Maria Raissi Dehkordi

Generation of pure endothelial cells from transgenic embryonic stem cells exhibiting an endothelial cell-specific expression of green fluorescent protein upon differentiation

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Maria Raissi Dehkordi

from Tehran, Iran

Giessen, 2014

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and

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This work is dedicated to

My Beloved Family

who have always been there for me in all stages of my life

by their unconditional loving care

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1 Introduction

1.1 Cardiovascular diseases

1.1.1 Definition and prevalence

Cardiovascular diseases (CVDs) are considered as the major cause of mortality globally, accounting for 17.3 million deaths per year, a number that is expected to exceed 23.6 million by 2030 (Mendis, Puska et al. 2011; Smith, Collins et al. 2012). Nearly 30 % of the deaths worldwide are attributed to CVDs (Mendis, Puska et al. 2011). These diseases currently account for half of non-communicable diseases and are considered as one of the major causes of morbidity around the world (Mendis, Puska et al. 2011; Laslett, Alagona et al. 2012). According to the report presented by the American Heart Association, the mortality rate caused by these diseases has decreased in North America during the last decade, though the morbidity and the socioeconomic burden caused by these ailments still remain high (Roger, Go et al. 2012). On the other hand, the distribution of CVDs, once considered to mainly affect developed countries, has now shifted to the developing countries due to urbanization, globalization, and industrialization (2010). In these countries, nearly 80% of the CVDs- and diabetes-related deaths usually occur at younger ages than in higher-income countries (Mendis, Puska et al. 2011; Smith, Collins et al. 2012). From 2011 to 2025, CVDs account for nearly 50% of projected cumulative economic losses due to non-communicable diseases in low- and middle- income countries (Laslett, Alagona et al. 2012).

Cardiovascular diseases are classified into atherosclerotic and non-atherosclerotic. The atherosclerotic category includes coronary heart disease, cerebrovascular disease, diseases of the aorta and arteries, including hypertension and peripheral arterial disease, and diseases of the veins including deep vein thrombosis. The non-atherosclerotic diseases include rheumatic heart disease, congenital heart disease, and cardiac conduction and valvular disorders.

1.1.2 Endothelial dysfunction and cardiovascular diseases

Different mechanisms participate in the pathogenesis of CVDs. These diseases are generally caused by stenosis or occlusion of the vessels supplying blood to the end

organs, primarily due to atherosclerosis (Libby and Theroux 2005; Jawad and Arora 2008). Endothelial cells (ECs) have been demonstrated to play a central role in many atherosclerotic diseases, including coronary heart disease (Heitzer, Schlinzig *et al.* 2001; Libby 2002; Bonetti, Lerman *et al.* 2003; Kullo and Malik 2007). Upon encountering risk factors such as dyslipidemia, glycoxidation products caused by diabetes mellitus, pro-inflammatory cytokines derived from adipose tissue, or bacterial by-products, ECs augment the expression of particular adhesion molecules such as vascular cell adhesion molecule (VCAM) and chemotactic factors such as macrophage chemoattractant protein-1 (MCP-1) (Steinberg 2002). This may lead to an increased attraction of leukocytes and monocytes, and the accumulation of resident macrophages at the subendothelial space (Libby 2002; Mestas and Ley 2008). In addition, the oxidation of low-density lipoprotein (LDL) particles and the resulting formation of foam cells may be partly promoted by ECs (Luo, Liu *et al.* 2012), leading to an increased expression of scavenger receptors by macrophages (Li and Glass 2002; Moore and Tabas 2011). The interaction between resident leukocytes, ECs, and smooth muscle cells (SMCs) migrating from the media to the intima leads to, sustains, and promotes the formation of atherosclerotic plaques (Libby and Theroux 2005; Sakakura, Nakano *et al.* 2013). Moreover, damage of the endothelial monolayer of atherosclerotic plaques can lead to further thrombotic events (Virmani, Burke *et al.* 2006; Sakakura, Nakano *et al.* 2013). Newer studies dissect novel roles for dysfunctional ECs in vascular calcification through the formation of osteoprogenitor cells (Yao, Jumabay *et al.* 2013).

Dysfunctional ECs and their deregulated stimulation and response play a central role in several other pathological conditions including diabetes mellitus (Taylor 2001; Tabit, Chung *et al.* 2010; Wong, Wong *et al.* 2010), hypertension (Versari, Daghini *et al.* 2009), aging and smoking (Brunner, Cockcroft *et al.* 2005), congestive heart failure (Kerem, Yin *et al.* 2010), allograft rejection (Rao, Yang *et al.* 2007), and pulmonary hypertension (Sakao, Tatsumi *et al.* 2009).

1.1.3 Therapeutic modalities for cardiovascular diseases

In addition to risk factor stratification at the early stages, pharmacological or interventional therapy may be necessitated in the management of CVDs (Deveza, Choi *et al.* 2012). Surgical procedures are usually preferred in patients with multiple vascular bed involvement (McFalls, Ward *et al.* 2004). However, these procedures may not be

always safely used in case of co-morbidities. Therapeutic catheterization procedures still remain the gold-standard strategies for the management of many CVDs with underlying vascular derangement, especially those with acute events or full vessel occlusion. Nevertheless, there are limitations due to restenosis and in-stent thrombosis, especially in patients with microcirculatory disorders (Libby, Schwartz *et al.* 1992; Thanyasiri, Kathir *et al.* 2007; Kim, Suh *et al.* 2008; Seiler 2010; Brilakis, Patel *et al.* 2013). Even under adequate anti-platelet therapy, different factors such as endothelial dysfunction may be correlated with thrombotic events after stent implantation (Fujisue, Sugiyama *et al.* 2013).

Body's own protective mechanisms may partly prevent the pathological conditions related to vascular stenosis. For instance, migration and proliferation of healthy ECs may contribute to the repair of damaged ECs (Ross, Bowen-Pope *et al.* 1982). This can be achieved by the body's innate physiological response to ischemia by upregulation of angiogenic growth factors and mobilization of circulating elements that enable new vessel formation through different mechanisms (Losordo and Dimmeler 2004). These mechanisms include vasculogenesis (*de novo* formation of primordial ECs from undifferentiated ECs or angioblasts), angiogenesis (sprouting of new blood vessels from preexisting blood vessels), and arteriogenesis (formation of arteries)(Yancopoulos, Klagsbrun *et al.* 1998; Heil, Eitenmuller *et al.* 2006; Penn 2008; Carmeliet and Jain 2011; Xu and Cleaver 2011; Deveza, Choi *et al.* 2012; Marcelo, Goldie *et al.* 2013).

Most of therapeutic studies have focused on angiogenesis (Lu, Pompili *et al.* 2011) through different mechanisms, including upregulation of vasculogenic/angiogenic genes, application of potent growth factors, and cellular therapy (Chu and Wang 2012). There are several reviews discussing the advances in, as well as hurdles of gene therapy approaches (Hedman, Hartikainen *et al.* 2011; Ishikawa, Tilemann *et al.* 2011). Directed application of selective growth factors, including vascular endothelial growth factor (VEGF) has been widely studied and tested in animal ischemia models as well as clinical studies (Folkman 1998; Losordo and Dimmeler 2004; Das, George *et al.* 2009; Lu, Pompili *et al.* 2011; Chu and Wang 2012). However, the efficiency and clinical benefit of these methods in the induction of a sustainable vasculature has not been largely confirmed in clinical trials (Deveza, Choi *et al.* 2012).

More recently, cellular therapy approaches or stem cell therapy has gained attention in the induction of neovascularization and treatment of CVDs (Kastrup 2010). Patients

with CVDs have been estimated to be the largest group benefiting from these approaches compared to other patient groups (Hotkar and Balinsky 2012). Cellular therapy in the treatment of CVDs may act both indirectly via induction of growth factors (paracrine effects), and directly through homing and proliferation at sites of injury (Srivastava and Ivey 2006; Adams, Xiao *et al.* 2007; Sun and Gerecht 2009; Leeper, Hunter *et al.* 2010). Most stem cells used in clinical trials have been harvested from bone marrow, as the most easily used source for autologous application. However, the disadvantages of bone marrow-derived stem cell therapy include the paucity of obtainable therapeutically useful stem cells and the need for substantial extended culture before use (Perin, Dohmann *et al.* 2003; Pittenger and Martin 2004; Horwitz 2008; Hotkar and Balinsky 2012).

Historically, scientists have divided stem cells into two major distinct categories: Embryonic stem (ES) cells and adult stem cells (Leeper, Hunter *et al.* 2010). Induced pluripotent stem cells (iPSCs), which are produced through the reprogramming of adult somatic cells into multipotent stem cells are another category of stem cells, which have recently gained attention in cell therapy.

1.2 Stem cells

1.2.1 Stem cell properties

The term “stem cells” originates in the literature as early as 1868 in the brilliant studies by the German biologist Ernst Häckel. He described stem cells as the unicellular organism from which a multicellular organism originates and portrayed “stem trees” to show the hierarchy of evolution (Ramalho-Santos and Willenbring 2007). Stem cells are defined by their characteristics of long-term self-renewal and differentiation potential (Gardner and Beddington 1988; Morrison, Shah *et al.* 1997). Self-renewal is the asymmetrical or symmetrical division of a stem cell to one or two daughter stem cells with at least one daughter cell having the developmental potential similar to the mother cell (He, Nakada *et al.* 2009). This property, though, is not unique to stem cells. For instance, self-renewal may also be seen in lymphocytes as terminally differentiated cells (Fearon, Manders *et al.* 2001), a small number of glial progenitor-type cells (Trentin, Glavieux-Pardanaud *et al.* 2004), and in endodermal cells derived from ES

cells in co-culture with mesenchymal cells (Sneddon, Borowiak *et al.* 2012). Nevertheless, this property, which occurs extensively in stem cells, can be generally distinguished from limited self-renewal processes in these restricted populations of progenitor-type or adult cells.

Differentiation is another characteristic of stem cells, which happens when the stem cells differentiate into one specialized cell type or a multitude of them, and lose their self-renewal potential.

1.2.2 Application of stem cell studies

A basic insight into the processes occurring during stem cell self-renewal and differentiation can be enormously helpful in understanding the *in vivo* mechanisms during growth and regeneration procedures. It may help us understand where is the point that the fate of a cell is determined to go through differentiation or stay at a more premature state. Through *in vitro* and *in vivo* stem cell studies, we may comprehend the body's repair mechanisms in confrontation with internal and external insults (Gardner 2007). This knowledge may translate into therapeutic approaches for a multitude of diseases, as well as understanding of the underlying aging mechanisms.

One of the aims of regenerative medicine is to provide adequate numbers of cells for therapeutic purposes, when the body's own mechanisms are not able to overcome the damages induced by genetic, chemical or physical factors. Cellular therapy has been most widely used for the regeneration of epidermis or cornea (Rama, Matuska *et al.* 2010; Chadli, Martin *et al.* 2011), therapy of spinal cord injury (Mothe and Tator 2013), as well as treatment of leukemia (Burt, Loh *et al.* 2008), Parkinson's and Huntington's disease (Lescaudron, Naveilhan *et al.* 2012; Nishimura and Takahashi 2013), diabetes mellitus (Chhabra and Brayman 2013), and CVDs (Bartunek, Behfar *et al.* 2013). Application of stem cells as models for understanding and treatment of cancer has also gained attention (Bajada, Mazakova *et al.* 2008). However, many hurdles should be overcome before reaching the optimal therapy conditions. One of the concerns is immunogenicity in "non- autologous transplantation". The other issue is defining a particular point of time that is ideal for therapeutic purposes, i.e. possess both the desired proliferative capacity and target cell properties *in vivo*. Comprehensive studies on stem cell dynamics and their milieu as well as clinical experiments shall provide an interface between basic science and medicine for the best targeted applications.

One of the new applications of stem cells is drug discovery. This process may benefit from the expandability and testability of these cells *in vitro*. Tissue engineering may serve to optimize the stem cell niche and improve the quality assurance of the tested drugs (Nirmalanandhan and Sittampalam 2009), by providing a well-designed three-dimensional milieu, mimicking the real *in vivo* circumstances.

1.2.3 Stem cell categories

Stem cells are generally classified in two ways: Based on the level of their plasticity i.e. the, meaning that they can give rise to all cell types, including extraembryonic tissuesability of a cell to become different cell types, and according to the tissue that they originate from. On the basis of plasticity, these cells can be divided into totipotent, pluripotent, and multipotent. Germ cells and embryos at the 1-or 2-cell stage are totipotent.

Adult stem cells and cord blood stem cells are examples of multipotent cells, giving rise to multipotent cells and a particular group of differentiated cells. These types of stem cells are undifferentiated cells found in some terminally differentiated tissues with a high turnover rate, such as blood and skin, or in organs with a significant regeneration capacity, like liver and pancreas (Rando 2006). Adult stem cells are deemed to persistently interact with their surrounding milieu or 'niche' (Scadden 2006; Greco and Guo 2010). The fact that they are isolated from a specific tissue or organ increases the chances to direct these cells into a restricted population of adult cells that they are destined to be. However, their scarce numbers (with the exception of hematopoietic cells), as well as their tendency to transdifferentiate into other cells types (Herzog, Chai *et al.* 2003; Kanji, Pompili *et al.* 2011; Fukata, Ishikawa *et al.* 2013; Scarlett 2013), make their isolation very difficult due to the possibility of contamination with other intervening cells types (Brignier and Gewirtz 2010). Cord blood cells as another type of multipotent stem cells have recently gained attention for therapeutic purposes (Bissels, Eckardt *et al.* 2013).

Embryonic stem cells and embryonic germ cells (EGCs) are examples of pluripotent stem cells. Pluripotent cells can give rise to all cells types, except for totipotent cells and extraembryonic tissues. Embryonic germ cells are isolated from primordial germ layers in the post-implantation period commencing at E 6.5 through a nuclear re-programming event (Hayashi, de Sousa Lopes *et al.* 2007; Saitou, Kagiwada *et al.* 2012). These cells

demonstrate the typical ES cell markers and have the ability to contribute to chimeras (Leitch, Blair *et al.* 2010). Embryonic stem cells, which are obtained from the inner cell mass (ICM) of the embryo, have gained enormous attention during the last 2 decades for developmental as well as experimental therapeutic purposes. In the next section, a brief overview about embryonic stem cells is discussed.

1.3 Embryonic stem cells

1.3.1 Origin of embryonic stem cells

After formation of the zygote, cells undergo division. At the 16-cell morula stage, polarization and compaction of the embryo occurs. At this time point, cells consist of two layers. The outer layer comprises the trophoectoderm, giving rise to the outer layer of placenta and umbilical cord. The inner layer gives rise to ICM, where all the cellular lineages of the embryo (ectoderm, mesoderm and endoderm), allantois, amnion, and yolk sac originate. At embryonic day 3 in mouse and embryonic days 5-6 in human, cavitation (blastocoel formation) occurs and ICM increases in size (Figure 1 A). Embryonic stem cells were first isolated in 1981 by separation and culture of cells from the ICM of blastocysts (Evans and Kaufman 1981; Martin 1981). The efficiency of mouse ES cell isolation strongly relies on the genetic background of the mice. In some inbred animals, the isolation efficiency is much more robust compared to others (Yu and Thomson 2008).

At day 4.5 in mouse and days 8-9 in human, the embryo implants in the uterus wall through the syncytiotrophoblast, which comprises the outer layer of the trophoblast (De Miguel, Fuentes-Julian *et al.* 2010). At this bilaminar stage (Figure 1 B), ICM is divided into two layers: hypoblast and epiblast. Hypoblast or the primitive endoderm contributes to the extraembryonic tissues such as the lining of the primitive yolk sac. Epiblast or the primitive ectoderm gives rise to all germ layers, including ectoderm, mesoderm, and endoderm, and is a source for epiblast-derived stem cells. These cells are pluripotent and unlike ES cells, are derived from the post-implantation embryo (Tesar, Chenoweth *et al.* 2007).

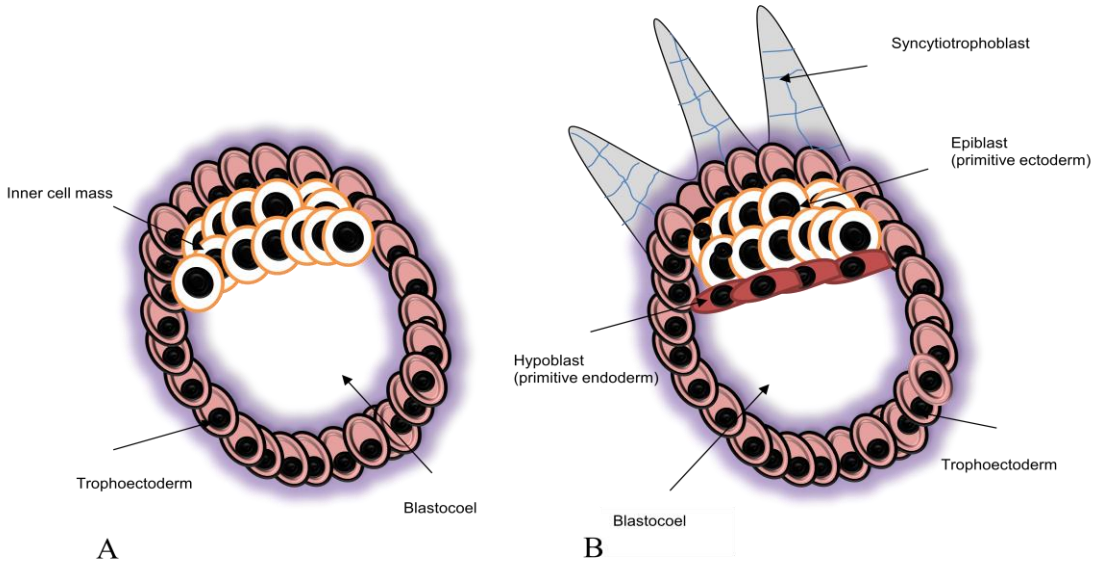

Figure 1 Schematic view of the mammalian embryo.

(A): Blastocyst stage; **(B):** Bilaminar stage. Adapted and modified from De Miguell *et al.* Stem Cell Rev 6(4), 633-649 (2010).

1.3.2 Pluripotency and self-renewal in mouse embryonic stem cells

At the time mouse ES cells were for the first time isolated, they were cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) to maintain their undifferentiated state (Evans and Kaufman 1981; Martin 1981). Later, analysis of the medium led to the identification of leukemia inhibitory factor (LIF) as a cytokine keeping mouse ES cells in their undifferentiated state (Smith, Heath *et al.* 1988; Williams, Hilton *et al.* 1988). Leukemia inhibitory factor is a member of the interleukin (IL)-6 family, which acts through heterodimerization of its receptor and glycoprotein (Gp)130. The LIF-induced activation of its receptor and Gp130 triggers a cascade of events via different signaling pathways, leading to the maintenance of pluripotency and self-renewal of ES cells through the activation of transcription factors (Zhang, Owczarek *et al.* 1997; Burdon, Smith *et al.* 2002) (Figure 2).

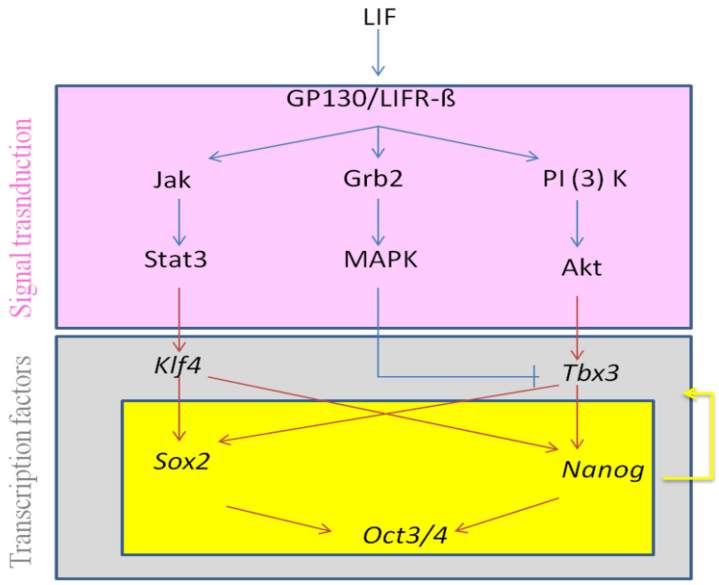

Figure 2 Parallel signaling pathways contributing to pluripotency in mammalian embryonic stem cells

The Jak–Stat3 pathway activates Klf4, while the PI(3)K–Akt pathway stimulates the transcription of Tbx3. The MAPK pathway antagonizes the nuclear localization of Tbx3. Klf4 and Tbx3 mainly activate Sox2 and Nanog, respectively, and maintain expression of Oct3/4. Transcription of all these transcription factors is positively regulated by Oct3/4, Sox2 and Nanog, conferring stable expression in the absence of all signals. Klf4: Kruppel-like factor 4; Tbx3: T box transcription factor 3; PI(3)K-Akt: phosphatidylinositide 3-kinases-Akt pathway; Oct3/4: octamer-binding transcription factor—3/4. Sox 2: sex determining region Y-box 2. Adapted and modified from Niwa H *et al.* Nature 460, 118-122 (2009).

An autoregulated circuit between some of the transcription factors such as octamer-binding transcription factor (Oct)-4, sex determining region Y-box (Sox) 2, and the homeodomain protein Nanog results in the induction and maintenance of stemness and self-renewal in ES cells (Boyer, Lee *et al.* 2005). Despite some common features, there are differences between mouse and human systems. For instance, c-Myc has a regulatory role in stemness in mice, but not in human (Kidder, Yang *et al.* 2008). Another difference is the existence of the stage specific embryonic antigen (SSEA)-1 in mice and SSEA-3/4 in human (De Miguel, Fuentes-Julian *et al.* 2010).

Embryonic stem cells have been typically cultured in serum-containing medium. Fetal bovine serum (FBS) contains a low amount of antibody and a high content of growth factors, making it a versatile condition for the culture of a variety of cells. Due to some unknown interactions between serum components and cells, some techniques have been more recently invented to keep cells in a more unanimously undifferentiated

state. Application of a combination of cytokines such as LIF and bone morphogenic protein (BMP)-4, as well as a mixture of inhibitors, namely GSK inhibitor (CHIR99021) and MEK inhibitor (PD0325901) in a serum-free condition has been shown to maintain ES cells in their ground state (Niwa, Ogawa et al. 2009; Li and Ding 2010; Kanda, Sotomaru et al. 2012; Lee, Chuang et al. 2012)(Figure 3). Despite the existing evidence for a higher expression level of markers of undifferentiated state in serum-free culture using small molecules, this method has not been shown to improve human ES cell derivation from blastocysts (Van der Jeught, O'Leary *et al.* 2013). Moreover, these studies have not been used in large scale, high-throughput systems. The high costs of these inhibitors and cytokines is another issue that must be taken into account. As a result, these methods have not been yet considered as ideal for high-throughput experiments.

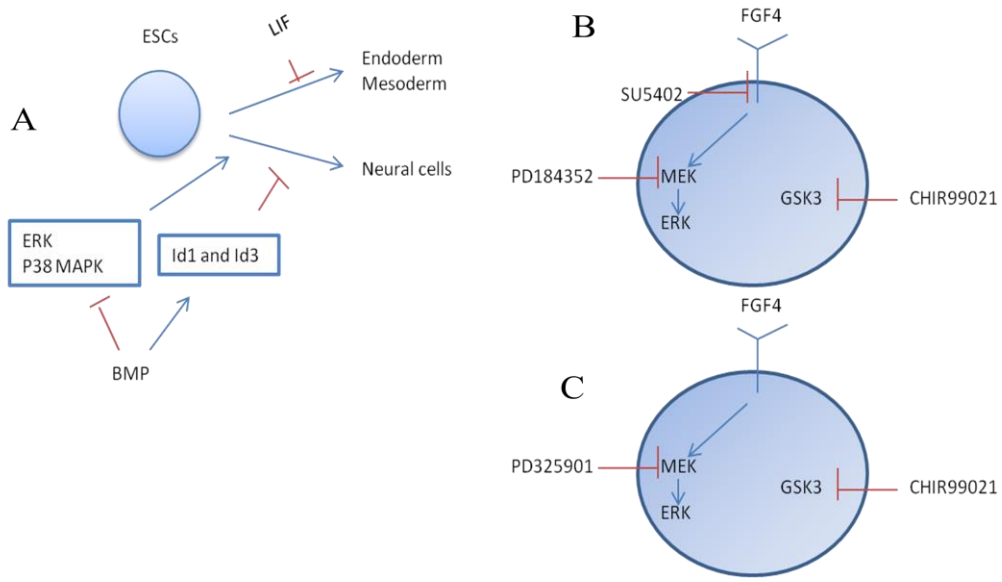

Figure 3 Maintenance of self-renewal in mouse embryonic stem cells using cytokines and chemical inhibitors

(A): Inhibition of differentiation by LIF and BMP **(B):** Inhibition of differentiation by 3 inhibitors (3i) **(C):** Inhibition of differentiation by 2 inhibitors (2i). LIF: leukemia inhibitory factor; BMP: bone morphogenic protein; ESCs: embryonic stem cells; FGF-4: fibroblast growth factor-4. Adapted and modified from Hirai H, *et al.* Biochem J. 438 (1) 11-23 (2011).

1.3.3 Differentiation of mouse embryonic stem cells

In the absence of MEFs and LIF in medium, ES cells spontaneously differentiate into various cell types (Robertson 1997). When ES cells are injected into the blastocyst, they

can undergo the process of differentiation just in a way similar to the native cells of the ICM (Bradley, Evans *et al.* 1984). Once ES cells are cultivated in suspension culture conditions, i.e. under circumstances that discourage their attachment to the culture plate, they tend to form aggregates that are called embryoid bodies (EBs)(Doetschman, Eistetter *et al.* 1985). The differentiation in EBs recapitulates cell differentiation in the ICM, where the outer layer forms a primitive endoderm and the inner cells undergo apoptosis and contribute to the amniotic cavity (Coucouvanis and Martin 1995; Sasaki, Fassler *et al.* 2004; Fujiwara, Hayashi *et al.* 2007). Upon differentiation of ES cells, a variety of different cell types arise. These cell types include cardiomyocytes (Wei, Juhasz *et al.* 2005), smooth muscle cells (Vazao, das Neves *et al.* 2011), osteogenic cells (Yamashita, Nishikawa *et al.* 2010), chondrogenic cells (Toh, Guo *et al.* 2009), neuronal cells (Hayashi, Guerreiro *et al.* 2010), endothelial cell (EC)s (Li, Hu *et al.* 2011), hepatic and pancreatic cells (Medine, Lucendo-Villarin *et al.* 2008; Schulz, Young *et al.* 2012; Sivertsson, Synnergren *et al.* 2013), and hematopoietic cells (Lim, Inoue-Yokoo *et al.* 2013) (Figure 4).

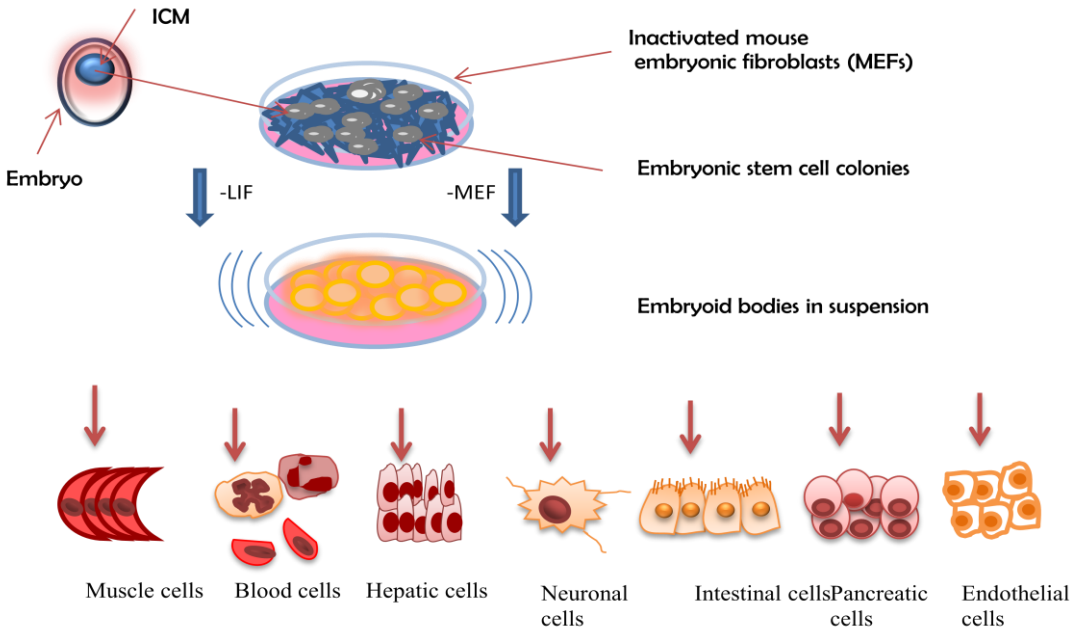

Figure 4 Schematic view of culture and differentiation of embryonic stem cells

Mouse embryonic stem cells are obtained from the inner cell mass of the embryo. In the absence of LIF and MEFs (feeder cells), mouse embryonic stem cells form embryoid bodies, which under certain culture conditions can give rise to different cell types. ICM: inner cell mass; LIF: leukemia inhibitory factor; MEFs: mouse embryonic fibroblasts.

