Fibroblasts were used for the co-culture of iPSCs, what presents image (b) from Fig.48. The decisive confirmation for the cells to be in the undifferentiated phase, was the lack of the signal coming from the Flk1 amplification. The displayed bands on the gel presented in the panel (a) of the Fig. 48, demonstrate signals imminent from the pluripotent markers, which should be expressed in all undifferentiated pluripotent stem cells. The mentioned markers are as follow: c-Myc, Klf4, Oct4, Sox2, Nanog. The GAPDH was used to verify cDNA synthesis efficacy by reverse transcription and as a control for mRNA. In the Fig. 48 the agarose gels displayed in the panels (b) and (c), are revealing bands originating from

undifferentiated ES cells. These bands allowed to compare the obtained pattern of expression, with the one of the undifferentiated iPS cells.

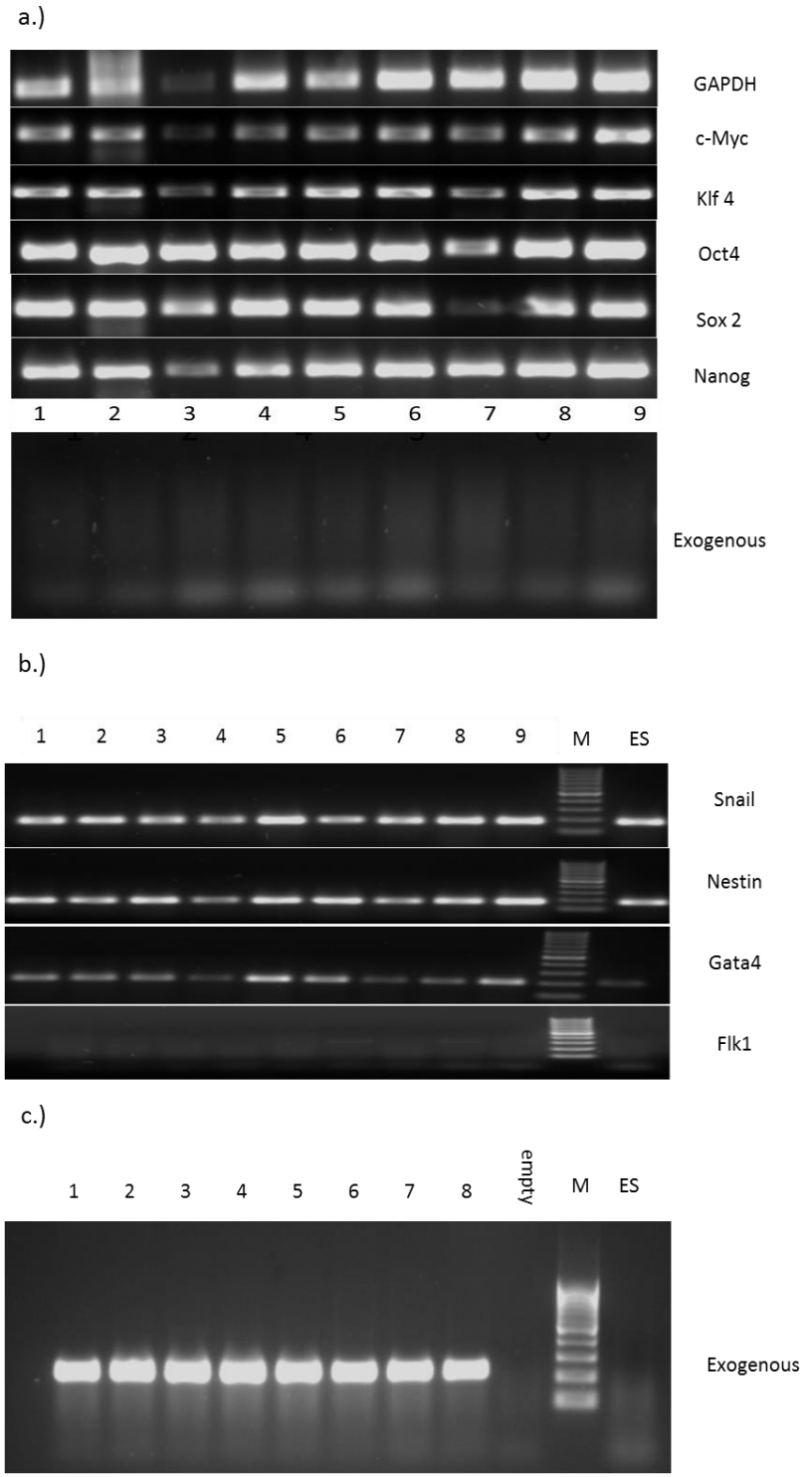


Figure 48: Expression patterns of various genes in the undifferentiated iPS cells. Panel: a.) expression of the pluripotent markers (c-Myc, Klf4,Oct4 and Sox2, Nanog), b.) expression of the lineage specific markers (Snail, Nestin, Gata4) and endothelial specific marker Flk1, c.) expression of the transgene from the genomic DNA. In the experiments b.) and c.) ES cells were used as a negative control. M - marker ladder (JenaBioscence).

In the Fig. 49 are data derived from the undifferentiated ES cells, which were expressing pluripotent markers. Observed bands are supporting the conclusion that iPS cells behave like ESCs, when it comes to the expression of the pluripotency transcription factors. It was proved that, there is no expression of the transgene (exogene) in the ES cells (wild type).

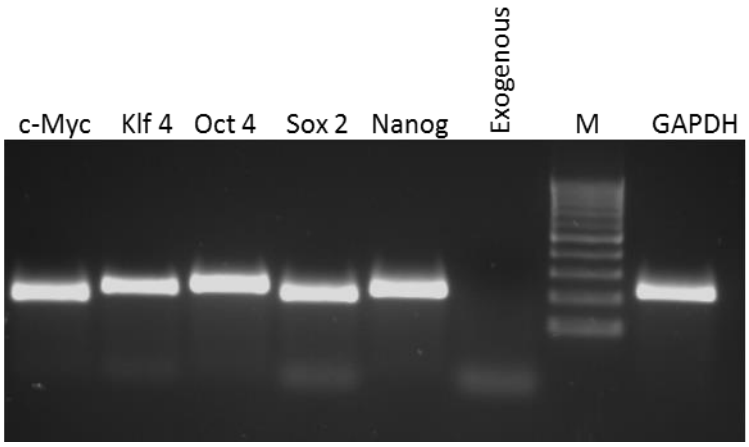


Figure 49: Expression pattern of pluripotency genes (c-Myc, Klf4, Oct4, Sox2, Nanog) in the undifferentiated mouse embryonic stem cells. M - marker ladder (JenaBioscence).

The presented lineage specific markers (Fig. 48 (b) and Fig. 50) are characteristic of differentiated pluripotent stem cells. The Snail protein is considered to be crucial for mesoderm formation in the developing embryo. Nestin is present in many developing cells, however it is assumed as a marker for the ectodermal differentiation. Gata4 is involved in the regulation of the genes important during embryogenesis and above all in myocardial differentiation (NCBI, www.ncbi.nim-nih.gov). The bands on the agarose gel, in the Fig. 50 are distinctive for the lineage specific markers in the differentiated iPS cells. Samples with cDNA were used to confirm, that there is no expression of the transgene, because the transgene got silenced. In this case the differentiated ESCs were used as a control to prove the similarity of the expression pattern concerning the lineage specific markers in iPS and ES cells. The expression of Flk1 was evident and suggested the differentiation towards endothelial or blood cells.

