Blood Generation from Hemogenic Endothelium Proven by Continuous Single Cell Imaging

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1. Table of Content

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2. Abstract

The generation of the first blood cells in the vertebrate embryo is spatially, temporally and molecularly closely linked to vasculogenesis pointing to a close developmental relationship between endothelial and hematopoietic cells. However, despite decades of long research, it still remains disputed whether blood cells arise directly from mesoderm, from a common endothelial-hematopoietic precursor or from hemogenic endothelium.

Employing a new imaging and tracking technology we address the question of the existence of hemogenic endothelium. This novel imaging technology allows one to constantly follow the fate of all cells in a culture at the single cell level over long periods of time. To examine whether endothelium can generate blood, we used a well established mesodermal differentiation system of mouse embryonic stem cells (ESCs) in addition to purified primary cells from mouse embryos, imaged the appearance of endothelial and hematopoietic cells from their mesodermal precursors by time lapse microscopy followed by single cell tracking.

We could observe that blood cells can be generated from endothelial cells. Clonal endothelial colonies arising from single mesodermal $Flk1^{+}$-cells contain hemogenic and non-hemogenic subsets of endothelial cells. Both share the same phase contrast morphology and the capacity to take up acetylated low density lipoprotein – exclusive characteristics of endothelial cells in ESC-derived cultures at this developmental stage. In addition the endothelial nature of the blood precursors was verified by the expression on vascular endothelial (VE)-Cadherin and the formation of tight junctions that contain the endothelial-specific protein Claudin-5.

Despite their typical hematopoietic morphology endothelium-derived blood cells expressed the pan-hematopoietic markers CD45 and partly Mac1, a surface molecule being detectable on cells of the myeloid lineage. In addition, we characterized the hemogenic endothelial cells by their expression of CD41, c-kit and Tie2. Not only the expression of markers but also the characteristic sequence of cellular behaviour with highly similar kinetics was highly reproducible between ESC-derived and embryo-derived cultures suggesting that this process is precisely regulated at the cellular and molecular level.

In summary we show direct evidence that endothelial cells can act as direct precursors of hematopoietic cells. By continuously observing the whole process of endothelial to blood cell transition at the single cell level, we can rule out the longstanding doubts about the existence of hemogenic endothelial cells and provide insights into the cellular and molecular processes involved in their regulation.

3. Introduction

3.1. The hematopoietic system: overview and function

The hematopoietic system is highly regenerative. In contrast to other organs in the body like the brain or the skeleton, blood and skin can recover very fast and efficiently if larger portions are removed. Most blood cells have a short life time and are continuously lost and regenerated. Each day an adult human loses about 10^{11} blood cells that have to be replaced (Gilbert, 2003). As the hematopoietic system recovers in a highly efficient manner, hematopoietic cells can be removed from the organism without harming it. This makes blood easily accessible for research and consequently has led to the broad knowledge about the hematopoietic system and its stem cell biology which made blood one of the best understood tissues.

The hematopoietic system of vertebrates consists of more than 9 lineages which can be subdivided in myeloid and lymphoid blood cells. Myeloid cells contain erythrocytes, megakaryoyctes, monocytes and granulocytes of different subtypes whereas the B and T cells as well as dendritic and natural killer cells are lymphoid. Erythrocytes perform oxygen and carbon dioxide transport to and from peripheral tissues and organs, whereas megakaryocytes are responsible for blood clotting. Monocytes and granulocytes fulfill the innate immune response by engulfing pathogens, dead cells and debris from the body or killing pathogens by secretion of cytotoxic substances. In contrast, lymphoid cells protect the body from invaders by the adaptive immune response (Janeway et al., 1999).

In accordance with these functions the hematopoietic system is a flexible system that is in constant flux and movement. This allows fighting invaders at different peripheric or central locations within the body, removal apoptotic cells and guarantees proper gas exchange in all areas.

3.2. Adult and embryonic hematopoiesis

3.2.1. Adult hematopoiesis

According to the current model of the stem cell hierarchy, all blood cells found in the adult organism originate from hematopoietic stem cells (HSCs). Based on their ability to either self-renew leading to the production of more stem cells, or to differentiate into cells of all different blood lineages, the HSCs can give rise to a complete new hematopoietic system, e.g. if transplanted into lethally irradiated recipients. This

distinguishes the long-term repopulating HSCs (LTR-HSCs) from more committed progenitors that have lost the potential to provide multi-lineage reconstitution for the entire life (Orkin, 2000).

The continuous production of sufficient but not excessive numbers of blood cells of all lineages and the equilibrium between undifferentiated and differentiated cells has to be tightly controlled as its loss leads to the acquisition of anaemia and leukaemia (Orkin, 2000).

The control of the blood stem cell pool is mainly maintained by the bone marrow, the niche for the HSCs as well as for many progenitors and differentiated blood cells (Zhang, J. et al., 2003). Containing a variety of cell types like fibroblasts, adipocytes, endothelial cells and osteoblasts (Rattis et al., 2004; Coultas et al., 2005), as well as the extracellular matrix components (Whetton et al., 1999; Arai et al., 2004) the niche is a specialized local microenvironment. Growth factors, cytokines, survival factors and other substances are secreted by cells in the niche that altogether support and control differentiation and proliferation (Morrison et al., 2008).

3.2.2. Embryonic hematopoiesis

The hematopoietic cell pool and the stem cell hierarchy is already formed during embryogenesis and was shown to be molecularly, spatially and temporally highly conserved between fish (Amatruda et al., 1999), birds (Godin et al., 2005), amphibians (Turpen, 1998) and mammals (Baron, 2003). In all vertebrates, embryonic hematopoiesis occurs in two waves, the primitive (or ventral) and the definitive (or dorsal) wave, which distinguishes transiently present (primitive) and adult type (definitive) blood lineages. In mammals, besides the yolk sac, the aorta-gonads-mesonephros (AGM) region, the placenta and the foetal liver are involved in the establishment of the hematopoietic system before the bone marrow takes over some time before birth (Figure 1.1) (Baron, 2003; Mikkola et al., 2006).

In the following, the establishment of the mouse hematopoietic system is explained.

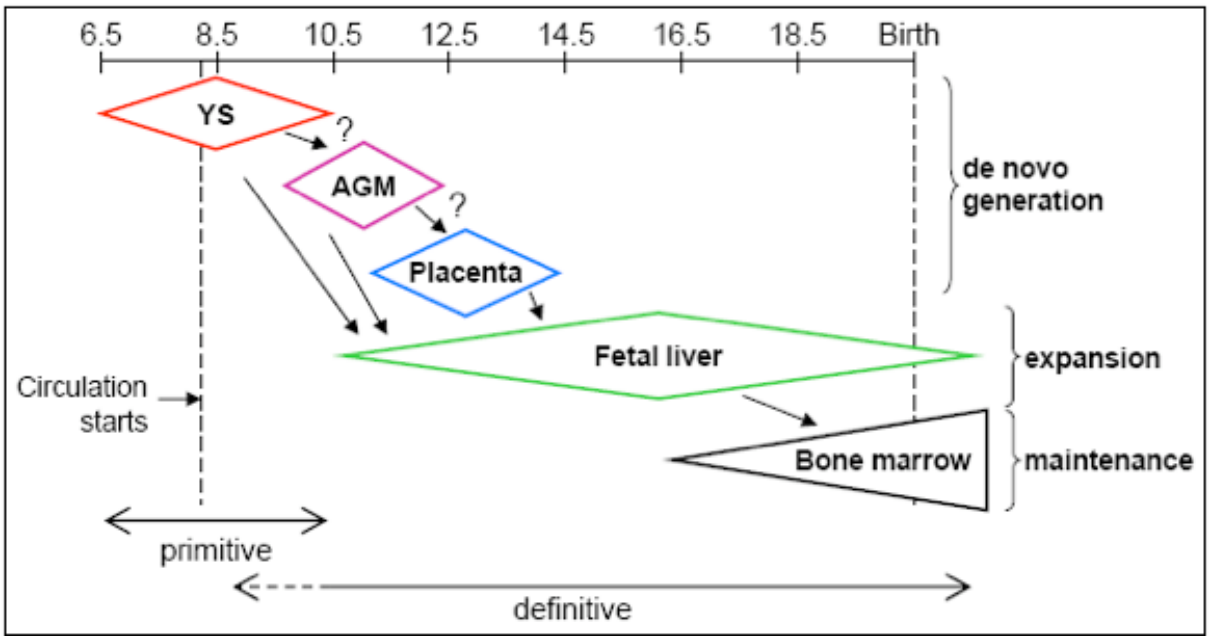

Figure 1.1: Embryonic hematopoiesis is established in many sites in the developing mouse. Hematopoiesis occurs in two waves, the primitive and the definitive. The yolk sac (YS) produces blood cells first, followed by the aorta-gonads-mesonephros region (AGM) and the placenta. The fetal liver expands the blood cell pool. Before birth, hematopoiesis shifts to the bone marrow where the blood stem cell pool is maintained. With the onset of circulation at around E8.25, blood cells start to circulate through the embryo. Figure modified from Baron, 2003 and Mikkola et al., 2006.

3.2.2.1. Primitive hematopoiesis

Primitive blood containing erythrocytes, macrophages and megakaryocytes, is short lived and only produced between embryonic day (E) 6.5 and E10.5 (Palis et al., 1999; Lichanska et al., 2000) These cells are named "primitive" as they morphologically resemble the blood cells in lower vertebrates. Primitive erythroid cells differ from their adult type counterpart by significantly larger size, an additional haemoglobin-type and by the presence of a nucleus. They can also enucleate between E12.5 and E16.5 of mouse development, though, and circulate as primitive erythroblasts until 5 days after birth (Kingsley et al., 2004).

Primitive megakaryocytes and macrophages are poorly characterized. Primitive macrophages share several characteristics with the adult macrophages as e.g. the expression of typical surface markers and the ability for phagocytosis. In contrast to definitive macrophages, they bypass the monocyte-stage, do not produce peroxidase and lysozyme enzymes. Macrophages in the embryo contribute to tissue remodelling by their capacity to engulf dead cells and debris (Lichanska et al., 2000; Shepard et al., 2000). Similarly, primitive megakaryocytes resemble their adult type counterparts

although they show reduced polyploidy. Their protein expression and their ability to produce platelets are comparable to adult megakaryocytes (Tober et al., 2007).

All primitive hematopoietic cells originate exclusively in the yolk sac (YS) (Palis et al., 1999; Lux et al., 2008). In contrast to the adult hematopoietic system, primitive blood cells are not thought to arise from a HSC but rather to differentiate directly from mesodermal precursors. These originate from the primitive streak, which forms with the onset of gastrulation at E6.5 at the region that will ultimately form the posterior portion of the embryo (Lawson et al., 1991; Huber et al., 2004; Gadue et al., 2005). With ongoing development, the primitive streak extends towards the distal end of the embryo and gives rise to the extraembryonic mesoderm that will differentiate into allantois, amnion, blood, endothelium and vascular smooth muscle of the YS (Kinder et al., 1999; Tam et al., 2004).

Primitive blood cells accumulate in blood islands which are structures embedded between visceral endoderm and mesothelium and consist of hematopoietic cells surrounded by endothelial cells. The latter fuse with ongoing development to form the first vascular plexus. Microscopic analysis suggested that blood islands form by E7.5 (Palis et al., 1995), but primitive erythroblast activity is detectable from E6.5 on (Palis et al., 1999). Consistent with the recent finding that blood islands are of polyclonal origin, this indicates that primitive blood cells arise before the development of blood islands which form later from accumulating angioblasts and primitive erythroblasts (Ferkowicz et al., 2005; Ueno et al., 2006).

3.2.2.2. Definitive hematopoesis

During definitive hematopoiesis, blood cells are generated that are morphologically, functionally and molecularly identical to hematopoietic cells produced in postnatal life. They can be found in the embryo as soon as day E9.0 and initially consist of myeloid cells before lymphoid cells are produced from day E10.5 on (Palis et al., 1999; Douagi et al., 2002; Yokota et al., 2006). Many anatomical sites participate in the establishment of definitive hematopoiesis: the yolk sac (YS), the aorta-gonads-mesonephros (AGM) region, the placenta and the foetal liver. However, as soon as the onset of circulation has started between E8.25 and E8.5, the question of where hematopoietic cells arise or migrate to is difficult to answer (Downs et al., 1998). In the line with this, the origin of the LTR-HSCs is still strongly debated (Dzierzak et al., 2008).

3.2.2.2.1. The yolk sac produces adult type blood cells

Apart from primitive blood, the YS can give rise to definitive type blood. Consisting of undifferentiated precursors and differentiated myeloid cells, definitive type blood is generated in the YS from day E7.0 on (Palis et al., 1999; Lux et al., 2008). However, whether these cells are produced from HSCs or whether they have the capacity to circumvent the stem cell hierarchy and directly differentiate from a mesodermal progenitor cell is unclear at the moment as controversial results exist about the potential of YS cells to generate HSCs. Initially, fusing a chicken YS to a quail embryonic body before the establishment of the circulatory system provided evidence that HSCs contributing to adult hematopoiesis are exclusively produced by the embryo proper. With the onset of circulation, chicken YS-derived cells share a common blood system with cells derived from the quail embryo. As identified by the distinct chromatin distribution in quail and chicken cells, the origin of adult type HSCs was found exclusively in the quail embryo whereas the YS did not contribute to the adult stem cell pool (Dieterlen-Lievre, 1975; Beaupain et al., 1979).