1.3.4 Differentiation of mouse embryonic stem cells to endothelial cells

Mesodermal cells in the early embryo, which are developed from the ICM of the embryo, are positive for brachyury. In 1998, Gordon Keller and his co-workers identified a precursor called blast-colony-forming cells (BL-CFC), as an *in vitro* equivalent of hemangioblasts (Choi, Kennedy *et al.* 1998). These cells are positive for both brachyury and fetal liver kinase (Flk)-1, and have the potential to develop into ECs. Under the influence of different transcription factors and environmental stimuli, the same precursor can develop into SMCs, as well as contribute to the definitive hematopoiesis (Lancrin, Sroczynska *et al.* 2010)(Figure 5). Definitive hematopoiesis occurs after the early wave of primitive hematopoiesis in the yolk sac and is mediated through the hemogenic endothelium. These cells originate from the dorsal aorta in close propinquity to the endothelium wall of the aorta (de Bruijn, Speck *et al.* 2000; Eilken, Nishikawa *et al.* 2009; Lancrin, Sroczynska *et al.* 2009).

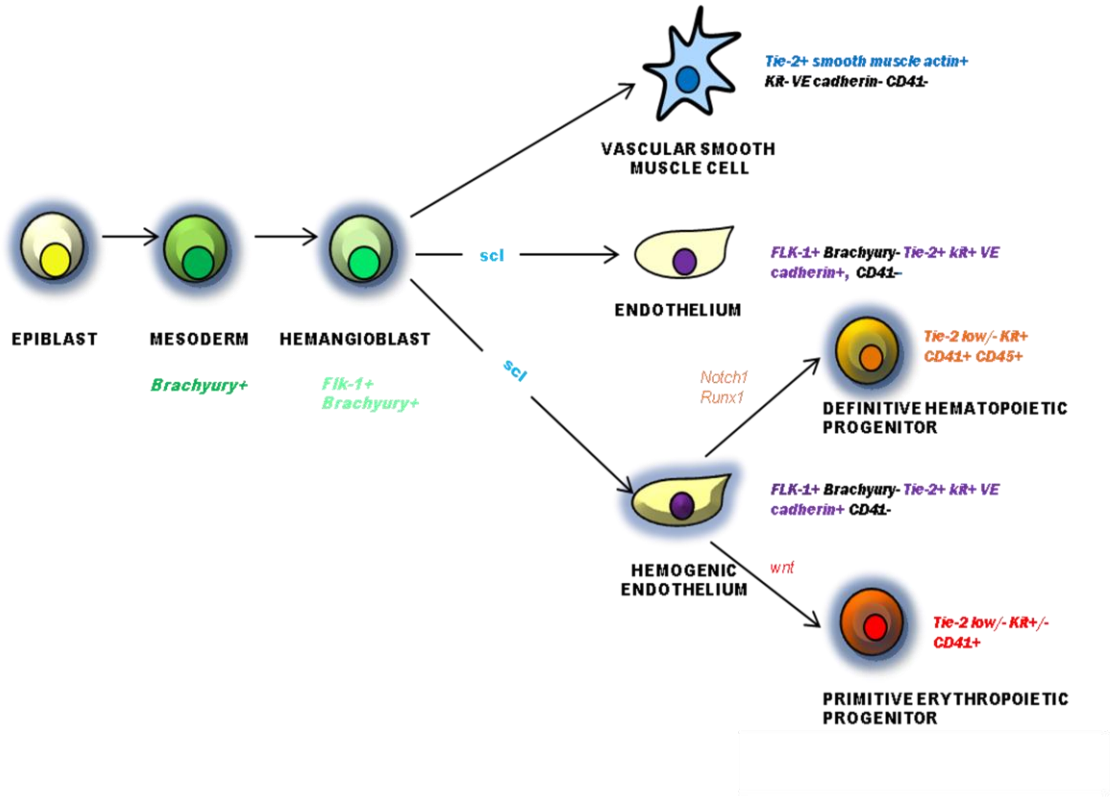

Figure 5 Model of endothelial and blood cell formation from the hemangioblast

In the figure, the specific phenotypes of differentiated cell populations derived from the hemangioblast, and the key regulators and transcription factors involved in endothelial and hematopoietic development are depicted (adapted and modified from Lancrin *et al.* J Mol Med; 88: 167-172 (2010)).

Derivation of ECs from ES cells has been widely based on EB formation and subsequent plating of EBs on different modified substrates in the presence of growth factors to enhance the formation of vascular networks. Blood islands and vascular networks in EBs are excellent models for vasculogenesis and angiogenesis in the early embryo, especially under pathological conditions such as hypoxia or tumor growth. These models underlie the ongoing studies on EC development as well as therapeutic applications and drug discovery (Kim, Kim *et al.* 2008; Huang, Niiyama *et al.* 2010; Kim, Bae *et al.* 2012).

1.4 Background to endothelial cells

Endothelial cells are a group of cells that originate from mesodermal cells in the body and line the inner part of the vasculature. The total number of ECs in adult human is approximately 1x 10^{13} (Sumpio, Riley *et al.* 2002). There are two general properties that apply to all ECs: 1) As an anatomical property, ECs adhere to each other to form a seamless inner lining of vessels in the body; 2) Functionally, these cells are selectively permeable and act as an active interface with body cells and fluids (Voelkel and Rounds 2009). Endothelial cells, once recognized as a homogeneous cell population, are now considered to be quite "heterogeneous", with broad morphological and functional variations (Aird 2012).

1.4.1 Ontogeny of the vasculature

The vasculature in the body is formed through two different major mechanisms: vasculogenesis and angiogenesis. These two mechanisms may also work in coordination to form body blood vessels (Figure 6). Angiogenic remodeling is another mechanism contributing to vasculature formation, which entails the reconstitution of the existing angiogenic plexus.

Vasculogenesis is defined as the *de novo* formation of primordial ECs from undifferentiated ECs or individual angioblasts, where blood vessels are formed through clustering of individual angioblasts into linear cords. This mechanism is followed by the formation of a patent lumen, which is also termed as tubologenesis (Risau and Flamme 1995; Xu and Cleaver 2011). Vasculogenesis is the primary mechanism through which the vasculature in the early embryo develops, including primitive blood vessels of the endocardium and primary aorta, as well as of the yolk sac. It implies a series of events

including angioblast proliferation, migration, adhesion, and differentiation (Voelkel and Rounds 2009).

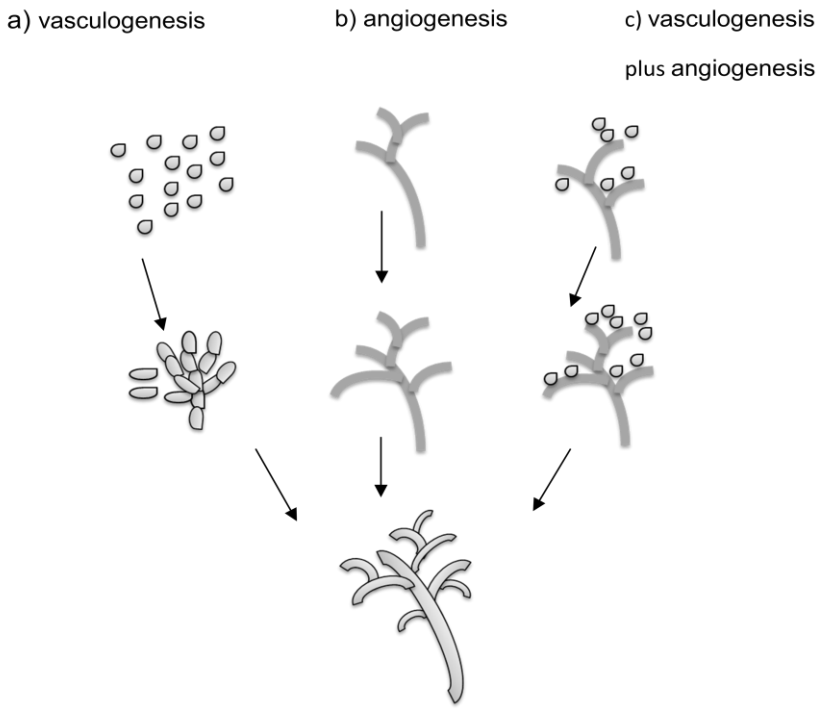

Figure 6 Schematic illustration of different mechanisms contributing to blood vessel formation.

a) Vasculogenesis is the *de novo* formation of endothelial cells through the aggregation of angioblasts in the mesoderm. b) Sprouting angiogenesis is the formation of new blood vessels through the formation of new sproutings out of the pre-existing vessels. c) Blood vessel formation through coordinated vasculogenesis and angiogenesis in the developing organs. Adapted and modified from The pulmonary Endothelium: Function in Health and Disease, Voelkel N and Rounds S, Wiley-Blackwell (2009).

The primary vasculature that has been formed through vasculogenesis is expanded through angiogenesis. Angiogenesis occurs through two mechanisms: Sprouting angiogenesis and angiogenic remodeling. Sprouting angiogenesis encompasses the elongation and sprouting of new blood vessels out of the pre-existing ones, leading to the expansion of vascular networks (Yancopoulos, Klagsbrun *et al.* 1998; Penn 2008; Carmeliet and Jain 2011; Marcelo, Goldie *et al.* 2013). This is mediated via proliferation of the quiescent cells at vessel walls and proteolytic degradation of the extracellular matrix, after which new sprouts starts to extend. Angiogenic remodeling is the mechanism through which the cells undergo changes in their size and shape, to adapt to cell demands and hemodynamic changes. An example in the early embryo is

the formation of the branching angiogenic tree out of the homogenous network, resembling a fisherman's net. Examples in the adult stage include remodeling in the female reproductive system, wound healing, and pathological conditions such as angiogenic tumors (Voelkel and Rounds 2009).

Arteriogenesis as a later maturation step leads to the formation of arteries, and is characterized by the coating of vascular beds via pericytes and SMCs (Heil, Eitenmuller *et al.* 2006). Angiogenesis and arteriogenesis play a major role in postnatal neovascularization procedures (Carmeliet and Jain 2011). Most of the therapeutic studies have been focused on angiogenesis (Lu, Pompili *et al.* 2011).

1.4.2 Transcription factors and key molecules in vascular development

The role of certain transcription factors in vascular development has been investigated in recent studies. Despite the redundancy of many of these transcription factors, a few of them such as Tal1 have shown a unique role in vascular development, especially in close interactions with Flk-1 and vascular endothelial (VE)-cadherin (Pham, Lawson *et al.* 2007). There is a narrow window for the expression of many of these transcription factors, which tend to be down-regulated, once the cells differentiate into a more adult phenotype (Kataoka, Hayashi *et al.* 2011). Some of these transcription factors such as Sox17 and Sox18 are essential for the specification of ECs to a particular phenotype (Marcelo, Goldie *et al.* 2013).

The VEGF family (VEGF A-E) and its receptors are key molecules in EC development, survival, and differentiation, as well as blood vessel formation. VEGF-A is highly expressed in the extraembryonic endoderm at the time of blood island formation in the yolk sac, as early as day 7.5 (Breier, Clauss *et al.* 1995). Absence of one of the VEGF-A alleles leads to early death of mice during embryogenesis, due to a variety of vascular defects (Carmeliet, Ferreira *et al.* 1996). On the other hand, VEGF-A overexpression is also lethal to the embryo (Miquerol, Langille *et al.* 2000). Different isoforms of VEGF-A (120, 164, 188 in mouse and 121, 145, and 165 in human) have distinct biological activities, the coordination of which leads to vascular development. VEGF-A 165 is an example of a smaller, more diffusible VEGF, which has been extensively used *in vitro*. Recent studies have shown that VEGF may principally induce

proliferation and propagation of ECs, rather than driving the cells into a special endothelial phenotype (Marcelo, Goldie *et al.* 2013). Flk-1 or vascular endothelial growth factor receptor-2 (VEGFR-2) is one of the most reliable markers for EC proliferation. Mice lacking Flk-1 die at E8.5 - E9.5 due to the lack of blood island and vascular plexus (Shalaby, Rossant *et al.* 1995). Likewise, ES cells lacking Flk-1 develop ECs, which are incapable of propagation (Schuh, Faloon *et al.* 1999).

BMP-4 is another key molecule in the induction of mesoderm, as well as its differentiation towards hematopoietic and endothelial fate (Marom, Levy *et al.* 2005; Chiang and Wong 2011). Fibroblast growth factor (FGF)-2 works downstream of BMP-4 and participates in hemangioblast formation in murine stem cells (Pearson, Sroczynska *et al.* 2008). However, no such role has been identified for FGF-2 in human ES cell studies (Kelly and Hirschi 2009).

1.4.3 Arterial versus venous versus lymphatic differentiation

Vessels can be classified into arteries, veins, and lymphatic vessels, based on their functional and anatomical properties, as well as the direction of fluids. This specification occurs very early during the development of primordial ECs of the yolk sac (Figure 7), according to a predefined pattern (Wang, Chen *et al.* 1998). On the other hand, assumption of a venous or arterial fate has been demonstrated to be plastic and reversible in line with the environmental stimuli and body demands (le Noble, Moyon *et al.* 2004; Nasu 2005).

The ephrin family of receptor tyrosine kinases is the largest family among the growth factor receptors, using membrane-tethered ephrin as their ligand (Marcelo, Goldie *et al.* 2013). EphrinB2 (EfnB2) ligand is expressed in arteries, while EphrinB4 receptor (EphB4) is enriched in veins. Activation of Flk-1 via VEGF may trigger the activation of EfnB2 and suppression of EphB4, thereby favoring an arterial identity (Lawson, Scheer *et al.* 2001). Chicken ovalbumin upstream promoter transcription factor (COUP TF)-II, on the other hand, has been shown to be one of the key genes for venous fate specification (You, Lin *et al.* 2005). Thus, an interrelation of all these genes and the reciprocal signaling may play a role in the specification of an arterial *vs.* venous phenotype.

The lymphatic circulation has an essential role in the immune system, as well as in returning the extravasated proteins and cells to the blood. Prospero homeobox protein (Prox)1 has been found to be highly expressed in the venous ECs, contributing to the lymphatic system (Srinivasan, Dillard *et al.* 2007). Venous ECs start to express Prox1 at day 9.5. By day 11.5, clusters of Prox1-positive venous ECs begin to emerge along the cardinal vein (Srinivasan, Dillard *et al.* 2007; Francois, Short *et al.* 2012).

The Sox18 gene, which is also essential for specification of a venous fate, has been shown to regulate Prox1 expression (Francois, Caprini *et al.* 2008; Marcelo, Goldie *et al.* 2013). However, the presence of Sox18 is not sufficient for lymphatic specification, as arteries also express Sox18 (Pennisi, Gardner *et al.* 2000). COUP-TFII, has also been shown to be highly expressed in lymphatic ECs (Lee, Kang *et al.* 2009), playing a role together with Sox18 in activation of Prox1 and the development of endothelial lymphatic progenitors (Srinivasan, Geng *et al.* 2010). Despite these proceedings, the exact mechanisms of arterial *vs.* venous *vs.* lymphatic endothelial differentiation still needs further declaration.

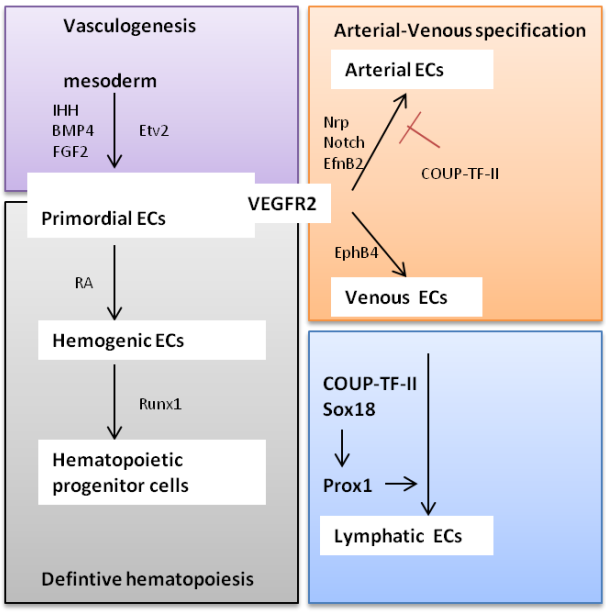

Figure 7 Specification toward an arterial *vs.* venous *vs.* lymphatic fate in the early embryo

Major extrinsic and intrinsic factors that regulate endothelial cell specification throughout embryonic vascular development. BMP4: bone morphogenetic protein 4; FGF2: fibroblast growth factor 2; IHH: Indian hedgehog; RA: retinoic acid; VEGFR2: VEGF receptor type II. Adapted and modified from Marcelo *et al.* Circ Res.112(9):1272-87 (2013).

1.4.4 Phenotypic diversity of endothelial cells

Apart from the specification of vessels to arteries, veins, and lymphatic vessels, there is a high level of endothelial phenotypic heterogeneity in various organs and vascular beds (Atkins, Jain *et al.* 2011). The basic mechanisms leading to the specification of a special EC phenotype in different organs are mostly unknown. Endothelial cells are generally classified into a continuous type, which is further subcategorized into non-fenestrated and fenestrated subgroups, and a discontinuous or sinusoidal type. The continuous non-fenestrated type is mostly found in the vessels of lung, brain, heart, muscle, and skin, and is characterized by tight and adherens junctions (Dejana 2004). The continuous fenestrated type is characterized by diaphragms, which allow the transport of water and small molecules, and is typically found in the capillaries of exocrine and endocrine glands and in the renal vasculature, where a high amount of transendothelial transport is required (Marcelo, Goldie *et al.* 2013). The discontinuous type is characterized by large fenestrae, and is found in certain sinusoidal vascular beds, including liver and bone marrow (Marcelo, Goldie *et al.* 2013). This diversity has an important impact in cellular therapy approaches.

1.4.5 Endothelial cell markers in early and late stages of endothelial cell differentiation

Endothelial cells are derived from a population of cells that are positive for brachyury (Lancrin, Sroczynska *et al.* 2010). Some endothelial-specific genes are expressed in both early and late stages of EC development with different expression levels and functional profiles. Flk-1, which is one of the earliest markers of ECs, may also give rise to a whole population of cells, including hematoendothelial cells and SMCs (Lancrin, Sroczynska *et al.* 2009). The high expression of this gene in ECs potentially makes it a versatile option for conventional purification of ECs. However, the expression of this marker in non-EC populations (Ahlbrecht, Schmitz *et al.* 2008) and the capacity of Flk-1-positive cells to give rise to non-ECs, including SMCs, hematoendothelial cells, and epithelial cells (Ishitobi, Wakamatsu *et al.* 2011), makes it less favorable for achieving pure populations of ECs. VE-cadherin, as another specific endothelial marker is predominantly expressed on more mature ECs, though its expression is also found on premature stages of EC development (Vittet, Prandini *et al.* 1996). Cluster of differentiation (CD)31 or platelet endothelial adhesion molecule

(PECAM)-1 as another EC marker is expressed in both early and late stages of EC differentiation, though its function may alter during the course of differentiation (Li, Wang *et al.* 2005; Mariappan, Winkler *et al.* 2009). Thus, most of these markers, though relatively specific to ECs, may not characterize definitie populations of early and late stages of EC development.

Endothelial progenitor cells (EPCs) are a distinct category of multipotent stem cells, which originate from the peripheral blood, cord blood, and bone marrow (Asahara, Murohara *et al.* 1997; Asahara, Masuda *et al.* 1999). These cells express both hematopoietic stem cell markers such as CD34 and CD133, and endothelial markers such as VE-cadherin, CD31, and Flk-1 (Hur, Yoon *et al.* 2004; Fadini, Losordo *et al.* 2012). Endothelial progenitor cells are divided into two subgroups of early and late EPCs (Hur, Yoon *et al.* 2004). Early EPCs, which appear after short-term culture of mononuclear cells from peripheral blood, express mononuclear cell markers and promote vasculogenesis through the production of cytokines and growth factors (Urbich, Aicher *et al.* 2005; Medina, O'Neill *et al.* 2010). Late EPCs, on the other hand, are involved in vasculogenesis and angiogenesis through physical incorporation into the vascular regeneration processes and highly express VE-cadherin and Flk-1 (Yoder, Mead *et al.* 2007; Medina, O'Neill *et al.* 2010).

In the experiments underlying my thesis, the promoter of VE-cadherin, as one of the most specific markers of ECs, was used for labeling and tracking of ECs by designing a vector in which the specific labeling protein GFP is expressed under the control of this promoter. On the other hand, due to the abundance and robustness of CD31 expression in different developmental stages of ECs, transgenic ES cell-derived ECs were selected based on the expression of this molecule for later experimental research such as cell purification for therapeutic purposes. Thereafter, the selective EC gene pattern during the course of differentiation in this population was assessed. In the next two sections, a brief overview of CD31 and VE-cadherin is provided.

1.4.5.1 VE-cadherin

VE-cadherin is the major endothelial-specific cell adhesion molecule, playing an important role in vascular development and growth (Lampugnani and Dejana 1997). This molecule is the cadherin number 5 among the cadherin family named cadherin -4 to 11, and is the only endothelial-specific among them. Different cDNA mappings have

shown a homology of VE-cadherin to the other known cadherins with the exception of differences within the cytoplasmic tail (Suzuki, Sano *et al.* 1991). Cloning of VE-cadherin has shown its specificity to the vascular system in embryonic tissue (Breier, Breviario *et al.* 1996; Larson, Wadman *et al.* 2004), so that mice lacking this gene die due to severe hemorrhage (Matsuyoshi, Toda *et al.* 1997). This molecule is represented as a dimer with extracellular domains 1-5, which interact with catenin proteins p120, β-catenin, and plakoglobin. β-catenin and plakoglobin connect to α-catenin, which is related to actin fibers (Figure 8). Some of the proteins that interact with VE-cadherin have enzymatic activity, while others have scaffolding properties. This interaction leading to the formation of complex structures, regulation of VE-cadherin activity, and signal transduction (Dejana, Orsenigo *et al.* 2008). The structural roles of VE-cadherin are crucial for the maintenance of the integrity of ECs and the barrier function.

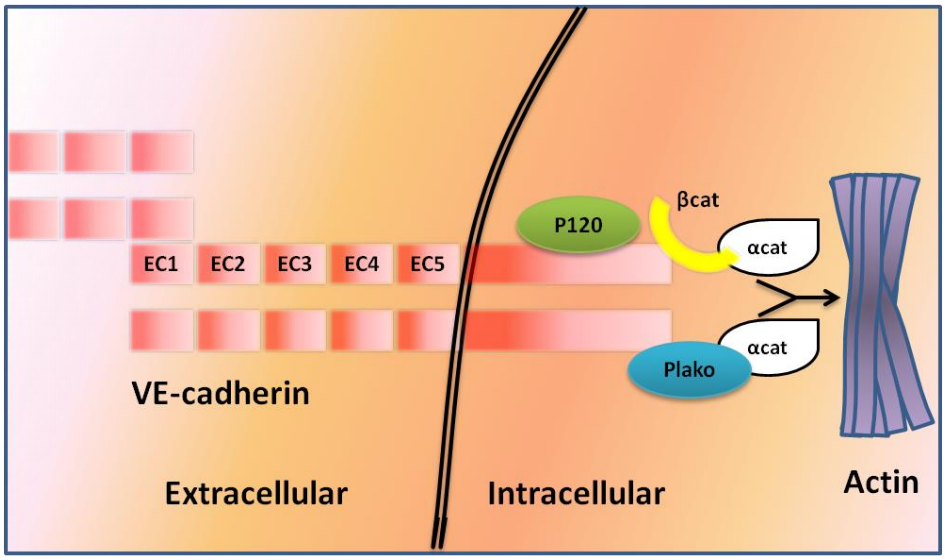

Figure 8 Molecular organization of VE-cadherin

VE-cadherin is shown as a dimer, the minimal functional unit of cadherins. EC1-EC5 are the homologous extracellular domains of VE-cadherin. The formation of multimolecular complexes, comprising signaling, regulatory, and scaffolding proteins is promoted by clustering of VE-cadherin.The interacting proteins include the catenin proteins p120, β-catenin (βcat) and plakoglobin (plako). β-catenin and plakoglobin connect directly with VE-cadherin and α-catenin (αcat). EC1-5: Extracellular domains. Adapted and modified from Dejana *et al.* J Cell Sci. 121(Pt 13):2115-22 (2008).

Apart from structural function, VE-cadherin has been shown to play a role in angiogenesis processes. This molecule is dispensable for the initial vasculogenesis, but is fundamentally required for the later angiogenesis and remodeling processes (Gory-Faure, Prandini *et al.* 1999; Bäumer, Keller *et al.* 2006). VE-cadherin supports the

survival signals of Flk-1 through transmission of antiapoptotic signals (Carmeliet, Lampugnani *et al.* 1999). This property has been used to design anti-VE-cadherin antibodies, which may potentially help to prevent the metastatic growth of tumors (Liao, Li *et al.* 2000). On the other hand, this molecule may play inhibitory functions on Flk-1 signaling, thus reducing the proliferation rate and contributing to vascular integrity (Vestweber 2008; Dejana and Giampietro 2012). A 2.5-kb region of the VE-cadherin promoter has been shown to direct an endothelial-specific reporter gene expression *in vivo* (Gory, Vernet *et al.* 1999).

1.4.5.2 CD31

CD31 is a type I transmembrane glycoprotein, consisting of an extracellular region with 6 immunoglobulin-like homology domains, a transmembrane domain, and a cytoplasmic tail. This molecule is highly expressed on ECs as a major constituent of the intercellular junctions (Newman 1997), and has a critical role in the maintenance of the vascular integrity in response to inflammatory stimuli (Carrithers, Tandon *et al.* 2005). This has been partly ascribed to its modulatory roles on other regulating molecules such as catenins and sphingosine-1-phosphate (S1P) (Komarova, Mehta *et al.* 2007). An anti-inflammatory role has been also attributed to CD31 through maintenance of vascular integrity, suppression of pro-inflammatory cytokines, inhibition of inflammatory signaling pathways (Carrithers, Tandon *et al.* 2005; Goel, Boylan *et al.* 2007), and inhibition of leukocyte transmigration through the rearrangement of the cytoskeleton (Chen and Tzima 2009).

In proportion to anatomic and physiological conditions, CD31 has been shown to be involved in seemingly contradictory functions. In areas where the blood turbulence and shear stress is disrupted, CD31 contributes to pro-inflammatory cascades (Harry, Sanders *et al.* 2008), while in other areas of the vasculature, the anti-inflammatory functions such as cytokine suppression predominate (Goel, Schrank *et al.* 2008). Some of the opposing functions of CD31 in inflammation might be ascribed to the isoform-specific actions of CD31 in different cell types due to alternative splicing, which may affect all the inflammatory functions, such as vascular integrity, angiogenesis, and leukocyte adhesion to ECs (Wang and Sheibani 2006; Bergom, Paddock *et al.* 2008). CD31 is also expressed in the hematopoietic lineage (Newman 1997; Newman and Newman 2003).

1.5 Gene transfer in embryonic stem cells and their derivatives

1.5.1 Non-viral methods

The ability of ES cells for clonal expansion makes them ideal candidates for gene modification studies and production of individually-modified ES clones. The methods used for transient transfection of ES cells include electroporation, use of cationic lipids and non-liposomal cationic vectors, and viral-mediated infection (Yates and Daley 2006). Chemical transfection includes introduction of the DNA material into cells via cationic lipids (such as lipofectamine®) and non-liposomal polycationic polymers (such as FuGENE®).

The disadvantages of these methods in ES cell transfection include low efficiency rates (as low as 1%) as well as poor integration into the genome and silencing. For these reasons, it is difficult to establish stably transfected ES cell lines using these methods (Liew, Draper *et al.* 2007). Electroporation leads to a temporary pore formation in the plasma membrane through exposure of the cells to voltage pulses and entry of DNA into the cells. This method has been considered successful for ES cell transfection. As an advantage of electroporation over chemical transfection, the size of inserted DNA is not a restrictive factor (Nolkrantz, Farre *et al.* 2002; Hohenstein, Pyle *et al.* 2008). One disadvantage, however, is its limitation for high throughput systems (Moore, van Laake *et al.* 2005).

1.5.2 Viral methods

1.5.2.1 Retroviral vectors

Viral vectors are frequently used in ES cell gene transfer studies. Previously, retroviral vectors were commonly used to establish stable transfection of ES cells and integration of the transgene into the host. However, the common problem of transcriptional gene silencing, resulting in a relative to complete loss of transgene function remained unsolved, especially in the case of replication-deficient retroviruses. On the other hand, the issue of proto-oncogene activation by retroviral insertional mutagenesis and tumorigenesis has been thoroughly investigated in previous studies with retroviral

constructs (Mikkers, Allen *et al.* 2002; Du, Spence *et al.* 2005). For these reasons, more advantageous methods are required as safer as well as more efficient alternatives compared to retroviral vectors.