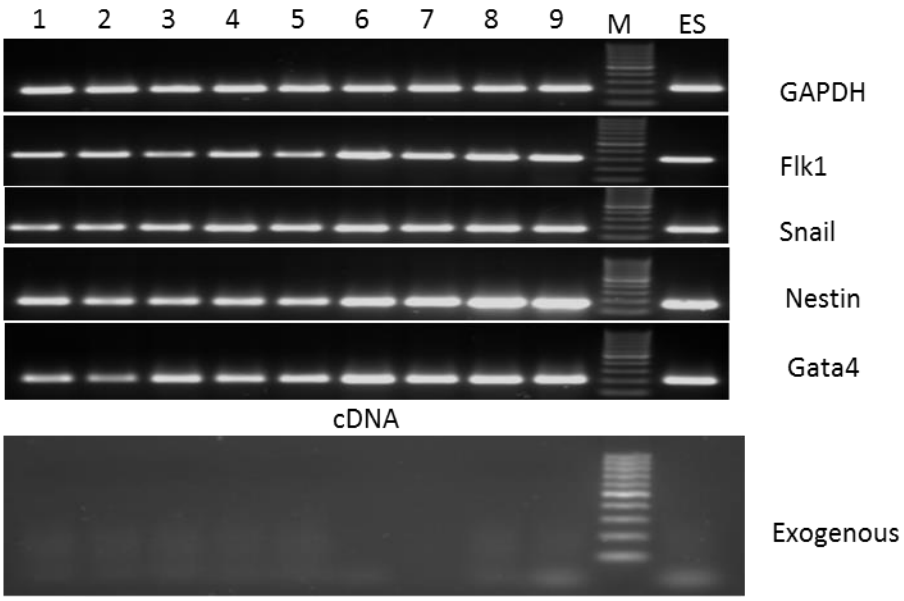


Figure 50: Expression pattern of the genes involved in the formation of the three embryonic germ layers (Snail. Nestin, Gata4) and endothelial marker Flk1 in the differentiated iPS cells. ES - differentiated ESCs were used as a positive control. M - marker ladder (JenaBioscence).

The expression of the pluripotent markers was in addition verified through the immunofluorescent staining with the specific antibodies: Oct4, Sox2 (Fig. 51).

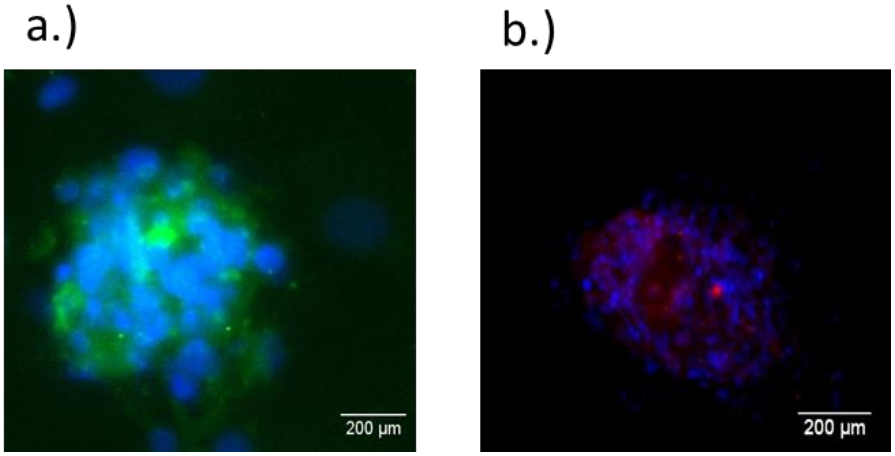


Figure 51: The figure presents fluorescent images of iPS cells (ES cell-like colonies). Panel: a.) staining with the Oct 4 antibody (Oct4/DAPI), b.) staining with Sox2 specific antibody (Sox2/DAPI), DAPI used for nuclear staining. Staining confirmed presence of pluripotent markers in iPS cells.

The biggest difficulty regarding the IDLV transduction is the concern of its silencing in the host. The possible cause may lie in the formed IDLV episomes, which in the host cell are becoming objects

of epigenetic silencing. These mentioned actions, implicate probable chromatin remodeling. the experiments carried out by (Pelascini et al., 2013), with HDACs inhibitors trichostatin A and sodium butyrate, aimed to test if the transduction efficiency and transgene yield will change accordingly, to the use or none of the above mentioned inhibitors.

In the course of further studies, experiments were carried with the HDACs inhibitors and the IDLV construct. Due to the lack of the reporter gene (GFP) in the vectors for inducing pluripotency (OKMS, OSK) the mentioned vectors, could not be tested. Instead, the CMV_GFP vector was used in the experiment. Pictures presented in Fig. 52 display increased GFP expression in transduced MEFs in comparison to the untreated cells, upon the trichostatin A application. The enrichment of the marker gene GFP was significant and that fact supported the claim, that HDAC inhibitors are the potential protectors against the IDLV silencing in the host genome.

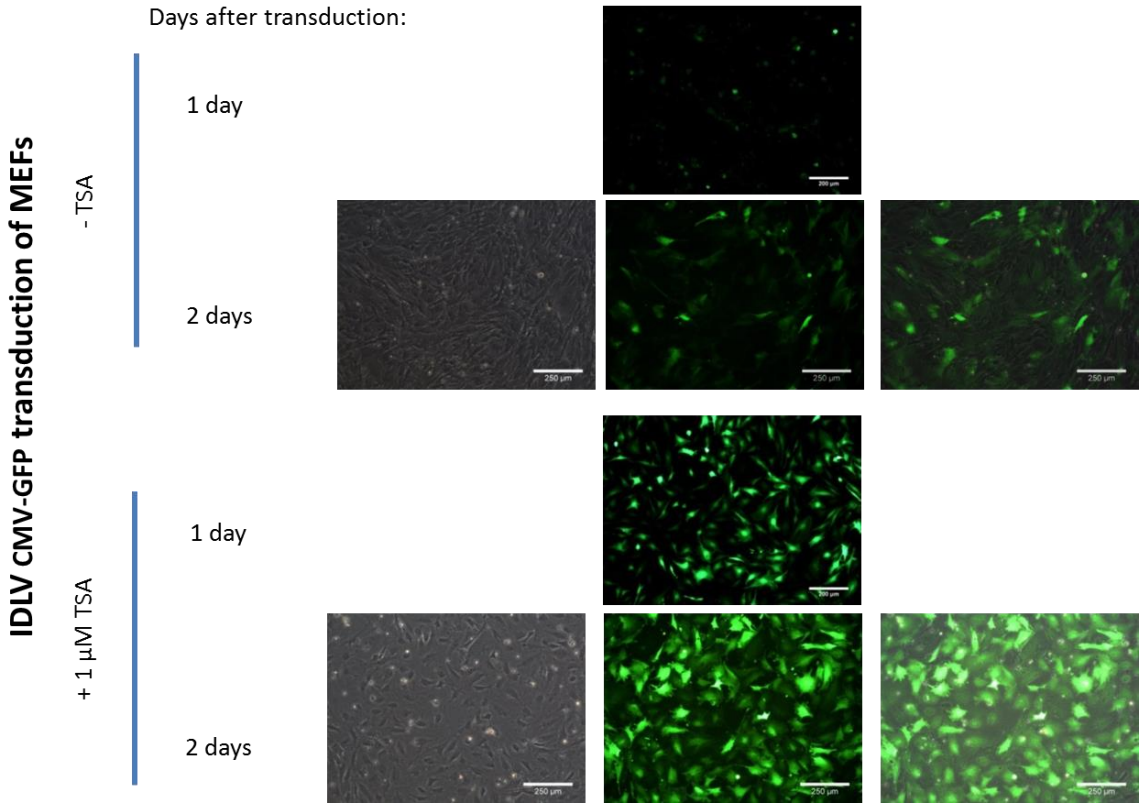


Figure 52: Transduced mouse embryonic fibroblasts showing the enhancement of the GFP expression upon the inhibitor tirchostatin A application. TSA treatment caused chromatin rearrangements, such as elevated histone acetylation and chromosome de-condensation as well as increased rate of RNA synthesis. (Magnification 25x)

4.3. Xenobiotic-free cell culture (2i culture)

Prior to be able to apply the desired cells for human therapies, one critical condition needs to be fulfilled. Cells must be cultured in the setting which does not contain any animal or animal-derived elements, including medium or the support on which they are being grown. That is why one of the aims of this doctoral project was the verification of xenobiotic-free culture conditions. The 2i medium was described thoroughly in the subparagraphs: 1.4.4. and 2.8.4. Fig. 53 displayes mESCs cultured in the 2i conditions. Process of adaptation assumed gradual feeder depletion by consecutive passages on only gelatin or Vitronectin XF™ coated culture plates. Adjustments of the new conditions included also progressive decrease in the amount of FCS in the medium, until the stage when the cells were seeded in the defined medium.