Opposing evidence was shown by Yoder and co-workers who transplanted murine E9.0 and E10.0 YS cells into livers of newborn recipients. Transplanted cells contributed to the lymphoid and the myeloid system in adult primary and secondary recipients which confirmed the presence of adult LTR-HSCs in the YS (Yoder et al., 1997a; Yoder et al., 1997b). However, the primary transplantation only succeeded when cells were injected into neonatal, but not adult mice, suggesting that the HSCs have not acquired the full potential for adult engraftment yet. Furthermore, as the circulatory system is established prior to the detection of HSCs, emigration of intraembryonically produced HSCs cannot be excluded and several independent groups obtained opposing results to Yoder's studies. As lymphoid cells are believed to arise from HSCs, the detection of lymphoid cells was taken as indicator for the presence of HSCs (Nishikawa et al., 2000). *In vitro* cultured YS tissue isolated before the onset of the heartbeat was shown not to give rise to B-lymphoid cells (Nishikawa et al., 1998b). Similarly, Yokota and co-workers used the lymphoid-specific protein RAG1 as a specific read out and were unable to detect $RAG1^+$ cells in YS of E8.0 embryos (Yokota et al., 2006). Likewise, transplantation of *in vitro* cultured E8.0 extra-embryonic YS cells into mice resulted in short-term repopulation ability limited to myeloid but not lymphoid potential pointing to the absence of multi-potent HSCs in the E8.0 YS (Cumano et al., 1996; Cumano et al., 2001). In addition, embryos lacking a functional vascular system confirmed the emergence of definitive type

erythro-myeloid but not lymphoid precursors in the YS: vascular endothelial (VE-) Cadherin-deficient embryos showing impaired endothelial adherence junctions are unable to establish a functional vascular system connecting the embryo proper to the YS. YS cells isolated from these mice at the age of E9.5 and E10.5 can produce definitive type multi-potent hematopoietic cells of the myeloid lineage but are devoid of lymphoid potential l(Rampon et al., 2003). Whereas these studies show the intrinsic capacity of the YS to give rise to adult type myeloid cells, they indicate that adult type multi-potent HSCs with lymphoid potential are not produced in the YS.

In contrast to these findings, recent results showed that YS-derived definitive type blood cells can contribute to the adult HSC pool. Lineage tracing of YS-derived blood cells labelled by a genetic marker at E7.5 allowed the identification of YS-derived cells in the adult bone marrow thereby verifying that cells emerging in the YS do participate in the adult hematopoietic system (Samokhvalov et al., 2007).

Taken together, this indicates that the mouse YS does have the intrinsic capacity to produce adult type hematopoietic cells of the myeloid and erythroid lineage but was not observed to have lymphoid potential. In addition, the YS probably also produces adult HSCs.

The generation of both primitive and definitive blood cells in the YS suggested that they directly relate to one another. Although both arise from $Flk1^+$ cells (Kennedy et al., 1997), a direct relation between both populations is unlikely. Lineage tracing experiments in Xenopus have shown that primitive and definitive blood cells already originate from different blastomeres in the blastocyst (Ciau-Uitz et al., 2000). However, while this proves that primitive and definitive lineages are not directly related in amphibians, it is still outstanding whether this is also the case in mammals.

3.2.2.2.2. The aorta-gonads-mesonephros (AGM) region produces hematopoietic stem cells (HSCs) and progenitors

In addition to the YS, blood cells were found to be produced from the para-aortic splanchnopleural (P-Sp) / aorta-gonads-mesonephros (AGM) region that exists from day E8.0 of mouse development. The splanchnopleural mesoderm initially differentiates into the P-Sp that gives rise to the aorta, the gonads and the mesonephros by E10.0 (Cumano et al., 1996; Medvinsky, A. et al., 1996). Multi-lineage progenitors were identified in the P-Sp between E7.5 and E8.0 which – in contrast to YS cells of mice with

the same age – even could produce cells of the lymphoid lineage (Cumano et al., 1996). Furthermore, explant cultures of AGM regions could show that adult type HSCs with the capacity to reconstitute adult lethally or sub-lethally irradiated mice not only reside in this area between E8.0 and E11.0, but are also generated there intrinsically, independently of YS or foetal liver tissues (Medvinsky, A. L. et al., 1993; Medvinsky, A. et al., 1996; Cumano et al., 2001). Whereas the urogenital ridges and the mesonephros are only weakly hemogenic, the aorta has high hematopoietic activity. Notably, histological analysis could identify hematopoietic cell clusters in the lumen of the aorta sticking to the ventral vessel wall (de Bruijn et al., 2000). Recent studies gained more knowledge about this specific localization and showed that, in contrast to the dorsal wall, only the ventral portion of the dorsal aorta could initiate the production and support the expansion of hematopoietic progenitors and stem cells at E10.5 and E11.5 (Taoudi et al., 2007).

However, the cellular origin of the hematopoietic clusters is unclear. Interestingly, hematopoietic activity of the AGM region was found beginning E8.5 - remarkably prior to the appearance of intraaortic hematopoietic clusters arising at E10.5 (Medvinsky, A. et al., 1996; Godin et al., 2005). Subaortic localized blood cells were identified in addition to the intraaortic hematopoietic clusters initially in birds, later also in mice (Jaffredo et al., 2000). Bertrand et al. found a continuous distribution of cells expressing hematopoietic markers between the sub-aortic mesentery and the aorta in mice which points to hematopoietic cells migrating between the aorta and the subaortic patches (Bertrand et al., 2005). In birds, these paraaortic foci were shown to originate from the intraaortic hematopoietic clusters (Jaffredo et al., 1998). This suggests that in birds, blood or hemogenic cells from the aortic compartment ingress into the underlying tissue. As hemogenic activity can be detected prior to the intraaortic clusters in mice, a different mechanism has been postulated in that hematopoietic cells or their precursors emerge in the subaortic patches and migrate towards the aorta to pass its wall and enter its lumen (Godin et al., 2005). The fact that blood cells in the clusters consist of Sca1-negative or -positive and CD41-negative and -positive cells, two markers eventually expressed on progenitor blood cells, may point to distinct origins of hematopoietic cells (de Bruijn et al., 2002; Bertrand et al., 2005). Thus, blood cells arising in the P-Sp/AGM region may have multiple cellular and spatiotemporal origins.

3.2.2.2.3. The placenta generates hematopoietic progenitors

The comparison of HSC numbers in the P-Sp/AGM region and in the foetal liver provided first evidence for an additional hematopoietic organ to produce HSCs (Kumaravelu et al., 2002). The placenta was first considered as a hematopoietic and possibly hemogenic tissue by Alvarez-Silva and co-workers who found multi-potent hematopoietic progenitor activity in this organ (Alvarez-Silva et al., 2003). The placenta is formed from the allantois and the chorionic plate which are of extraembryonic mesodermal origin deriving from the primitive streak and the proximal epiblast, respectively. Both fuse at E8.5 to give rise to the labyrinth of the placenta (Rossant et al., 2001).

In recent studies the placenta was identified to be an additional niche of HSCs in the developing foetus (Gekas et al., 2005; Ottersbach et al., 2005). Besides cells expressing Sca1, thus hinting for the presence of multi-potent progenitors (Ottersbach et al., 2005), transplantations of placental cell solutions into adult irradiated recipients could prove the existence of long-term repopulating HSCs in the placenta between E10.5 and E13.5 (Gekas et al., 2005; Ottersbach et al., 2005).

The placenta possesses the intrinsic capacity to produce hematopoietic cells as erythroid, myeloid as well as c-kit$^+$ and CD41$^+$ multi-lineage hematopoietic progenitors were isolated from the allantois dissociated from the embryo before the chorio-allantoic fusion had occurred and before the circulation has established (Zeigler et al., 2006; Corbel et al., 2007). The endogenous ability to produce multi-potent hematopoietic cells that can give rise to myeloid and lymphoid cells if cultured *ex vivo* was also discovered in mice lacking a circulatory system (Rhodes et al., 2008). This indicates that the placenta is indeed a major hematopoietic organ producing blood cells *de novo* although the final proof of the intraplacental emergence of LTR-HSCs is still missing.

3.2.2.2.4. Foetal liver and bone marrow are major hematopoietic organs during late embryogenesis

The foetal liver is the main hematopoietic organ in the developing conceptus, but does not produce hematopoietic cells *de novo* in contrast to YS, AGM, placenta. Thus, it is seeded by E9.5 by blood cells that have been produced elsewhere and supports the expansion of the existing blood cell pool by providing the right environment. It harbours high numbers of foetal type HSCs, undifferentiated and committed progenitors which are thought to terminally differentiate in the foetal liver (Mikkola et al., 2006). Little is known about the foetal liver microenvironment. It is probably diverse and besides supporting

rapid expansion of the foetal liver HSCs it also promotes the differentiation of more committed blood cells (Moore et al., 1997).

Beginning around E11.0 adult hematopoietic niches are colonized, first thymus and spleen and finally the bone marrow around E15.0 (Godin et al., 2005). At this time the skeletal system has fully matured and hematopoiesis shifts to the bone marrow which provides a suitable niche for adult HSCs and progenitors. In mice, some hematopoietic activity resides in the foetal liver for several days after birth before the bone marrow becomes the main site of hematopoiesis.

3.3. What is the cellular precursor of blood cells?

The YS, the P-Sp / AGM region and the placenta produce hematopoietic cells *de novo*. In addition to the hemogenic sites many intra- and extra-cellular molecules were discovered that promote hematopoiesis (Godin et al., 2005), but the precise developmental steps leading to the *de novo* generation of blood cells are still unknown. To produce hematopoietic cells or agents acting on blood cells for clinical approaches, it is highly important to understand the biology and development of blood cells. The investigation of hemogenic sites is necessary to gain knowledge about the environment determining the hematopoietic fate. It is thus of special interest to know and investigate the direct cellular precursor of blood cells that is embedded in the hemogenic organs and acquires hemogenic or hematopoietic fate in response to intrinsic and extrinsic molecules provided by neighbouring cells and the local microenvironment. To study the precursor cell would significantly contribute to a better understanding of the biology of hematopoietic cells and allow the identification of molecules that support *de novo* hematopoiesis and might be of interest for therapy.

Interestingly, the identity of the direct cellular precursor of hematopoietic cells is unknown. The past 20 years have gained a lot of knowledge about hematopoietic cell biology, marker expression and functional properties and have opened new technical possibilities. However, the question of the direct blood cell precursor is still highly controversial and strongly debated. Although all hemogenic sites are of mesodermal origin, it is questionable whether blood cells are directly generated from mesoderm.

Remarkably, in hemogenic locations, nascent blood cells were always observed close to endothelial cells: hematopoietic and endothelial cell appearance could spatially and temporally be correlated in the YS, in the AGM region and in the placenta (Figure 1.2): blood cells in the YS are surrounded by endothelium to form the blood islands (Ferkowicz

et al., 2005), in the AGM region, hematopoietic cells form clusters sticking to the endothelial cells lining the aorta lumen (Pardanaud et al., 1996; Tavian et al., 1996; de Bruijn et al., 2000; Pouget et al., 2006) and blood cells emerge in the vascular lumen of the placenta labyrinth (Ottersbach et al., 2005; Rhodes et al., 2008). In addition, molecular analysis revealed that both cell types share the expression of many molecules. Surface markers such as CD34, Tie2, Flk1, Sca1, PECAM-1, c-kit, □4-integrin and Endomucin were found on embryonic hematopoietic and endothelial cells (Dumont et al., 1995; Sanchez et al., 1996; Takakura et al., 1998; Yoshida et al., 1998; Ogawa et al., 1999; de Bruijn et al., 2002; Park et al., 2004; Matsubara et al., 2005). The same holds true for several transcription factors like Runx1/AML1, GATA-2, SCL/tal1, Notch1 and GATA-1 (Kallianpur et al., 1994; Minegishi et al., 1999; Fujimoto et al., 2001; North et al., 2002; Kumano et al., 2003; Ling et al., 2004). This provides strong evidence that endothelial and blood cells are developmentally closely related to one another. However, the precise steps leading to divergence of these pathways are not yet fully understood.