1.5.2.2 Lentiviral vectors

Lentiviral constructs as a modified subclass of retroviruses have emerged as means to provide a durable and permanent expression of transgenes in both germ line and somatic cells, including non-dividing ones (Naldini, Blomer *et al.* 1996; Lois, Hong *et al.* 2002; Nguyen, Oberholzer *et al.* 2002). Lentiviral vectors may minimize silencing compared to retroviruses. Furthermore, random integration of the transgene into the genome, as a drawback of retroviruses, is significantly reduced in lentiviruses. In addition, tumorigenicity as one of the negative aspects of retroviruses has not been frequently reported with lentiviruses (Montini, Cesana *et al.* 2006; Cattoglio, Facchini *et al.* 2007). This method of genetic modification is used for both experimental research and therapeutic purposes. The genome of the virus is in the form of RNA, which is reverse-transcribed to DNA upon entering the cell. The formed proviruses are able to integrate into the host genome and replicate indefinitely.

Most currently available lentiviral constructs are based on the second or third generation lentiviral vectors, designed for improved safety and infectivity (Sakuma, Barry *et al.* 2012). The second generation transduction methods entail use of the main construct, packaging vector, and envelope vector in separate plasmids (Figure 9). Packaging plasmids encode the virion proteins, including the reverse transcriptase and capsid. The respiratory syncytial virus (RSV) promoter upstream of 5' long terminal repeat (LTR) in the lentivector allows efficient production of viral RNA, reducing the number of genes from human immunodeficiency virus (HIV)-1 that are used for packaging, replication and transduction to three genes (gag, pol, rev). The envelope plasmid encodes a heterologous envelope for vesicular stomatitis virus-G (VSV-G) to pseudotype the vector. Furthermore, a deletion in the enhancer of the U3 region of 3'LTR ensures self-inactivation of the lentiviral construct after transduction and integration into the genomic DNA of target cells. More recently, third generation lentiviral vectors have been generated. The four plasmids used to generate these vectors are a packaging construct containing only gag and pol genes, a plasmid expressing rev, an envelope plasmid (VSV-G), and the main transgene construct (Escors and Breckpot

2010; Sakuma, Barry *et al.* 2012; Schambach, Zychlinski *et al.* 2013). The third generation lentiviral systems are considered to further decrease the biohazard risks of transduction systems as well as the risk of insertional mutagenesis. Lentiviral genetic material is generated in the packaging cells (Human embryonic kindney (HEK) 293T cells) (Figure 9).

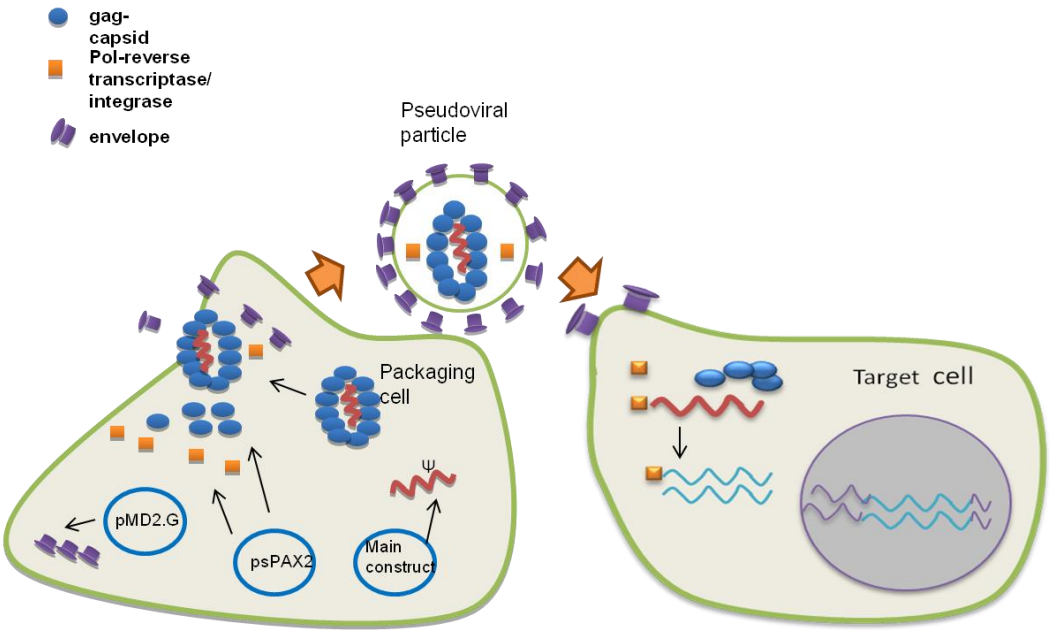

Figure 9 Mechanism of lentiviral transduction using second generation vectors.

Pseudoviral particles are produced in packaging cells through a combination of the main lentivector, packaging vector, and envelope plasmid. Reverse transcriptase and capsid are encoded by packaging vector (psPAX2), while envelope is encoded by a separate plasmid (pMD2.G). Another vector carries the genetic material, which is transcribed to produce the single-stranded RNA demonstrated by ψ (psi) sequence. The produced pseudoviral particles are used for the transduction of target cells.

1.6 Labeling and purification of embryonic stem cell-derived endothelial cells

The ability to follow and study cell survival, migration and differentiation both *ex vivo* and *in vivo* is fundamental for the success of cell-based therapies. Some of these methods include imaging through nanoparticles (Bhirde, Xie *et al.* 2011), magnetic resonance imaging (Srinivas, Boehm-Sturm *et al.* 2012), and genome technology

engineering methods. The latter includes construction of reporter genes, for instance green fluorescent protein (GFP), under the control of a promoter of interest using different vector backbones.

Establishment of ES cell clones with reporter gene expression under the control of markers of the undifferentiated state of ES cells has been previously performed successfully (Cao, Lin *et al.* 2006). In order to study the ES cell-derivatives, it is required to design a construct, whereby the reporter gene is activated under a promoter of interest upon differentiation. For instance, ES cell-derived ECs require a specific reporter gene system, which will be exclusively expressed upon differentiation to ECs. On the other hand, cells need to be differentiated into a highly pure population of cells, in order to overcome the problem of teratoma formation, as well as differentiation into other cell types. Up to now, different techniques have been applied for the purification of ES cell-derived ECs. One of these methods is fluorescence activated cell sorting (FACS) using antibodies against various endothelial markers (Yamashita, Itoh *et al.* 2000; Levenberg, Golub *et al.* 2002; McCloskey, Lyons *et al.* 2003). One of the disadvantages of mechanical sorting procedures of wild type ES cells based on endogenous endothelial markers is the need for an additional labeling process for tracking purposes.

Selection of ECs based on a drug resistance gene, as well as FACS-sorting of the cells based on GFP expression under a specific promoter has also been used, yielding in varying and usually relatively low degrees of pure endothelial-like cell populations (Marchetti, Gimond *et al.* 2002; Li, Wu *et al.* 2007; Kim and von Recum 2009). There have been few reports about the selection of cells based on the simultaneous expression of antibiotic-resistance genes and GFP under the control of different specific promoters using conventional non-viral transfection systems. These studies have been associated with the disadvantage of unstable expression of GFP due to gene silencing (Kim and von Recum 2010).

Labeling and selection methods of ES cell-derived ECs shall allow us to study pure populations of cells with a high degree of robustness and reliability, and to provide means for precise developmental studies and experimental research.

1.7 Aims of the project

Studies on ES cells enable us to address a significant number of unresolved questions about regeneration and self-renewal in the human body, as well as development and differentiation into adult tissues. Furthermore, these cells serve as an extremely precious population for studies on cell-based therapeutic approaches. Endothelial cells are one of the target populations derived from ES cells that have gained great interest due to their potential use in various diseases originating from vascular lesions.

As for basic research studies and therapeutic purposes, it is fundamental to track cells during their proliferation, development, differentiation, and migration. One of the aims of this study was to establish an efficient approach for the generation of an ES cell line with specific expression of GFP under the control of the VE-cadherin promoter. This eliminates the need for repetitive steps of cell labeling, which should be performed in addition to cell purification processes. With the lentiviral system, the issue of silencing related to conventional reporter systems is also alleviated. Moreover, it was aimed to produce high amounts of a pure population of cells expressing endothelial-specific GFP, as a prerequisite for experimental therapeutic applications, as well as developmental studies.

In order to achieve these aims, the underlying objectives were followed:

- Establishment of optimal growth conditions for ES cells in their undifferentiated stage as well as during their differentiation to ECs
- Establishment of a lentiviral vector with expression of GFP under the control of the VE-cadherin promoter for transduction of ES cells
- Validation of the generated ES cell line expressing GFP under the control of the VE-cadherin promoter using different visualization techniques along with molecular methods
- Utilization of different purification procedures for an efficient selection of ES cell-derived ECs
- Study the purification of ES cell-derived ECs using antibiotic selection
- Purification of the transgenic ES cell-derived ECs expressing GFP under the control of the VE-cadherin promoter based on CD31 as another specific marker for ECs
- Comparing the sorting efficiency using GFP and CD31 in achieving functional ECs based on molecular biology and cell culture methods

- Analysis of the pattern of selective endothelial-specific gene expression together with stem cells in undifferentiated state and early differentiation in the sorted populations over time
- Definition of a distinct time point for the robust and accurate isolation of ES cell-derived ECs based on flow cytometry, gene analysis, and microscopy
- Culture and analysis of pure ECs with a specific GFP expression *in vitro*

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

Cell counter Neubau chamber (Marienfeld, Germany)

Cell culture dishes (2.5-, 5-, 10-, and 20-cm) and plates (6-, 12-, 24-, 48-, and 96- well) (Greiner Bio One, Germany)

Cell culture incubator (Nuaire, USA)

Chamber slides (Thermo Scientific, Germany)

Cold centrifuge (Thermo Scientific, USA)

Confocal microscope (Zeiss, Germany)

Disposable pipettes (2 ml, 5 ml, 10ml, 25 ml, 50 ml) (Greiner Bio-One, Germany)

Dissecting instruments (F. S. T. Fine Science Tools, Germany)

Electrophoresis system (Peqlab, Germany)

Electroporator gene pulser (Biorad, Germany)

Eppendorf tubes (0.5 ml, 1.5 ml, 2.0 ml) (Eppendorf, Germany)

FACS machine (BD-Bioscience, Germany)

Falcon tubes (15 ml and 50 ml) (Greiner Bio-One, Germany)

Fluorescence micrsocope (Leica, Microsystems, Germany)

Gel documentation (Intas, Germany)

Heating block (HLC, UK)

Heating chamber (Heraeus, UK)

Incubator shakers (New Brunswick Scientific, Germany)

Laminar flow (Nuaire, Germany)

Microwave (Sharp, Germany)

Microcentrifuge 22 R (Hettich, Germany)

PCR machine (Eppendorf, Germany)

Petri dishes (Greiner Bio-One, Germany)

Pipetboy (Integra bioscience, Germany)

Pipettes tips: 2; 20; 200; 1000 µl (Eppendorf, Germany)

Photometer (SmartSpec Plus, Bio-Rad, Germany)

RT-PCR machine (Biorad, Germany)
Special accuracy weighing machine (Denver Instruments)
Spectrophotometer- Nanodrop (Peqlab, Germany)
Vacuum pump (KNF lab, USA)
Vortex machine (VWR, Germany)
Water bath (Microm, USA)
Weighing machine (A&D, USA)

2.1.2 Reagents and chemicals

100 base-pair DNA ladder (New England Biolabs, USA)
1 Kb base-pair DNA ladder (New England Biolabs, USA)
Acetone (Roth, Germany)
Agarose (USB Corporation, USA)
ß-Mercaptoethanol (2-ME) (Sigma, Germany)
DNase I, RNase free (Fermentas, Germany)
dNTPs (Invitrogen, Germany)
Dimethyl sulfoxide (DMSO) (Serva, Germany)
DNA gel loading buffer (Roti®-Load DNA Roth, Germany)
Ethanol (EtOH) (Roth, Germany)
Ethidium bromide (EthBr) (Roth, Germany)
Ethylenediaminetetraacetic acid (EDTA) (Fluka, Germany)
Glycerol (Sigma, Germany)
HEPES (PAA, Gemany)
Isopropanol (Roth, Germany)
Kalium chloride (Roth, Germany)
Kalium hydrogen phosphate (Roth, Germany)
LB-Agar (Lenox) (Roth, Germany)
LB-Medium (Lenox) (Roth, Germany)
Methanol (Roth, Germany)
Mowiol (Sigma Aldrich, Germany)
n-Buthanol (Roth, Germany)
Natriumhydrogenphosphat dihydrate (Roth, Germany)
Paraformaldehyde (Roth, Germany)

Random primer (Invitrogen, Germany)

Reverse transcriptase (RT)-Buffer (Invitrogen, Germany)

Sodium hydroxide (Roth, Germany)

Sodium chloride (Roth, Germany)

Super optimal Broth medium (SOB) (Roth, Germany)

Sybr Green fluorescein mix (ABgene, Germany)

Tris (hydroxymethyl) aminomethane (TRIS) base (Roth, Germany)

Triton X 100 (Roth, Germany)

Trypan blue (Sigma Aldrich, Germany)

Tween 20 (Sigma Aldrich, Germany)

2.1.3 Antibodies

Table 1 List of primary antibodies

Antibody	Company
CD31 rat anti-mouse	BD, Germany
CD34 rat anti-mouse	BD, Germany
copGFP rabbit anti-mouse	Evrogen, Germany
Dynabeads M280 sheep anti-rat IgG	Invitrogen, Germany
Flk-1 at anti-mouse	BD, Germany
Rat IgG2a, isotype control	BD, Germany
SMA Clone 1 A 4	AbD serotec, Germany
Turbo-GFP rabbit anti-mouse	Evrogen, Germany
VE-Cadherin rat anti-mouse	BD, Germany

The secondary antibodies included Alexa-488, -555, and -647 and PE- conjugated anti-rat and anti-rabbit antibodies.

2.1.4 Primers

Table 2 Primer sequences used for polymerase chain reactions

Gene	Primer sequence
Brachyury fw	GGA GAG CGA GCT GTG GCT GC (20 bp)
Brachyury rev	TGA GGG TGG GAG CTG GCA GG (20 bp)
CD31 fw	CCC TTG AGC CTC ACC AAG CTC TG (23 bp)
CD31 rev	TTG GGC CTT CGG CAT GGA ACG (21 bp)
CD34 fw	CGA GAA GTG AGG TTG GCC CAG G (22 bp)
CD34 rev	GGG AGC AGA CAC TAG CAC CAG C (22 bp)
CD45 fw	TGG CCT TTG GAT TTG CCC TTC TGG (24 bp)
CD45 rev	AAG AGT TGT GAG GCT GGC ACC ATC (24 bp)
C-kit fw	AGC GTC TTC CGG CAC AAC GG (20 bp)
C-kit rev	AGC AGC GGC GTG AAC AGA GTG (21 bp)
c-Myc fw	GCA GAC AGC CAC GAC GAT GCC (21 bp)
c-Myc rev	GAC CAG TGG GCT GTG CGG AG (20 bp)
Ephb2 fw	CTA CGA CAG CAA CGG CTG AGC TG (23 bp)
Ephb2 rev	CCG GAT GAA TTT GGT CCG CAG CC (23 bp)
Ephb4 fw	TCA GCC AAA GTG AGG CGG CG (20 bp)
Ephb4 rev	TGC GGA CGC TGT GCT GTT CC (20 bp)
Flk-1 fw	AAA CCT CTT GGC CGC GGT GC (20 bp)
Flk-1 rev	AGG GCT CGA TGC TCG CTG TG (20 bp)
Id1 fw	CGC CTC AAG GAG CTG GTG CC (20 bp)
Id1 rev	TGG AAC ACA TGC CGC CTC GG (20 bp)
Mesdc2 fw	TTG TGC CTT ACG CTG GGC AGT C (22 bp)
Mesdc2 rev	TGG GGT TCC CAG ACA CGG TGA C (22 bp)
Nanog fw	CTT GCT CTT TCT GTG GGA AGG CTG C (25 bp)
Nanog rev	GGC CTG GCT GCT CCA AGT TGG (21 bp)
Nestin fw	CAG GAG CGC AGA GAG GCG C (19 bp)
Nestin rev	GGG ATG GGA GTG CTG GCC AAG (21 bp)
Nos3 fw	TGG GTT TAG GGC TGT GCG GC (20 bp)
Nos3 rev	GCC TGG GCA CTG AGG GTG TC (20 bp)
Oct4 fw	GCC CCA ATG CCG TGA AGT TGG AG (23 bp)
Oct4 rev	GGG GCC GCA GCT TAC ACA TGT TC (23 bp)
PBGD fw	ATG TCC GGT AAC GGC GGC (18 bp)

PBGD rev	GGT ACA AGG CTT TCA GCA TCG C (22 bp)
Prox1 fw	CAC CCA GCA CCG CAG AAG GAC (21 bp)
Prox1 rev	GCA TGT TGG AGC TGG GGT AGC G (22 bp)
Sox2 fw	TGC ACT TCG CCC GTC TCG AG (20 bp)
Sox2 rev	CAG GGC GCT GAC GTC GTA GC (20 bp)
Sox17 fw	TAA ATG GGA GGG AGG GTC ACC ACT G (25 bp)
Sox17 rev	CTA TGG CCA CGG GAC ACG CC (20 bp)
Sox18 fw	ACC GCG CAG CCC CGA ATC (18 bp)
Sox18 rev	CAC GCT TTG CCC AGC ATC TTG C (22 bp)
SSEA1 fw	ACG CAC GGA TAA GGC GCT GG (20 bp)
SSEA1 rev	TGC CCA GGG GGA CGA GAA CC (20 bp)
Tal 1 fw	GGC CGA GCG CTG CTC TAT AGC (21 bp)
Tal 1 rev	CTG TTG GTG AAG ATG CGC CGC (21 bp)
Tie1 fw	GCT GCG CTT TGC CAG TGA TGC (21 bp)
Tie1 rev	ATG GCC ATC CAA CGC ACA GGG (21 bp)
Tie2 fw	GGG TTA CGG ATG GAT GCC GCC (21 bp)
Tie2 rev	GCC TCG GTG TTC ACA TGC TCC C (22 bp)
VE-cadherin fw	GCT CTC CAC AAA GCT CGG CCC (21 bp)
VE-cadherin rev	GCC CAG GAA GGC TCC CAA AGC (21 bp)

2.1.5 Restriction endonucleases

Table 3 List of restriction endonucleases

Restriction endonuclease	Sequence
BamHI	g|gatcc
ClaI	at|cgat
MluI	a|cgcgt
XbaI	t|ctaga
BspTI	c|ttaag
EcoRI	g|aattc
NheI	g|ctagc
SalI	g|tcgac
XhoI	c|tcgag

All restriction enzymes were purchased from Fermentas, Germany.

2.1.6 Vectors

Table 4 List of Vectors

Vectors	Definition
pGZ-CMV	backbone construct
psPax2	packaging plasmid
PMD2.G	envelope plasmid
p-VE-cadherin	(initial vector used for the isolation of the VE-cadherin promoter)
pGZ-VE cadherin	generated vector

2.1.7 Enzymes used for polymerase chain reactions and cloning

Table 5 List of enzymes for molecular biology techniques

Enzyme	Company
MangoMix	Bioline, Germany
Pfu Polymerase	Fermentas, Germany
T4 Ligase	Fermentas, Germany
Taq Polymerase	Bioline, Germany

2.1.8 Cell culture reagents

ß-mercaptoethanol (Sigma-Aldrich, Germany)
Collagenase B (Roche, Germany)
DNase I (Roche, Germany)
Dulbecco's modified eagle medium containing 4.5 g/l glucose (DMEM) (Invitrogen, Germany)
Fetal bovine serum (FBS) (PAA, Germany)
Gelatin (Sigma-Aldrich, Germany)
Heparin (Sigma-Aldrich, Germany)

Iscove's modified Dulbecco medium (IMDM) with stable glutamine (Biochrom-AG, Germany)
Leukemia inhibitory factor (LIF) (ESGRO®, Germany)
L-Glutamine, 2mM (Invitrogen, Germany)
Matrigel (BD, Germany)
MCDB 131 medium (Sigma-Aldrich, Germany)
Mitomycin C (Sigma-Aldrich, Germany)
Non-enzymatic dissociation solution (Millipore, Germany)
Non-essential amino acids (NEAA) (Invitrogen, Germany)
Opti-MEM (Invitrogen, Germany)
PBS Ca & Mg - free (PAA, Germany)
Penicillin/Streptomycin (Pen/strep) (PAA, Germany)
Polybrene (Hexadimethrin Bromid) (Millipore; Germany)
Poly (2-hydroxyethyl methacrylate) (polyHEMA) (Sigma-Aldrich, Germany)
Sodium pyruvate (PAA, Germany)
Trypsin-EDTA (Gibco, Germany)
Vascular endothelial growth factor (VEGF) (Peprotech, Germany)
Zeocin (invivogen, Germany)

2.1.9 Cell lines/Cells

E14Tg2a.4 mouse ES cells
Endothelioma cells
HEK 293 T cell line
Mouse embryonic fibroblasts

2.2 Cell culture techniques

2.2.1 Preparation of cell culture media, buffers, and solutions

1% PBST

1000 µl Triton X-100 +
100 ml 1x phosphate buffered saline (PBS)

10 x PBS

2g KH_2PO_4

12.5g Na2HPO4

2g KCl

Dissolved in 1 l ddH_2O, PH set to 7.4

Collagenase B Solution

0.2% Solution in distilled H_2O+

10 µg/ml DNase I

Differentiation medium

IMDM

15% FBS

1% NEAA, 1% Pen/Strep

1mM Pyruvate

100 µm ß-Mercaptoethanol

ES cell medium

Differentiation medium + LIF 1000 U/ml

FACS Buffer

3 mM EDTA +

5% FBS in PBS

Freezing medium

DMEM

50% FBS

10% dimethyl sulfoxide (DMSO)

HEK 293T cell Medium

DMEM

10% FBS

1% Pen/Strep

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) Buffer

21.21 g NaCl

1.2 g KCl
0.18 g KH2PO4
0.24 g Na2HPO4×7H2O
3 g Glucose
0.03 g Phenol red
14.3 g HEPES

Dissolved in 2.5 L Cell culture water, PH set to 7.3, autoclaved, and stored in the fridge.

Mouse embryonic fibroblasts medium (MEF Medium)

DMEM
15% FBS
1% NEAA
1% Pen/Strep

Mouse endothelioma cells medium (mEndo Mediuhhm)

MCDB 131
15% FBS
1% L-Glutamin
1% Pen/Strep
50 µg/ml VEGF
100 µg/ml Heparin

Medium for the inactivation of mouse embryonic fibroblasts (Inactivation medium)

DMEM
5% FBS
1% Pen/Strep
1% NEAA
10 µg/ml mitomycin C

Soc Medium

SOB (Super Optimal Broth, Roth) medium filter sterilized with 20mM Glucose

Tris-EDTA (TE) Buffer

For 500ml solution:

5ml 1M Tris pH 8

1ml 0.5M EDTA pH 8

496ml dH2O

Autoclave to sterilize

2.2.2 Cell culture routine

All cells were maintained in humidified 37°C incubators provided with 7% CO_2 and centrifuged at 200 g x 5 min, unless otherwise stated. Except for HEK 293 T cells, all culture plates were pre-coated with 0.2 %-0.5% gelatin solution in cell culture water (HEK 293 T cells easily adhere to the surface of adhesive culture plates; therefore, no substrates are required for plating of these cells).

2.2.2.1 Thawing

The frozen cell cryovials were taken from -196°C liquid nitrogen tank and immediately thawed in 37°C water bath. The cells were carefully mixed with MEF medium and centrifuged at 200 g x 5 min. The cell pellet was subsequently resuspended in an appropriate amount of medium for further experiments or culture.

2.2.2.2 Freezing

Cells were harvested by trypsinization and pelleted by 200 g x 5 minutes. Afterwards, they were carefully resuspended in 500 µl freezing medium and placed in cryovials. The vials were immediately transferred to -80°C freezers, and after overnight storage, transported to -196°C liquid nitrogen tanks for long-term storage.

2.2.2.3 Passaging

When cells reach confluency, they can be passaged on a new culture plate/flask. To begin with passaging, the medium was aspirated and cells were thoroughly washed with PBS (HEPES buffer for ES cells). Two methods were adopted for passaging: In the first method, cells were treated with 0.25% trypsin –EDTA and left in the 37°C incubator for 1 minute. Following the aspiration of trypsin-EDTA, cells were resuspended in an appropriate amount of medium and cultured onto new plates. In the second method, cells were kept in the incubator for a longer period after treatment of trypsin-EDTA

(approximately 5 minute, or until the cells detached). Subsequently, cells were resuspended in 5-times the volume of trypsin in a medium containing 10% serum, harvested by centrifugation (200 g x 5 min), and splitted according to the cell density.

2.2.3 Inactivation of mouse embryonic fibroblasts

The first passage MEFs were a kind gift from Dr. Sven Becker. These cells had been originally isolated from the embryos of the midgestation pregnant mice. The first passage MEFs were subcultured to produce the second passage MEFs, which were inactivated and used as feeder cells for ES cell culture. To prepare mitotically inactivated feeder cells, 2 vials from passage 1 MEFs were thawed on a 145 mm culture plate. After reaching confluency, cells were splitted on a total number of 10 culture plates (145 mm) over 2 passages. Cells were subsequently treated with inactivation medium. After 2-4 hours, they were thoroughly washed, harvested, immediately transferred to -80°C for overnight storage, and kept in a liquid nitrogen tank for long term. Normally, the 2 initial first passage mouse embryonic fibroblast cryovials gave rise to 50 vials of inactivated feeders. Upon demand, feeder cells were thawed and plated in ES medium on culture plates pretreated with 0.2% gelatin. One vial should generally suffice the coverage of the surface of a 6-well plate. One day after plating, ES cells were cultured on feeder cells, which were preconditioned with ES cell medium one hour prior to ES cell culture.

2.2.4 Embryonic stem cell culture

2.2.4.1 Undifferentiated state

E14Tg2a.4 cell line obtained from 129P2/OlaHsd mice was used for all ES cell experiments. The cells were thawed on a 60mm tissue culture plates pre-coated with a layer of inactivated MEFs. Upon confluency, ES cells were splitted with a dilution of 1:50-1:20 onto 6x35 mm or a 6-well plate pre-coated with inactivated MEFs. Routinely, the ES medium was exchanged every day, and ES cells were splitted every second day. Too big ES cell colonies, may lead to a decreased pluripotency potential. On the other hand, too low ES cell confluencies, may decrease the propagation capacities of the cells. In general, ES cells should be splitted when ES colonies reach a confluency of around 70%.

2.2.4.2 Differentiation culture

Embryonic stem cells start to differentiate upon removal of LIF and feeders. Usually, differentiation is induced through the formation of three-dimensional spheroidal structures, called EBs. In the current experiments, the method of embryoid body formation in suspension was used, which is one the most commonly used methods for EB formation.

Prior to differentiation, MEF depletion was performed by dissociation of ES cells and their subsequent plating in differentiation medium (ES cell medium without LIF) with a dilution of 1:300 on gelatin-coated plates for 45 minutes. Feeder cells adhere more rapidly than ES cells, so that the supernatant contains a high proportion of pure ES cells. To form EBs, a total number of 1500000 cells from the supernatant were resuspended in 15 mL differentiation medium (100000 cells/mL), transferred onto non-adhesive 10-cm culture dishes, and incubated on a rotator orbital shaker with a speed of 25 rpm at 37°C /7% CO2 for 4 days. The medium was exchanged at the 4^{th} day of EB formation. For medium exchange, the medium containing EBs was transferred to 50 ml Falcon tubes. Embryoid bodies were allowed to settle down in Falcon tubes for 10 minutes. The supernatant was carefully aspirated and replaced with new medium. Afterwards, EBs were either plated on gelatin-coated plates or kept in suspension for time course studies. Alternatively, ES cells underwent a predifferentiation stage before EB formation. The MEF-depleted ES cells were cultured for 2 days on gelatin-coated plates. This stage is suggested to increase the percentage of Flk-1-positive cells.

2.2.5 *In vitro* tubule formation assay

Cells were cultured on matrigel (BD) to test the ability of both dissociated EBs and isolated ECs to form vascular tubular structures *in vitro.* Matrigel coating was performed according to the manufacturer's protocol.

2.2.6 HEK 293 T cells

HEK 293 T cells were used for the production of pseudoviral particles. These cells were cultured on adhesive plates without the need for gelatin pre-coating. HEK 293 T cells were thawed, propagated once, and grown to a confluency of 70% before virus production.

2.2.7 Endothelioma cells

Mouse endothelioma cells were used for the validation of the pGZ-VE-cadherin construct. These cells were cultured on gelatin-coated plates in mouse endothelioma medium. The medium was exchanged at least twice a week. Prior to passaging, cells were kept in PBS for 15 minutes, in order to facilitate cell detachment.