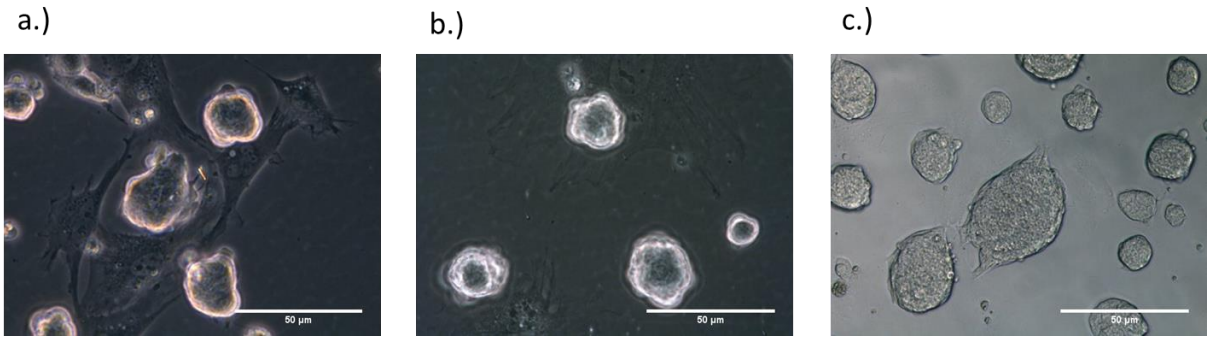


Figure 53: Gradual adaptation of mES cells to the xenobiotic-free conditions. Panel: a.) mESC second day after passage and feeder depletion, b.) mESC fifth day after passage and feeder depletion, c.) mESC in 2i medium at the day seven after passage and feeder depletion.

The mESCs maintained in the 2i medium were stained with specific antibodies to verify if the new media conditions did not impact pluripotent capacities. The used markers were as follows: Oct4, Sox2 and SSEA1 (Stage-Specific Embryonic Antigen-1 (CD15)). The CD15 is expressed on murine ES cells, however it decreases upon differentiation. The outcomes presented in the Fig. 54, confirmed the naïve state of the cells kept in the defined media conditions.

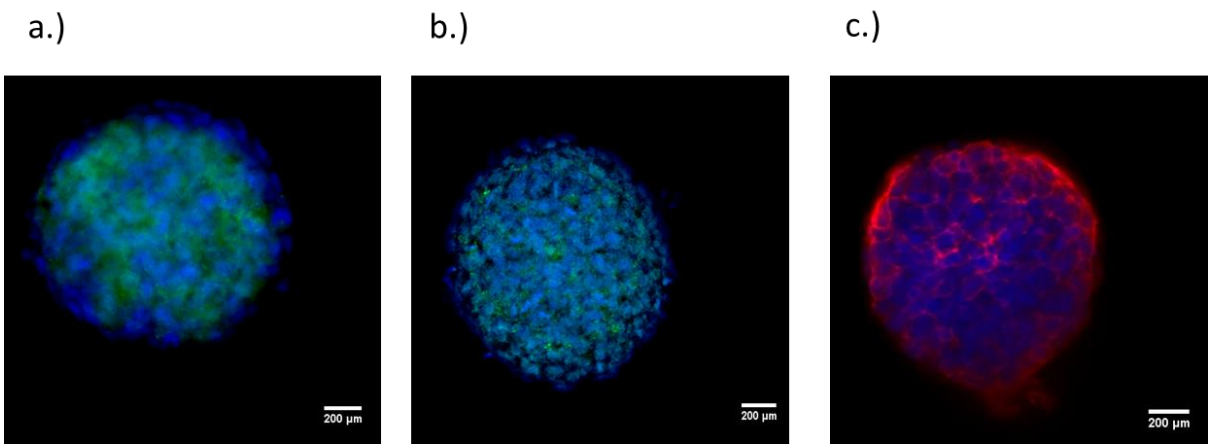


Figure 54: The mES cells maintained in the defined media conditions, xenobiotic-free media (2i), expressing defined pluripotency markers, panel: a.) Oct4 (Oct4/DAPI), b.) Sox2 (Sox2/DAPI), c.) SSEA1(SSEA1/DAPI). DAPI used for nuclear staining.

The unique for embryonic stem cells transcription factors (Oct4, Sox2) as well as markers for the three germ layers (Gata4, Nestin) and for endothelial differentiation (Flk1), were validate as well. The specific features of the mESCs, cultured in the 2i medium, were verified by means of PCR. The bands specific for each marker can be observed on the agarose gel presented in the Fig. 55. The expression patterns of the mentioned markers are presented in four different experimental conditions. The mRNA was extracted from the following cells: row 1 (i) feeder cells, row 2 (ii) mES cells co-cultured with iFCs in the conventional medium, row 3 (iii) mES cells cultured without feeder support in the defined medium, row 4 (iv) mES cell line expressing GFP, maintained in the 2i culture. The visible band from Flk1 expression, in the first row, was most probably originated by the fibroblast on which the mESCs were cultured. That band is not present in the other rows proving that the cells were in the undifferentiated state. The presented results proved that the expression pattern of the chosen markers was the same for the mESCs cultured in conventional medium containing FSC, like for the cells maintained in the defined media conditions. The strong band of Nestin expression in the second and third row does not have to indicate the differentiation, but according to the literature, it can be also a sign of cell proliferation (Wiese et al., 2004).

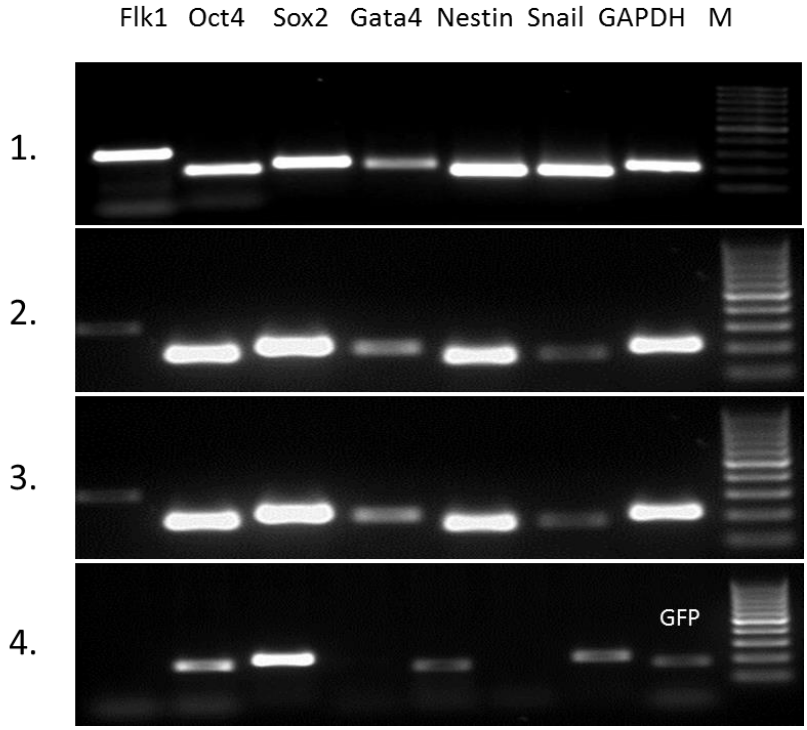


Figure 55: Screen of mESCs in different media conditions, concerning expression patterns of pluripotency markers (Oct4, Sox2) and lineage specific markers (Gata4, Nestin, Snail) and endothelial marker (Flk1). Rows: 1.) iFCs maintained in the standard conditions, 2.) mESCs co-cultre with iFCs and complete medium, 3.) mESCs maintained in the 2i medium, 4.) ES cell line (expressing GFP) cultured in 2i medium. M - marker ladder (JenaBioscence).