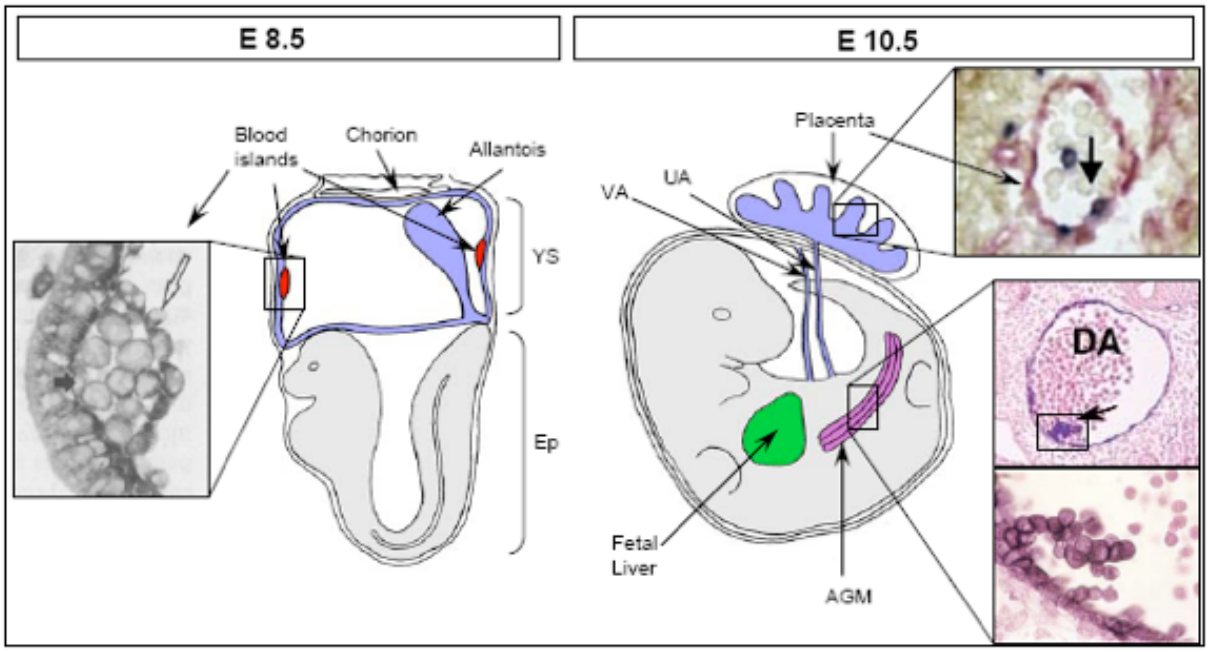

Figure 1.2: Endothelial cells localize in close proximity to blood in hemogenic sites. In the extraembryonic yolk-sac (YS) arise hematopoietic cells in the lumen of blood islands which are lined by endothelial cells at E8.5. Similarly, hematopoietic cells arise in the placenta vascular labyrinth and in the aorta-gonads-mesonephros (AGM) region as clusters sticking to the ventral wall of the dorsal aorta (E10.5; DA). Blood clusters were also identified in the vitelline (VA) and umbilical (UA) arteries. The foetal liver does not generate blood but supports expansion of the blood cell pool (Urness et al., 2000; Yoder et al., 2001; Rhodes et al., 2008).

3.3.1. Characteristics of endothelial cells

Endothelial cells are lining the intra-luminal wall of vessels. As a barrier they separate the vascular lumen from the perivascular space and control the permeability of plasma solutes, the extravasation of leukocytes and the communication between adjacent cells (Bazzoni et al., 2004; Liebner et al., 2006). The vasculature is established from mesoderm as one of the first functional systems in the developing conceptus. Initially, mesodermal cells differentiate into angioblasts (vasculogenesis) in the extra-embryonic YS around day 6.5 of development that fuse to form the first vascular plexus.

Endothelial cells are a very heterogeneous population of cells in the mammalian body and different types contribute to the formation of lymphatic vessels as well as to arterial and venous blood vessel. Diverging subsets of endothelium are already observable in the developing embryo. Studies of chicken-quail chimeras have shown that endothelial cells are of different developmental origin leading to a heterogenous population of endothelial cells that contribute to vascular systems of different tissues. The somatopleural endothelium derives from the somatic mesoderm, colonizes the vasculature of the body wall and forms the lymphatic vessels whereas the splanchnopleural endothelial cells arises from the splanchnic mesoderm and gives rise to the vasculature of the visceral organs. Both of them participate in the formation of the aorta which is formed by the fusion of the initially paired aortae (Pardanaud et al., 1996). During the fusion, the vascular wall undergoes extensive remodelling which is closely link to the time- and location-restricted capacity of the aortic wall to contribute to the formation of hematopoietic clusters (Pouget et al., 2006). Interestingly, blood clusters only form at the ventral wall of the aorta where splanchnopleural endothelial cells localize that are replaced by somatopleural endothelial cells after the AGM region has lost its hemogenic potential (Pardanaud et al., 1996).

3.3.2. Evidence for the existence of the hemangioblast

Since hematopoietic and endothelial cells arise at same time and locations and share the expression of molecules, suggested that both derive from a common origin and are produced from a common precursor cell, the hemangioblast.

First evidence for the hemangioblast was suggested from histological analysis of chicken YS blood islands (Sabin, 1920). Immunohistochemistry of blood islands has shown that same markers are expressed by luminal blood cells and by endothelial cells which hints to a common origin. In addition, the loss of some molecules impairs both, the

endothelial cell and the blood cell development. Mice lacking the gene encoding the foetal liver kinase (Flk1, VEGF-R2) die between E8.5 and E9.5 due to the lack of blood islands in the YS (Shalaby et al., 1995). They do not only fail to establish primitive or definitive hematopoiesis but also a functional vascular system (Shalaby et al., 1997). In addition, the absence of SCL/tal1 (stem cell leukaemia, T cell acute leukaemia 1) leads to a defective hematopoiesis as well as to an impaired vascular remodelling of the YS (Robb et al., 1995; Shivdasani et al., 1995; Visvader et al., 1998). The common origin of blood and endothelium was also proven by using different embryonic stem cell (ESC) lines that were differentiated *in vitro*. Choi and co-workers observed that both lineages are generated from single cells thereby suggesting that both share a common origin (Choi et al., 1998). Altogether this hints to the existence of a common precursor of blood and endothelial cells.

However, the molecular characterisation and isolation of the putative hemangioblast is still poor. Firstly, it is probably only present very transiently, because mesodermal cells rapidly differentiate into committed angioblasts and hematopoietic cells (Kinder et al., 1999). Secondly, the hemangioblast is a cell that exclusively gives rise to endothelial and blood cells but not to cells of other lineages. Even though the hemangioblast can be enriched by the expression of Flk1, SCL/tal1 and Brachury (Chung et al., 2002; Fehling et al., 2003; Huber et al., 2004), cells expressing these markers can give rise to other tissues apart from blood and endothelium, as well (Yamashita, J. et al., 2000; Ema et al., 2003; Schroeder et al., 2003; Kubo et al., 2004; Bussmann et al., 2007). Thus, although a bi-potent hemangioblast is probably present in the embryo, it is still poorly characterized and thus difficult to distinguish from multi-potent mesodermal cells.

3.3.3. Hypothesis of the cellular origin of blood cells

The close developmental relationship between endothelial and blood cells is evident by their synchronous appearance at same sites in the embryo and by the expression of identical molecules. Meanwhile it is generally believed that both share a common mesodermal precursor (Choi et al., 1998; Nishikawa et al., 2000; Mikkola et al., 2002). According to previous publications, the two possibilities of how blood and endothelial cells could be related to one another are displayed in Figure 1.3 (Nishikawa et al., 2000): blood and endothelium could directly arise from a bi-potential hemangioblast independently from one another. Alternatively, the hemangioblast could first give rise to

endothelium that differentiates into blood. In this case, the endothelial cell would be hemogenic because it has acquired hemogenic potential.

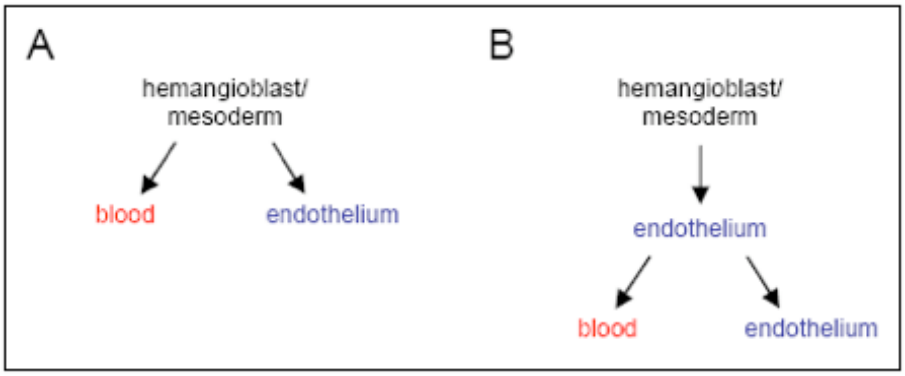

Figure 1.3: Two hypothesis displaying the possible relation between endothelial and blood cells in the developing embryo. Blood and endothelial cells can either share a common progenitor that independently generates both, endothelium and blood (**A**). In contrast, a mesodermal progenitor could first differentiate into endothelium which then produces blood and endothelial cells (**B**). Modified from Nishikawa et al. 2000.

3.3.4. Evidence for the existence of hemogenic endothelium

Endothelial characteristics as vascular endothelial (VE-) Cadherin-expression or uptake of acetylated low density lipoprotein (DiI-Ac-LDL) were transiently found on nascent embryonic blood cells expressing CD45, a pan-hematopoietic marker. This indicated that blood cells might differentiate from mesoderm through an endothelial intermediate, the hemogenic endothelium.

Labelling of chicken embryonic endothelial cells by DiI-Ac-LDL injection, a modified lipoprotein that is taken up by endothelial cells, *in ovo* before hematopoietic clusters became visible in the aortic lumen, resulted in a DiI-Ac-LDL$^+$ vascular tree. Shortly thereafter, DiI-Ac-LDL$^+$CD45$^+$ blood cells were detectable in the circulation (Jaffredo et al., 1998). This suggested that endothelial cells had taken up DiI-Ac-LDL and differentiated into blood cells that are released into the circulation. Furthermore, endothelial cells were tagged by retroviral infection before AGM hematopoietic clusters were observed in the chicken, and slightly later, virally transfected hematopoietic cells were found (Jaffredo et al., 2000).

In *ex vivo* cultures, mouse VE-Cadherin$^+$ cells purified from E9.5 embryos could give rise to VE-Cadherin$^-$CD45$^+$ blood cells (Nishikawa et al., 1998b). Consistent with this, *in vitro*

studies using ES cell differentiations could show that ESC-derived VE-Cadherin$^+$ cells can give rise to CD45$^+$ hematopoietic cells (Nishikawa et al., 1998a; Hashimoto et al., 2007). Evidence for the existence of hemogenic endothelium in mice was strengthened by *in vivo* analysis. Sugiyama et al. could identify DiI-Ac-LDL$^+$ erythrocytes and multi-potent hematopoietic precursors in E10.5 mouse embryos that were pulsed with DiI-Ac-LDL before the onset of the circulation (Sugiyama et al., 2003). In addition, a very small number of VE-Cadherin$^+$CD45$^+$ cells was identified in E9.5 or E11.5 AGM regions (Fraser et al., 2003; Taoudi et al., 2005) and VE-Cadherin$^+$ cells co-expressing embryonic haemoglobin were found in E6.75 blood islands (Ema et al., 2006). Kim et al postulated that virtually all foetal liver HSCs co-express VE-Cadherin at E13.5 which suggests an endothelial intermediate step in the development of HSCs (Kim et al., 2005).
In human embryos, the existence of hemogenic endothelial cells was also suggested. Upon their cultivation purified endothelial cells gave rise to blood cells (Oberlin et al., 2002).
Collectively, these data indicate the possible existence of hemogenic endothelial cells.

However, doubts exist whether the endothelial markers VE-Cadherin and DiI-Ac-LDL uptake are indeed exclusively expressed by endothelial cells. Although VE-Cadherin was shown to be expressed by endothelial cells (Breier et al., 1996; Nishikawa et al., 1998b), the detection of VE-Cadherin on embryonic blood cells also led to the suggestion that VE-Cadherin may not be restricted to endothelium (Dumont et al., 1995; Drake et al., 2000; Kim et al., 2005). Furthermore, DiI-Ac-LDL might not exclusively label endothelial cells but also macrophages which are present from day 7.0 dpc on (Palis et al., 1999; Sugiyama et al., 2003). Concentrating on single markers, all previous lineage tracing experiments did not show convincingly that hemogenic endothelium exists. In addition, the lack of morphological identification of cells leaves considerable room for speculations. Most importantly, all previous analysis performed end-point analysis which cannot rule out the existence of possible intermediate stages. Thus, strong doubts exist about the existence of hemogenic endothelial cells.

3.3.5. Requirement for time lapse microscopy

Although hematopoiesis in the developing embryo has been in the focus of research for a very long time, the controversy over the exact relation between blood and endothelium could not be solved yet. Most publications dealing with the differentiation of mesoderm into blood cells used approaches that couldn't answer this question precisely because of the following difficulties: (i) performing end-point analysis only allows studying of the outcome. The differentiation of mesoderm into endothelium and blood is a continuously changing process that might involve intermediate stages that are not considered in these analyses. (ii) As pointed out before, the presumptive precursor of blood and endothelium is poorly characterized. This automatically leads to the use of impure populations which can lead to false read-outs if all cells are analysed as a bulk. (iii) The simultaneous use of molecular markers, morphological appearance and cellular behaviour can significantly contribute to the identification of cells. Lineage tracing experiments in *in vivo* studies failed to analyse the morphology in combination with molecular or functional markers.