2.3 Molecular biology techniques

Molecular biology techniques were used for the construction of transgenes, as well as analysis of transgenic cells in both undifferentiated and differentiated stages.

2.3.1 PCR

PCR reactions were used for both generation of the transgenes and analysis of the bacterial colonies. Mango mix (containing Taq polymerase) was used for the screening of bacterial colonies after cloning procedures, while Pfu polymerase was applied for the amplification and subcloning of VE-cadherin promoter into the lentiviral vector. The VE-cadherin promoter had been previously extracted from the genomic DNA of the black six mice by Dr. Sven Becker in our lab and incorporated into a conventional non-viral vector. In the present experiments, the VE-cadherin promoter (insert) was amplified and subcloned into the lentiviral vector. The oligonucleotides that were used for this purpose consisted of 36 base pairs: primers (25-base), restriction sites (6 bases), and random bases (5 bases) (Figure 10). Cloning was designed using Vector NTI program.

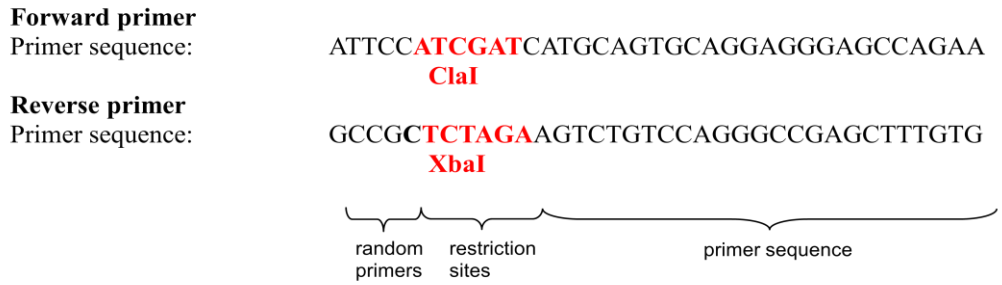

Figure 10 Forward and reverse primers used for amplification of VE-cadherin promoter

VE-cadherin promoter was amplified according to the underlying PCR reaction and settings:

PCR protocol

H2O	36 µL
10 x Buffer	5 µL
dNTPs	2 µL
fw primer	2.5 µL
rev primer	2.5 µL
template DNA	1 µL
Pfu polymerase	1 µL
---	---
Total	50 µL

Thermal cycler program

95 ° C	2 min	
---	---	
95 ° C	30 s	x35
59 ° C	30 s	
72 ° C	5 min 30 s	
---	---	
72 ° C	15 min	
---	---	
4 ° C	∞	

2.3.2 Restriction digestion

Restriction digestion was used for subcloning of VE-cadherin promoter into the lentiviral vector by replacing the cytomegalovirus (CMV) promoter. Restriction enzymes were purchased from Fermentas and digestion procedures were done based on the manufacturer's instructions. A total of 5 µg of vector and 1 µg of insert were used in the reactions with 2 restrictions enzymes in a total volume of 30 µl. The reaction has been demonstrated on the next page.

Insert (VE-cadherin promoter)	
H2O	9 µL
1xTango buffer	5 µL
XbaI	1 µL
ClaI	1 µL
template DNA	16 µL
---	---
Total	30 µL

Plasmid (pGZ-CMV)	
H2O	22.8 µL
1xTango buffer	5 µL
XbaI	1 µL
ClaI	1 µL
template DNA	2.2 µL
---	---
Total	30 µL

2.3.3 PCR clean-up

This step was done to purify DNA molecules from dNTPs, salts, and primers after PCR and enzymatic digestion reactions. The procedure was performed based on manufacturer's protocol (Qiaquick PCR purification kit).

2.3.4 Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose in Tris EDTA (TE) buffer at concentrations of 0.8-2.5%. After adding ethidium bromide, agarose was cooled, and gels were poured into casting trays. Electrophoresis was performed in Mupid gel tanks at 100-140V in 0.5x TE buffer. The bands were visualized by UV transillumination; images were captured using Intas image software.

2.3.5 Gel extraction

Nucleic acids were loaded on 0.8%-1% agarose gels. After electrophoresis, bands were cut using a sharp scalpel on a transilluminator using QIAquick Gel Extraction Kits (Qiagen) and DNA was isolated according to the manufacturer's protocol. The isolated DNA was eluted using 30 μl distilled H20, and subsequently used for ligation.

2.3.6 Dialysis

Dialysis was performed after digestion of the amplified insert and gel extraction of the vector. This procedure modified DNA purity by decreasing salt concentrations.

2.3.7 Ligation

After digestion of the vector and insert with the same enzymes, the compatible ends were coupled, using a reaction catalyzed by T4 ligase. This leads to the formation of the phosphodiester bond between the free 5'-phosphate und 3'- hydroxy groups. This reaction was performed according to the manufacturer's directions (Fermentas) in a total volume of 20 µl. For this reaction, a total amount of 100ng vector DNA with a vector/insert molar ratio of 1:3 was used.

This reaction can occasionally lead to the formation of different products. For instance, the vector DNA that is extracted from the gel may be contaminated with non-digested vector. In some other instances, a re-ligation of the vector DNA may happen.

To control these effects, a negative control reaction was assigned. As the negative control, a ligation reaction was performed with no insert in the reaction. Ligation was performed according to this formula:

Ligation	**pGZ-VE construct**	**Ligation control**
H2O	13.4 µL	14.7 µL
10x ligation buffer	2 µL	2 µL
pGZ-CMV (vector)	1.3 µL	1.3 µL
VE-cadherin promoter (insert)	1.3 µL	0
T4 DNA ligase	2 µL	2 µL
Total	20 µL	20 µL

Figure 11 shows a schematic view of the generation of a construct with VE-cadherin promoter driving the expression of GFP and a zeocin resistance gene from a lentiviral backbone vector with a CMV promoter.

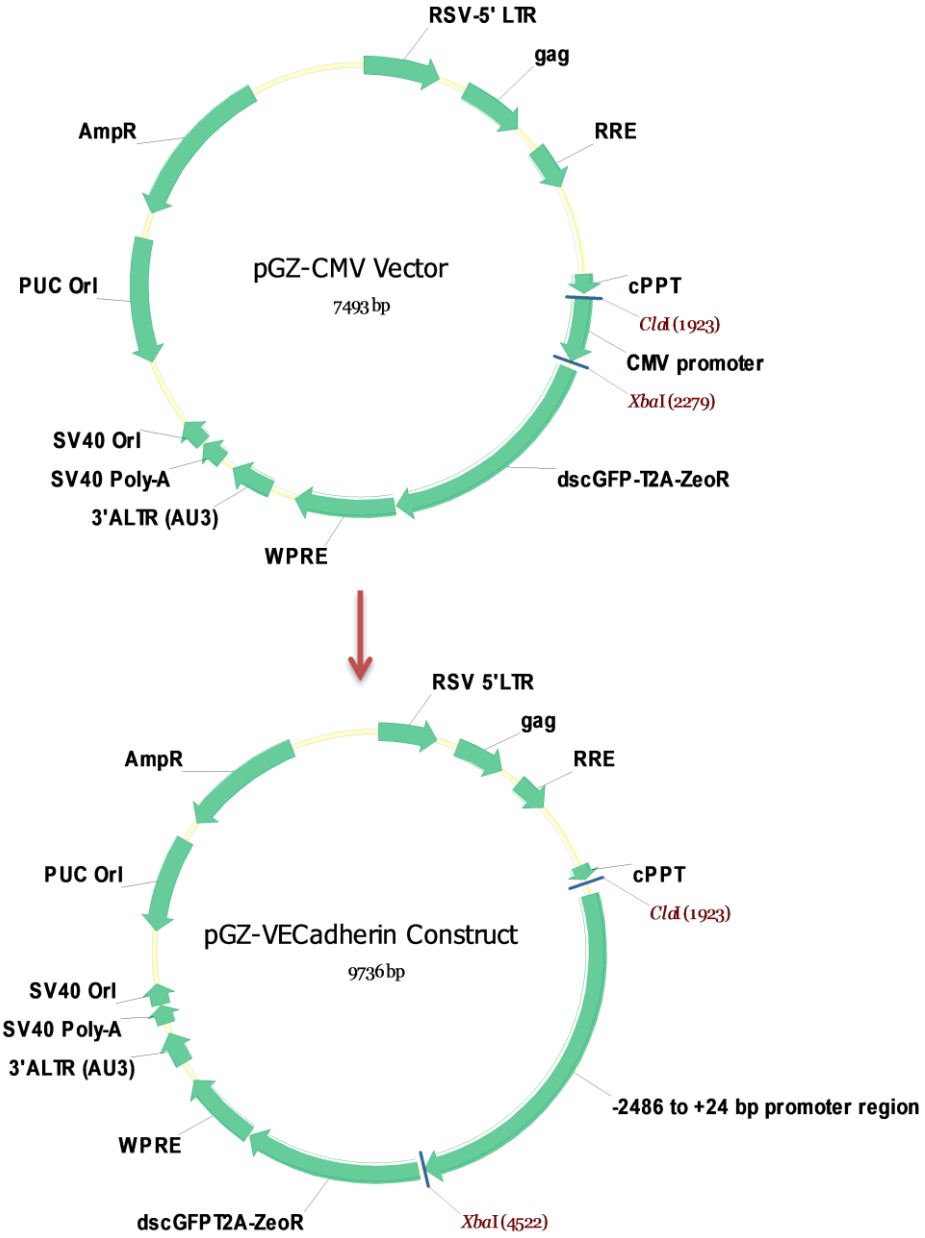

Figure 11 Generation of pGZ-VE cadherin construct from pGZ-CMV vector.
RSV-5'LTR: Hybrid from the promoter of the respiratory Syncytial Virus and long terminal repeat, gag: packaging signal, RRE: rev responsive element, cPPT: central polypurine tract; copGFP: GFP from Copepodes, Zeo: Zeocin-Resistence gene; WPRE: posttranscriptional regulatory element of woodchuck hepatitis virus, Amp: Ampicillin resistence, ori: Start point of the Replication, pA: Polyadenylation signal.

2.3.8 Bacterial transformation

One hundred microliters of Competent XL1-Blue *E. Coli* (*Escherichia coli)* bacteria were thawed on ice. The XL-1 bacteria are tetracycline resistant and endonuclease deficient, leading to an increased quality of DNA samples. Furthermore, these bacteria are recombinant deficient, improving the stability of the insert. Twenty microliters of

plasmid DNA was mixed with bacteria on ice. The mixture was then transferred to electroporation cuvettes and electroporated at 1.8 kV; 25 μFD; 200 Ohm; capacitance extender 250). The transformed bacteria were immediately mixed with 800 µl pre-warmed Soc medium. After 40 minutes incubation on 37°C heating blocks shaking at 500 rpm, bacteria were plated on selective LB agar plates containing ampicillin. Agar plates were kept overnight in 37°C incubator and checked for growth of bacterial colonies. Colonies were picked the next day and checked for accuracy by gel runs.

2.3.9 Plasmid purification

Single bacterial colonies were picked from plates and put into growth Luria Broth (LB) medium containing 100µg/ml ampicillin on a shaker overnight at 37 °C. Mango mix, which contains Taq polymerase, was used for screening of positive bacterial clones. Plasmid DNA was isolated by Miniprep (QIAprep Spin Miniprep Kit (Qiagen) according to the manufacture's protocol and the bacteria were frozen in -80°C. The Miniprep reactions gave rise to an average of 20µg DNA. Sequences were validated using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing system. Maxipreps were later prepared from the Minipres, using the NucleoBond® Xtra Maxi Kits (Macherey Nagel). This led to the production of an average amount of 1mg total DNA, which was stored at -20°C and used for the transduction of ES cells.

2.3.10 RNA extraction

RNA isolation was performed using Fermentas kit. Up to 10 million cells were trypsinized, washed in PBS, and re-suspended in lysis buffer, which was supplemented with ß-mercaptoethanol. The lysates were immediately subjected to RNA isolation according to the manufacturer's protocol. A maximum of 100 µL distilled water was used to elute RNA. The eluted RNA concentration was measured using spectrophotometer (nanodrop).

2.3.11 Reverse transcription

High-capacity cDNA reverse transcriptase kit (Applied Biosystems) with random primers was used for reverse transcription, according to the manufacturer's protocol. A minimum of 100n g RNA was used as template. The synthesized cDNAs were either

kept at -20 °C or used freshly. A 1:10 dilution of cDNA was used for PCR and qRT-PCR reactions.

2.3.12 Quantitative real-time RT-PCR (Sybr Green realtime RT-PCR)

Sybr Green-based quantitative real-time RT-PCR (qRT-PCR) employs Sybr Green as a dye, which emits fluorescence upon binding the double stranded (ds)DNA. The intensity of fluorescence correlates with the amount of dsDNA in the reaction. Primers were designed using Primer-Blast. All of the primers were designed to have a melting temperature of approximately 60°C (59°C-61°C). The amplicon length was 50-250 base pairs (bp) and the final primer concentration was 400 nanomolar (nm). To avoid amplification of genomic DNA, intron-spanning primers were used. The other inclusion criterion was having a maximum of 1 GC clamp at the 3' end of the primer. Reactions were performed in a standard 25µl final reaction mix. As RT-PCR controls, the template was replaced with PCR-grade water. The process was performed using a standard cycler program. MasterMix was pipetted into 96-well reaction plates to which 2µL cDNA was added directly. The list of primers is provided in table 2. All experiments were performed at least in triplicates. Prism 5 was used for the analysis of the qRT-PCR results. Student's t-test as well as one-way ANOVA with Tukey's post hoc was applied for the statistical analysis. Results are expressed either as ΔCt or fold change in gene expression, using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCt= (Ct, target – Ct, housekeeping gene)$_{population1}$ – (Ct, target – Ct, housekeeping gene)$_{population2}$.

PCR settings for qRT-PCR

PCR master mix	12.5 µL
Fw primer	0.5 µL
Rev primer	0.5 µL
sterile water	9.5 µL
cDNA	2 µL
---	---
Total	25 µl

2.4 Lentiviral transduction

Lentiviral transduction system is an efficient method for the stable integration of transgenes into the genome (Figure 12). The 5' and 3' LTRs promote the transcription and polyadenylation of the virion RNA. Central polypurine tract (cPPT) is responsible for transporting HIV provirus into the nucleus. The addition of this element between the LTRs improves the transduction efficiency. The RSV promoter upstream of the 5' LTR allows efficient Tat-independent production of viral RNA, reducing the number of genes from HIV-1 in the packaging system. Packaging signal is produced by gag in the packaging plasmid. Rev response element (RRE) binds gag and is involved in packaging of viral transcripts. Woodchuck hepatitis virus post-transcription regulatory element (WPRE) promotes RNA processing and maturation, enhances vector packaging, and increases viral titer. The envelope plasmid contains pseudotyping viral vectors with VSV-G, enabling the transduction of an extensive range of cell types from different species. This constellation makes lentiviral transduction an efficient method for transgenesis. Lentiviral transduction consists of 2 consecutive steps: transfection of HEK 293 T cells and transduction of target cells.

2.4.1 Transfection of HEK 293 T cells

HEK 293T cells were cultured on adhesive plates. After reaching 90% confluency, the cells were transfected with the main construct (pGZ-VE-cadherin) together with packaging and envelope plasmids, psPax2 and pMD2.G, respectively (Figure 12). Serial amounts of DNA were diluted in 400µl serum-free DMEM medium and used for transfection optimization. Turbofect (transfection reagent) was added to the respective samples, mixed by pipetting, and left at room temperature for 20 minutes. The mixture was dropwise added to HEK 293 T cells. Supernatants containing pseudoviral particles were collected 48 and 72 hours after transfection. The successive stages of transfection, as well as the amounts of DNA and transfection reagent are provided in tables 6 and 7.

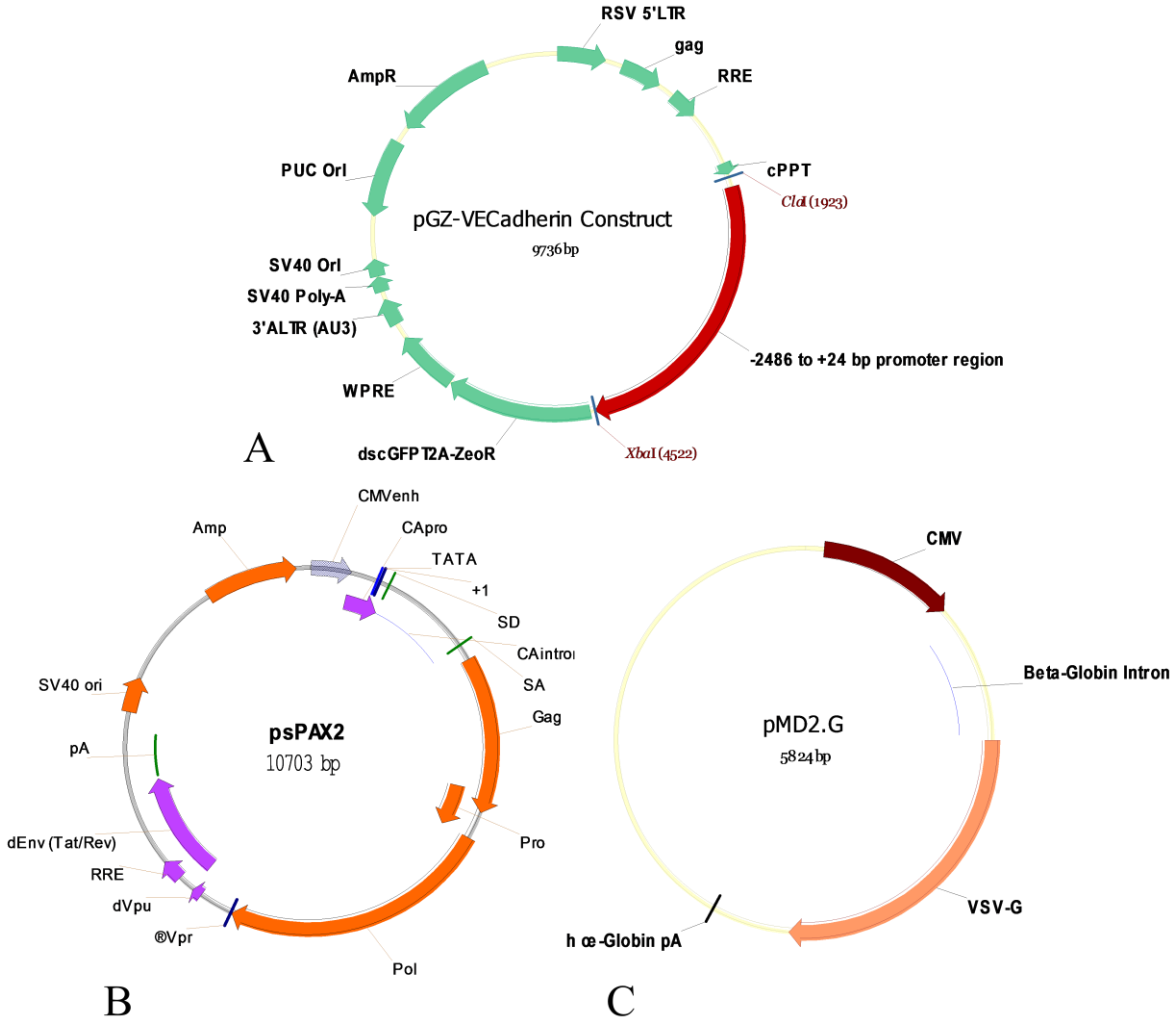

Figure 12 Vectors used for transfection of HEK 293 T cells; a combination of the main construct (pGZ-VE-cadherin), packaging vector (psPax2), and envelope vector (pMD2.G).

(A): PGZ-VE-cadherin construct (description in Figure 11). **(B): Packaging plasmid** CMVenh: Cytomegalovirus enhancer; CApro: Chicken beta actin Promotor, CAintron: Chicken beta actin Intron, Gag: group antigen, Pro: Protease, Pol: Polymerase; dEnv: envelope proteins including the genes for Tat und Rev, RRE: rev response element, pA: Polyadenylation signal, SV40 ori: simian virus 40 origin, Amp: Ampicillin resistance gene. **(C): Envelope plasmid** CMV: Cytomegalovirus promoter, VSV-G: Glycoprotein of the vesicular stomatitis virus, pA: Polyadenylation signal, Amp: Ampicillin resistence gene.

Table 6 Transfection of HEK 293 T cells and production of pseudoviral particles

Time	Procedure
Day 1	Seed HEK 293T cells on plates (Confluency should reach 70%-90% by day 2)
Day 2	Combine the main vector/construct, envelope and packaging plasmids with serum-free medium and transfection reagent (as in table 7)
Day 3	18 hours post transfection: Check for GFP expression; replace medium with high serum medium (50% FBS)
Day 4	24 hours after medium exchange: Harvest virus, Spin at 3000 rpm /5 min at room temperature; filter through 0.22 µm filters; replace with high serum medium (50% FBS)
Day 5	24 hours after harvest 1: Harvest virus, discard packaging cells, freeze the pseudoviral particles at -80°C or use freshly on target cells

Table 7 Ratio of vectors and reagent for the transfection of HEK 293 T cells with total amounts of 8µg and 18 µg DNA

8 µg DNA			18 µg DNA		
12 µl Turbofect			25 µl Turbofect		
pGZVE-Cad (µg)	psPAX2 (µg)	pMD2.G (µg)	pGZVE-Cad (µg)	psPAX2 (µg)	pMD2.G (µg)
4	2.67	1.33	9	6	3

The ratio of the pGZ-VE-cadherin: psPAX2: pMD2.G is 3:2:1.

2.4.2 Transduction of embryonic stem cells

A total number of 2000 single undifferentiated mouse ES cells were cultured in 500µl ES medium containing double concentration of LIF on a 24-well plates and transduced with a total amount of 8µg and 18µg virus DNA and 16 µg/ml polybrene. After 12 hours, ES cells were subcultured onto 10-cm feeder-coated plates and left at 7% CO2 incubator for 5-7 days to grow to ES cell colonies.

2.4.3 Clone picking

After the ES cells had grown to confluent colonies, 192 ES colonies were picked, dissociated with trypsin, and cultured onto MEF-coated 96-well plates. After reaching confluency, ES cells were passaged onto duplicate MEF-coated 96-well plates. Half of the cells were frozen at -80°C, and the rest were grown on gelatin-coated plates in ES differentiation medium as the predifferentiation stage. Two days later, cells were dissociated with trypsin and transferred to polyhema-coated 96-well plates to form EBs. At day 4, EBs were cultured on gelatin-coated 96-well plates with 5ng/mL VEGF. After 2 days, clones were selected based on the appearance of GFP-positive vessel-like structures, using high throughput screening with a fluorescence microscope. The GFP-positive ES cell clones that were kept in liquid nitrogen were then thawed and propagated and the rest were discarded. A total number of 24 clones out of the 192 frozen clones were positive for GFP-positive vessel-like structures. The positive clones were propagated on 10cm plates, harvested, and frozen in liquid nitrogen for further experiments.

2.4.4 Safety measures

In addition to the safety consideration in designing the vector to neutralize the infectivity of HIV particles, the following measures were taken to decrease the risk of contamination with pseudoviral particles: Wearing double gloves and lab coat was mandatory. The work was performed in a special S2 lab with Class II laminar flow hood. Care was taken to minimize the creation of splashes or aerosols. Work surfaces were decontaminated at least twice a day and after any spill of viable material. All cultures, stocks, and other regulated wastes were decontaminated before disposal by autoclaving. Materials were kept in a durable, leakproof, properly marked and sealed waste.

2.5 Immune-based techniques

2.5.1 Immunohistochemistry

Immunofluorescence staining was carried out using different primary antibodies and Alexa fluor-conjugated secondary antibodies. Whole EBs as well as dissociated EBs were washed with 0.01% PBST, fixed in ice-cold methanol or 4% PFA, and permeabilized using 0.1% Triton X. Cells were washed with 0.01% PBST and incubated in 10% goat serum in 0.01% PBST for 1 hour at room temperature as the blocking step. Primary and secondary antibodies were diluted in the blocking solution. The dilution of the primary antibodies was as follows: VE-cadherin (BD), CD31 (BD), Flk-1 (BD): 1:100; α-SMA (Sigma): 1:500; anti-TurboGFP (Abcam) and anti-cop-GFP (Evrogen): 1:1000. Cells were incubated in primary antibody solution either for 3 hours at room temperature or overnight at 4°C. Secondary antibody incubation was performed under dark conditions in room temperature for 1 hour with a dilution of 1:500. Secondary antibodies included anti-rat and anti-rabbit antibodies conjugated with Alexa fluor -488, -555, and -647 (all BD). Nuclear counterstaining was carried out using Sytox blue (Life Technologies), Draq-5 (eBiosciences) and DAPI (Life Technologies) with a dilution of 1:1000 for a maximum of 20 minutes at room temperature. Cells and EBs were mounted using Mowiol. Samples were analyzed using Zeiss confocal and Leica fluorescence microscopes.

2.5.2 Flow cytometry

2.5.2.1 Analysis of cell populations

Whole EBs were dissociated using 0.05% trypsin/0.2mM EDTA, non-enzymatic cell dissociation solution, or collagenase B (0.3 PZ U/ml) at 37°C supplemented with DNase (0.01 mg/mL; Sigma). All the next steps were performed on ice. Dissociated cells were passed through 40 µm cell strainers, washed with FACS buffer, and blocked with 10% FBS in FACS buffer for 30 minutes on ice. A total number of 500000 cells were treated with primary antibody with a dilution of 1:100 in 100 µL of blocking solution and incubated on ice for 30 minutes. Cells were afterwards washed with FACS buffer by cold centrifugation at 1000 rpm for 5 minutes. Subsequently, cells were incubated in 100µL of blocking solution containing secondary antibodies with a dilution of 1:500 for

another 30 minutes. Secondary antibodies were coupled to fluorophors, which were identified and analyzed using the FACS machine. Cells were washed with FACS buffer 3 times using cold centrifuge at 1000rpm for 5 minutes before the analysis. IgG staining and unstained populations were regarded as control. FACS analysis was performed using FACS LSR II machine (BD).

2.5.2.2 Cell sorting

EBs were dissociated as discussed in the previous section. Single cells were sorted to GFP-positive and -negative populations using FACS Aria sorter. Wild type EBs were used to set parameters for GFP expression in each stage. Dead cells were excluded pre- and post-sorting using Topro-3 staining. Sorted cells were separated, counted, and later used for culture as well as direct RNA analysis.

2.5.3 Sorting based on magnetic beads

2-12 day old EBs were dissociated using collagenase B (0.3 PZ U/ml) and DNase (0.01mg/mL-Sigma) on a shaker in 37°C for 40 minutes. Subsequently, cells were washed in HEPES buffer by centrifugation at 1000 rpm for 5 minutes. The pellet was resuspended in HEPES buffer, passed through 40 µm cell strainers, and blocked in FBS. Afterwards, the cell suspension was filled up to 10ml with 0.3% BSA in PBS. After counting, cells were pelleted and resuspended in FBS with a final concentration of $1\times10^6/100\mu$l. CD31 antibody (BD) was added according to the manufacturer's protocol (4μl solution/1×10^6 cells) and cells were incubated on ice for 20 minutes. After washing, cells were resuspended in 500μl buffer and incubated with 25μl sheep anti-rat secondary antibody conjugated with magnetic beads (Dynal beads, BD) on a shaker in 4°C. The cells were subjected to a strong magnetic field and selected after several stages of washing. The negative and positive isolated cells were either directly cultured or used for further RNA analyses.

3 Results

3.1 Culture and differentiation of embryonic stem cells

Embryonic stem cells were cultured on inactivated MEFs in medium containing LIF (Figure 13 A). In the absence of LIF, ES cells differentiate and form homogenous EBs in suspension culture (Figure 13 B).

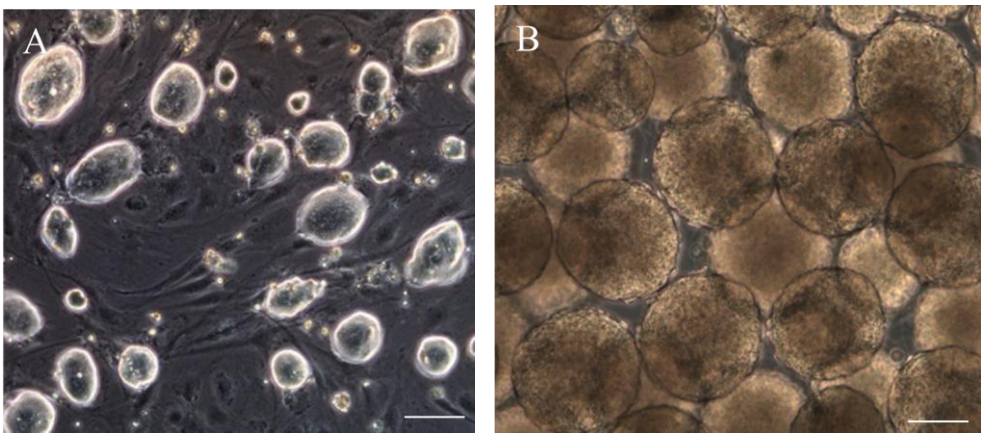

Figure 13 Undifferentiated and differentiated states of embryonic stem cells

(A): Embryonic stem cell colonies grown in the presence of leukemia inhibitory factor on inactivated mouse embryonic fibroblasts. **(B):** Day 3 embryoid bodies grown in suspension culture in the absence of leukemia inhibitory factor and inactivated mouse embryonic fibroblasts. Bar size represents 100 µm in A und 150 µm in B.