To further verify the performance of mESC in the defined media conditions, the comparison of cells from conventional culture method was carried by flow cytometry (Fig. 56). The presented data were selected from two experiments and are displaying the percentage of stained cells with specific pluripotency markers. In the study the IgG controls were used along. The narrow peaks, of the presented histograms, are indicating that the antigens are expressed at the same level. These findings underline the homogenous nature of the tested cell subsets. In case of the ESCs co-cultured with feeders in the medium containing FSC, the less homogenous population can be observed and lower percentage of stained cells. The double staining exhibited even higher heterogeneity in the ES cells maintained in the conventional conditions in comparison to the cells cultured in 2i medium.

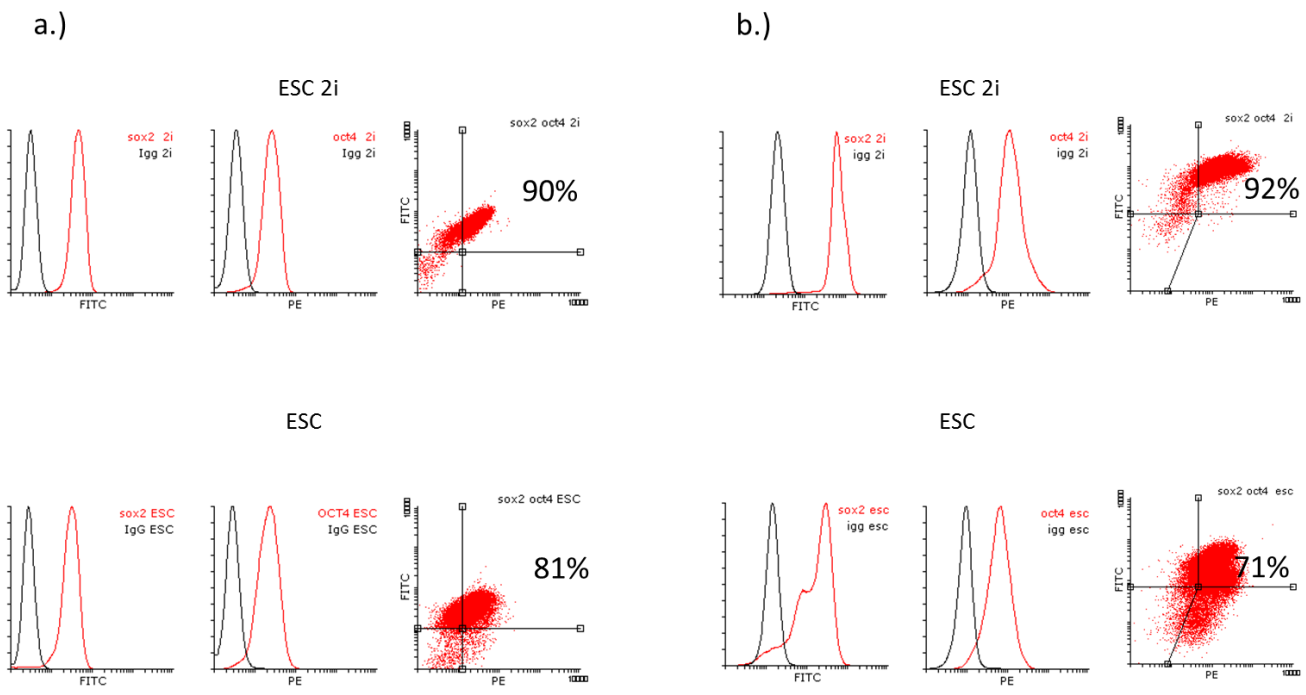


Figure 56: Flow cytometry analysis concerning expression of pluripotency markers. Comparison of expression patterns in the mESCs cultured in the 2i medium (ESC2i) or in the conventional culture conditions (ESC). The exhibited data are conceived from two different experiments a.) and b.). In each experiment, mESC obtained from both culture conditions, were stained with specific antibodies Oct4 or Sox2 and the IgG control or both antibodies were applied together. In case of double staining, the percentage of double positive cells in the cell subset are presented.

In order to investigate the expression levels of particular genes in the mESCs culture in the two culture conditions, the mRNA was extracted from the cells. The mRNA was pulled down from: cells in cell culture (i) day zero (D0) and from (ii) the EBs day one to day eight. The 3-D aggregates, from both starting cultures, were maintained in the medium containing FSC nevertheless some differences in the level of gene expression were observed (Fig. 57). The graphs from the Fig. 57 present mean ΔCt values.

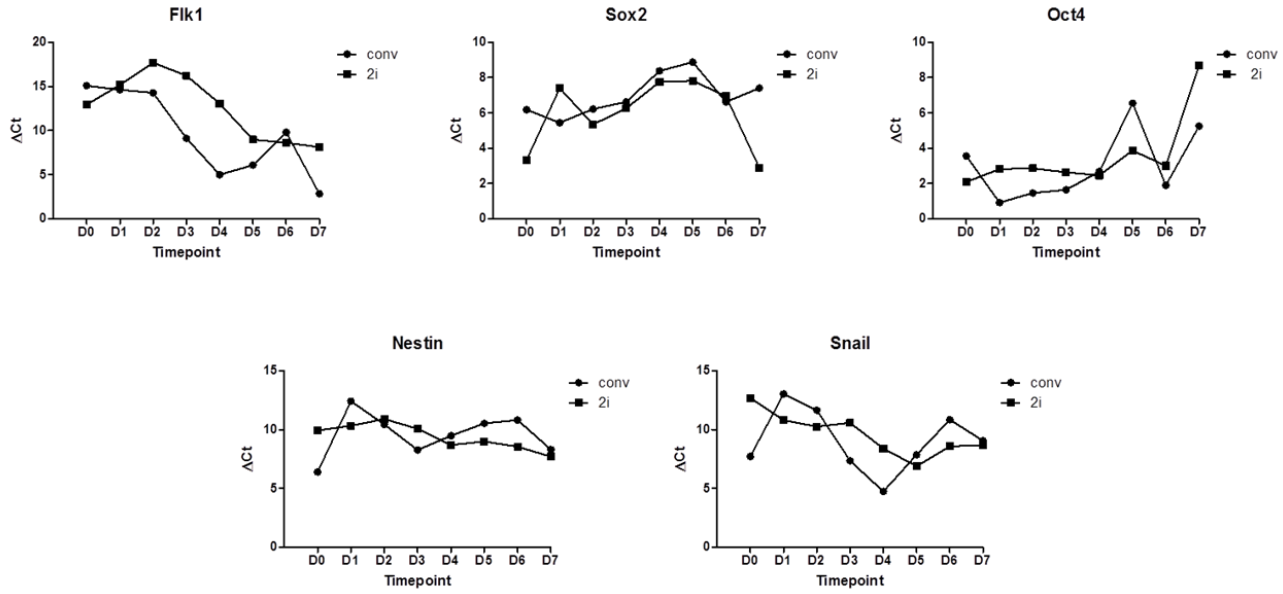


Figure 57: The Δ Ct data (mean values) showing the different gene expression in the mESCs cultured in the defined media (2i) or in the conventional conditions (conv). The real time PCR was applied to visualize and compare the expression tendency of pluripotency markers (Oct4, Sox2) and lineage specific markers (Gata4, Nestin, Snail) and endothelial marker (Flk1) in mESCs maintained in both culture conditions.