Consequently, a different approach is required that allows the continuous observation of single cells while they are generated to be able to clarify the differentiation pathway and to identify intermediate stages leading to a differentiated blood cell (Schroeder, 2005).
Thus, a novel imaging and tracking system was developed in our lab in which a culture is continuously observed using timelapse microscopy. Images of a high quality allowing the identification of subcellular structures are acquired in regular time intervals for up to 14 days. During acquisition, the specimen is kept in an atmosphere identical to a tissue culture incubator which ensures viability of cells. The employment of an automatic microscope stage permits recording of images at different positions and the detection of large areas of a culture. In addition, fluorescent signals to identify cell of certain lineages can be detected in regular time intervals without cytotoxic effects (Rieger et al., 2008; Schroeder, 2008).
After collecting the images, they are analysed frame-by-frame using a single cell tracking system that allows to manually follow individual cells and their progeny. Their proliferation and differentiation behaviour is displayed in pedigrees that contain information like cell division, apoptosis, generation times, morphologies, migration speed, behaviours in co-culture and fluorescence that altogether contribute to the identification of cells of a certain lineage as well as fate choices and ancestral relationship of each individual cell. Thus, the combined use of high throughput bio-

imaging and single cell tracking allows the efficient detection and precise analysis of many single cells providing indispensable information for the understanding of the differentiation process (Schroeder, 2008).

3.4. *In vitro* differentiation of embryonic stem (ES) cells

3.4.1. *In vitro* differentiation of embryonic stem (ES) cells: a model system to study embryonic hematopoiesis

Studying mouse embryogenesis at the cellular level *in vivo* is often limited by the difficult accessibility of tissues, low numbers of cells of interest and lack of knowledge about interactions or signalling from neighbouring tissue. The same holds true for embryonic hematopoiesis. Although the YS is part of the extraembryonic portion its observation is challenged by the internal localization of the embryo within mother and uterus. Even more complicated is the analysis of intraembryonic locations which can only be analysed after destroying the embryo.

To overcome these problems, embryonic stem cell (ESC) differentiation systems are used since several decades to investigate cellular processes of mouse development. ES cells derive from the inner cell mass of blastocyst-staged embryos (Evans et al., 1981; Martin, 1981). They are pluri-potent and have the capacity to differentiate into all different cell lineages in the body (Niwa, 2001; Chambers et al., 2004). Besides their unlimited expansion in culture and their comparably easy genetic manipulation, ES cells can be differentiated in defined culture conditions thereby allowing the control of the differentiation (Keller, G. M., 1995; Smith, 2001).

The *in vitro* differentiation of ESCs was shown to be highly similar to embryonic development *in vivo*. Defects resulting from the loss of transcription factors observed in *in vivo* studies could be replicated by ESC differentiations (Weiss et al., 1994; Porcher et al., 1996; Lacaud et al., 2002; Lacaud et al., 2004). In addition, the presence of cells with identical surface marker profile and lineage potential found in the embryo could be identified in ESC differentiation systems, as well (Nishikawa et al., 1998a; Yamashita, J. et al., 2000; Mitjavila-Garcia et al., 2002; Mikkola et al., 2003; Hashimoto et al., 2007). This demonstrates that the sequence of the cellular development as well as the kinetics of molecular and functional marker expression is highly similar to embryonic development. Thus, ESC differentiation is widely accepted as valid model system to study embryonic processes (Mikkola et al., 2002; Kitajima et al., 2003; Keller, G., 2005).

Besides differentiating ES cells to study embryonic development *in vitro*, ES cell differentiations can be used to easily and quickly generate cells of all different lineages. This is of special interest for clinical applications and therapy since putatively transplantable cells can be produced in large numbers. As pluri-potent cells can now be generated *in vitro* from somatic cells by nuclear transfer or by lentiviral transfection (Yamanaka, 2007; Hanna et al., 2008) the generation of differentiated cells from pluri-potent cells might become an important tool to cure diseases and cancers, e.g. anaemia or leukaemia. Although all hematopoietic lineages can be differentiated from ESCs *in vitro*, their precise differentiation pathway is not yet fully understood as a LTR-HSC could not be detected in ESC differentiation systems so far. Although this limits the use of ESCs to produce LTR-HSCs, other ESC-derived cells have successfully been transplanted into recipients thereby showing that in principle functional adult type hematopoietic cells can be differentiated from ESCs (Keller, G., 2005).

In all differentiation systems, the differentiation of ESCs into lineages of interest is based on the withdrawal of LIF (leukaemia inhibitory factor), a cytokine that efficiently prevents differentiation, and optionally the addition of factors that induce the differentiation into cells of interest (Chambers et al., 2004). To induce mesoderm formation, ESCs are either differentiated in embryoid bodies, on type IV collagen coated dishes or on OP9 stromal cells (Keller, G., 2005). As cells of all three germ layers are produced in the embryoid body differentiation, the ratio of mesodermal derivatives is very low (Nishikawa et al., 1998a; Zhang, W. J. et al., 2005). In contrast, type IV collagen and OP9 cells preferentially support mesodermal differentiation (Nakano et al., 1994; Nishikawa et al., 1998a).

3.4.2. Co-culture system of mouse embryonic stem cells and OP9 stroma cells

ES cells cultured on OP9 stroma spontaneously differentiate first into mesodermal cells which upon re-plating give rise to a variety of mesoderm-derived cell types. The culture is performed in the presence of FCS (foetal calf serum) which provides a multitude of cytokines and growth factors. SCF (stem cell factor, kit ligand, steel factor) is added as it was shown to enhance growth and differentiation of stroma-dependent cultures (Lyman et al., 1998). Also OP9 are thought to support survival and induce differentiation by the

secretion of a variety of factors (Yoshida et al., 1990; Matsumura et al., 2003; Groger et al., 2004).

Besides cardiomyocytes, mural cells and endothelial cells, a wide range of blood cells belonging to the primitive and definitive lineages are produced on OP9 stromal cells (Kodama et al., 1994; Nakano et al., 1994; 1996; Hirashima et al., 1999; Yamashita, J. et al., 2000; Schroeder et al., 2003). The generated hematopoietic cells can be primitive and definitive erythrocytes, granulocytes, macrophages, megakaryocytes and cells of the B-lymphoid lineage (Nakano et al., 1994; 1996). OP9 cells were established as a polyclonal cell line from the calvaria of neonatal osteopetrotic mice (op/op) lacking the gene encoding M-CSF (macrophage colony stimulating factor) (Yoshida et al., 1990). In contrast to other stroma cell lines used to support hematopoiesis, OP9 cells are devoid of secreting M-CSF which prevents the macrophage-dominated differentiation over other blood cell lineages (Nakano et al., 1994).

In contrast to the embryoid body differentiation, the OP9 co-culture is expanded in only 2 dimensions thereby allowing the visualization of each individual cell contained in the culture by microscopic approaches. Thus, the OP9 differentiation system provides an ideal tool to analyse the differentiation of ESC-derived cells into hematopoietic cells by time lapse microscopy, to study embryonic hematopoiesis and to clarify the developmental pathway leading from a mesodermal starting cell to differentiated blood cells.

4. Goals of the Thesis

The existence of hemogenic endothelium was postulated many decades ago but is still under dispute and strongly debated.

In my thesis, the longstanding controversy of the existence of hemogenic endothelium should be clarified by continuous observation at the single cell level. ESC-differentiation on OP9 cells should be employed as a model to study embryonic hematopoiesis, recorded by time lapse imaging and analyzed by single cell tracking. The combination of multiple lineage markers should unequivocally prove the nature of endothelial and blood cells observed and thus eliminate any doubts about cell identities. Furthermore, the presumptive hemogenic endothelial cell should be analysed in respect to its surface marker profile. In addition, the results obtained from *in vitro* studies should be replicated by imaging of mesodermal cells purified from mouse embryos differentiating into hematopoietic cells.

5. Results

5.1. Generation and purification of embryonic stem cell-derived mesodermal cells and time lapse imaging of their progeny

Investigating the putative existence of hemogenic endothelium requires to continuously follow the development and differentiation of individual cells to clarify their cellular origin. To solve whether endothelial cells can give rise to blood, the embryonic stem cell (ESC) differentiation on OP9 was observed by time-lapse imaging and analyzed by single cell tracking.

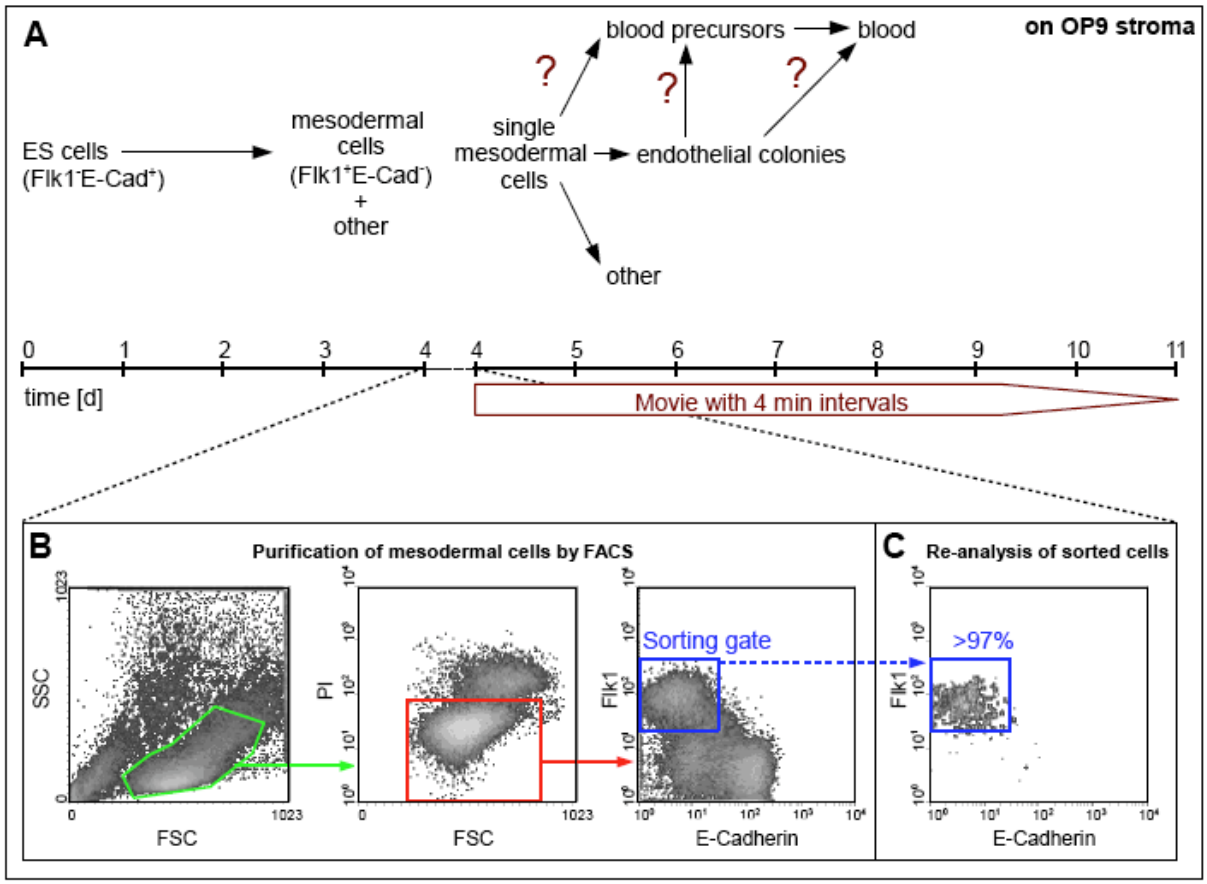

Figure 5.1: Experiment setup. A: ESCs are differentiated on OP9 stroma into mesodermal cells within 4 days of culture. Purification of mesodermal cells by FACS and re-culture on OP9 induces their differentiation into endothelial, hematopoietic and other mesodermal derivatives within another 5 days of culture. While mesodermal cells differentiate into blood and endothelium a time lapse video is recorded for up to 7 days. **B:** Purification of mesodermal cells by FACS. Of ESC-derived cells of the right size (FSC) and granularity (SSC, green) only living cells (PI^-) are gated (red) and $Flk1^+$E-$Cadherin^-$ cells are sorted (blue). **C:** Re-analysis of sorted cells is performed to confirm a high purity of sorted cells.

A detailed overview of the experimental setup is depicted in Figure 5.1. At first, ESCs were differentiated on OP9 stroma cells to mesoderm that up-regulates the surface marker Flk1 by day 4.0 of differentiation (Figure 5.1 A) (Nakano et al., 1994; Kataoka et al., 1997). Purified $Flk1^+$ cells are known to possess the potential to differentiate into a multitude of mesodermal derivatives including blood, endothelium, cardiomyocytes and smooth muscle cells upon re-culture on OP9 stroma (Nakano et al., 1994; 1996; Hirashima et al., 1999; Yamashita, J. et al., 2000; Schroeder et al., 2003; Hashimoto et al., 2007). $Flk1^+$ cells are a well known population that represents undifferentiated precursors of endothelial and blood cells. Thus, this population is a suitable starting population for the time lapse experiments to address the question of whether endothelial cells can produce blood cells.