Embryoid bodies spontaneously developed vessel-like structures upon plating on gelatin-coated culture dishes. The expression of EC markers such as Flk-1, CD31 and VE-cadherin was assessed using immunofluorescence microscopy (Figure 14 A-F). As shown in the figures, α-SMA, which is a marker of myofibroblasts, is also markedly expressed in the plated EBs.

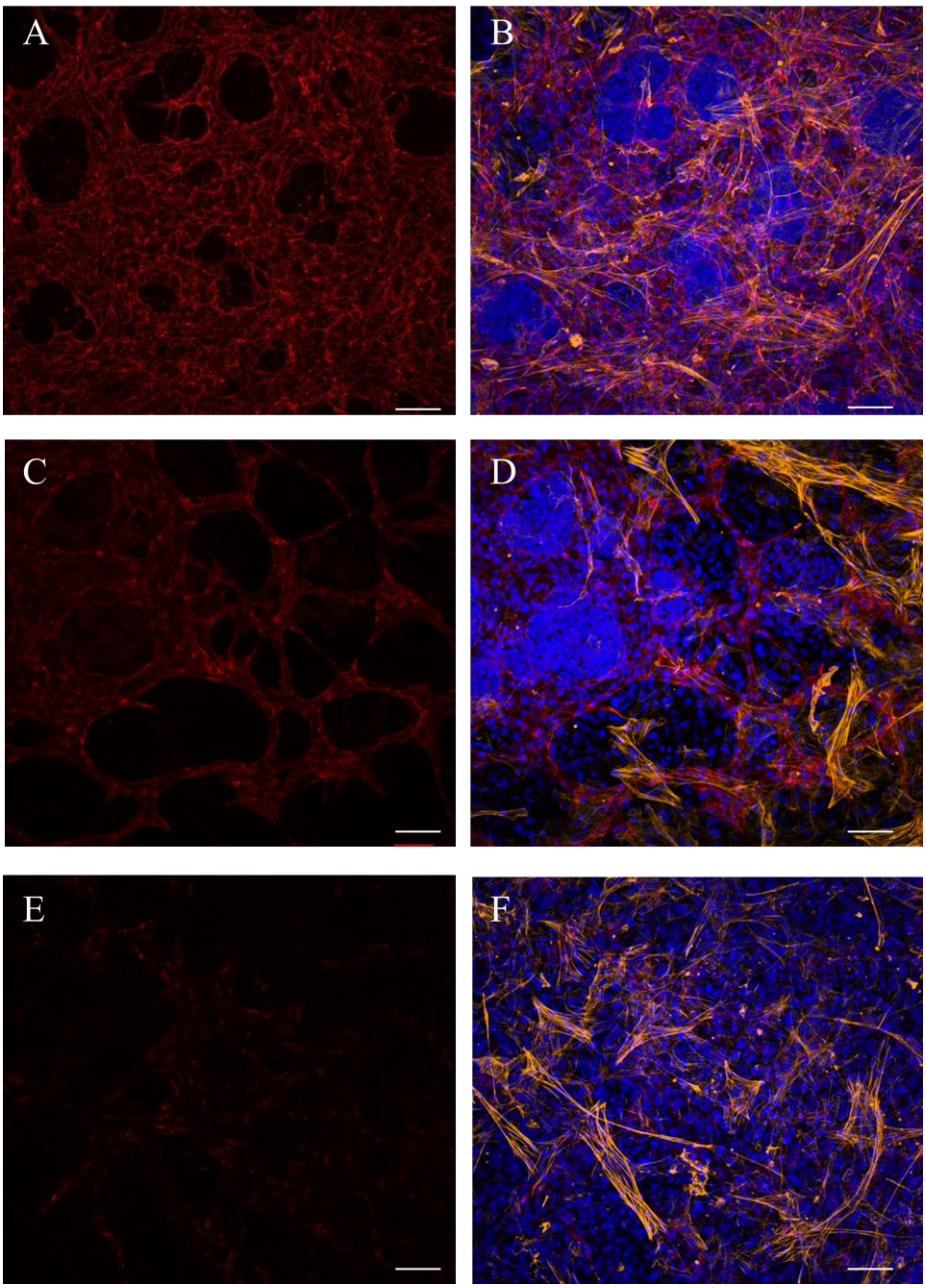

Figure 14 Characterization of different endothelial cell markers in embryoid bodies day 4 plated for 4 additional days in differentiation medium on gelatin-coated plates

(A): VE-cadherin (red); **(B):** VE-cadherin (red) merged with α-SMA (yellow) and nuclear counterstaining with Sytox-blue (blue)**. (C):** CD31 (red) **(D):** CD31 (red) merged with α-SMA (yellow) and nuclear counterstaining with Sytox-blue (blue)**. (E):** Flk-1 (red); **(F):** Flk-1 (red) merged with α-SMA (yellow) and nuclear counterstaining with Sytox-blue (Blue)**.** Bar sizes represent 50 µm.

3.2 Validation of the functionality of GFP expression in the backbone lentiviral pGZ-CMV vector

The backbone lentiviral construct expressed GFP and a zeocin resistance gene under the control of the CMV promoter (pGZ-CMV vector). To investigate the functionality of this vector in driving GFP expression in ES cells upon differentiation, HEK 293 T cells were infected using pGZ-CMV vector, together with the packaging and envelope plasmids. Pseudoviral particles were used to transduce ES cells. After transduction, EBs were derived from ES cells and were monitored for GFP expression (Figure 15). At the initial stages of EB formation, a robust GFP expression under the control of the CMV promoter could be detected (Figure 15 A). Over time, GFP expression was reduced (Figure 15 B), most probably as a result of silencing and/or the dominant autofluorescence in the older transgenic EBs.

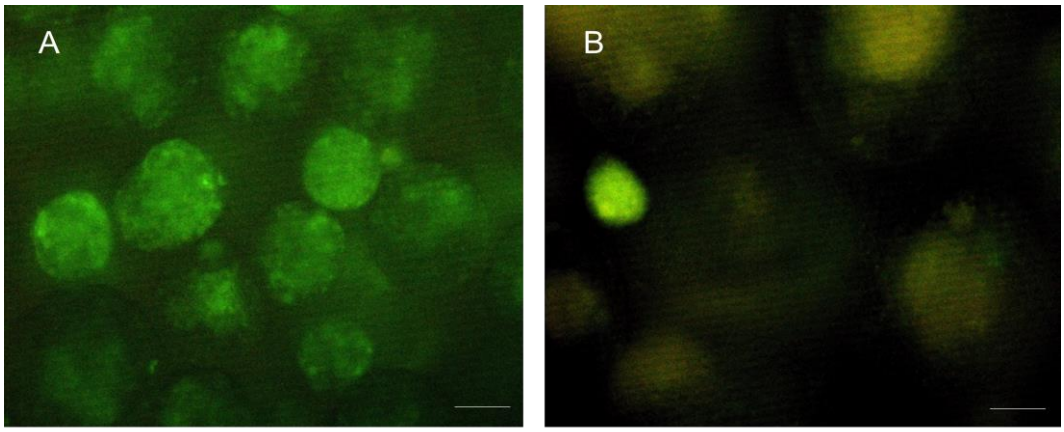

Figure 15 Assessing GFP expression in embryoid bodies derived from embryonic stem cells that were transduced with pGZ-CMV vector

(A) CMV-GFP+ embryoid bodies day 4; **(B)** CMV-GFP+ embryoid bodies day 10. Bar sizes represent 150 µm in A and 200 µm in B.

3.3 Validation of the pGZ-VE-cadherin construct and optimization of transduction conditions

To validate the generated, lenitiviral-based pGZ-VE-cadherin construct and to optimize the transduction conditions, different amounts of DNA were used for the infection of HEK 293 T cells and subsequent transduction of MEFs and mouse endothelioma cells, as negative and positive control, respectively. Transduction efficiency was tested in the

presence and absence of polybrene. Mouse endothelioma cells transduced with the construct showed the strongest GFP expression, when a total amount of 8µg DNA (4µg pGZ-VE-cadherin, 1.33µg envelope plasmid and 2.67µg packaging plasmid) in the presence of polybrene was used. In contrast, transduced MEFs showed virtually no GFP expression, thus confirming the specificity of the transgene (Figure 16 A, B).

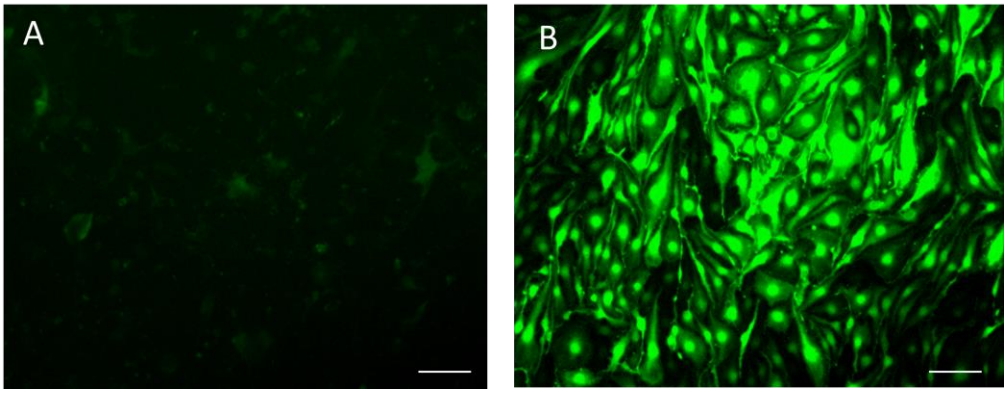

Figure 16 GFP expression in mouse embryonic fibroblasts (MEFs) and mouse endothelioma cells transduced with the pGZ-VE-cadherin construct

(A) MEFs as negative control; **(B)** mouse endothelioma cells as positive control. Scale bars represent 100 µm.

3.4 Transduction of embryonic stem cells with the pGZ-VE-cadherin construct and visualization of the GFP-positive vessel-like structures

The optimized conditions were applied to transduce single ES cells with pseudoviral particles produced by HEK 293T cells. Following transduction, 192 ES colonies derived from single ES cells were picked carefully and directed to differentiation. EBs were monitored based on the intensity of GFP expression and the visualization of vessel-like structures. From day 8 of differentiation, GFP-positive vessel-like structures started to appear. Before plating, GFP was expressed in the center of the EBs, while after plating of EBs on gelatin-coated plates and their treatment with 5ng/mL VEGF, GFP was expressed in the sproutings inside and around the EBs. From day 6 differentiation on gelatin-coated plates, 12.5% of the clones showed GFP-positive vessel-like structures. One of the clones displayed the highest level of GFP expression in association with vessel-like structures and the highest survival capacity in culture (Figure 17 A-D) and

was thus used in all the subsequent experiments. Figure 17 D shows the tubular structures derived from dissociated EBs originating from this clone, which were re-plated on matrigel. The image was taken 48 hours after culture of the cells.

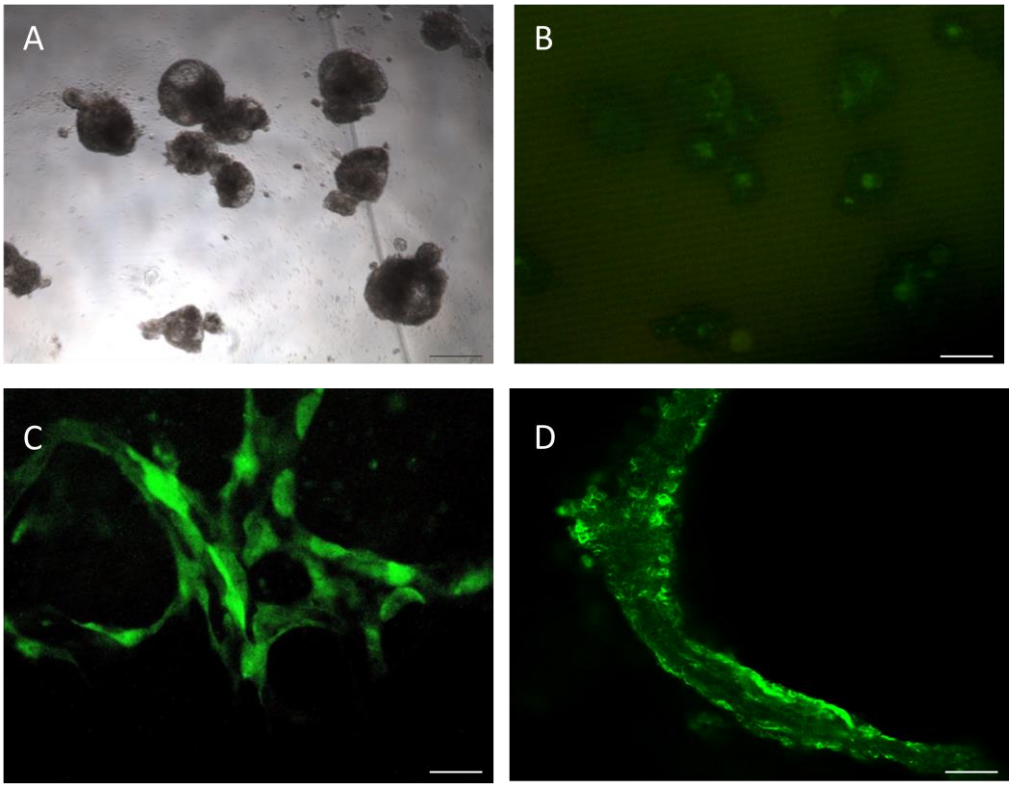

Figure 17 GFP expression in mouse embryoid bodies (EBs) derived from mouse embryonic stem (ES) cells transduced with the pGZ-VE-cadherin construct both in suspension culture and plated on gelatin

Mouse ES cells were transduced with a total amount of 8 µg DNA in the presence of polybrene. EBs at differentiation day 4 (bright field **(A)**, GFP channel **(B)**) and EBs at differentiation day 6 plated for 2 additional days on gelatin-coated plates **(C)** are shown**. (D):** Vascular tube formation assay in day 6 dissociated EBs cultured on matrigel for 2 additional days. Scale bars represent 100 µm in A and B, 50 µm in C and 20 µm in D.

3.5 Characterization of the GFP-positive embryonic stem cell-derived endothelial cells in both whole plated bodies and dissociated state

3.5.1 Whole embryoid bodies

In order to investigate the co-expression of GFP and vascular markers in ES cell-derived vascular structures, immunofluorescence staining was performed on plated EBs

at day 8 of differentiation. Figure 18 (A-F) shows the co-expression of GFP with VE-cadherin, as well as with CD31, in plated whole EBs at differentiation day 8.

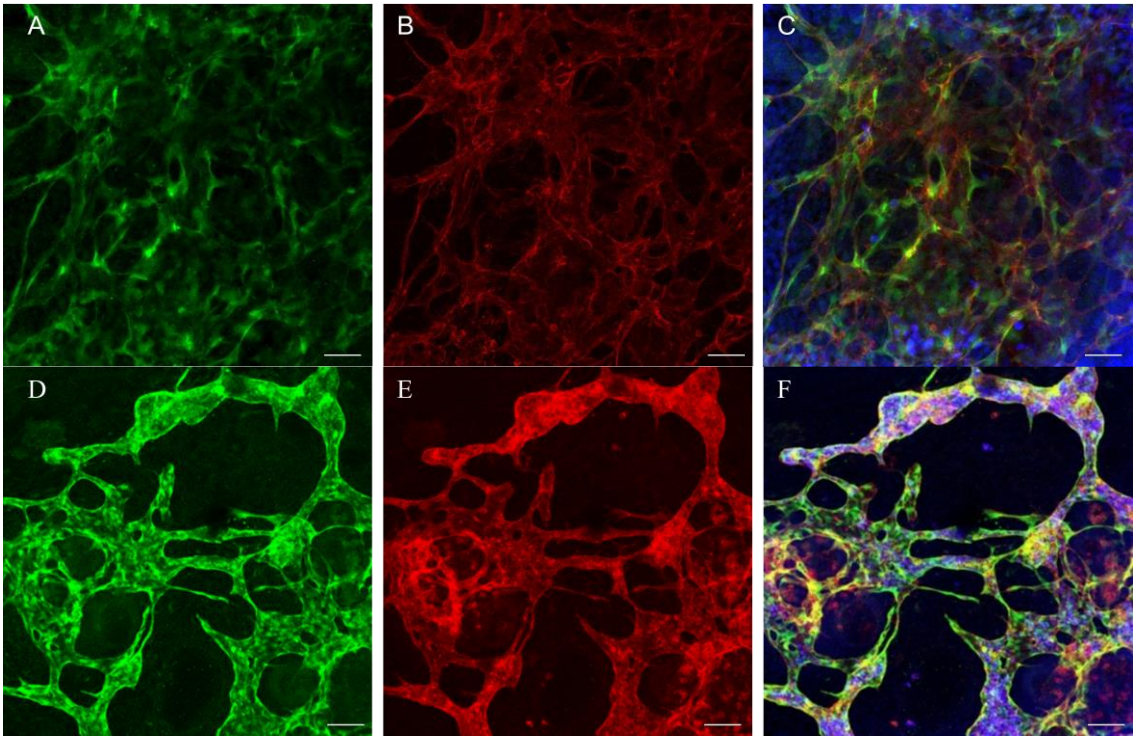

Figure 18 Co-localization of GFP and endothelial cell markers in vascular structures observed in plated embryoid bodies that were derived from transgenic embryonic stem cells

(A-C): Co-localization of GFP (A) and VE-cadherin (B) in day 4 whole embryoid bodies plated on gelatin-coated culture dishes for 4 additional days in the presence of 20ng/mL VEGF; merged figures counterstained with Sytox blue (C). **(D-F):** Co-localization of GFP (D) and CD31 (E) in day 4 whole EBs plated on gelatin-coated culture dishes for 4 additional days in the presence of 20ng/mL VEGF; merged figures counterstained with Sytox blue (F).Scale bars represent 100 µm.

3.5.2 Dissociated embryoid bodies

In order to study ES cell-derived ECs as single cells while preserving cell surface expression of VE-cadherin to the utmost, EBs were dissociated utilizing different dissociation solutions. Some of the dissociation solutions such as accutase and EDTA-based dissociation solutions led to a great loss of the surface markers upon dissociation. Conversely, collagenase B resulted in the highest degree of surface marker preservation compared to other tested solutions, and was therefore used together with DNase for single cell-dissociation of EBs (Figure 19). After dissociation, EBs were re-seeded on gelatin-coated plates. A high association of GFP with VE-cadherin and CD31 localized to tube-like structures was noted in the dissociated and re-seeded EBs (Figure 20, A-F).

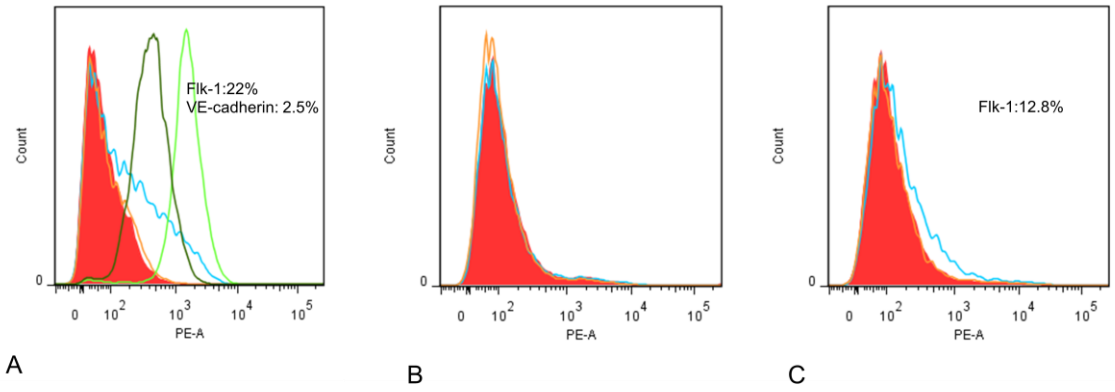

Figure 19 Expression patterns of endothelial markers upon dissociation of day 5 embryoid bodies with different dissociation solutions

(A): Collagenase B; **(B):** Accutase; **(C):** Non-enzymatic cell dissociation solution. Red: IgG Isotype control; Yellow: VE-cadherin; Blue: Flk-1; Dark Green: VE-cadherin positive control; Bright green: Flk-1 positive control.

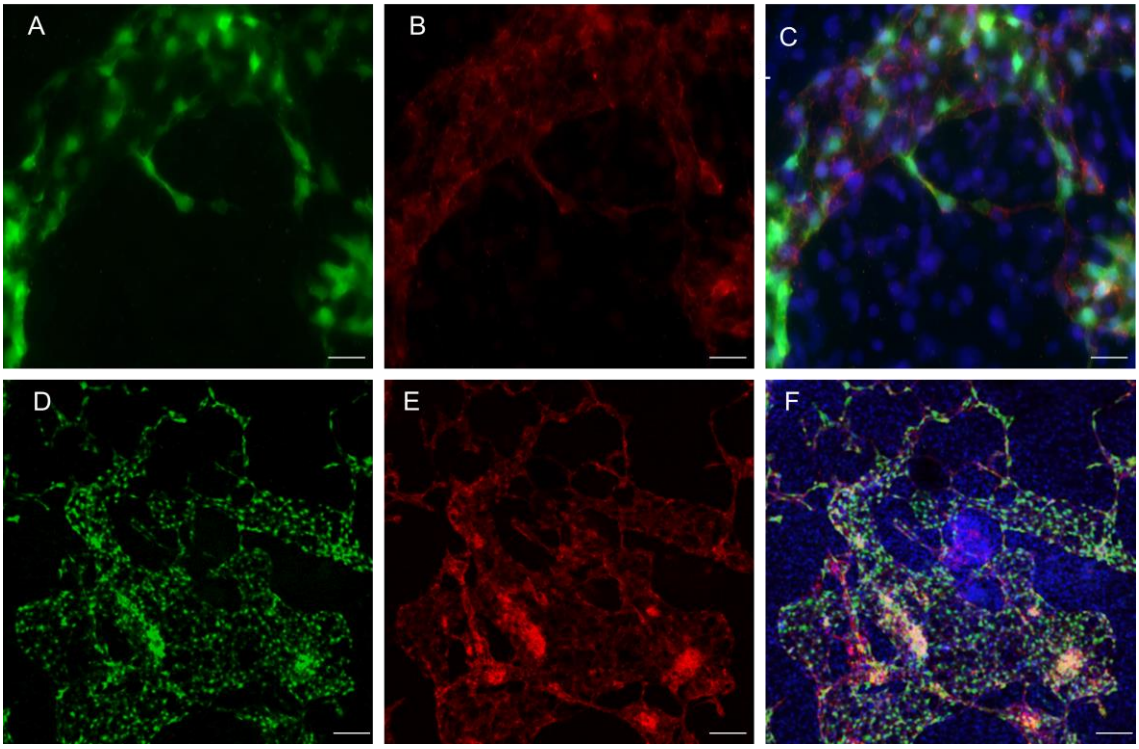

Figure 20 Co-localization of GFP and endothelial cell markers in vascular structures observed in dissociated and re-plated embryoid bodies derived from transgenic embryonic stem cells

(A-C): Co-localization of GFP (A) and VE-cadherin (B) in day 5 dissociated EBs plated on gelatin-coated culture dishes for 4 additional days in the presence of 20 ng/mL VEGF; merged figures counterstained with Sytox blue (C). **(D-F):** Co-localization of GFP (D) and CD31 (E) in day 5 dissociated EBs plated on gelatin-coated culture dishes for 4 additional days in the presence of 20ng/mL VEGF; merged figures counterstained with Sytox blue (F). Scale bars represent 25 µm in A-C and 100 µm in D-F.

3.6 Selection and characterization of GFP-positive cells representing endothelial cells

3.6.1 Treatment of cells with antibiotic (zeocin)

As the generated construct also expressed a zeocin resistance gene under the control of the VE-cadherin promoter, it was next attempted to select GFP-positive cells by treating the EBs derived from transgenic ES cells with different concentrations of zeocin. However, even with a concentration as high as 1 mg/mL, fibroblasts/myofibroblasts were not completely eliminated and the transgenic cells representing ECs appeared to be unhealthy with only weak GFP expression (Figure 21). Therefore, I opted for other methods which facilitated a more efficient selection.

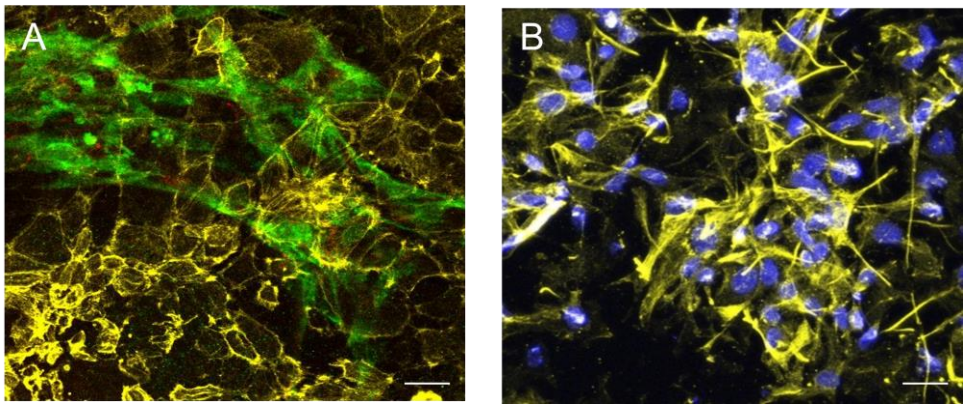

Figure 21 Treatment of cultured dissociated embryoid bodies expressing GFP and a zeocin resistance gene with different concentrations of zeocin

(A): Dim and temporary expression of GFP (green) as well as α-smooth muscle actin (yellow) in dissociated embryoid bodies at differentiation day 10 (2 days after treatment with 400 µg/ml zeocin). **(B):** Even with concentrations as high as 1 mg/mL some fibroblasts were still present in culture.

3.6.2 Flow cytometric analysis of the GFP-positive cells representing endothelial cells

In preparation for immune-based sorting of ECs, a time-course expression analysis of GFP, CD31, and the hematopoietic marker CD34 was performed in transgenic EBs at differentiation days 2.5-12 (Figure 22). There was some expression of GFP and CD31 in day 2.5 EBs, which did not represent a distinct population. Day 6 represented the highest number of CD31-positive cells (30%). On day 8, a GFP- and CD31-double positive population became distinct. Afterwards, the expression of both GFP and CD31

decreased significantly. Distinct CD34 expression became evident from day 10. Only a small population of these cells co-expressed GFP (2.15%) (Figure 22).

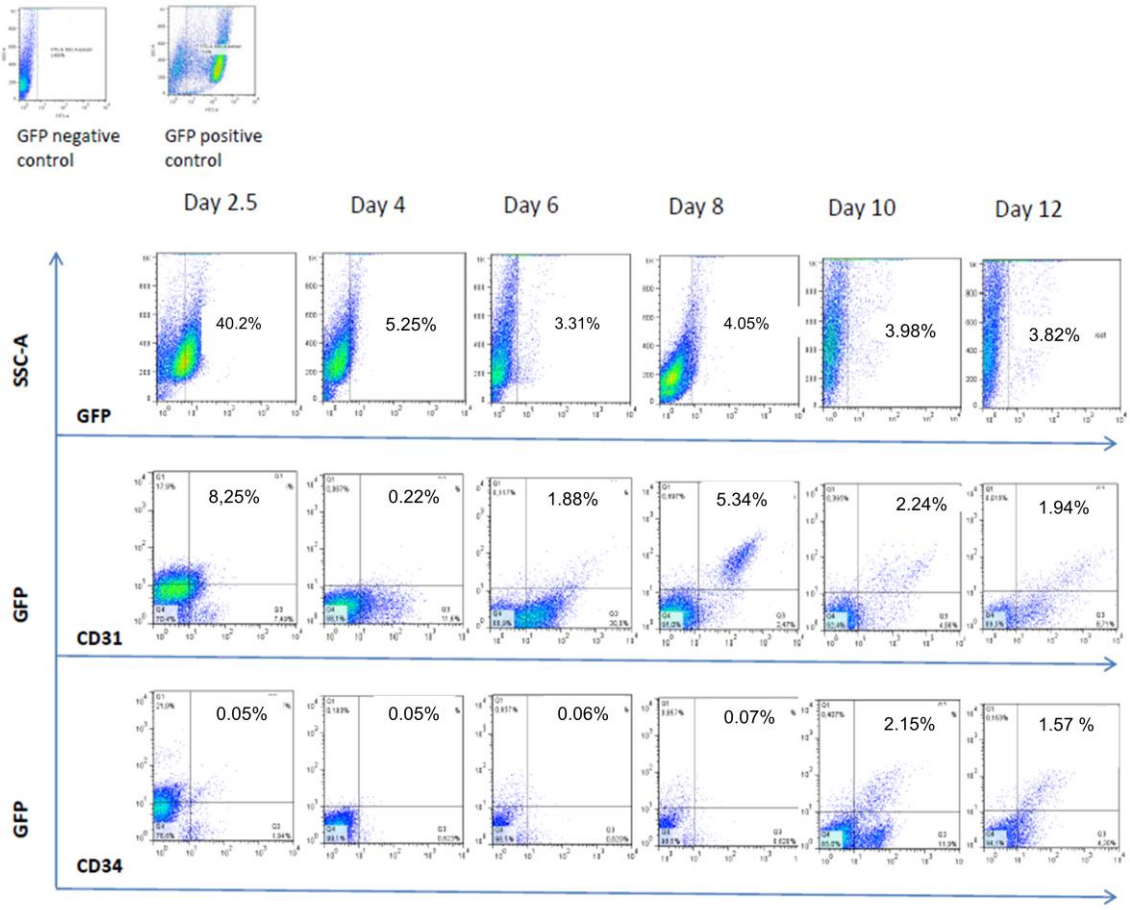

Figure 22 Flow cytometric time-course analysis of GFP, CD31 and CD34 in embryoid bodies (EBs) derived from transgenic embryonic stem (ES) cells at differentiation days 2.5-12

In the top left corner, the negative and positive controls for GFP are demonstrated (wild type EBs and lentivirally-transduced EBs with a CMV-GFP construct, respectively). Top panel: Pattern of GFP expression in EBs day 2.5 to day 12, expressing GFP under the control of the VE-cadherin promoter. Middle panel: Pattern of CD31 versus GFP expression in EBs day 2.5 to day 12, expressing GFP under the control of the VE-cadherin promoter. Bottom panel: Pattern of CD34 versus GFP expression in EBs day 2.5 to day 12, expressing GFP under the control of the VE-cadherin promoter.