The genes, which were tested, were as follows: Flk1, Sox2, Oct4, Nestin and Snail. As expected the expression of the Flk1 with time decreased. That case was already previously spotted in the experiments with the GFP kinetics (Fig. 34 and Fig. 35). This state is connected with the maturation of the probable endothelial or blood cells. The Sox2, Nestin and Snail expression patterns, in both conditions, displayed the same tendency and rather high expression. The trend for Nestin and Snail genes is in accordance with possible germ layers differentiation in the developing EBs. Likewise, the Sox2 expression maybe related to the emergence of the ectoderm and neural progenitor populations, which can be true for some EBs in the population. The Oct4 pattern is insignificantly different in the both conditions. The observed tendency is correct, with time Oct4 expression may get even higher, marking the differentiation towards mesoendoderm. This fact stays in agreement with the possibility of formation of early endothelial or blood cells, triggered as a default by FSC in the medium. The observed imbalance of Sox2 and Oct4 expression was assumed to be common, as the cells start to differentiate and are losing their pluripotent potential.

5. Discussion

5.1. Embryonic stem cells

The mouse ES cells were chosen as an object for the experiments presented in this dissertation. The boundless capabilities of the mouse ESCs constitute a platform to better comprehend the science of human embryonic stem cells. The unlimited potential of ES cells to generate any, from the desired cells of the organism for therapeutic usage and the opportunity to create models of the mammalian development, make the ESCs exceptional and hard to replace.

To be able to manage their capacity and the process of differentiation, which would lead the cells towards particular pathways, these actions need to be first recognized and characterized. Several pathways had been described, due to the advances in the embryology, which stimulated progress in visualization of the gastrulation process in culture and initiation of the three germ layers formation. The mechanisms mentioned-above helped to recognized and derive various progenitor cells. The biggest test will be passed by application of these cells in *in vitro* assessments, as well as in the clinical trials (Murry and Keller, 2008). The ES cells had unclosed entirely different door for the biomedical science. Credited to ESCs, it became possible to study the growth and role of the cells or tissues in healthy and pathological conditions *in vitro*. There is also a prospect to indicate determinants, which play crucial role in the embryogenesis and to asset those factors that may have influence on the human organism in the future life (Power and Rasko, 2011). However promising all the statements appear, there are still matters concerning ES cell science, which are perceived as major hurdles that need to be overcome, before the cells could be even considered for use in cell replacement therapies (Laura E. Sperling, 2013). The ability of ESCs to propagate infinitely, turned out to be a double-edged sword. That feature of stem cells, may cause formation of a tumor after the cell transplantation. It had been reported that inserted undifferentiated ESCs evoke teratomas.

The aim of this study was to generate progenitor cells and to utilize these cells for therapeutic reasons.

Before applying the differentiated ES cells in the regenerative medicine, the cell subset needs to reach 100% purity (Laura E. Sperling, 2013; Murry and Keller, 2008). The most promising method for purifying the heterogeneous cell populations is the method established on the ground of expression of detectable markers. Such markers are under control of the lineage specific promotors (Murry and Keller, 2008). That kind of approach had been tested during the course of experiments conducted during this doctorate program. As described in the experiments of Murry and Keller, apart from the antibiotic resistance also the fluorescent marker gene was applied for the selection procedure. Power and Rasko discussed the creation of suitable and imitating the real conditions environment for development of desired cells, tissues or ultimately organs (Power and Rasko, 2011). This matter was also approached during this doctorate. The main obstacle was the construction of specific bioreactors, which could yield satisfactory amount of differentiated cells.

5.2. Endothelial differentiation

The common way to differentiate ESCs into hematopoietic precursors assumes the use of cells from culture with feeders and medium comprising FSC, as well as formation of EBs. That approach is not free from pitfalls such as undefined cell culture setting, which makes the study of a single cell outcome difficult. Batch-depending variations of the serum composition make the serum an unreliable ingredient to use. Utilization of feeders carry the risk of possible contaminations or presence of pathogens. The supporting cells release numerous molecules, which may have an impact on unrestrained lineage commitment towards undesired phenotypes. That is why, in order to obtain hematopoietic precursors, stable and defined conditions must be created. There is also a demand for introduction of specific molecules, which would direct the fate of differentiation for the particular cells. An example of that molecule is bone morphogenetic protein 4 (BMP4), which displays high potential for deciding about the cell's destiny. The ideal system of differentiation needs apart from defined conditions, also a possibility to observe that process in a straightforward manner. The suspension culture of EBs formation is also not optimal. It lacks monitoring of the cell to cell occurring interactions or following the secretion of substances. The important issue concerning the differentiation is the selection of desired cell subsets at various stages. The suspension culture may not truly reflect the conditions needed for the differentiation towards the wanted cell type (Chiang and

Wong 2011). It is crucial to mimic the topography of the tissue extra cellular matrix which is not one-dimensional. This vital detail cannot be omitted, especially when it comes to endothelial cells, where shear stress plays crucial role in the healthy arteries. Numerous proofs exist to confirm the importance of the interactions between the cells, and the character of the surface they are based on. It is highly probable, that the topographical signaling can induce endothelial cells lining up and intensification of its propagation after the damage. The vascular endothelium is placed on the basement membrane which is significantly influencing ECs functions (Hatano et al., 2013).

All the mentioned above factors had been analyzed during the experimental plan of this PhD program. The downfalls had been recognized and the suggestion had been prepared. One of the considered optimizing methods assumes implementation of the BMP4 molecule. This protein is thought to increase, by impacting the Gata2 gene, the probability of the differentiation towards hemogenic and angiogenic precursors in the time-dependent mode. Chiang and Wong proved that destiny towards hemogenic differentiation is categorical at the very beginning of cell development and is decided in a cell self-governing mode. Szabo et al. in 2010 investigated the causes underlying the alterations of adult cells into the blood precursors in the responsiveness to direct change. The successful recognition of such factors, could help to eliminate the need of viral delivery of the transgene into the host cell (McCloskey et al., 2006, Chiang and Wong, 2011).

The questions raised by this dissertation concern the plausibility of implementation of the murine endothelial precursors and to which extend the identified processes can be translated in to the human ES or iPS cells. The findings provide a foundation for further improvements of *in vitro* schemes of action in order to generate human ES/iPS cell-derived endothelial cells.

5.3. Genetic manipulations

Gene therapies emerged after 1970 when the recombination of DNA became attainable. The enormous amounts of time and expenses invested into the scientific and medical research, brought achievements in the field of cell replacement therapies. Everything became reachable in gratitude to ESCs discovery and countless trials to improve their usage for human therapies. Patients gained access to treatments

using autologous transplants, which highly decreased risk of immune rejection or even death. The next step was the generation of iPS cells. Reprogramming gave new sources and capabilities for cell transplants and minimized the exposure to aggressive treatments (Fox et al., 2014).