E-Cadherin (epithelial Cadherin) was included in the sorting scheme because it is expressed by undifferentiated ES cells some of which are still present after 4 days of culture. As these cells should be excluded from the sorting population, only E-$Cadherin^-$ cells were selected (Hirashima et al., 1999; Yamashita, J. et al., 2000). In detail, by gating propidium $iodide^-$ (PI) cells of small size and low granularity the sorted fraction of the cell mixture was limited to living ESC-derived cells. Live, $Flk1^+$E-$Cadherin^-$ cells were selected and sorted into differentiating medium (Figure 5.1 B). A high sort quality was confirmed by re-analysing few sorted cells. If more than 97% of events could be detected in the sorting gate, cells were used for further analysis (Figure 5.1 C).

After purification, cells were counted and re-suspended in stem cell factor (SCF) – containing differentiating medium that had been previously equilibrated with CO_2. Enrichment of differentiating medium was sufficient to maintain 5% CO_2 for another week. Upon seeding living cells on fresh OP9, the culture was incubated for 1 hour to obtain gas exchange. After tightly sealing the flask, time-lapse imaging was started and performed for up to 7 days.

5.2. Cells with endothelial morphology can generate blood cells

5.2.1. Endothelial and blood cells exhibit specific morphologies

The differentiation of ESCs on OP9 performed has been intensely described previously (Nakano et al., 1994; 1996; Nishikawa et al., 1998a; Hirashima et al., 1999; Fraser et al., 2002a). It has been routinely used for several years and well understood. The identification of ESC-derived cells by their unique morphology and their ability to form distinct colonies has been analyzed in several studies in the past that confirmed that

free-floating cells are hematopoietic and adherent, flat cells with prominent nucleus that form sheet-like colonies are endothelial (Figure 5.2) (Nakano et al., 1994; 1996; Hirashima et al., 1999; Fraser et al., 2002a). This prompted us initially to perform time-lapse imaging in phase contrast only using morphological identification to address the question of the existence of hemogenic endothelium.

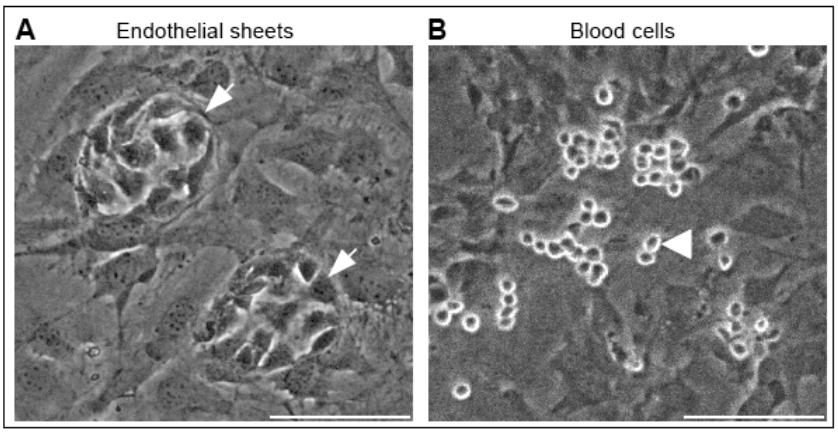

Figure 5.2: Morphology of ESC-derived endothelial cells and blood cells cultured on OP9. A: Two endothelial sheet colonies are depicted by arrows. **B:** Free-floating blood cells (arrowhead). Scale bar: 100 µm.

5.2.2. Imaging of wildtype embryonic stem cell-derived cells points to the existence of hemogenic endothelial cells

In order to analyze whether endothelial cells are capable of producing hematopoietic cells ESC-derived mesodermal cells were purified and imaged as described above.

As depicted in Supplementary Movie 5.1 and Figure 5.3, one single mesodermal cell (Figure 5.3 A) proliferates and differentiates into endothelial cells forming a sheet-like colony within 2 days after the start of the movie (Figure 5.3 C). Although all endothelial cells contained in the colony were morphologically indistinguishable from one another (Figure 5.3 C, enlarged section), some endothelial cells embedded into the sheet detach from the surrounding cells, become semi-adherent and move to the surface of the adherent colony still maintaining contact to the endothelial layer (Figure 5.3 D). Connections between semi-adherent and adherent endothelial cells are formed and loosened again, resulting in elongated looking cells moving on the endothelium (Figure 5.3 E). Upon adopting a semi-adherent appearance cells do not insert into the endothelial sheet anymore but round up around day 8 of ESC-differentiation, detach

from the endothelial sheet and free-float in the culture (Figure 5.3 F). The fact that nascent free-floating cells keep dividing eliminates the possibility that these cells were apoptotic.

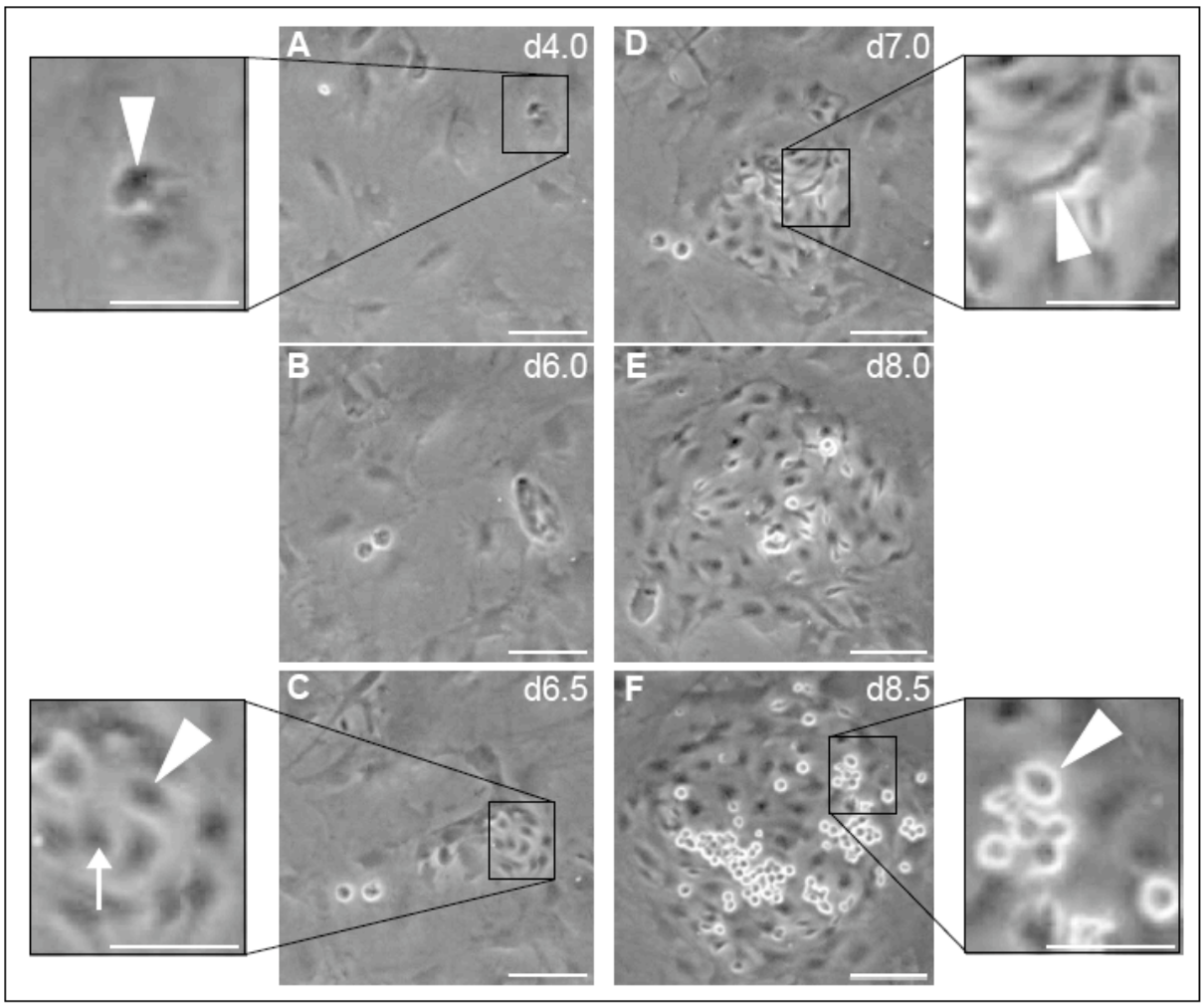

Figure 5.3: Cells with endothelial morphology can produce blood cells. Images extracted from the time lapse experiment shown in Supplementary Movie 5.1. Wildtype ESC-derived mesodermal cells were seeded on OP9 and recorded by time lapse imaging. Time after start ESC-differentiation is indicated. **A:** One single wild-type ESC-derived mesodermal cell (arrowhead in enlarged image) proliferates **(B)** and differentiates into an endothelial sheet colony **(C)** of that some cells detach from the endothelial sheet and are semi-adherent on the endothelial colony **(D, E)**. After some time the semi-adherent cells loose contact to endothelial cells and are present as free-floating blood cells **(F)**. Arrowheads in enlarged images of C, D and F depict the morphologies of adherent endothelial, semi-adherent and free-floating blood cells. The arrow in C highlights a non-hemogenic endothelial cell.
Scale bars: 100 µm and 50 µm in zoomed images.

Besides endothelial cells with hemogenic potential, non-hemogenic endothelial cells developed from the same mother cell. A morphological difference between both types of endothelial cells was not visible. Instead, all endothelial cells contained in the colony at day 6.2 of ESC differentiation exhibited the same typical endothelial morphology as

depicted in the enlarged part of Figure 5.1 C. While all offspring of hemogenic endothelial cells transformed into blood, non-hemogenic endothelial cells maintained their morphology and sheet formation.

These findings indicate that (i) hemogenic endothelial colonies exist in ESC-derived cultures, (ii) a hemogenic endothelial colony of monoclonal origin contains hemogenic and non-hemogenic subsets of endothelial cells, both of them sharing the same morphology, and that (iii) hemogenic endothelial cells transform into putative hematopoietic looking cells by going through a semi-adherent intermediate phase.

5.2.3. Imaging of embryonic stem cell-derived cells with constitutive nuclear label allows reliable continuous single cell tracking of all cells in a colony

To clarify the exact relationship of cells within the hemogenic endothelial colony to one another it is indispensable to follow each individual cell starting from the mesodermal mother until hematopoietic-looking cells have developed.

Tracking single cells in movies of wild-type ESC-derived cultures proved to be very challenging, because endothelial precursors often form 3-dimensional aggregates making it difficult to recognize single cells (Figure 5.1 B). To improve their identification wild-type ES cells carrying a constitutive nuclear label were established that allows their identification in the culture. A plasmid encoding a Histone2BVENUS (H2BVENUS) fusion and a puromycin selection cassette controlled by the chicken beta actin (CAG) promoter (Okita et al., 2004) was electroporated into wildtype ESCs. Selection for Puromycin resistant cells resulted in stably transfected ES cells that were pooled to obtain a polyclonal cell line constitutively expressing the fusion protein H2BVENUS. To ensure that the cell line produced behaves similar to wild-type ES cells its differentiation capacity was verified by culturing it on OP9 stroma. The differentiating cells were analyzed by FACS analysis and microscopy in regard to the presence and ratio of generated cell types and compared to equally treated wild-type cells (data not shown).

Phase contrast time lapse imaging of differentiated H2BVENUS ESC-derived mesodermal cells confirmed that endothelial cells can give rise to presumptive blood cell as observed in videos obtained from differentiated wild-type ESCs: an endothelial colony of monoclonal origin contained hemogenic subsets that produced free-floating cells through

a semi-adherent intermediate. By acquiring fluorescence images in regular intervals it was not only possible to confirm the monoclonal origin of the colony but also to track single cells starting from the mesodermal founder cell until free-floating cells were visible at day 8.5 of differentiation (Figure 5.4 and Supplementary movie 5.2).