3.7 Sorting of dissociated EBs expressing GFP under the control of the VE-cadherin promoter

3.7.1 GFP-sorting

After profiling the endothelial markers at different stages of the differentiation of EBs, cells were sorted on different stages of EB development based on GFP expression using

a flow cytometer. As the highest percentage of GFP-positive cells was at day 8, sorted cells on day 8 were used for RNA isolation and subsequent expression analyses of endothelial-specific markers. GFP-positive cells demonstrated a significantly higher expression of the majority of the investigated EC markers compared to the GFP– negative population (Figure 23). However, after a few days in culture, cells were weak and fragile, so that their maintenance for an extended period of time was not possible.

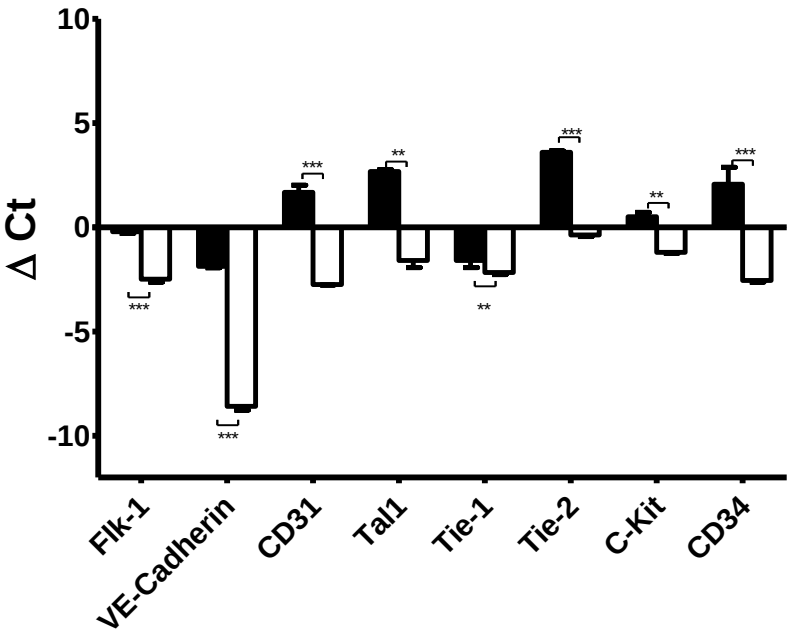

Figure 23 Gene expression analysis in GFP-sorted transgenic embryoid bodies

Transgenic embryoid bodies (EBs) at day 8 of differentiation were sorted based on GFP expression and subjected to gene expression analyses of endothelial cell-specific markers. Results are represented as ΔCT values for the GFP-positive (closed columns) *vs.* –negative (open columns) populations. PBDG served as the housekeeping gene. Data are presented as mean ± SD. Results were assessed for statistical significance using student's t-test $^{**}P < 0.01$, $^{***}P < 0.001$.

3.7.2 CD31-sorting

In the next step, magnetic beads were used for the selection procedure based on CD31 expression at different stages of EB development (days 2-8). The dissociated cells were first treated with rat CD31 antibody, and in the second step coated with sheep anti-rat secondary antibody conjugated with magnetic beads. Afterwards, the tubes containing cell solution were transferred into a strong magnetic field and the CD31-positive cells were selected. Figure 24 demonstrates the CD31-selected cells conjugated with magnetic beads in culture.

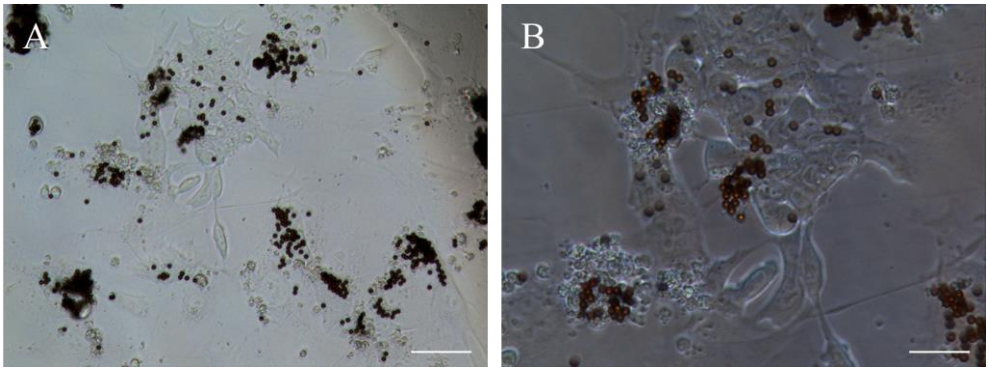

Figure 24 CD31-positive cells derived from dissociated embryoid bodies expressing GFP under the control of the VE-cadherin promoter

The isolation was performed based on magnetic beads. The images represent the sorted CD31+ cells after 2 days in culture. Bar sizes represent 100 µm in A and 50 µm in B.

3.8 Gene analysis of the transgenic embryonic stem cell-derived embryoid bodies expressing GFP under the control of the VE-cadherin promoter based on CD31

qRT-PCR reactions were performed on CD31-positive *vs.* CD31-negative populations for different markers expressed early and late during the course of differentiation of ES cells to ECs with an endothelial-specific GFP expression (Figure 25).

From day 3 on, the known vasculogenesis transcription factors such as c-kit and Tal1 showed an increased expression in the CD31-positive *vs.* CD31-negative populations. Additionally, many other EC markers along with genes involved in the development of ECs were expressed to a higher degree in CD31-positive *vs.* CD31-negative populations. Most pronounced gene fold increases were noted for VE-cadherin, Tyrosine kinase with immunoglobulin-like and EGF-like domains (Tie)2, and Sox18. On day 4, the biggest fold increase in endothelial-specific gene expression in the CD31-positive *vs.* CD31-negative population was observed for Tie-1, followed by Tie-2 and Tal 1 (Figure 25 C). From day 6 on, all the investigated earlier and late stage EC differentiation markers, as well as transcription factors of vascular development were markedly higher in CD31-positive *vs.* CD31-negative populations. The highest gene fold increase was noted for Tie-1 (43.9 fold) followed by Sox18 (39.9 fold), VE-cadherin (27.7 fold), and Nitric oxide synthase (Nos)3 (25.8 fold). These differences became more pronounced at day 8 of differentiation with Nos3 and VE-cadherin

representing a 604-fold and a 565-fold increase in CD31-positive *vs.* CD31-negative populations, respectively (Figure 25 D and E).

In these studies, the pattern of expression of 2 markers of hematopoietic cells namely CD34 and CD45 in the CD31-positive *vs.* CD31-negative population was also examined. The results showed a decrease in the fold gene expression of CD45 on day 8 in the CD31-positive *vs.* CD31-negative population, along with the specification to ECs. CD34 showed a trend to an increased gene fold expression in later days. A markedly increased expression of this marker in the CD31-positive *vs.* CD31-negative population could be expected in the later days of differentiation (from day 10), based on the later overall expression of CD34 (Figure 22) according to a later commitment to hematopoietic cells.

Compared to the GFP-positive population, the expression of most of the studied endothelial markers was slightly higher in the CD31-positive population at the same time point (Figure 25 F).

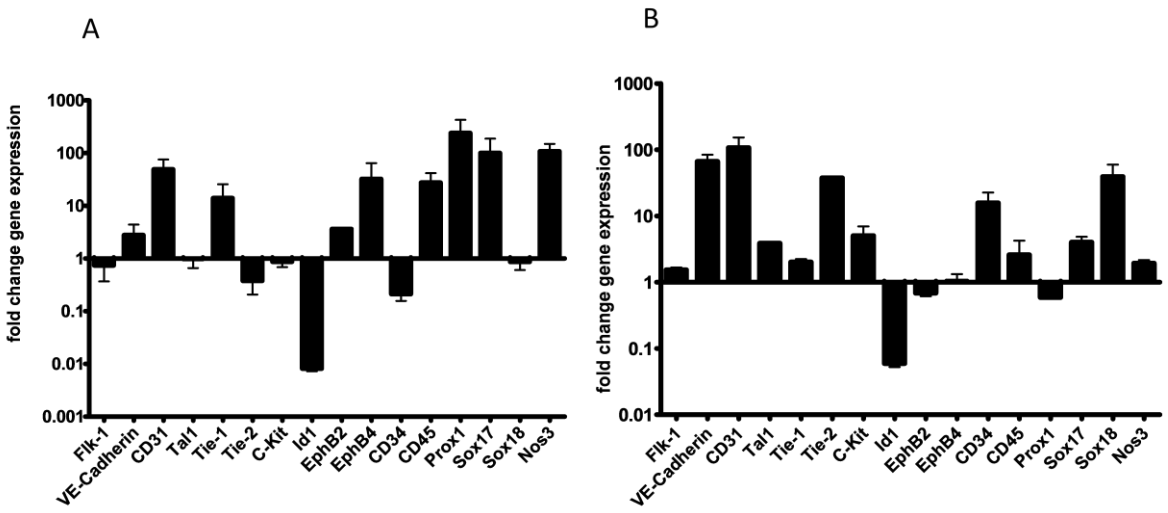

Figure 25 Gene expression analysis in CD31-sorted transgenic embryoid bodies (EBs)

(A, B): Transgenic EBs at days 2 (A) and 3 (B) of differentiation were sorted based on CD31 expression and subjected to gene expression analyses of endothelial cell specific markers. Results are expressed as n-fold change in gene expression in the CD31-positive *vs.* CD31-negative populations using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct$= (Ct, target – Ct, housekeeping gene)$_{CD31pos}$ – (Ct, target – Ct, housekeeping gene)$_{CD31neg}$. PBDG served as the housekeeping gene.

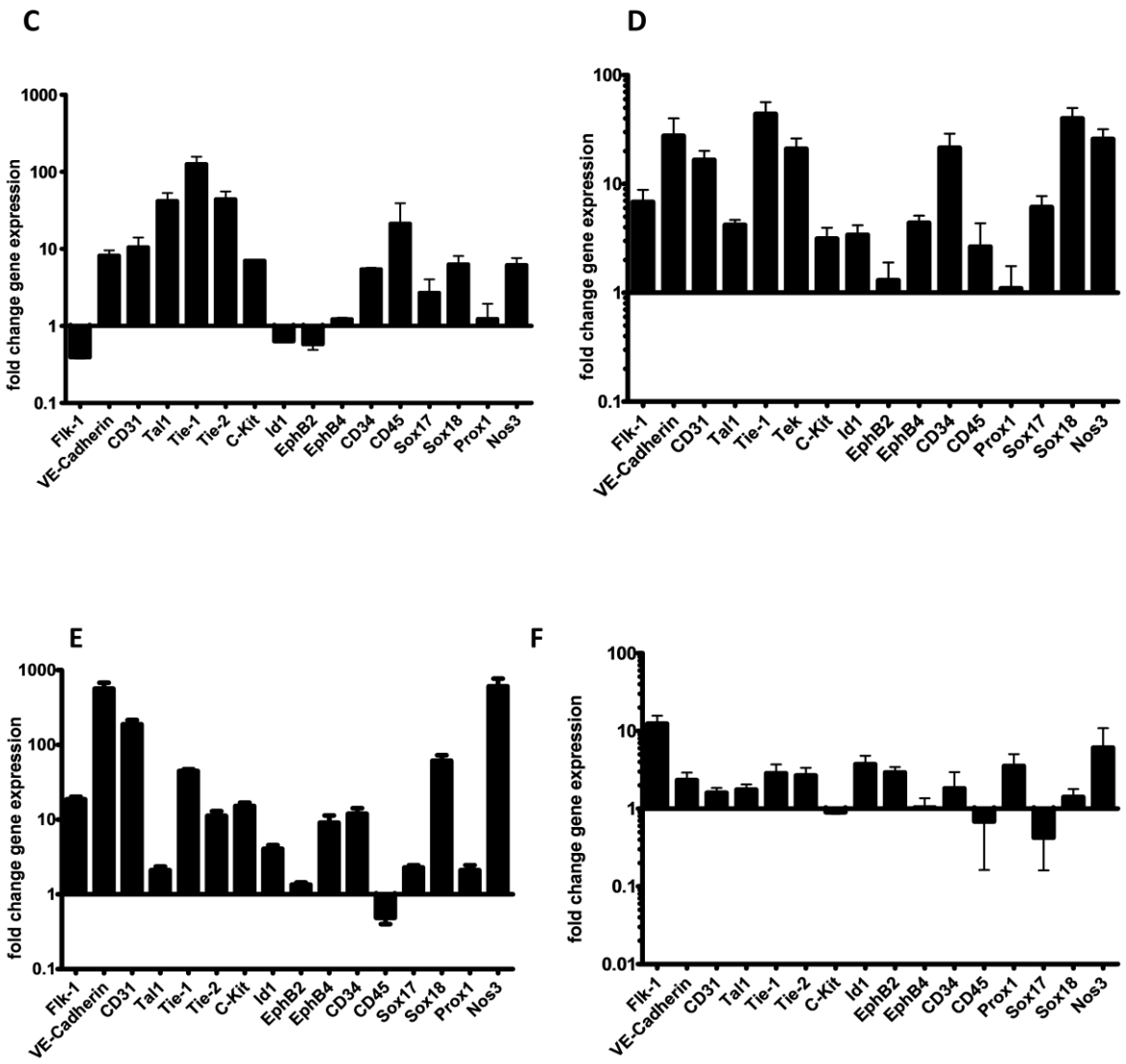

Figure 25 (continued) Gene expression analysis in CD31-sorted transgenic embryoid bodies (EBs)

(C-F): Transgenic EBs at day 4 (C), 6 (D), and 8 (E) of differentiation were sorted based on CD31 expression and subjected to gene expression analyses of endothelial cell specific markers. Results are expressed as n-fold change in gene expression in the CD31-positive *vs.* CD31-negative populations using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct$= (Ct, target – Ct, housekeeping gene)$_{CD31pos}$ – (Ct, target – Ct, housekeeping gene)$_{CD31neg}$. **(F):** N-fold change in gene expression of endothelial cell specific markers in the CD31-positive *vs.* GFP-positive populations as assessed by the $2^{-\Delta\Delta Ct}$ method. PBDG served as the housekeeping gene.

Next, the time course expression of EC surface markers in the CD31-positive population regardless of the CD31-negative population was evaluated. The expression of Flk-1, Tie-2, Tie-1, and VE-cadherin increased in the CD31-positive populations over time. The surge in the expression of Flk-1, Tie-2, and VE-cadherin happened from day 2 to day 3 (Figure 26 A). A second surge in the expression of VE-cadherin occurred

from day 6 to 8. For Tie-1, there was a tendency for increased expression in later stages as compared to the earlier stages of EC differentiation.

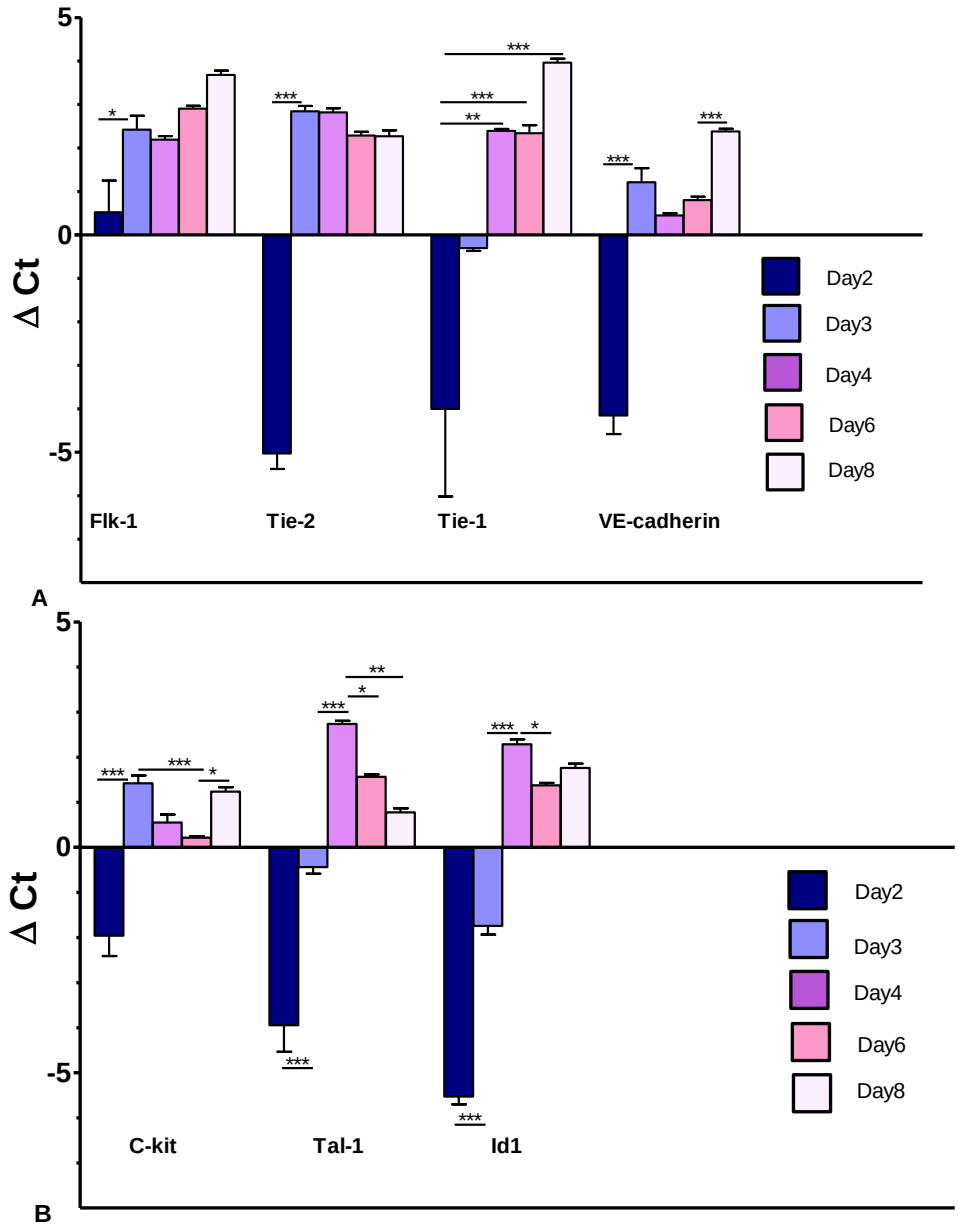

Figure 26 Time course gene expression analysis in the CD31-positive cell population

The CD31-positive cell population derived from transgenic EBs at days 2-8 of differentiation was subjected to gene expression analyses of **(A)** endothelial cell surface markers and **(B)** transcription factors of vasculogenesis Results are expressed as ΔCT values. PBDG served as the housekeeping gene. Data are presented as mean ± SD. Results were assessed for statistical significance using one-way ANOVA with Tukey's multiple comparison test **P* < 0.05, ***P* < 0.01, ****P* < 0.001)**.**

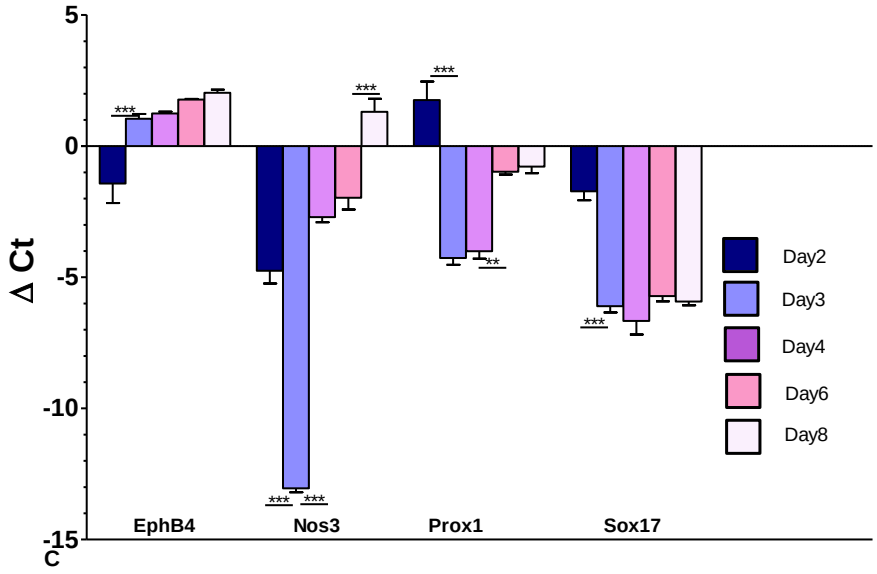

Figure 26 (continued) Time course gene expression analysis in the CD31-positive cell population

The CD31-positive cell population derived from transgenic EBs at days 2-8 of differentiation was subjected to gene expression analyses of **(C)** markers that characterize particular vessel types. Results are expressed as ΔCT values. Data are presented as mean ± SD. PBDG served as the housekeeping gene. Results were assessed for statistical significance using one-way ANOVA with Tukey's multiple comparison tests $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$**.**

The transcription factors involved in vasculogenesis showed a distinct pattern of expression (Figure 26 B). C-kit showed a sharp increase in expression from day 2 to 3 and from day 6 to 8 after a down-regulation in the intermediate phase. Tal-1 was up-regulated up to day 4, following by a steady decrease in expression in the later stages. Id1 showed a pattern comparable to c-kit with the highest expression level on day 4. Additionally, some markers characterizing particular vessel types (arterial *vs.* venous *vs.* lymphatic) such as EphB4, Nos3, Prox1, Sox17 and Sox18 were examined. These markers demonstrated a higher overall expression in the CD31-positive *vs.* CD31-negative population. EphB4 showed a significantly increased expression from day 3 compared to day 2. Following a sharp down-regulation of Nos3 at day 3, there was an increasing expression in the later days of differentiation in the CD31-positive population, with the highest level on day 8. The expression of Prox1, as a marker of lymphatic ECs, decreased on days 3 and 4 and later again slightly increased, while the expression of Sox17 decreased from day 3 and stayed at a low level during the later differentiation days in the CD31-positive population (Figure 26 C).

Eventually, the time course expression of markers of the undifferentiated state of ES cells and early differentiation genes in the isolated CD31-positive population as well as in comparison to the CD31-negative population at various differentiation stages was dissected (Figures 27 and 28).

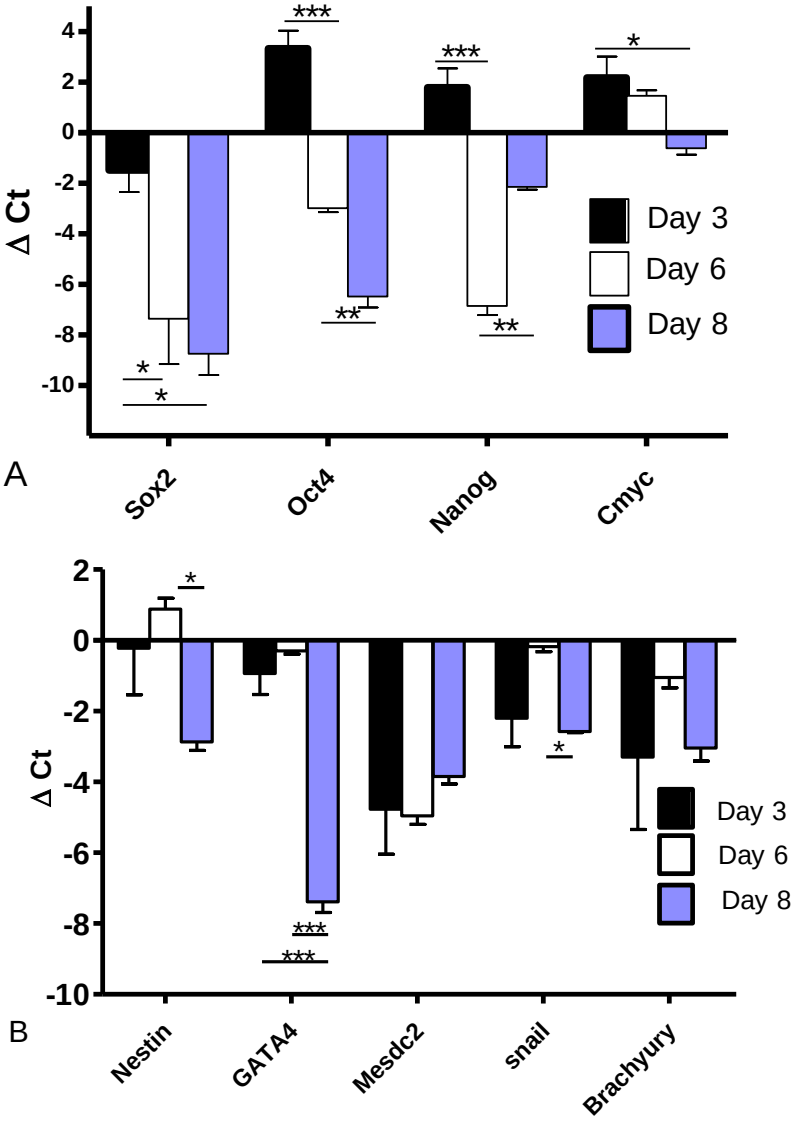

Figure 27 Gene expression analyses in CD31-sorted transgenic embryoid bodies (EBs) for embryonic stem cell markers as well as markers of early differentiation

Time course gene expression analysis in the CD31-positive cell population The CD31-positive cell population derived from transgenic EBs at days 3, 6, and 8 of differentiation was subjected to gene expression analyses of **(A)** markers of the undifferentiated state of ES cells, and **(B)** early differentiation genes. Results are expressed as ΔCT values. PBDG served as the housekeeping gene. Data are presented as mean ± SD. Results were assessed for statistical significance using one-way ANOVA with Tukey's multiple comparison tests **P* < 0.05, ***P* < 0.01, ****P* < 0.001**.**

The expression of markers of the undifferentiated state such as Sox2, Oct-4, Nanog, and c-Myc decreased from the early to the later stages of development (day 3 to 8; Figure 27 A). The expression of some of the examined early differentiation markers such as snail, nestin, and GATA4 showed a statistically significant down-regulation in the later differentiation days in the CD31-positive population (Figure 27 B). The pattern of expression of these markers was also evaluated at different time points in CD31-positive *vs.* CD31- negative populations (Figure 28). Except for c-Myc, day 8 of differentiation represented a lower level of expression of all these markers in CD31-positive *vs.* CD31-negative populations underscoring the rather mature typical, endothelial-like characteristics of the CD31-positive versus CD31-negative population at that time point as compared to the earlier stages of development, where CD31 may also be present in non-ECs.