However, to make these aims approachable on a larger scale, effort must be still placed on making the derivation of specific cells from hES or iPS cells possible. In the next subchapter one of the approaches to facilitate the generation of specific cells will be characterized, the lentiviral transgene delivery (refer to paragraphs: 1.3.1 and 1.6.1).

5.3.1. Lentiviral transduction of mESCs

The main objective of this doctorate was the generation of stable mES-cell lines by means of the lentiviral system of gene delivery. The aim had been realized, though the cloning procedures were not free from mistakes (T2A sequence, subchapter: 3.1.12.) or obstacles to overcome like for example difficulties with cloning of the murine pGP-mVe-cadherin vector. Numerous trials lead ultimately to the decision of removing the resistance gene sequence from the pGN-mVe-cadherin vector and then sub-cloning into that place sequence coding puromycin resistance gene.

The great progress in the field of lentiviral vectors made these vectors an effective, safe and solid instrument, which can be used for stable gene delivery into various cell types. Another benefit of this gene transfer is the fact that LVs can infect also non-quiescent cells, which puts them above retroviruses. Over decades, lentiviruses which originated from HIV-1 have been advanced to improve safety and to add new functionalities. One of the desired features is the fact that LVs are not transferring sequences responsible for coding the proteins belonging to the packaging vector.

This ability reduces the danger that the cells transduced by lentiviral vector could become prone to the attacks carried by virus-specific cytotoxic T lymphocytes (Merten and Rubeai, 2011; Sakuma et al., 2012).

All the improvements introduced to the LVs created a safe and reliable transduction instrument. These features are essential for the vector, which should be used for derivation of clinically functional cells and that is why lentiviruses were used during this PhD program.

5.3.1.1. Integrase deficient lentiviral vectors

The vectors originated from lentiviruses are currently the most widely used tool for gene therapies.

The danger connected to the insertional mutations pressed the need to find the alternative, which appeared to be the non-integrating LVs. That kind of vector may be obtained by two methods:

- introduction of point mutation in the sequence of the viral protein integrase (IN),
- alteration of the two conserved CA residues in the site of attachment of the virus LTRs.

Those intramolecular modifications effect in formation of circular DNA episomes and transgene expression (Cornu and Cathomen, 2007).

In this dissertation the IDLV vector was generated by means of site-directed mutagenesis. The innovative use of SDM, helped to introduce the point mutation to the viral protein sequence. The amino acid residue aspartic acid (D) was substituted by valine (V), what disabled the function of the targeted protein. The test carried out to verify the functionality of this vector showed promising result (paragraph 4.2). The generated episomes, in transduced cells, were subsequently lost through cell division (Fig. 52). As the episome is not replicated, it only remains in one of the dividing cells. However, in case of non-dividing cells the expression is stable.

The IDLV based vectors can prove to be very beneficial when it comes to the vascular gene transfer, what correlates with increased level of safety. Reports showed that the genotoxicity and integration system of IDLVs presents significantly diminished number of induced modifications of the genes in the host cells (Chick et al., 2012).

5.3.2. Induction of pluripotency

The development of mammals is a one-way route through which a gradual deficiency in developmental capacities occurs. The development process originates from a single cell, the diploid zygote and terminates when all of the 220 highly specific cells of the mammalian organism are established. During this path of development, cells pass through few stages characterizing their potential (Hochedlinger and Plath, 2009):

- totipotency - possibility to differentiate into all cell types of the organism,
- pluripotency - capacity to differentiate into any of the three germ layers,
- multipotency - ability to differentiate into multiple but limited cell types (lineage specification),
- unipotency - capability to differentiate into only one cell type.

Fig. 58 presents the decrease of the differentiation capacities during the normal development of the organisms.

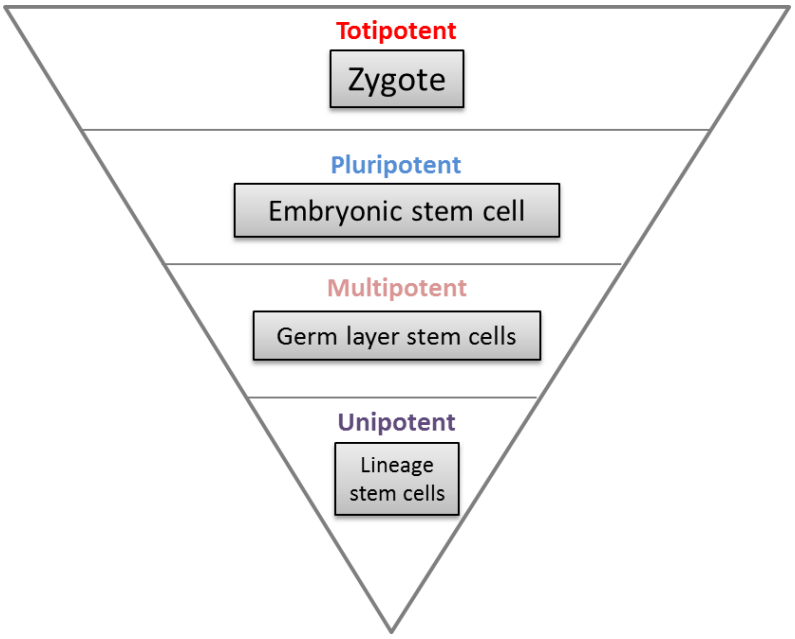


Figure 58: The gradual loss of differentiation potential by cells of the developing organism. The scheme is depicting the origin of cells and their potencies.

The possibility to artificially produce pluripotent stem cells *in vitro* became probable by the application of a cocktail of the transcription factors: Oct4, Sox2, Klf4 and c-Myc. The science of iPS cells demonstrates an uncharted territory of unlimited capabilities carried by these cells. The patient-specific cells would elevate the need of taking immunosuppressive medications throughout patient life. This method is not free from drawbacks and the procedures to generate iPS cells need various improvements and optimization. Probable genomic modifications emerged during the reprogramming or differentiation process of the iPSCs into the desired cell type, may impact the immunological phenotype of these cells. Other factors like incomplete (partial) reprogramming or genetic variability can also influence the possible rejection of transplanted cells. Reports indicated that

iPS cells possess "epigenetic memory", meaning that the cells can carry outputs from parental cells or acquired during reprograming process. This specific memory may effect potential of iPS cells to differentiate and moreover to retain in the state of a therapeutic cell. To overcome all the hurdles the "ground" state of iPS cells need to be first established and then subsequent clinical trials will become feasible (de Almeida, P.E. et al., 2014).

The cocktail of TFs used for induction of the pluripotency included: Oct4, Sox2, Klf4, c-Myc. After their application around one to two weeks are needed to observe reprogrammed cells and even though not all of the reprogrammed cells will reach the state of pluripotency. Nowadays, many scientist turn to the view that in order to successfully reprogram cells, a particular stepwise approach is needed. However, each step yields less and less fully reprogrammed cells, which may be due to unknown underlying actions. Comprehending the impact of the particular transcription factors during the different step of reprogramming will allow to uncover molecular mechanisms responsible for the pluripotency induction. The target genes in iPS cells, similar to the gene discovered in ESCs are Oct4 and Sox2, which co-reside promotors of highly expressed genes, as well as their own promoters. Klf4 deal with around half of its targets with the two above-mentioned TFs. The c-Myc activity was established through the analysis of limited target overlap and concluded that its function is distinct from the functions of Oct4, Sox2 and Klf4. The c-Myc targets are related with control of cells proliferation, metabolisms and biosynthetic pathways. The remaining three TFs are responsible for the governance of the transcriptional and developmental factors in the pluripotency organization. It can be thus concluded, that c-Myc is not needed for the up regulation of the pluripotency system at the last step of reprogramming. There is also an assumption that c-Myc enhances the discharge of promotor-proximal interruption of Pol II (RNA polymerase) and hence boosts transcriptional elongation instead of attracting the Pol II to the promotors. It is established that Oct4 and Sox2 are the master pluripotency genes. The controversy regards presence of Klf4 and c-Myc. The overexpression of c-Myc leads to elevated levels of the p53 protein. On the other hand expression of Klf4 impacts the amount of p21 protein, which is a cyclin-dependent kinase inhibitor. The p21 is involved in the inhibition of the proliferation and also decreases levels of the p53 in the cell. Therefore, it was proposed that c-Myc and Klf4 are reciprocally dependent and the balance between

their actions may guarantee good reprogramming (Hochedlinger and Plath, 2009; Yamanaka, 2012; Miyazaki et al. 2012; Muchkaeva et al. 2012).