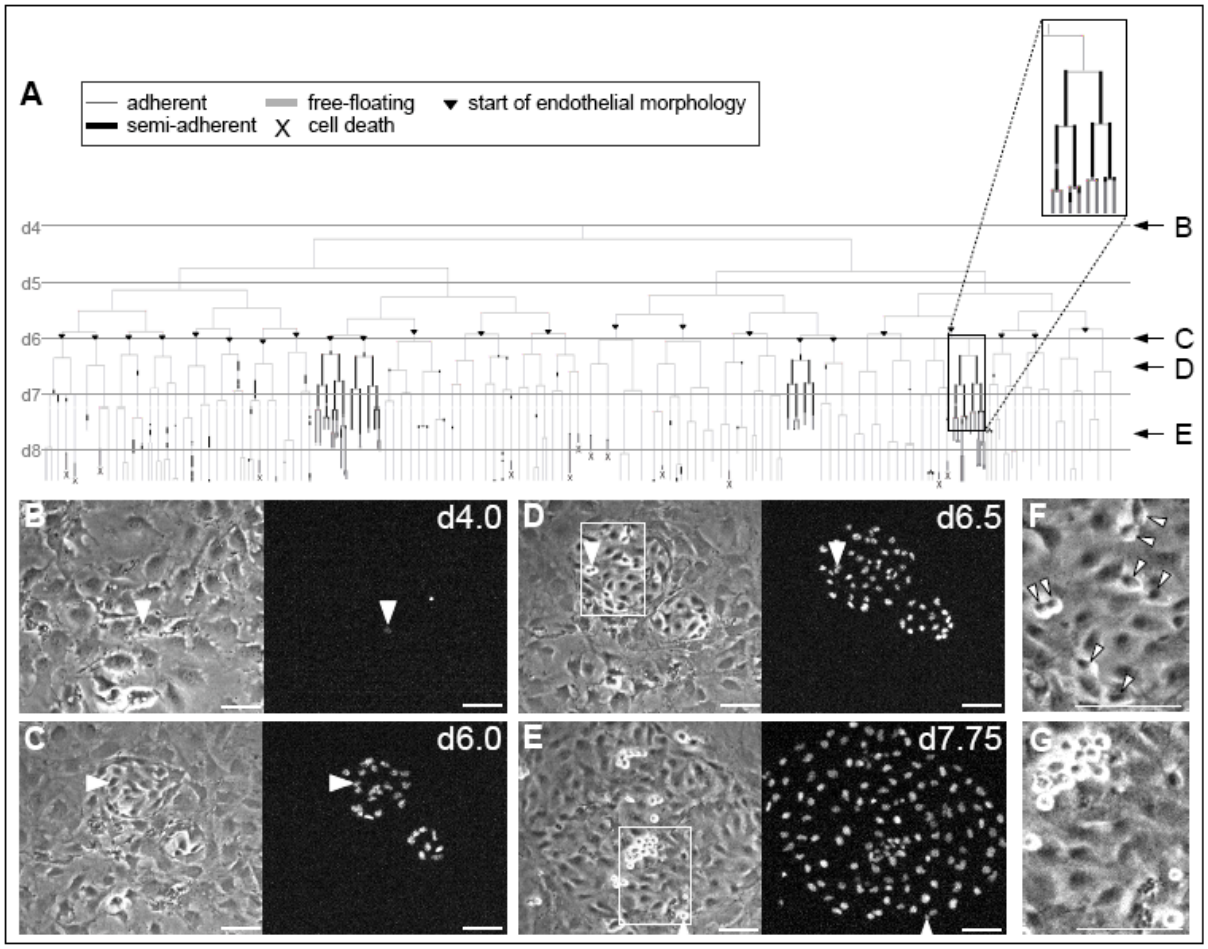

Figure 5.4: Single cell tracking of a hemogenic endothelial colony shows that endothelial cells can generate blood cells. Flk1^{+}E-Cadherin^{-} cells constitutively expressing H2BVENUS were cultured on OP9 stroma for 4 days while recording a time lapse video. Days of ESC differentiation are indicated. **A:** Pedigree corresponding to the colony shown in the images B-G and Supplementary Movie 5.2. **B-G:** Images extracted from the Supplementary Movie 5.2. Phase contrast images are shown left, fluorescent images detecting H2BVENUS are shown on the right. One mesodermal cell **(B)** produces an endothelial colony **(C)**, of that some cells become semi-adherent **(D, enlarged in F)** and finally free-floating **(E, enlarged in G)**.
Scale bar: 100 µm

The corresponding pedigree is depicted in Figure 5.4 A. The Flk1^{+}E-Cadherin^{-} sorted mesodermal starting cell (Figure 5.4 B) proliferates and in the 5th generation around day 6 of ECS-differentiation cells form a colony containing cells with typical endothelial morphology, as highlighted by triangles in the tree. By day 6.3 of differentiation all cells

look and behave identically and no difference between individual cells was obvious. Hemogenic and non-hemogenic endothelial cells show typical endothelial morphology at around the same time (Figure 5.4 C).

Shortly after acquiring endothelial morphology, four cells irreversibly become semi-adherent cells, two of them being sisters (Figure D and F). After two to three cell divisions all daughters of the hemogenic endothelial cells detach from the surface and are free-floating (Figure E and G).

Besides of their morphological appearance, endothelial and blood cells differ in the length of their cell cycle. With the onset of semi-adherence nascent free-floating blood cells show shorter and uniform generation times (generation time 11.2 hours ± 3.5 hours; n=69), whereas the non-hemogenic endothelial cells divide slower and in a very irregular fashion (generation time 18.0 hours ± 8.5 hours; n=185). This strengthens the point that adherent and suspension cells of monoclonal origin are indeed different cell types.

Blood cells were not tracked until the end of the movie since the rapid movements and the three-dimensional expansion of free-floating cells makes it difficult to reliably follow individual cells – despite the nuclear label. However, free-floating cells were followed and were not observed to incorporate into the endothelial sheet anymore. This is strong evidence that suspension cells have lost the potential to integrate into the endothelial colony.

Some endothelial cells were observed to transiently detach from the endothelial sheet as well. In most cases this was due to the cell division process that is accompanied by short detachment during cytokinesis observable in many adherent cell types (e.g. OP9). Detachment of adherent endothelial cells was additionally visible towards the end of the movie which can be explained by an increased number of apoptotic events of adherent cells leading to free-floating cell clumps.

Increased cell death of endothelial cells with proceeding differentiation could be observed occasionally within this study and is probably due to the age of the culture and the limited space in the adherent cell layer which might lead to increased apoptosis.

Although the three hemogenic sub-trees of the pedigree visible in Figure 5.4 are not directly related to one another, all of them show the similar length and length of semi-adherence and the same onset of free-floating morphology. Furthermore, the temporal sequence of the transition was identical to the ones analyzed in wild-type movies. Strikingly, endothelial to blood cell transitions were never observed at later time-points

than described above. This suggests that endothelial cells may possess hemogenic potential only within a very limited time window of differentiation.

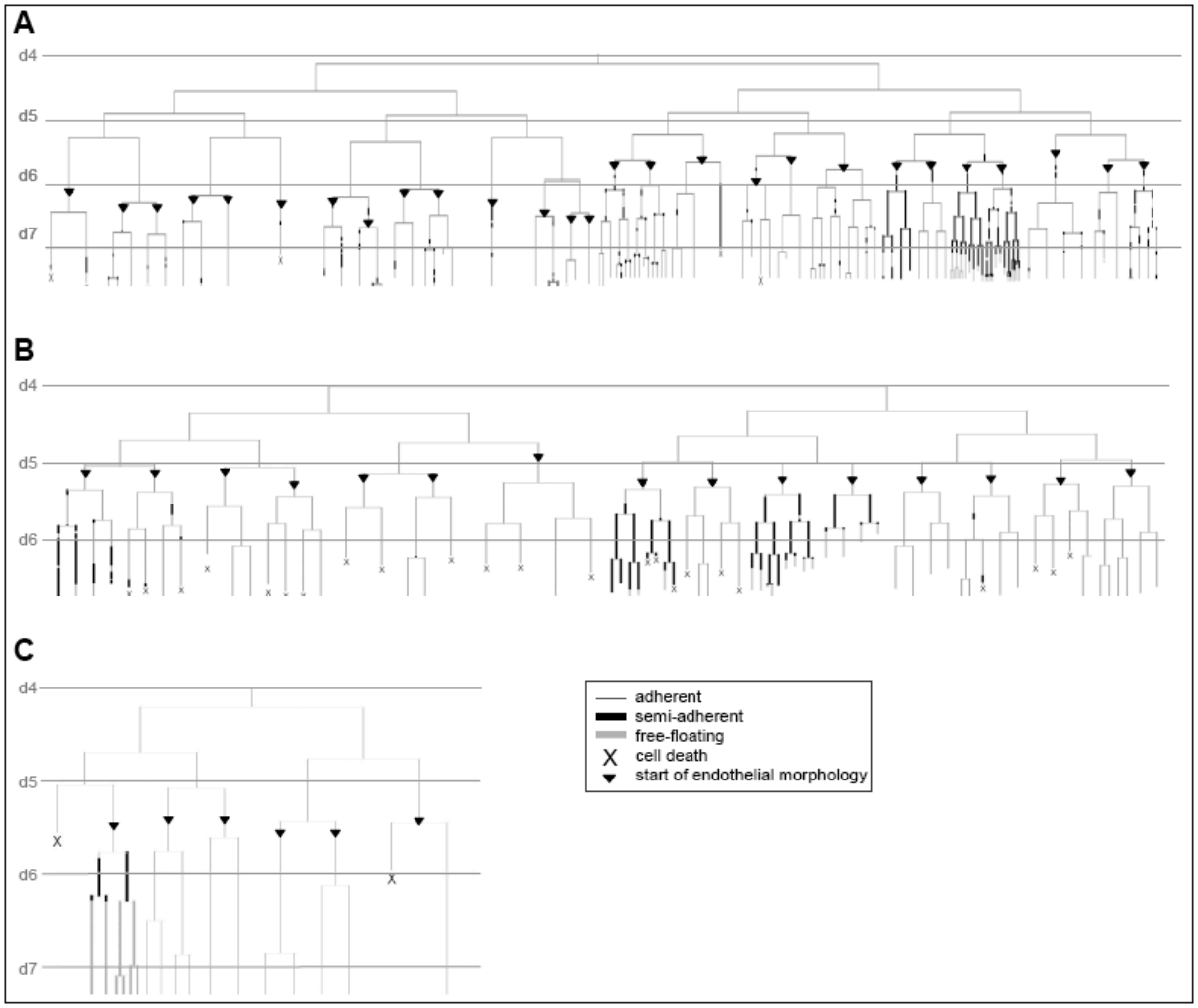

Figure 5.5: Additional pedigrees depicting endothelial cell – blood cell transitions (compare Figure 5.4). Kinetics and cellular behaviour are reproducible between all pedigrees shown in A-C and Figure 5.4. In contrast, the total size of the hemogenic endothelial colony and the number of hemogenic endothelial cells are variable.

More than 100 hemogenic endothelial colonies producing blood cells were observed in 16 independent experiments that were performed with 4 different ESC-lines under different culture conditions throughout this study. All of them displayed the same sequence of cellular behaviour – timing of endothelial cell maturation, detachment of hemogenic endothelial cells and their transition into free-floating blood cells as visible in some additional pedigrees displayed in Figure 5.5. Although some variability can be seen in colony size and the number of hemogenic endothelial cells within each colony, colonies consisting of 4 to up to 20 cells at the time point of hemogenic transition were able to

produce hemogenic endothelial cells. Similarly, endothelial colonies could contain 1 to 4 hemogenic endothelial cells. In contrast, the sequence of cellular behaviour and the kinetics of nascent free-floating cells appearing were highly reproducible. This suggests the presence of a mechanism that is strongly controlled on the cellular and molecular level.

5.2.4. Non-hemogenic endothelial colonies can morphologically not be distinguished from non-hemogenic endothelial cells in hemogenic endothelial colonies

To compare whether endothelial cells of non-hemogenic endothelial and hemogenic endothelial colonies show a major difference in their behaviour, single cell tracking of non-hemogenic endothelial colonies was performed in H2BVENUS expressing ESC-derived cells.

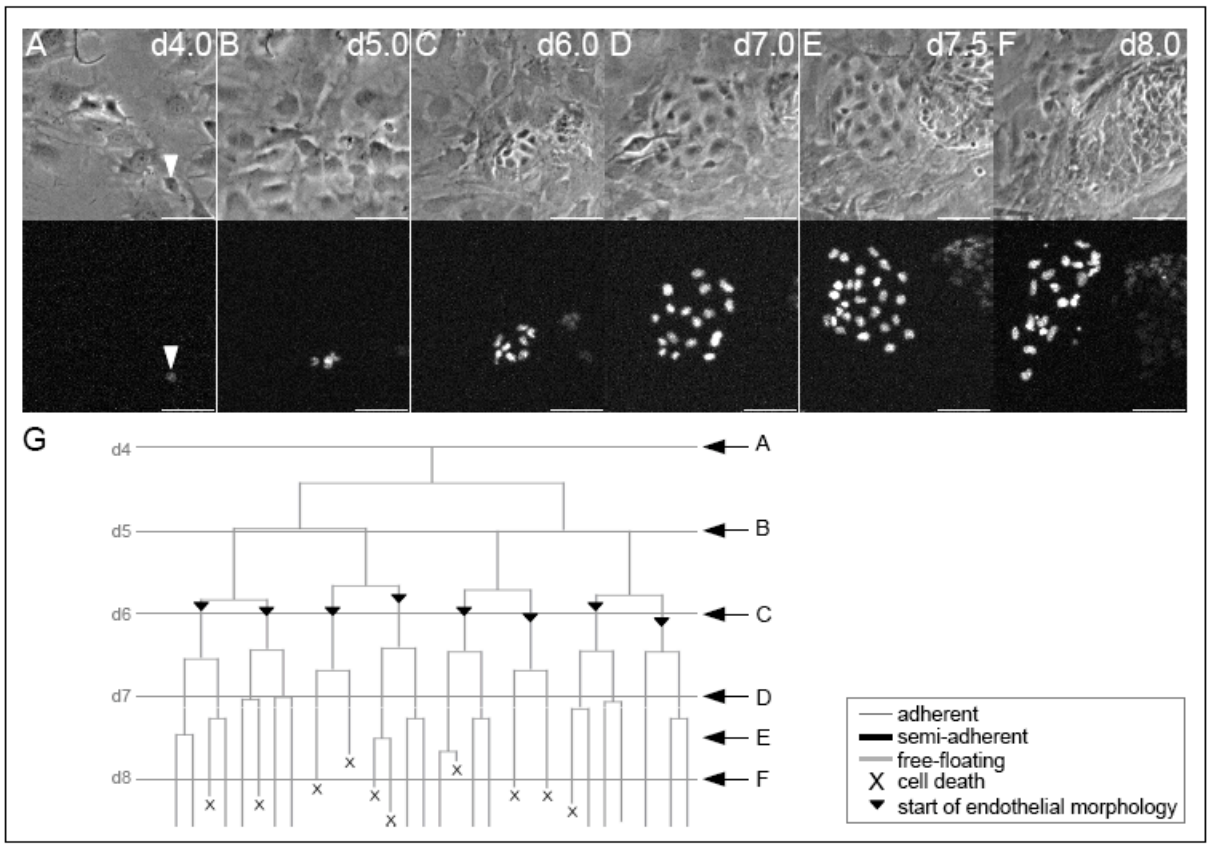

Figure 5.6: Single cell tracking of endothelial cells in non-hemogenic colonies. Indicated is the time after induction of ESC-differentiation. **A-F:** Images extracted from a movie. The upper panel show phase contrast, the lower panel shows fluorescent images detecting H2BVENUS signals. **G:** Pedigree corresponding to the images in A-F.
Scale bar: 50 µm.