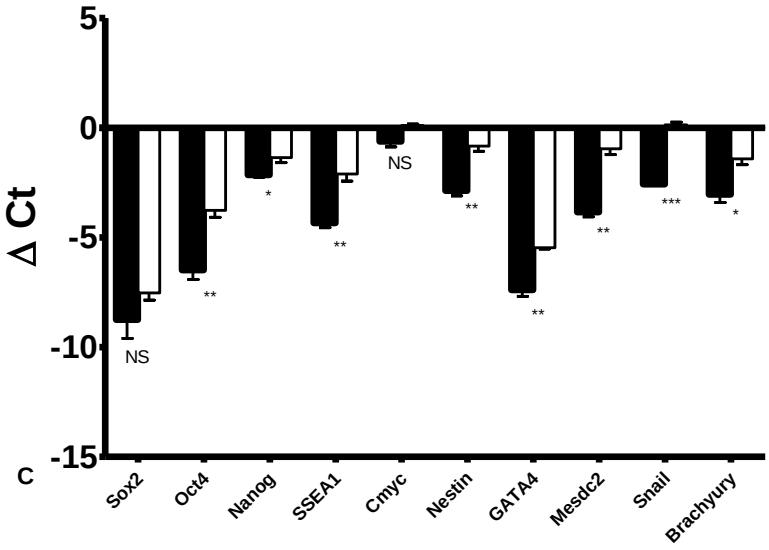

Figure 28 Difference in the expression of markers of the undifferentiated state and early markers of differentiation in the CD31+ (closed columns) *vs.* CD31- (open columns) at differentiation day 8

Results are expressed as ΔCT values. PBDG served as the housekeeping gene. Data are presented as mean ± SD. Results were assessed for statistical significance using student's t-test. $*P < 0.05$, $**P < 0.01$. NS: Not significant.

3.9 Culture of sorted cells

According to flow cytometry, day 8 represented a distinct population with the highest percentage of cells co-expressing CD31 and GFP (Figure 22). qRT-PCR confirmed a markedly higher expression of EC-specific markers on days 6 and 8 in the CD31-positive *vs.* CD31-negative populations (Figure 25). EB with endothelial-specific GFP expression were sorted and cultured at differentiation days 6 and 8 based on CD31 expression.

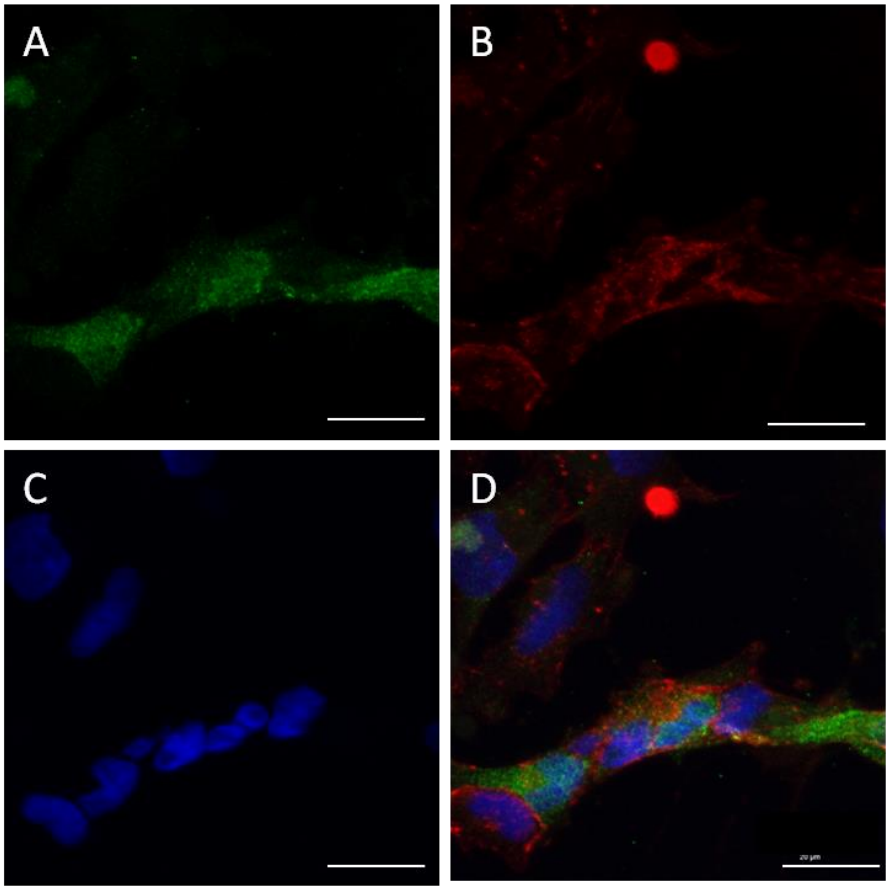

Figure 29 Culture of CD31-positive cells derived from EBs at differentiation day 8 on matrigel

(A): GFP; **(B):** VE-cadherin; **(C):** Nuclear counterstaining with draq 5; **(D):** Merged figures. Cells were treated with 20 ng/mL VEGF**.** Scale bars represent 20 µm.

Embryoid bodies that were sorted on day 8 and cultured on matrigel contributed to sproutings with GFP and VE-cadherin co-expression, rather than forming a cobblestone morphology (Figure 29). Embryoid bodies that were sorted and cultured on day 6 developed a characteristic EC cobblestone morphology, co-expressed GFP and different

endothelial markers, and contributed to a high-purity EC culture (Figure 30 A-L). These cells were passageable and maintained a good level of GFP expression in the next passages.

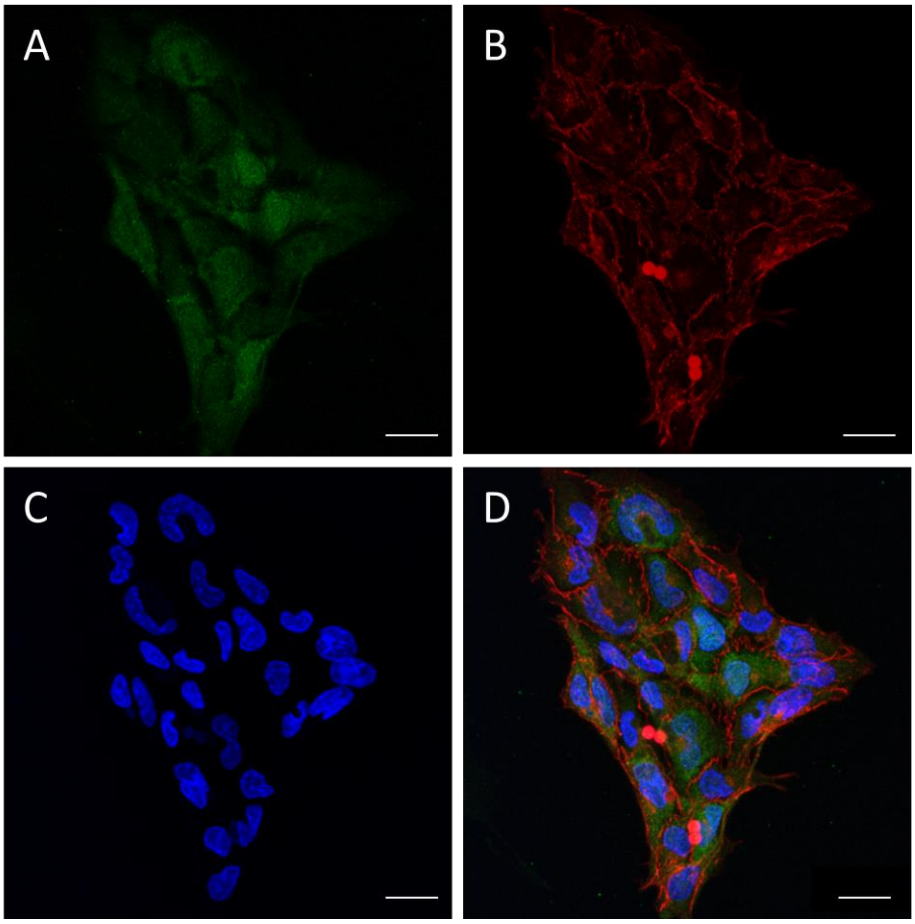

Figure 30 Isolation of CD31-positive cells derived from EBs on differentiation day 6 and their culture on gelatin-coated plates

(A): GFP; **(B):** Flk-1; **(C):** Nuclear counterstaining with draq 5; **(D):** Merged figures. Cells were treated with 20 ng/mL VEGF**.** Scale bars represent 20 µm.

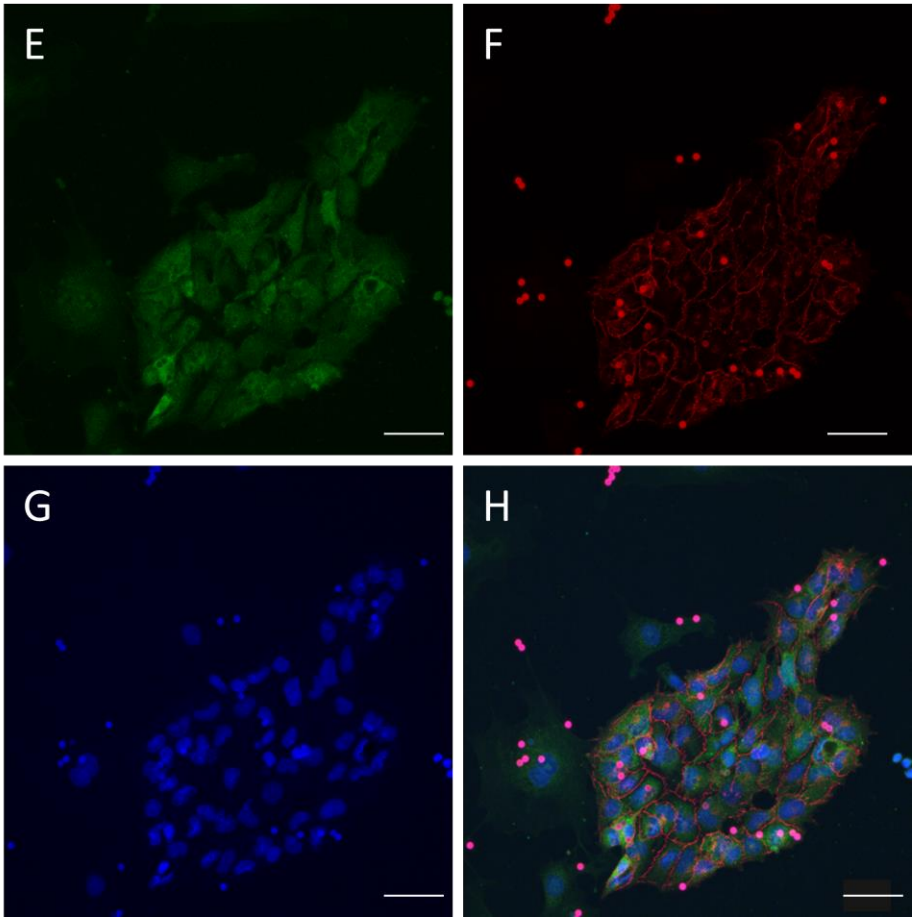

Figure 30 (continued) **(E):** GFP**; (F)** VE-cadherin**; (G)** nuclear counterstaining with draq 5; **(H)** Merged figures. Cells were treated with 20 ng/mL VEGF. Scale bars represent 50 µm.

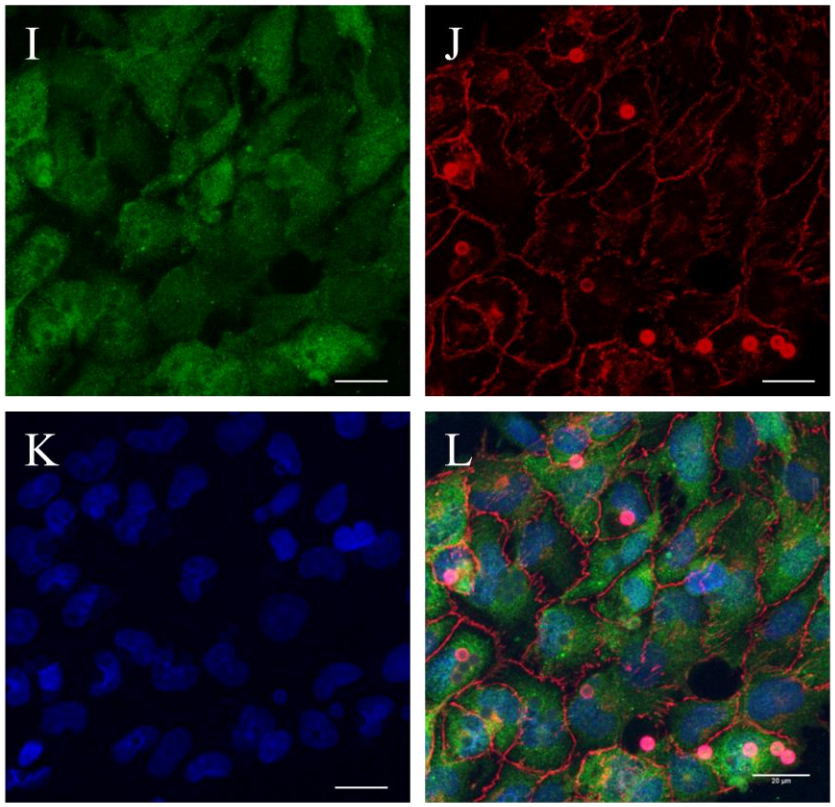

Figure 30 (continued) (I) GFP**; (J)** VE-cadherin**; (K)** Nuclear counterstaining with draq 5; **(L)** Merged figures. Cells were treated with 20 ng/mL VEGF. Scale bars represent 20 µm. This image is the magnification of the image on the previous page, representing a pure group of GFP -labeled cells in a cobblestone structure, typical of ECs.

Endothelial cells isolated on day 6 also eventually contributed to tube-like structures (Figure 31).

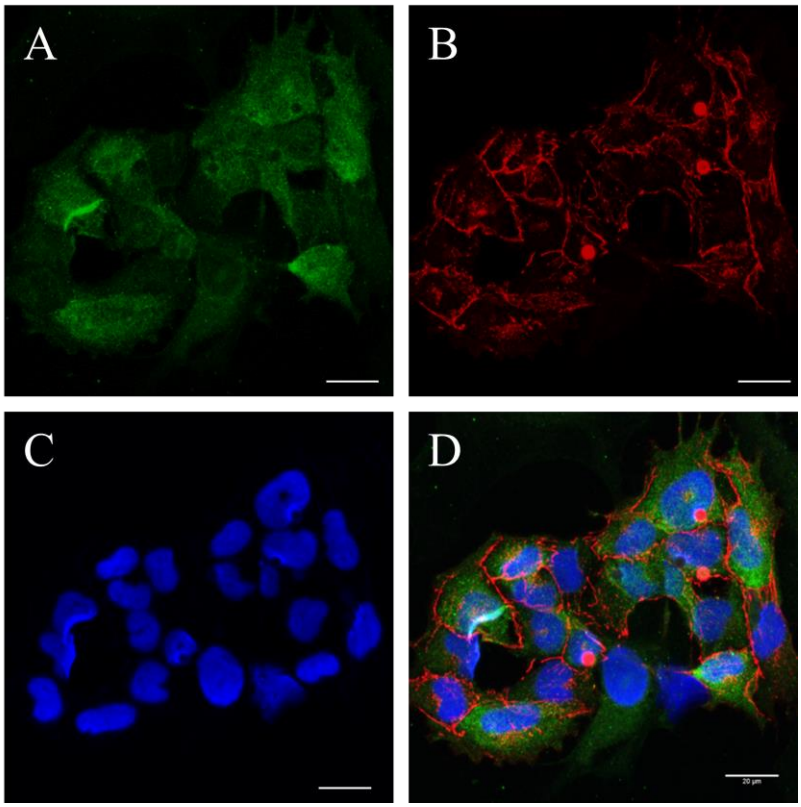

Figure 31 Formation of GFP-positive tube-like structures in CD31-positive cells

(A): GFP; **(B):** VE-cadherin; **(C):** Nuclear counterstaining with draq 5; **(D):** Merged figures. Cells were treated with 20ng/mL VEGF**.** Scale bars represent 20 µm.

4 Discussion

Embryonic stem cells are established from the inner cell mass of blastocysts (Evans and Kaufman 1981; Martin 1981) and when grown in the presence of LIF, form pluripotent ES cell colonies (Hirai, Karian *et al.* 2011). Upon LIF removal, ES cells spontaneously differentiate into three-dimensional structures, termed embryoid bodies, which contain derivatives of the three primitive germ layers and are a potential source for all ES cell lineages (Keller 1995; Murry and Keller 2008).

Embryoid bodies contain blood island- and vascular-like structures, mimicking vasculogenesis and angiogenesis in the embryo. These three-dimensional structures are one of the most commonly used means for the development of ES cell-derived ECs (Risau, Sariola *et al.* 1988; Wang, Clark *et al.* 1992; Kim, Kim *et al.* 2008). Embryonic stem cells can serve as an abundant source for functional ECs both in progenitor and mature stages with applications in regenerative medicine (Huang, Niiyama *et al.* 2010), vascular developmental studies (Glaser, Gower *et al.* 2011), as well as drug discoveries (Kim, Bae *et al.* 2012). The structure of EBs is an amalgam of different cell types, which interferes with the aforementioned applications. In this study, a lentiviral-based construct was generated, with the promoter of VE-cadherin as a specific EC marker (Vestweber 2008) driving the expression of GFP and a zeocin resistance gene for labeling and selection purposes. Thereafter, the functionality of the transgenic cells in developing vascular structures was examined. To obtain a pure EC population, as well as to dissect the EC gene profile on different days of EB differentiation, the cell populations were purified based on CD31 expression, as another specific marker for ECs (Ilan and Madri 2003; Newman and Newman 2003; Woodfin, Voisin *et al.* 2007).

4.1 Generation of a lentiviral construct (pGZ-VE-cadherin) for labeling and later characterization of embryonic stem cell-derived endothelial cells

Silencing is one of the general problems usually encountered with ES cells being transfected with the conventional non-lentiviral vectors, especially during maturation processes. In order to overcome this hurdle, viral-based vectors such as adenoviruses and retroviruses have been put into use. In recent years, lentiviruses, which are derived

from the viral family *Retroviridae*, have been successfully used for transgenesis purposes. Lentiviral transduction is an efficient method for the insertion of exogenous genetic material into cells. This method leads to a stable integration of as big as 10 kb cDNA inserts into the genome of both non-dividing and dividing cells, and clonal distribution in dividing cells (Freed and Martin 1994; Barde, Salmon *et al.* 2010; Sakuma, Barry *et al.* 2012). These vectors lack viral particles and are devoid of replication competent viruses. Additionally, they have the advantage to transduce non-dividing cells (Escors and Breckpot 2010; Sakuma, Barry *et al.* 2012).

Use of lentiviral vectors with reporter gene expression under the control of a constitutively active promoter or ubiquitin has been shown in both undifferentiated and differentiated states of ES cells or iPSCs without silencing during the course of proliferation and differentiation (Pfeifer, Ikawa *et al.* 2002; Huang, Niiyama *et al.* 2009; Huang, Niiyama *et al.* 2010; Jiang, Lin *et al.* 2010; Huang, Okogbaa *et al.* 2012). Clonal selection and expansion of ES cells leads to the development of clones of interest with a target transgenic pattern. Lentiviral vectors have been successfully used for this purpose, which is greatly facilitated when an antibiotic resistance cassette is expressed under a constitutively active promoter.

For the selection of ES-cell derived ECs, an EC-specific promoter is required, driving the expression of a reporter and/or antibiotic resistance gene upon its activation during differentiation. VE-cadherin, as one of the most specific EC adhesion molecules, is expressed on EBs day 5 (Vittet, Prandini *et al.* 1996). In addition to adhesive roles, other functions of VE-cadherin include anti-apoptotic and angiogenic effects, as well as regulation of leukocyte transmigration (Carmeliet, Lampugnani *et al.* 1999; Gory, Vernet *et al.* 1999; Bäumer, Keller *et al.* 2006; Dejana and Giampietro 2012). In a study by Li and his colleagues, the promoter of this molecule was used to generate a conventional non-lentiviral vector expressing GFP under the control of the VE-cadherin promoter for transfection and production of ES cell clones, which expressed GFP upon differentiation to ECs (Li, Wu *et al.* 2007). The use of other markers of later stages of EC development such as Tie-1 has also been reported in previous studies (Iljin, Petrova *et al.* 2002; Marchetti, Gimond *et al.* 2002; Kim and von Recum 2010). Depending on application, more progenitor type promoters like Flk-1or Tie-2 have also been used for the isolation of ECs (Hirai, Ogawa *et al.* 2003; Magid, Martinson *et al.* 2003).

In this study, the VE-cadherin promoter was used in a lentiviral construct to generate ES cell clones expressing GFP upon activation of the VE-cadherin promoter, leading to the labeling of ES cell-derived ECs. VE-cadherin–driven GFP expression of human ES cell-derived ECs in a lentiviral vector has been also recently shown by James and his co-workers (James, Nam *et al.* 2010; James, Zhan *et al.* 2011). The authors describe a consistent amount of the transgene in ES cells through passaging of transduced ES cell colonies in parallel cultures (James, Nam *et al.* 2010). Later, they demonstrate a method of selection, which was facilitated by a preliminary FACS-sorting of the clones that temporarily showed an unspecific GFP expression in undifferentiated ES cells, possibly leading to enrichment of clonal derivatives with viral integration. Transgenic clones were later characterized by the identification of GFP-positive, vessel-like structures during differentiation (James, Zhan *et al.* 2011)**.** However, the reason for this temporary GFP expression in undifferentiated ES cells and its possible impact on later cell functionality could not be dissected. In the current experiments, undifferentiated ES cells did not express any GFP, while a temporary GFP expression in day 2 EBs was observed, prior to the long-lasting GFP expression in later differentiation days. Our applied approach precluded the initial unspecific GFP sorting. It is not known whether this initial GFP expression has a relevance to the later specific GFP expression. In addition, unlike the method taken by James and his colleagues which was based on transduction of ES cell colonies, ES cells in the present study were transduced at a single cell level. Following the formation of ES colonies, they were passaged onto 10cm MEF-coated plates. After reaching confluency, the whole grown ES colonies were picked manually with a 100 µL pipette tip, using no dissociation solution. In our view, this may confer a higher clonal integrity and purity, avoiding cross-contamination of different ES cell colonies.

4.2 Characterization of embryonic stem cell-derived endothelial cells with specific GFP expression in both whole plated and dissociated embryoid bodies

In the present experiments, formation of vascular-like structures started from day 8 differentiation of EBs or 4 days after plating of day 4 EBs. This correlates with the later expression of VE-cadherin in EBs, compared to the earlier markers of EC

differentiation such as Flk-1, Tie-2, and CD31. Before that time, GFP-positive structures appeared in non-specific, poorly organized structures essentially concentrated in the center of EBs.

In order to analyze the cells at a single cell level, various dissociation solutions were used to test the resistance of EC surface markers under enzymatic and non-enzymatic procedures and to optimize the dissociation procedures. Among endothelial markers, VE-cadherin is particularly sensitive to proteolytic enzymatic activity, especially due to metalloproteinases, elastase, and cathepsin G released during inflammation (Xiao, Allison *et al.* 2003; Luplertlop, Misse *et al.* 2006). Minimal amounts of trypsin even in the presence of calcium also lead to the elimination of all surface VE-cadherin molecules in cell culture (Lampugnani, Resnati *et al.* 1992). Furthermore, EDTA-based dissociation solutions disassemble VE-cadherin by chelating calcium, leading to the dysfunction and dissociation of the molecule (Gao, Kouklis *et al.* 2000). The best results regarding the preservation of VE-cadherin molecule was obtained with collagenase B.

Internalization, destabilization and diminishment of VE-cadherin molecules at the cell surface has been reported with conditions such as Flk-1 activation through VEGF exposure and FGF signaling inhibition, leading to dissociation of EC contacts (Gavard and Gutkind 2006; Murakami, Nguyen *et al.* 2008). It is therefore not an unexpected finding to temporarily or permanently lose a proportion of the extracellular domain of VE-cadherin during dissociation to single cells. In the present study, following dissociation, VE-cadherin expression started to reappear after 2 days in culture. This period was possibly required for retrieval of the internalized domains of VE-cadherin. However, on rare occasions, GFP expression was not accompanied by VE-cadherin expression, possibly explained by the degradation of the molecule, promoter switch-off, or transdifferentiation processes due to paracrine effects during stress imposed by degradation procedures (Xiao, Allison *et al.* 2003). Decreased VE-cadherin expression has been demonstrated to be associated with endothelial-mesenchymal transdifferentiation in endothelial monolayers and intimal inflammation in sites of neovascularization (Bobryshev, Cherian *et al.* 1999; Frid, Kale *et al.* 2002). These observations suggest that regulated changes in VE-cadherin levels have important consequences on endothelial function and pathophysiology.

4.3 Selection and characterization of embryonic stem cell-derived endothelial cells

4.3.1 Treatment of cells with antibiotic

Pure cell populations are essential for cell-based therapies and should alleviate the problem of teratoma formation when ES cells are implanted *in vivo* (Blancas, Lauer *et al.* 2008). Antibiotic selection of pure mature cells, especially ECs from ES cells, has not been in extensive use before. A Tie-1 promoter driving a puromycin resistance gene has shown to be effective in selecting ECs upon treatment with puromycin (Kim and von Recum 2010).

As a zeocin resistance gene was also designed under the control of the VE-cadherin promoter, EBs were also treated with different concentrations of zeocin for the purification of GFP-expressing cells. The addition of antibiotic to the whole 3-dimensional structures of EBs was not efficient enough to penetrate through the EBs. Accordingly, EBs were dissociated, re-plated at different confluencies, and treated with different concentrations of zeocin. However, even with high zeocin concentrations, it was not possible to completely eliminate cells with a fibroblast/myofibroblast phenotype as indicated by α-SMA expression in culture. Previous studies have also shown the presence of these cells, which may be derived from EPCs or due to a transdifferentiation step with increased reactivity to transforming growth factor (TGF)-β and laminin (Yamashita, Itoh *et al.* 2000; Marchetti, Gimond *et al.* 2002). This may also partly result from the suboptimal efficiency of antibiotics in the elimination of non-transgenic cells. There are studies about increasing the stringency of selection based on zeocin resistance gene by introduction of different initiation codons or smaller peptides at the beginning of the zeocin resistance gene (Van Blokland, Hoeksema *et al.* 2011). In these experiments, GFP-positive cells after zeocin treatment in culture showed a dim GFP expression with no overall healthy phenotype. A reason might be that the recombinant cells are not completely protected from the adverse effects of zeocin such as break and methylation of DNA strands (Oliva-Trastoy, Defais *et al.* 2005). In selection of cells with antibiotics, special measures must be undertaken to increase the efficiency of antibiotics, while at the same time protecting the cells from their harmful effects.

4.3.2 Selection of cells based on GFP expression

Due to the suboptimal results of EC selection with antibiotic treatment, the cells were next sorted on different days of EB differentiation based on GFP expression.

In a series of studies by Kim and his colleagues, the cells that were selected with antibiotics expressed some of the EC markers in culture. Though, GFP, which was concomitantly expressed under EC-specific promoters, was not detectable in the selected cells. In their experiments, as well as in the studies by Marchetti and his colleagues, VE-cadherin was a weaker promoter than its counterparts like Tie-1 (Marchetti, Gimond *et al.* 2002; Kim and von Recum 2010). Moreover, purification of ES cell-derived ECs based on GFP expression under the control of the VE-cadherin promoter was not possible because of the weak GFP expression and differentiation plasticity of the cells (Kim and von Recum 2010). In the present experiments, GFP expression in vascular structures was quite strong, and the EC gene profiling studies of the GFP-sorted EBs confirmed the functionality of the construct as well as the feasibility of sorting. However, maintenance of GFP-positive cells in culture after sorting procedures for an extended period of time was not possible due to their weakness and fragility after protracted FACS procedures.

4.3.3 Selection of endothelial cells based on CD31 expression

In the next step, magnetic bead-sorting based on CD31 expression was applied for the purification of ECs in order to overcome the difficulties in getting sufficient numbers of viable cells after the harmful dissociation procedures and protracted course of FACS. This approach also made it possible to identify cells double-positive for GFP and CD31, thus facilitating the characterization of ES cell-derived ECs based on more than 1 marker (Kim and von Recum 2010). Further, early and late markers of EC development in the CD31-sorted populations derived from could be analyzed EBs in time course studies. Hence, an optimal time point for the selection of functional ECs could be dissected. A multitude of data have challenged the concept of hemangioblast as a common precursor for both endothelial and hematopoietic cells by showing the hemogenic endothelium giving rise to hematopoietic cells (Choi, Kennedy *et al.* 1998; Chung, Zhang *et al.* 2002; Huber, Kouskoff *et al.* 2004; Vogeli, Jin *et al.* 2006). In view of that, a significant proportion of hematopoietic cells are developed from an

endothelial origin (Goldie, Lucitti *et al.* 2008; Zovein, Hofmann *et al.* 2008; Boisset, van Cappellen *et al.* 2010; Zape and Zovein 2011). Therefore, labeling and purification of ES cell-derived ECs based on dissimilar markers may additionally bring more insight into the identification and later application of functional cells (Hirschi 2012).