Specific small molecules had been used to indicate that repressive chromatin states support the stability of the differentiated cells. The whole mechanism is not yet fully recognized and it is unknown whether if the changes in chromatin structure are global or just are related to the particular gene. The HDACs (see paragraph 4.2) are typically connected with chromatin condensation and suppression of transcription. The HDACs inhibitors trichostatin A (TSA), butyrate and valporic acid (VPA) markedly enhance the reprogramming efficacy in murine and human fibroblasts. This statement is supporting the results obtained from the experiment presented in the Fig. 52. Similar improvement of reprogramming was observed when the c-Myc was excluded from the TFs cocktail but the VPA was used (Plath and Lowry, 2011).

The success in induction of pluripotency in MEFs with the use of LV and OSK vectors, conducted during this doctorate, is brining closer the answers. The lack of reprogramming of the adult cells with the generated in house integrase deficient lentiviral vector is fully justified. That rationale can be based on the discussed in this subchapter difficulties, concerning the matter of the successful cell differentiation.

After the strenuous efforts to gain the insight into the genetic and epigenetic mechanism underlying reprogramming and pluripotency, scientists are only getting a grip of that complicated networks.

The proposed solutions which could be employed, are either establishment of a technique that would yield enormous amounts of reprogrammed cells or identification of early epigenetic indicators which would designate only the cells that will undergo the full reprograming and could achieve pluripotency (Plath and Lowry, 2011).

5.4. Xenobiotic-free medium

Cell culture has a vital task to recapitulate physiological conditions of the one found *in vivo*. This specific microenvironment needs to provide suitable temperature, pH, oxygen and nutrient supplies (Brunner et al., 2010). Another important aspect is the determination of the appropriate cell attachment, like gelatin or other coating agent, or supporting cells (feeder cells). These factors can also impact the performance of the growing cells.

In this thesis improvements, which are able to create fully defined conditions for the sustainable cell-growth and are minimizing the risk of contact with animal photogenes, were investigated. In order to be able to use the generated hES or hiPS cells for the therapeutical treatments, such specific conditions have to be created (see paragraph: 1.4.4 and 4.3).

Traditionally, the hES or hiPS cells had been co-cultured with mouse fibroblast in the serum supplemented medium, thus several solutions had been proposed:

- medium with serum replacement,
- addition of growth factors mixtures,
- fibronectin or Matrigel matrices for growing cells.

A short comparison of the use of serum and serum replacement, in the cell culture medium is presented in Table 10.

Table 10: Summary of the benefits of the serum and serum replacement application in the cell culture media. Adapted from: Brunner et al.,2010.

Benefits of serum:	Benefits of serum replacement:
• supplies growth factors and hormones • assures proteins transport • impacts attachment and help in spreading factors • source of additional amino acids, vitamins and trace elements, fatty acids • reduction of shear stress	• chemically defined and controlled culture conditions • reduced variability in culture medium composition • reduced risks of microbial contamination • reduction of fetuses and animals suffering • independence of commercial supply

In this study yet another approach concerning xenobiotic-free media was tested the 2i (two inhibitors) medium (refer to subchapter 1.4.4. and chapter 4.3). The obtained results proved that the maintenance of mESCs in these conditions does not impact negatively the performance of the tested cells. Neither, lack of serum or serum replacement, nor the absence of feeder cells showed undesirable functioning of the mES cells (refer to Fig. 55 and Fig. 57). Moreover, it was noticed that the cells in 2i conditions

were growing slightly faster and the propagation process had been facilitated. The passage of cells does not need additional washing step of the cells, before trypsinization, due to the lack of serum in the medium.

In regard to the reprogramming the continuous attempts are undertaken, in order to determine and define further small molecules which are impacting the reprogramming method. Hopefully those endeavors will result in establishment of proficient and accurately defined maintenance conditions for iPS cells. Due to the difficulties concerning the complex matter of iPS differentiation towards specific cell types, maybe it would be more beneficial to generate: (i) transitional lineage specific stem cells and (ii) progenitors or (iii) differentiate desired cells by means of chemically characterized conditions. Chemical methods are attracting interest and may prove to be useful, especially in the field of regenerative medicine (Li and Ding, 2010).

5.5. Conclusion

The generated stable cell lines were suitable for the groundwork purposes, before the experiments concerning m/hiPS cells could be implemented. The employed endothelial specific promoters were useful as cell markers. Due to the presence of the GFP fluorescent protein, which is expressed under the control of applied endothelial promoters, the differentiated cells were traceable. The newly established cell lines can be used in the angiogenesis models. One of the proposed angiogenesis assay assumes injection of SK-MLE 5 cell line (Human Skin Melanoma) into the nude mice, where these cells will form a malignant melanoma. Next, the selected and purified endothelial cells would be subcutaneously implanted to the immune-deficient mice with the developed tumor. Subsequently, the endothelial cells are expected to migrate and proliferate in order to form the new blood vessels or to integrate into the existing ones. The histological studies would definitely show the sites of integration of the implanted endothelial cells, due to the presence of the reporter gene GFP.

The subsequent goal, of the presented doctoral program, concerned induction of the pluripotency. The generated miPS cells gave the insight into the extremely complex nature of the cell reprogramming. The achieved results are solely restricted to the induction with the use of lentiviral gene delivery. The generated miPSCs were used as a study of the pluripotency and allowed to tackle

and approach the difficult matter of the maintenance of that kind of cells. The task of the pluripotency induction with the integrase deficient lentivirus was not entirely realized. The vector carrying the point mutation in that specific viral protein was generated in house and was subjected to numerous experiments.

Most importantly, a novel tool had been developed, the newly generated stable cell lines can be used in the research for the pilot experiments in the field of regenerative medicine. Investigations carried out on the non-human live material, allow for the progress without ethical controversy, nevertheless this development is to a certain degree limited. The restrictions concern mostly similarities and the extent of the comparability of the rodent and human systems. Presented data can serve as a platform or interface between the bench and bedside and bring closer to the personalized medicine.

In this doctoral thesis various assessments to maximize the profits from *in vitro* methods were presented. On the other hand, the *in vivo* experiments are indispensable in order to truly explore the multiplicity of the roles which endothelial cells play in the vascular system of the human beings. The interactions occurring in health and pathological conditions cannot be envisaged and fully recapitulated *in vitro*. The experiments conducted with living organisms are also not free from disadvantages: (i) advancement of the techniques, (ii) length of the time needed for the assay, (iii) restricted amount of animals that can be used per possible tests, (iv) inconstancy within the specimens and between the animals coming from the same species.

The newly established cell lines were attainable in the time frame of four weeks. These cell lines were applied for tracking the endothelial differentiation and verification of the antibiotic resistance. The conducted experiments showed that the utilized system of antibiotic selection did not bring the anticipated results. Despite the noticeable enrichment of the GFP expression, the weak point of this procedure was most probably insufficient number of the endothelial cells in the population. Another objective, that needs to be raised, is the choice of the suitable differentiation method, which would yield high amount of the ESC-derived endothelial cells.