Non-hemogenic endothelial colonies do not show any difference in proliferation, numbers of cell divisions, generation times nor in onset of endothelial morphology. This shows that non-hemogenic endothelial cells in hemogenic colonies behave as other non-hemogenic endothelial cells in the culture.

5.2.5. Hemogenic endothelial colonies appear in very low frequencies in OP9 differentiation cultures

In the movies, several different types of monoclonal colonies could be identified: colonies with endothelial sheet morphology, with blood morphology (suspension and adherent blood cells generated by a non-endothelial sheet colony) and colonies with unequivocal morphology (cardiomyocytes, pericytes or other). The frequency of all morphologically-identifiable types of colonies counted in 6 independent time-lapse experiments of ESC-derived mesodermal cells is shown in Table 5.1. Only 1.2% of all observed colonies are endothelial sheet colonies which produce blood. Vice versa, most blood cells observed are produced from non-endothelial cells (82.9%) whereas only 17.1% derive from endothelial sheet colonies.

Table 5.1: Colonies originating from one ESC-derived mesodermal cell were identified by morphology in time lapse experiments. Average colony numbers from 6 independent experiments are shown. No difference could be observed in the resulting colony frequencies when using 3 different ESC lines or different culture conditions (see below). Other: cardiomyocyte colonies and colonies without unequivocal morphology.

Colony type	Average colony number / experiment	Mean %
Blood colony	30 (± 12)	5.8
Non-hemogenic endothelial sheet colony	231 (± 61)	44.4
Hemogenic endothelial sheet colony	6 (± 4)	1.2
Other	253 (± 129)	48.6

This very low frequency of endothelial cell to blood cell transitions were reproduced in 16 independent experiments collected from differentiations of 4 different ES cell lines.

It is known from other studies that in addition to endothelial colonies with sheet-like morphology endothelial clusters with a less apparent morphology are present in these cultures (Hirashima et al., 1999; Matsumura et al., 2003; Hashimoto et al., 2007). This means that probably more endothelial colonies producing hematopoietic cells are present

that were scored as pure blood colonies here. Therefore, our analysis represents a conservative evaluation of the frequency of blood generation from endothelium.

5.3. Endothelial and hematopoietic cells can be distinguished by specific fluorescent markers in time lapse experiments

The reliable identification of endothelial and blood cells is crucial to prove the existence of hemogenic endothelium. Although both differ considerably in their morphological appearance, it had to be excluded that the observed hemogenic endothelial cell is of a different celltype that localizes within the endothelial sheet colony. Vice versa, since angioblasts have been shown to float in peripheral blood (Asahara et al., 1997) the possibility of free-floating cells that are not hematopoietic had to be excluded. Thus, the hemogenic transition of endothelial cells has to be verified by showing the presence of specific endothelial and hematopoietic markers on these cells. As hemogenic endothelial cells can only be identified by continuous time-lapse imaging and single cell tracking so far, the used markers not only have to be exclusive but also applicable in long term imaging of *in vitro* cultures.

5.3.1. Cells with endothelial morphology exhibit typical functional and molecular endothelial characteristics

To confirm that blood precursors are endothelial, the identity of these cells can in addition to the morphology be evidenced by the incorporation of acetylated low density lipoprotein (Ac-LDL), by the expression of VE-Cadherin and by the presence of functional tight junctions.

5.3.1.1. Endothelial cells specifically take up DiI-Ac-LDL

Endothelial cells exhibit an increased uptake of acetylated low density lipoprotein (Ac-LDL) compared to other cell types. Its addition to living cultures or its injection into living animals results in specific binding and engulfment of the modified lipoprotein by endothelial cells (Voyta et al., 1984; Yablonka-Reuveni, 1989; Bollerot et al., 2006). DiI-labelled Ac-LDL (DiI-Ac-LDL), when used in living cultures, is internalised, enzymatically degraded and the lipophilic DiI accumulates in the endosomal membranes thereby labelling the vesicles of the endothelial cells (Voyta et al., 1984; Lodish et al., 1999).

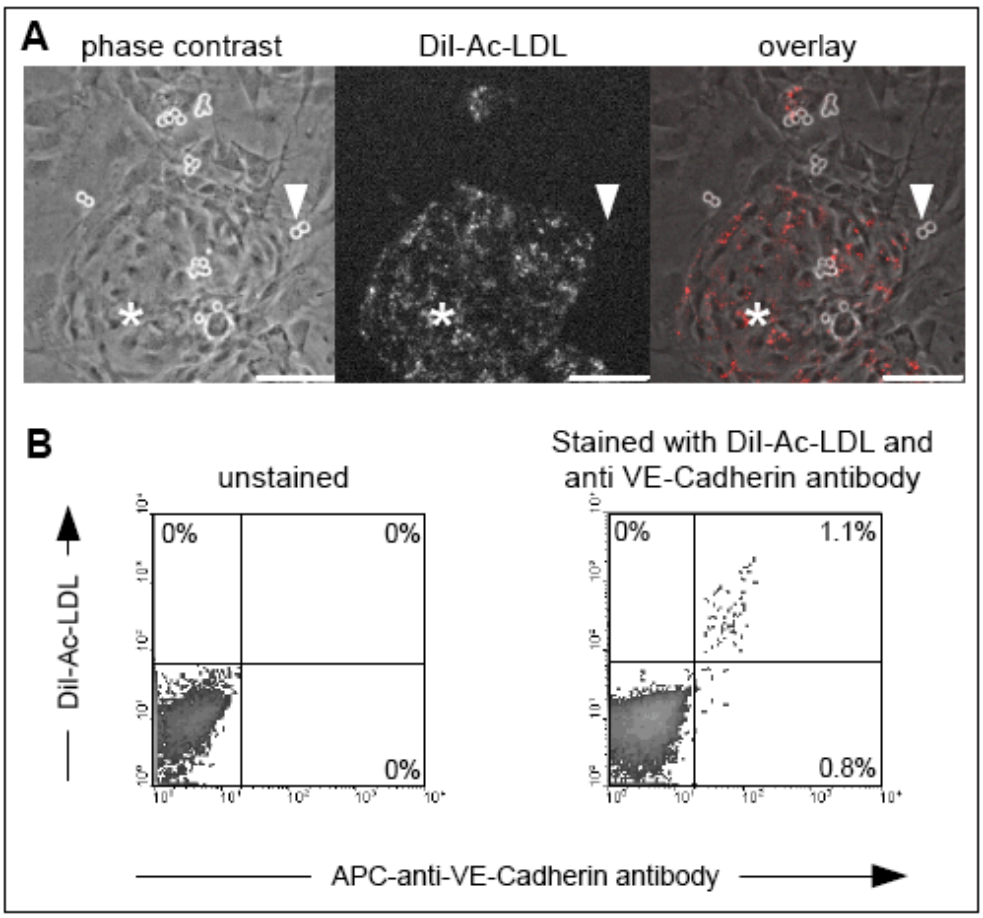

Figure 5.7: DiI-Ac-LDL specifically labels endothelial but not hematopoietic cells. ESC-derived mesodermal cells were cultured on OP9 in the presence of DiI-Ac-LDL and analyzed at day 8 (A) and day 6.5 (B) of differentiation. **A:** Endothelial cells forming a sheet colony are DiI-Ac-LDL$^+$ (asterisk), free-floating blood cells are DiI-Ac-LDL$^-$(arrowhead). **B:** FACS analysis showing that all in culture stained DiI-Ac-LDL$^+$ cells can be detected by an anti-VE-Cadherin antibody. Left hand side: negative control; right hand side: DiI-Ac-LDL containing culture stained with an anti-VE-Cadherin antibody.
Scale bar: 100 µm.

It was tested if DiI-Ac-LDL added to the normal differentiating medium is specifically enriched in endothelial cells even in the presence of serum-derived LDL contained in the culture medium is present. As shown in Figure 5.7 A, it exclusively stained endothelial sheet colonies. Upon their differentiation, cells of sheet-like endothelial colonies accumulate DiI-Ac-LDL in their vesicles as was evaluated in a time lapse experiment detecting DiI-Ac-LDL present in the culture. In contrast, DiI-Ac-LDL staining was not visible in blood cells (Figure 5.7 A, image extracted from a movie at day 6.5 of ESC-differentiation) or cells with cardiomyocytic morphology (not shown). Microscopically comparing colony frequencies with cultures lacking DiI-Ac-LDL verified that cell fates are not changed by the permanent presence of DiI-Ac-LDL. However, as the signal intensity

was very bright the concentration of the modified lipoprotein in the culture was decreased to 50 ng/ml to minimize any potential toxic effects.

5.3.1.2. Endothelial cells specifically express vascular endothelial (VE-) Cadherin and form tight junctions

As endothelial cells are an integral part of vessels, they separate the vascular lumen from perivascular tissue. Their function relies on the formation of cell-cell junctions to maintain cellular polarity, permeability to plasma solutes, leukocyte extravasation and communication between adjacent cells (Bazzoni et al., 2004; Liebner et al., 2006). Adherence and tight junctions consist of a multitude of integral membrane proteins, most of them being expressed by both epithelial and endothelial cells. Two proteins, vascular endothelial Cadherin (VE-Cadherin) and Claudin5 are restricted to endothelial adherence and tight junctions, respectively (Lampugnani et al., 1995; Dejana, 1996; Morita et al., 1999).

Therefore, to identify endothelial cells, the expression of VE-Cadherin and the presence of tight junctions were analyzed. Recently, Hisatsune et al. (Hisatsune et al., 2005) have cloned the regulatory elements directing the endothelial-specific expression of VE-Cadherin. Here, this promotor was initially cloned in front of a mitoVENUS fusion gene (Okita et al., 2004) and stable transfected into ES cells. Although stable transfected monoclonal ESC lines were established within my thesis (data not shown), these cells were not employed in time lapse videos. Guo and co-workers subsequently created another ESC line that uses the endothelial-specific VE-Cadherin promotor to drive a VENUSClaudin5 fusion protein exclusively in endothelium (VE-Cadherin::VENUSClaudin5 ES cells) (Guo, R. et al., 2007). Upon differentiation, the ESC-derived cells produce VENUSClaudin5 protein only in endothelial cells expressing VE-Cadherin. As endothelial cells form intercellular tight junctions they integrate the VENUSClaudin5 fusion protein into the junctions thereby labelling cell membranes.

In order to elucidate which cells in our system express VE-Cadherin and tight junctions the VE-Cadherin::VENUSClaudin5 ESC line was differentiated on OP9 and analyzed by morphology and FACS. In Figure 5.8 A an image taken at day 6.5 of differentiation depicting the specificity of these markers is shown: an endothelial sheet colony is not only expressing VE-Cadherin visible by the detectable fluorescence signals but is in addition forming tight junctions with surrounding endothelial cells as the VENUSClaudin5

fusion protein integrates into the cell membranes. In contrast, hematopoietic suspension cells do not express VE-Cadherin and cannot integrate VENUSClaudin5 into tight junctions.