CD31 is a 130-kDa glycoprotein with six extracellular immunoglobulin-like domains, a transmembrane domain and a cytoplasmic tail, which is highly expressed on ECs, and at lower levels on blood cells such as platelets (Newman 1994). Some of the functions of endothelial CD31 include maintenance of EC monolayer integrity, cellular signaling, mechanoception, and leukocyte transmigration (Muller, Ratti *et al.* 1989; Newman and Newman 2003). CD31 has been used for the derivation of human ES cell- and iPSC-derived endothelial lineages (Levenberg, Golub *et al.* 2002; Glaser, Gower *et al.* 2011; Li, Hu *et al.* 2011). The non-endothelial CD31-positive population in human bone marrow has also been reported to contribute to vasculogenesis/angiogenesis (Kim, Cho *et al.* 2010). Unlike human system, which shows peak expression of CD31 in EBs days 13-15, the first expression of CD31 in mouse EBs occurs on day 4, after a transient expression of some isoforms in ES cells (Li, Wang *et al.* 2005) and the following down-regulation in early EBs, possibly related to splicing of different CD31 isoforms (Li, Wang *et al.* 2005; Mariappan, Winkler *et al.* 2009). In mice, this occurs in a successive manner after the expression of Flk-1, followed by Tie-2, Tie-1 and VE-cadherin both *in vitro* and *in vivo* (Dumont, Fong *et al.* 1995; Vittet, Prandini *et al.* 1996).

In the present experiments, CD31 expression peaked on day 6, while a population co-expressing GFP was characterized 2 days later. This was comparable to previous developmental studies, including the investigations performed with a Tie-1 promoter driving GFP in murine ES cells (Vittet, Prandini *et al.* 1996; Marchetti, Gimond *et al.* 2002).

4.4 Specific gene profiling of the sorted populations

Development of ECs based on the isolation of CD31-positive cells in day 8 mouse EBs has been shown in a study previously (Mariappan, Winkler *et al.* 2009). However, characterization of the early and late markers during the course of EC differentiation in the CD31-sorted populations and on the basis of that, the best time point for the

isolation and culture of functional ES cell-derived ECs has not been investigated in detail before. Accordingly, several markers of different vessel phenotypes, such as EphB4, Prox1, and homeobox genes such as Sox17 and Sox18, cell surface receptors such as Flk-1, Tie-1, and Tie-2, transcription factors involved in vasculogenesis such as Tal1, c-kit, and Id1, hematopoietic markers such as CD34 and CD45, and VE-cadherin as one of the most specific adhesive molecules were analyzed. The gene expression analyses demonstrated a pronounced surge in the expression of EC surface markers and transcription factors involved in vascular development between differentiation days 2 and 3 in the isolated CD31-positive population, yielding a mature cell population at the later differentiation days 6 and 8. This was characterized by a high expression of endothelial-specific markers, and a decreased expression of markers of the undifferentiated stage and early differentiation. Furthermore, at these later developmental stages, the expression of surface markers of ECs and transcription factors of vascular development in the CD31-positive *vs.* CD31-negative population was significantly higher, indicating the efficiency of CD31-based selection in driving functional ECs. Moreover, the results of gene expression analyses comparing CD31-positive versus GFP-positive populations suggested the superiority of the CD31-based purification.

Accordingly, differentiation days 6 and 8 were chosen for culture of CD31-sorted EBs, whereby the sorted cells particularly developed typical EC cobblestone morphology at day 6 and eventually formed tube-like structures. The pattern of expression of some of the investigated EC-specific genes in the sorted populations and their possible roles in vascular development are discussed in the following section.

4.4.1 Early markers of vascular development

Flk-1 as well as Tie and Eph family belong to receptor tyrosine kinases and have a critical role in vascular development (Adams and Alitalo 2007). Flk-1 is a cell surface receptor, mediating the responses by VEGF. Flk-1-positive cells have been shown to give rise to hemangioblasts with the potential to differentiate into endothelial, hematopoietic, and smooth muscle cells from murine ES cells (Nishikawa, Nishikawa *et al.* 1998; Adams and Alitalo 2007; Blancas, Lauer *et al.* 2008). Tie-2 is a surface molecule mediating a vascular protective role through both angiostasis and angiogenesis depending on the spatial localization and interactions with its ligand (Fukuhara, Sako *et*

al. 2008; Heinke, Patterson *et al.* 2012). When murine ES cells are put into differentiation, a robust expression of Flk-1 and Tie-2 starts around day 3 (Vittet, Prandini *et al.* 1996). According to the results of this study, Flk-1 and Tie-2 showed a remarkable surge on day 3 in CD31-positive cells, with a steadily high expression level during the later differentiation days. In human, on the contrary, Flk-1 and Tie-2, as well as the hematopoietic marker CD34 are highly expressed in undifferentiated ES cells (Li, Wilson *et al.* 2009).

In these studies, a continuous increase in expression of Flk-1 in the CD31-positive *vs.* CD31-negative population was observed over time, suggesting its association with a more EC-specific characteristic in the later stages compared to the earlier stages, at which Flk-1 may also contribute to non-EC populations, including SMCs and hematopoietic cells. The highest fold increase in the expression of Tie-2 in the CD31-positive *vs.* CD31-negative populations was observed in the intermediate differentiation period (days 3-6), in association with the beginning of specification to ECs, along with CD31 peak expression. This association was also noted in a recent study (Gu and Shively 2011). Hematopoietic cells expressing Flk-1 or Tie-2 have the capacity of re-endothelialization, which may have impact in cellular therapy approaches (Nowak, Karrar *et al.* 2004; Sarkar, Rey *et al.* 2012).

4.4.2 Transcription factors involved in vasculogenesis and endothelial cell differentiation

Tal1(Scl) is one of the key regulators for the development of hemangioblasts and of hematopoiesis which is driven by GATA2. GATA2 as a potent factor also gives rise to Flk-1-positive mesodermal cells, as well as regulates CD31 expression. A Population of double Flk-1-/ Tal1-positive cells as blast colony-forming units leads to the induction of EC-specific genes and generation of ECs (Gering, Rodaway *et al.* 1998; Chung, Zhang *et al.* 2002; Dooley, Davidson *et al.* 2005; Lugus, Chung *et al.* 2007).

Despite the known role of Tal1 in hemangioblast formation, its role has been considered to be dispensable for vasculogenesis. It has been shown that ECs or hematopoietic cells can still be characterized after Tal1 ablation or knockout in the face of vascular or hematopoietic defects, indicating possible compensatory pathways for vascular differentiation and/or hematopoiesis (De Val 2011). In my study, Tal1 expression was markedly higher in the CD31-positive *vs.* CD31-negative population

from day 3 EB formation, with the biggest difference, as well as the highest Tal1 expression in the CD31-positive population alone, at day 4. This was concomitant with a peak expression of Id1. Id1 is a transcription factor from the loop-helix-loop family with a major role in maintaining ES cell self-renewal (Romero-Lanman, Pavlovic *et al.* 2012) as well as EPC proliferation upon VEGF stimulation (Ciarrocchi, Jankovic *et al.* 2007; Wang, Yu *et al.* 2010; Li, Wang *et al.* 2012). In the human system, Id1 is essential for the TGF-β inhibition-mediated growth and maintenance of ES cell-derived ECs (James, Nam *et al.* 2010). However, there are no studies on the possible role of Id1 in murine ES cell-derived ECs. Along with the specification of CD31 to a more endothelial-specific identity on day 6, Id1 also showed a higher expression in the CD31-positive *vs.* CD31-negative population, suggesting a possible role in the induction of ES cell-derived ECs in the mouse system, which requires further investigation.

According to previous studies, a population of c-kit-positive mesodermal cells gives rise to ECs, vascular SMCs and cardiac progenitors with a high proliferation and expansion capacity (Wu, Fujiwara *et al.* 2006; Tallini, Greene *et al.* 2009). Furthermore, c-kit has been shown to be essential for the maintenance of hematopoiesis in bone marrow (Kimura, Ding *et al.* 2011). The c-kit-positive population isolated from amniotic fluid has been demonstrated to produce some phenotypes of ECs (Benavides, Petsche *et al.* 2012). According to the present results, the expression of c-kit was higher in the CD31-positive *vs.* CD31-negative population from day 3 on, with its peak level of expression in the CD31-positive population on day 3, which was 1 day earlier than the peak expression of Id1 and Tal1. A second increase in expression occurred on day 8 in a manner comparable to Id1. This may indicate a role for c-kit in the induction of EC development both at early and later stages of differentiation.

4.4.3 Specific markers of different vessel phenotypes

The venous and arterial vascular systems demonstrate distinct anatomical, functional, as well as molecular differences (Aranguren, Luttun *et al.* 2007; Yamashita 2007). The venous vascular system, which might be the default EC differentiation target (Red-Horse, Ueno *et al.* 2010), is characterized by EphB4 and COUP-TFII expression. Arterial differentiation, on the other hand, is characterized by EfnB2, delta-like (Dll)-4, Notch1 and 4, Jagged-1, and connexin-40 expression. Prox1 is considered as the most specific lymphatic endothelial marker (Wang, Chen *et al.* 1998; Swift and Weinstein

2009; Salvucci and Tosato 2012). In addition to the known role of ephrin family ligands and receptors in venous and arterial specification, their function has been shown to be also particularly important for vascular development and angiogenesis (Swift and Weinstein 2009; Salvucci and Tosato 2012).

In the present study, some of the above-mentioned factors were assessed in populations of ECs isolated based on CD31 expression on different days of differentiation of EBs. EphB4 showed a statistically significant increase in expression on differentiation days 3 to 8 in comparison to day 2. The expression pattern of EphB2 was also analyzed, which showed a higher expression only in the earlier isolated CD31-positive population (not shown). This gene as another member of the ephrin receptor family has been implicated in vascular SMC proliferation (Woods, Blystone *et al.* 2002), and may regulate EC branching and chemotaxis in orchestration with EphB4 (Salvucci, de la Luz Sierra *et al.* 2006).

Nos3 is a mediator of commitment of EPCs to ECs, and is up-regulated during ES cell-derived EC development (Rossig, Urbich *et al.* 2005). Furthermore, down-regulation of Nos3 may lead to dilated and aberrant vasculature in CD31- knockout mice (Dimaio, Wang *et al.* 2008). The present experiments revealed a 604-fold increase in the expression of Nos3 in CD31-positive *vs.* CD31-negative populations on day 8. This may underscore the efficiency of CD31-sorting and subsequent development of functional vessel structures in later days of EB differentiation.

Prox1 is a master regulator of lymphatic EC specification and maintenance (Wigle, Harvey *et al.* 2002; Johnson, Dillard *et al.* 2008). The transcription factors Sox18 and Coup-TFII are critical for the induction of Prox1 expression in lymphatic EPCs located within the embryonic veins (Francois, Caprini *et al.* 2008). In the present experiments, the expression of Prox1 in the CD31-positive population was down-regulated from day 3, with a slight up-regulation on days 6 and 8. Sox18 had a significantly higher expression in the CD31-positive population vs. CD31-negative population from day 3, especially at later differentiation days. Sox17 together with Sox18 has been shown to have a role in arteriovenous specification. Morphants have been reported to have vasculature defects with aberrances in the arterial EC-related gene patterns, as well as defects in lymphatic vasculature (Cermenati, Moleri *et al.* 2008; Francois, Caprini *et al.* 2008; Pendeville, Winandy *et al.* 2008; Francois, Short *et al.* 2012). The modifying effect by Sox*7* and Sox17 in lymphangiogenesis in Sox18-deficient mice has been

demonstrated in previous studies (Hosking, Francois *et al.* 2009). In the present thesis, similar to Sox18, the expression of Sox17 was higher in CD31-positive cells *vs.* CD31-negative population. However, its expression level in the CD31-positive population alone decreased significantly from day 3. The exact interacting mechanisms of these transcription factors in orchestration with other genes in the induction of arterial *vs.* venous *vs.* lymphatic differentiation during the course of EC development is a complex network, which requires specific detailed investigations in future studies. This may have an impact in cellular therapy approaches for the derivation of a special phenotype of vessels based on the underlying pathological vascular lesion.

4.4.4 Late markers of endothelial cell differentiation

Tie-1 and VE-cadherin are 2 examples of EC markers, which are expressed later during the course of differentiation of ES cells to ECs. Tie-1 is expressed in day 5 EBs following Flk-1 and CD31 (Vittet, Prandini *et al.* 1996). The results of this study showed a higher overall expression of Tie-1 in CD31-positive *vs.* CD31-negative populations, with the highest expression in the CD31-positive cells alone on day 8. Tie-1, which is activated by hypoxia or VEGF treatment, has been shown to be involved in angiogenesis and neovascularization (McCarthy, Crowther *et al.* 1998; Seegar, Eller *et al.* 2010). Therefore, it is not expressed in mature vessels, where no neovascularization takes place. This is in obvious contrast to Tie-2, which is expressed ubiquitously in all ECs and is important for proliferation and maintenance of ECs in all stages (Dumont, Gradwohl *et al.* 1994; Seegar, Eller *et al.* 2010). The peak expression of Tie-1 in CD31-positive cells at day 8 may implicate the high angiogenic activity at this point. In line with these considerations, cell culture also confirmed the angiogenic sprouting in day 8 CD31-positive cells cultured on matrigel, while an earlier time point resulted in a more proliferative state of ECs.

VE-cadherin as another late marker of ECs and the major endothelial specific cell adhesion molecule (Lampugnani and Dejana 1997) has several structural, as well as functional role involved in angiogenesis (Bäumer, Keller *et al.* 2006; Dejana, Orsenigo *et al.* 2008; Vestweber 2008). In a previous study, gene analysis of the VE-cadherin-positive *vs.* -negative cells had shown an increased expression of EC markers in the VE-cadherin-positive cells in day 6.5 EBs. VE-cadherin expression at an earlier stage was associated with hematopoietic markers (Nikolova-Krstevski, Bhasin *et al.* 2008). In my

study, VE-cadherin showed a statistically significant steady increase in expression during the course of differentiation in the CD31-positive population. At day 8, there was a 560-fold increase in VE-cadherin expression in CD31-positive *vs.* CD31-negative populations. The high level of association of VE-cadherin and CD31 may indicate the robustness of taking advantage of the VE-cadherin promoter and CD31 for labeling and selection purposes, respectively. In my view, EC selection based on CD31 was advantageous to sorting based on GFP, which rendered the cells fragile following the detrimental, lengthy FACS sorting procedures.

4.5 Culture of sorted cells

It was possible to obtain a significant number of CD31-positive/GFP-positive cells representing ECs with a high purity level by treating the cells with 20 ng/mL VEGF prior to and after isolation, and during culture. Isolation of the cells on day 6 and further culture with VEGF for two days led to a robust endothelial phenotype in culture, which is in agreement with the gene expression analyses showing the most pronounced expression of EC-specific genes in CD31 sorted cells on day 8 of differentiation.

Treatment of isolated cells with growth factor cocktails in serum-dependent conditions may enhance both immature vascular structures containing SMCs and hematopoietic cells (Boyd, Dhara *et al.* 2007; Pearson, Sroczynska *et al.* 2008; Sun, Cheng *et al.* 2009; Irion, Clarke *et al.* 2010). A growth factor cocktail of VEGF, FGF-2, IL-6, and erythropoietin, as well as VEGF alone, have been shown to increase the percentage of CD31-positive cells within the EBs, as well as the cellularity and number of EBs (Vittet, Prandini *et al.* 1996; Marchetti, Gimond *et al.* 2002; Sun, Cheng *et al.* 2009). A combination of other growth factors such as BMP-4 and Activin A has also demonstrated to increase the primitive vasculogenesis outgrowths (Boyd, Dhara *et al.* 2007; Pearson, Sroczynska *et al.* 2008), along with hematopoietic development (Irion, Clarke *et al.* 2010). As the development of hematopoietic cells was not desirable, EBs were treated with VEGF alone, which led to a significant level of CD31/GFP-double positive populations. Recently, Chiang and his colleagues defined a new system for the efficient development of murine ES cells to hemangioblast and angioblast fates and an efficient production of ECs through successive treatment of the cells to accurately-scaled doses of growth factors (Chiang and Wong 2011). Though very promising, these

systems require further optimization for the high-throughput production of ECs with applications in cellular therapy.

While the effect of different growth factors on the formation of vascular networks has gained widespread interest, less is known about the mechanical forces of the environment on the growth of ECs and development of vascular networks. The traction forces exerted by some substrates like fibronectin may lead to an enhanced endothelial-endothelial connectivity (Califano and Reinhart-King 2009). These compliant substrates mimic the normal healthy conditions, while in pathological conditions the substrate becomes stiffer, leading to gaps between the cells (Krishnan, Klumpers *et al.* 2011). Matrigel is derived from Engelbreth-Holm-Swarm mouse sarcoma cells (Kleinman and Martin 2005). The main components of matrigel are laminin and collagen, as structural proteins. However, matrigel is also enriched with growth factors that promote the formation of angiogenic tubules. In line with these findings, the results of this project also demonstrated the development of variant EC phenotypes upon culture of the sorted cells on different substrates. The results of these experiments showed that the culture of ES cell-derived ECs on matrigel leads to the formation of angiogenic sprouts, while culture on gelatin-coated plates gave rise to EC cobblestone morphology. These differences may have an impact in tissue engineering in providing the proper milieu for therapeutic vasculogenesis.

5 Conclusions and future prospects

In this study, an ES cell clone was generated carrying an integrated VE-cadherin transgene expressing GFP upon differentiation to ECs using a lentiviral-based construct. This model can be applied to trace the activity of VE-cadherin promoter in single cells within embryoid bodies. Moreover, the VE-cadherin/GFP-positive clones may constitute a proper model for observing the effects of several pro- or anti-angiogenic factors. This may aid to understand the molecular mechanisms regulating VE-cadherin promoter activity under physiological and pathological condition. Additionally, the introduction of an antibiotic resistance gene under the control of the VE-cadherin promoter for the selection of ECs may be used as model to generate other constructs with various antibiotic resistance genes and potentially more efficient cell selection. In these studies, the robustness of EC selection based on the expression of CD31, as another EC-specific marker could be demonstrated. Based on the analysis of some established and candidate genes involved in the early and later stages of EC differentiation, as well as pattern of growth in cell culture, a specific time point for efficient, high purity selection of GFP-labeled ECs from differentiating ES cells using CD31-based cell sorting could be determined.

These studies may serve as a fundament for future investigations on the detailed mechanisms of ES cell development and differentiation into ECs, as well as a model for drug discovery and prospective cellular therapy approaches in various diseases associated with vascular damage. The GFP-labeling may allow us to track and study the cells as they migrate, proliferate, or differentiate at sites of vascular injury. The sorted cells at different developmental stages may be applied to coat the inner layer of many devices in models of neovascularizarion, as well as of the artificial devices such as extracorporeal membrane oxygenation (ECMO) machines to provide a physiological environment for the circulation of oxygen and nutrients. Tissue engineering may aid to construct a proper milieu for further therapeutic vasculogenesis.

Despite the huge benefits about stem cell research, there are limitations about their application. For instance, the issue of immunogenicity in stem cell studies must be taken into account in all therapeutic models with embryonic stem cells. Moreover, the results of successful treatment of various diseases using embryonic stem cells in animal models may not be extrapolated to human studies due to ethical issues. For this reason, the

reprogramming of autologous human somatic cells to produce induced pluripotent stem cells may offer a cell resource for the differentiation of desired target cells, thus alleviating the ethical as well as immunogenicity issues. The methods and results of this project may be used as a model for applicative studies on the derivation of pure ECs and functional vasculature from induced pluripotent stem cells in human.

6 Summary

Embryonic stem (ES) cell-derived endothelial cells (EC)s may be used as a therapeutic option in experimental models of diseases originating from vascular lesions. Moreover, studies on these cells may provide insight into EC development and differentiation in the human body. In this regard, it is fundamentally required to label, track, and finally isolate a pure population of ES-cell-derived ECs. In this study, a murine ES cell line was established, which expressed green fluorescent protein (GFP) as well as a zeocin resistance gene under the control of the murine Vascular Endothelial (VE)-cadherin promoter after lentiviral transduction of single ES cells. 192 ES colonies derived from single transduced ES cells were picked randomly and directed to differentiation. From day 6 of differentiation, 12.5% of the clones showed GFP-positive vessel-like structures. Immunofluorescence microscopy demonstrated the co-expression of various EC markers (VE-cadherin, CD31) on ES cell-derived vascular structures. Based on flow cytometry, the highest GFP expression level could be observed in embryoid bodies at differentiation day 8. Flow-cytometric cell sorting at this time point revealed a significantly higher level of expression of the majority of investigated EC markers in the GFP-positive compared to the GFP-negative population. In addition, magnetic beads were used for the isolation of ECs based on CD31 expression. The sorted cells were subsequently subjected to gene profiling, in order to determine the optimal time point for the isolation and subsequent culture of ECs. In the sorted cells on days 6 and 8 of differentiation, all investigated markers of EC differentiation and transcription factors of vasculogenesis demonstrated a markedly higher expression in the CD31-positive versus CD31-negative population. Cultured CD31-positive cells at differentiation day 6 developed a characteristic EC cobblestone morphology, co-expressed GFP and different endothelial markers, and eventually formed tube-like structures. In conclusion, generation of ES cell clones expressing GFP upon differentiation to ECs, and their sorting based on CD31, provides a feasible method for the production of pure labeled ECs. This system may serve as a powerful tool for studies on the differentiation of ECs from ES cells and induced pluripotent stem cells, as well as prospective cellular therapeutic approaches in various diseases associated with vascular damage.

7 Zusammenfassung

Aus embryonalen Stammzellen (ES-Zellen) abgeleitete Endothelzellen können in Modellen zur Therapie von Erkrankungen, die mit vaskulären Läsionen assoziiert sind, eingesetzt werden. Außerdem geben ES Zellstudien wichtige Einblicke in die Entwicklung und Differenzierung von Endothelzellen im menschlichen Organismus. Für diese Zwecke ist es essentiell, die aus ES-Zellen abgeleiteten Endothelzellen zu markieren und nachzuverfolgen und die differenzierten Zellen schließlich in eine reine Zellpopulation zu überführen. In der vorliegenden Arbeit wurde eine murine ES-Zell-Linie generiert, welche nach lentivirusbasierter Transduktion von ES Zellen das grün fluoreszierende Protein (GFP) sowie ein Zeocin-Resistenzgen unter Kontrolle des murinen vaskulären endothelialen (VE)-Cadherin-Promotors exprimiert. 192 aus einzel-transduzierten ES-Zellen abstammende ES-Zellkolonien wurden zufällig selektiert. Ab Tag 6 der Differenzierung zeigten 12,5% der Klone GFP-positive vaskuläre Strukturen mit Co-Expression verschiedener vaskulärer Marker (VE-Cadherin, CD31). Die durch Durchflusszytometrie am Tag 8 der Differenzierung selektierten GFP-positiven Zellen zeigten eine signifikant höhere Genexpression verschiedener Endothelzellmarker als die GFP-negative Zellpopulation. Darüber hinaus wurde ein weiteres, auf der Expression von CD31 basierendes Verfahren zur Selektion der aus ES Zellen abgeleiteten Endothelzellen angewendet und das Genprofil der selektierten Endothelzellen zu verschiedenen Zeitpunkten der Differenzierung analysiert. Auf diese Weise wurde der optimale Zeitpunkt für die Isolierung und anschließende Kultivierung und Charakterisierung dieser Zellen festgelegt. Alle untersuchten Marker sowie Transkriptionsfaktoren der Endotheldifferenzierung zeigten eine deutlich höhere Expression in der CD31-positiven gegenüber der -negativen Zellpopulation am Tag 6 und 8 der Differenzierung. Die Kultivierung der CD31- positiven Zellen am Tag 6 der Differenzierung führte zu einer für Endothelzellen charakteristischen Kopfsteinpflastermorphologie mit Co-Expression des GFPs mit verschiedenen endothelialen Markern. Schließlich bildeten die kultivierte Zellen tubuläre Strukturen aus. Zusammenfassend stellt die Generierung embryonaler Stammzellklone, welche während der Differenzierung zu Endothelzellen GFP exprimieren, und die Selektion dieser Zellen auf Basis der Expression von CD31, eine effiziente Methode für die Entwicklung reiner Endothelzellen dar. Diese Studien repräsentieren darüber hinaus die Voraussetzung für zukünftige detaillierte Studien zur Differenzierung von

Endothelzellen aus ES Zellen und induzierten pluripotenten Stammzellen, und für die Anwendung Zell-basierter experimenteller therapeutischer Strategien bei verschiedenen vaskulären Erkrankungen.

Abbreviations

BL-CFC	Blast colony forming cells
BMP	Bone morphogenic protein
bp	base pairs
CD	cluster of differentiation
CMV	Cytomegalovirus
COUP-TF-II	Chicken ovalbumin upstream promoter transcription factor IIS
cPPT	Central polypurine tract
CVDs	Cardiovascular diseases
DMEM	Dulbecco's modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAse I	deoxyribonuclease I
dsDNA	double stranded DNA
EBs	embryoid bodies
ES	embryonic stem
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EfnB2	EphrinB2
EGCs	Embryonic germ cells
EPCs	endothelial progenitor cells
EphB4	EphrinB4 receptor
ECMO	extracorporeal membrane oxygenation
FBS	Fetal Bovine Serum
FGF	fibroblast growth factor
FACS	fluorescence activated cell sorting
Flk-1	Fetal liver kinase-1
GFP	Green fluorescent protein
Gp	Glycoprotein
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HIV	human immunodeficiency virus

ICM	inner cell mass
IMDM	Iscove's Modified Dulbecco's Medium
IL	Interleukin
iPSCs	induced pluripotent stem cells
LB.	.Luria Broth
LDL	low-density lipoprotein
LIF	Leukemia inhibitory factor
LTR	long terminal repeat
MCP-1	macrophage chemoattractant protein
MEFs	mouse embryonic fibroblasts
NEAA	Non-essential amino acids
Nm	Nanomolar
Nos3	Nitric oxide synthase 3
Oct4	Octamer-binding transcription factor
PBS	phosphate buffered saline
PECAM-1	platelet endothelial adhesion molecule-1
Pen/Strep	penicillin/Streptomycin
Prox1	Prospero homeodomain transcription factor 1
RRE	rev response element
RNA	ribonucleic acid
RSV	respiratory syncytial virus
SMA	smooth muscle actin
SMCs	smooth muscle cells
SOB	Super optimal Broth medium
Sox	Sex determining region Y-box
S1P	Sphingosine-1-phosphate
TGF	Transforming Growth Factor
Tie	Tyrosine kinase with Ig-like and EGF-like domains
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VEGF-R	vascular endothelial growth factor receptor
VSV-G	vesicular stomatitis virus-G
WPRE	woodchuck hepatitis virus post-transcription regulatory element

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Declaration of Intent

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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16. Manuscript in preparation: Raissi M, Becker S, Markart P, Martin U, Werner Seeger W, and Voswinckel R. Purification and characterization of endothelial cells derived from murine embryonic stem cells transduced with a lentiviral vector expressing GFP under the control of the VE-cadherin promoter

Poster/Oral presentations

1. Endothelial Cells developed from Embryonic Stem Cells for cell-based Therapy– Institute's Colloquium. Max Planck Institute for heart and lung research October 2012.
2. Driving murine embryonic stem cells toward endothelial phenotype for cell-based therapy. Poster presentation. UGMLC seminar on lung diseases, poster presentation Marburg, Germany, June 2012.

3. VE-Cadherin as a Specific Promoter for Genetic Labeling and Selection of Pure Embryonic Stem Cell-Derived Endothelial Cells for Therapeutic Purposes. Poster presentation. Bremen DGP Meeting. Pneumologie 2012; 66 - A703
4. VE-Cadherin as a Specific Promoter for Genetic Labeling and Selection of Pure Embryonic Stem Cell-Derived Endothelial Cells for Therapeutic Purposes. American Thoracic Society San Francisco May 2012. Poster presentation ATS journals Chapter DOI: 10.1164/ajrccm
5. Driving murine embryonic stem cells toward endothelial phenotype for cell-based therapy**.** Poster presentation. Dresden DGP Meeting Pneumologie 2011; 65 - P407.
6. Development and purification of embryonic stem cells-derived endothelial cells for therapeutic purposes. Poster presentation at the Keystone Symposia, Stem Cells, Development and Homeostasis Santa Fe, USA. February 2011.
7. Effect of body mass index in the outcomes of stenting for the treatment of coronary artery lesions, International Congress of the Iranian HeartAssociation in collaboration with the American College of Cardiology on Heart Disease (November 2008)
8. Outcomes of percutaneous coronary intervention in patients aged 65 and over *vs.* those under the age of 65, International Congress of the Iranian Heart Association in collaboration with the American College of Cardiology on Heart Disease (November 2008)
9. Comparison of sirolimus-*vs.* paclitaxel-eluting stents for the treatment of coronary artery lesions, International Congress of the Iranian Heart Association in collaboration with the French Society of Cardiology (October 2006).

Medical Translations (English-Persian)

1. Skeleton: Discover the evolution and structure of bones (2009)
2. Cecil's Infectious diseases (2007)
3. Harrison's Rheumatologic diseases (2005)
4. Cecil's Musculoskeletal and Connective tissue diseases (2004)
5. Harrison's Rheumatologic diseases (2001)
6. Adams Orthopaedics principles (2001)
7. Habif's Dermatology textbook (1996)

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