Additional hurdle to overcome after differentiation is the indication of positive/stable clones. The process is a vicious circle, because only upon differentiation the transduced cells can be screened,

in search of the specific GFP expression. The efficiency of this procedure was extremely low, few clones out of generally 96 picked clones from among $3(x)10^3$ transduced mES cells turned out to be positive. The ratio of lentiviral transductions was reaching 1-3% of the success rate. This fact is widely reported in the literature. However, the production of viral particles for the successful transductions was achieved. The idea of testing different concentrations of the DNA and transfection factor in combination with the determined number of HEK cell brought satisfactory results. The next step, in the optimization of that procedure, was the creation of the stock of validated viral particles, ready-to-use in upcoming experiments, what was successfully implemented.

Until now, the core issue of the application of the integrase deficient lentiviral vector remains unresolved. The attempts with HDAC inhibitors brought new hope, yet it is too early to state if the achieved results would benefit the induction of pluripotency with the OKMS or OSK vectors.

To summarize, the collected data need careful consideration and application of strengthening arrangements in order to bring closer the idea of derivation endothelial cells from hES or hiPS cells. Possibility to use genetically amended, but yet autologous cells, will tremendously minimize the risk of implant rejection by autoimmune response. Pluripotent stem cells are endless, but still not fully discovered, source of potentially applicable remedies to be used in the regenerative medicine. Gene transfer and cell replacement therapies are still in their infancy, though they are closer than ever before.

6. Outlook

Methods which could be applied are described in this chapter and will be considered for future experiments in order to improve the derivation of the endothelial cells.

The risk of the random insertion of the exogenous gene into the rodent genome can cause many unexpected events, like for example default functioning of critical genes. The introduced gene may be silenced, which in consequence may result in unclear phenotype. One of the possible solutions might be the Rosa26 permissive locus (Fig. 59).

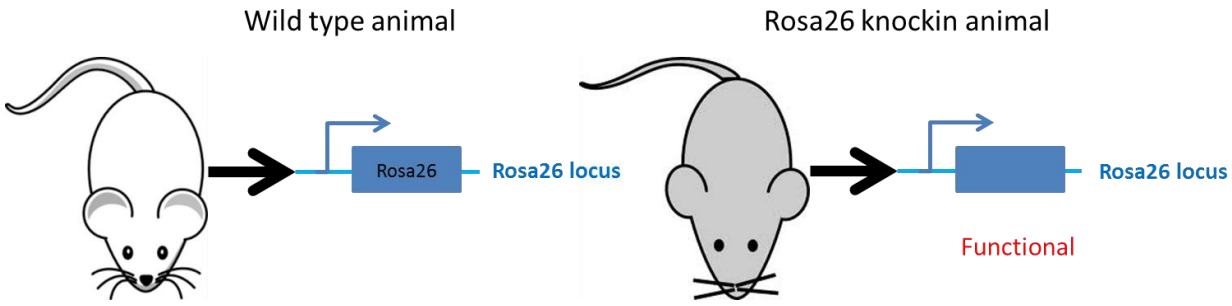


Figure 59: The cartoon depicting Rosa26 locus functionality. Graphic modified after genOway website: http://www.genoway.com/.

The Rosa26 locus in mice is very useful when it comes to genetic modifications, because it might be targeted with tremendous efficacy and is expressed in almost all cell types. The gene directed to the Rosa26 locus by homologous recombination in ES cells can be utilized in order to obtain constitutive and ubiquitous expression of the introduced gene (Perez-Pinera et al., 2012). The existence of the Rosa26 locus was proved by targeting the tdRFP (red fluorescent protein), cDNA to this locus by means of homologous recombination. The expression of tdRFP in the various lineages derived from human ES cells was then observed, proving that the hypothesis was right (Irion et al., 2007; Friedrich and Soriano, 1991).

Another approach to be considered is the application of adeno associated virus integration site 1 (AAVS1). The AAVS1 is also known as a PPP1R2C locus on human chromosome 19 and is referred to as a "safe harbor". This means, that it can anchor exogenous DNA of an anticipated

function. What is characteristic for this site is the fact that it possesses an open chromatin structure and is capable of transcription. There are no reports concerning unwanted consequences for the cell resulting from the introduced DNA sequence. In order to achieve the integration at the safe genomic locus one more element needs to be introduced the transcription activator-like effectors (TALEs). TALEs belong to a group of bacterial plant pathogen proteins, which identify exact DNA sequences and alter the gene behavior. Upon binding with the a FokI cleavage domain, the naturally occurring restriction endonuclease, TALE nucleases (TALENs) identify particular sequences of DNA, which were previously determined through the internal tandem repeats conveyed in the TALEs. Connection of the two TALENs and the desired DNA enables FokI to dimerize. Through that action, a break in the aimed chromosome is initiated and in this way homologous recombination at the site of the cleavage is promoted. The TALEs paired with the nucleases (Fig. 60), when applied in combination with the AAVS1 donor vectors, which comprises homologous recombination flanking arms, may serve as tool for exogenous gene insertion to the defined and safe genomic harbors (Juillerat et al., 2014).

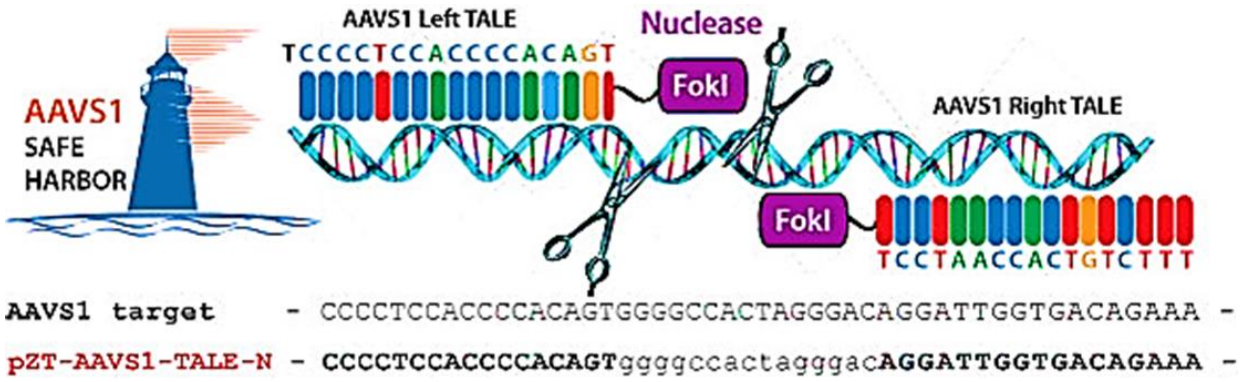


Figure 60: Scheme illustrating targeted integration at a safe genomic locus AAVS1 Safe Harbor TALE-Nuclease. Reprinted from System Biosciences website: http://www.systembio.com/aavs1.

Making the introduction of new genes into the mammalian cells a common technique, would tremendously facilitate the basic science. Majority of the methods for the stable introduction of genes in to host cells apply random integration of the transgene, accompanied by antibiotic selection and screening processes, which would indicate cell subset carrying the transgene. The random integration method, however, causes many undesired side-effects. Finding the right tools, which would ease

the exact insertion of the transgene at the precise locus in the host genome, could elevate all unwanted consequences of random integration (Perez-Pinera et al., 2012).

The two presented alternatives, for introducing an exogenous DNA sequence into the host genome, sound promising. These methods may bring closer to reach the desired cell subsets, thanks to the transgenic animals from which these cells could be obtained.

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9. Declaration

Eidesstattliche Erklärung

Hiermit erkläre ich, Anita Jolanta Golec, dass ich die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt habe, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten.

Gießen,

Anita Golec