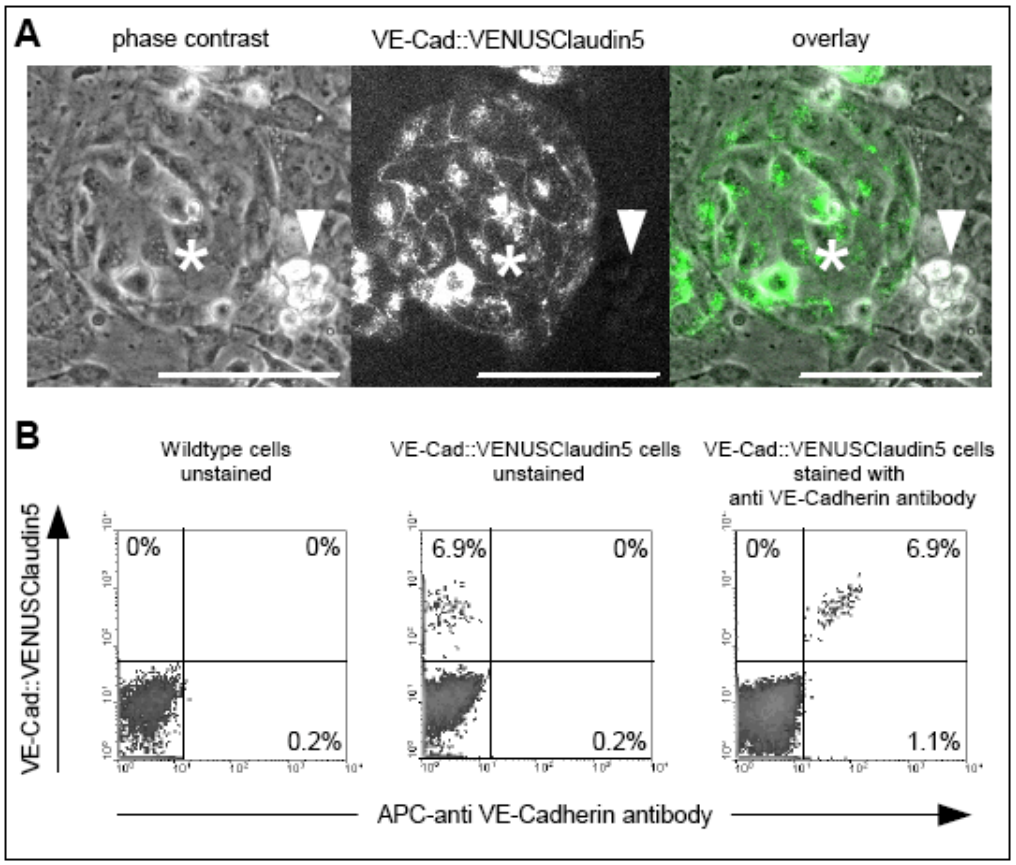

Figure 5.8: Endothelial cells specifically express VE-Cadherin and form tight junctions. A: VE-Cadherin::VENUSClaudin5 ESC-derived mesodermal cells were cultured on OP9 for 4 days. VENUSClaudin5 fusion protein is produced and incorporated into tight junctions by cells with endothelial morphology (asterisk) but not by blood cells (arrowhead). **B:** FACS analysis confirming that cells expressing the VENSClaudin5 fusion also express endogenous VE-Cadherin antigen. As negative controls differentiated unstained wildtype (left panel) and VENUSClaudin5 (middle panel) cells are shown. After culture of VE-Cadherin::VENUSClaudin5 ESC-derived mesodermal cells for 2.5 days, cells were stained with an antibody detecting VE-Cadherin (right panel).
Scale bar: 100 µm.

5.3.2. Proof of blood cell character by immunostaining in the living culture

5.3.2.1. In culture immunostaining against blood surface markers specifically labels free-floating blood cells

Blood cells can be identified by the specific expression of surface markers. The distinction of different blood cell types by the expression of certain surface molecules is routinely used in basic research as well as in clinical diagnostics to characterize and purify different populations from a mixture of cells (Nakano et al., 1994; 1996; Choi et al., 1998; Fraser et al., 2002a; Fehling et al., 2003).

To ensure that free-floating cells arising from endothelium are in fact blood cells we took advantage of the well characterized profile of surface marker expression on ESC-derived blood cells (Fraser et al., 2002a). As it is necessary to identify free-floating cells after they have been stained, staining in the movie is crucial to maintain the architecture of the culture and the position of individual blood cells. To prevent the loss of identity or the difficulties of identifying single cells but to make antigens visible while performing time-lapse imaging we tested whether the addition of fluorescently-labelled antibody to the culture medium of the living culture can specifically stain cells without affecting the differentiation procedure.

Initial experiments in differentiation cultures were performed with a labelled anti-CD45 antibody, because it recognizes the pan-hematopoietic surface molecule CD45. This resulted in brightly stained cells within 30 minutes after the addition of diluted PE-anti-CD45 antibody. Cells that were previously named blood cells according to their free-floating morphology, brightly stained for CD45 (Figure 5.9 A). The addition of PE-labelled isotype-matched control antibodies to the living culture did not bind to any cell within the culture (data not shown) thereby showing antibody specificity.

To confirm that the staining in the culture medium at 37°C over days is still highly specific, cells were flushed off the stromal layer and only stained with an anti-Mac1 antibody recognizing cells of the monocytic-granulocytic lineage (Springer, 1971). As shown in Figure 5.9 B most cells detectable with CD45 stained in culture co-express Mac1 antigen thereby showing that in culture immunostaining against CD45 stains blood cells of the myeloid lineage.

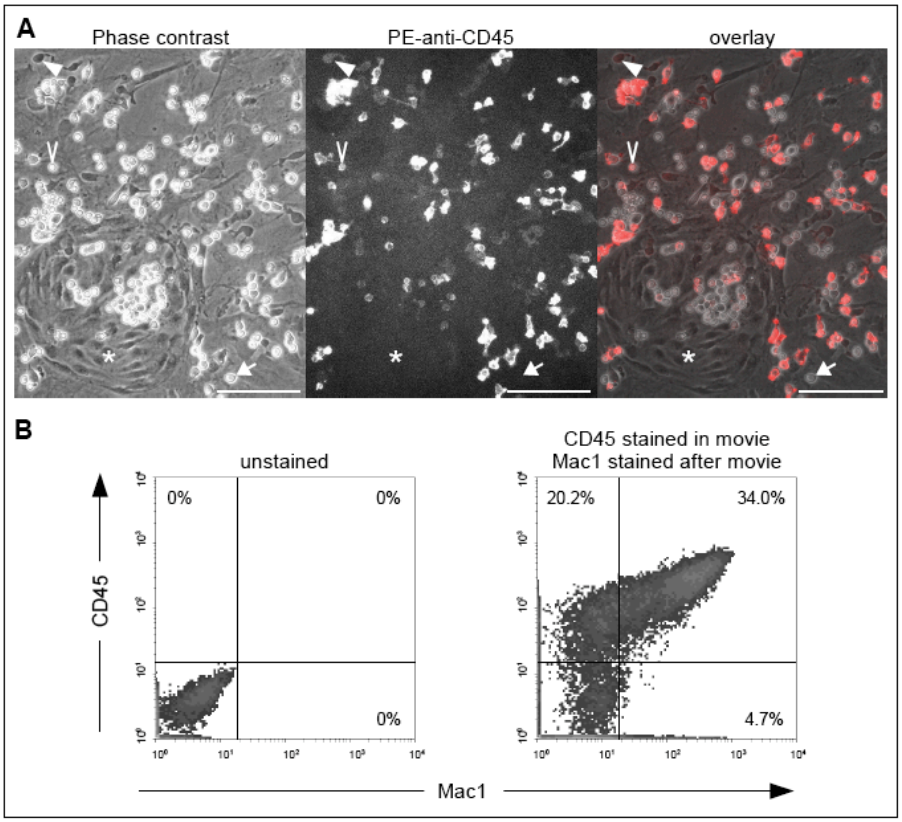

Figure 5.9: In culture immunostaining against CD45 specifically labels hematopoietic cells. ESC-derived Flk1^{+}E-Cadherin^{-} cells were cultivated in the presence of a PE (A) or APC (B) labelled anti-CD45 antibody. **A:** Anti-CD45 antibody added to the living culture can specifically bind and label cells with free-floating (open arrowhead) or adherent (closed arrowhead) hematopoietic morphology although not all cells to be expected to be blood cells are stained (arrow). Cells contained in a colony with endothelial sheet morphology cannot be detected by the antibody (asterisk) **B:** The majority of CD45^{+} living blood cells co-express the myeloid hematopoietic marker Mac1 at day 8.5 of ESC differentiation. At day 8.5, two days after endothelial cell to blood cell transitions occurred, suspension cells were flushed off. They were stained only with a PE-Cy7-anti-Mac1 antibody and analyzed by FACS. Propidium iodide negative, living cells are shown.
Scale bar: 100 µm.

Preliminary experiments to test efficiency, specificity and concentrations were successfully performed with PE- or APC- conjugated anti-CD45, PE- or FITC-anti-CD41, PE- and FITC-anti-Mac1, FITC-anti-PECAM-1, PE-anti-Tie2 and APC-anti-c-kit antibodies. Their titration revealed that the best signal to noise ratio could be obtained if all antibodies were used in a final concentration of 20 ng/ml or 100 ng/ml for anti-c-kit.

Not all antibodies tested were found to be suitable for in culture immunostainings. Ter119 antibody used to distinguish erythroid cells from other blood cells did not successfully stain cells in culture even if high concentrations or different fluorescent dyes (PE or APC) were used.

Antibody-containing cultures were kept for several days (up to 7 tested) the staining was still present and specific. Furthermore, it could be excluded that the addition of antibody is increasing cell death, influencing the proliferation behaviour or affecting the differentiation process.

5.3.2.2. In culture immunostaining does not label dead cells

Besides living blood cells dead cells of different origins can be found in the supernatant of differentiation cultures. The possibility of antibody binding by dead free-floating cells could be eliminated by analysing movies of differentiation cultures containing anti-CD45 antibody in the culture. Live cells which are dividing and actively moving were compared to passively floating cells in their ability to bind anti-CD45 antibody (Table 5.2). Of more than 1100 dead cells counted only 1 was positively stained. This shows that the antibody does not bind to dead cells.

On the other hand 1601 of 2154 living suspension cells analyzed expressed the surface marker CD45. $CD45^-$ living suspension cells are probably blood cells as well. The lack of a CD45 signal hints to unequal antibody binding capacity of different blood cell types or the presence of $CD45^-$ cells belonging the erythroid lineage (Kina et al., 2000). However, to stringently read-out the hematopoietic identity of endothelium-derived suspension cells, the analysis was restricted to CD45 expressing cells in following experiments.

According to the results obtained from experiments addressing the specificity of DiI-Ac-LDL uptake, VE-Cadherin expression and tight junctions formation, living suspension cells never expressed the endothelial markers VE-Cadherin::VENUSClaudin5 and/or DiI-Ac-LDL.

Table 5.2: In culture immunostaining against CD45 only labels living free-floating cells. VE-Cadherin::VENUSClaudin5 ESC-derived mesodermal cells were cultivated on OP9 in medium containing DiI-Ac-LDL and APC-anti-CD45 antibody while recording the movie. Staining of cells was evaluated on day 9 of ESC differentiation. Living cells were identified by the following criteria: morphology, proliferation and active movement in the movie.

Phenotype of cells analyzed	Cells / total number analyzed
VE-Cadherin::VENUSClaudin5$^+$ and/or DiI-Ac-LDL$^+$ live suspension cells	0 / 2154
$CD45^+$ endothelial cells in sheets (adherent)	0 / ~ 7000
$CD45^+$ live suspension cells	1601 / 2154
$CD45^+$ dead suspension cells	1 / 1139

5.3.2.3. In culture immunostaining against blood-surface markers does not label endothelial cells

To extend the analysis of specific antibody binding in culture time-lapse experiments of cultures containing all endothelial markers (VE-Cadherin::VENUSClaudin5, DiI-Ac-LDL) and anti-CD45 antibody were performed.

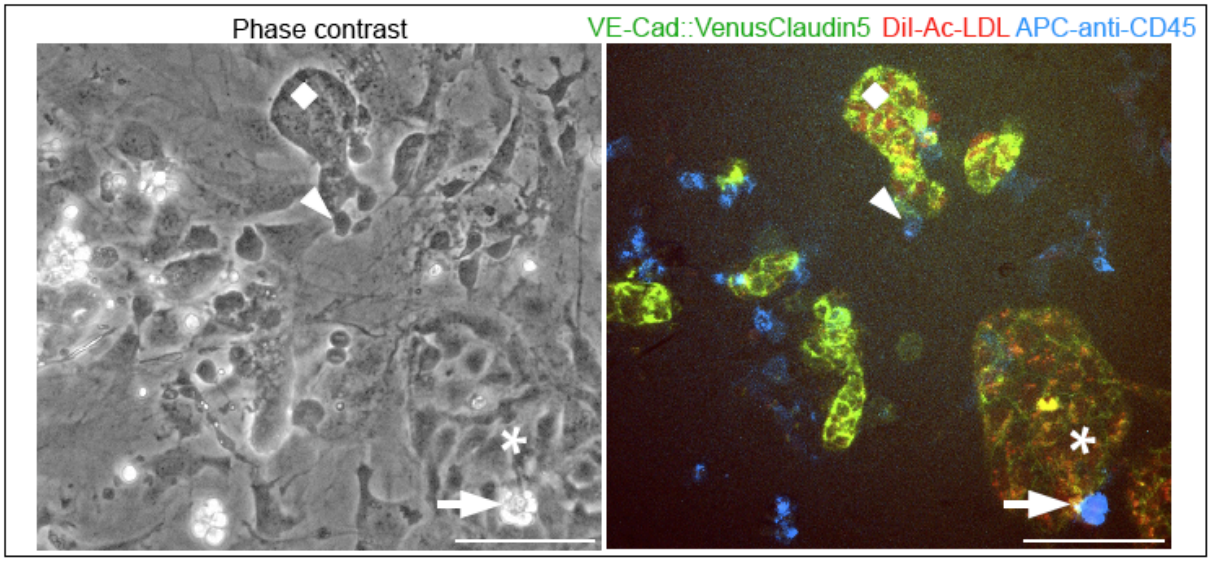

Figure 5.10: Identification of endothelial and hematopoietic cells by molecular markers in living ESC-derived cultures. VE-Cadherin::VENUSClaudin5 ESC-derived mesodermal cells were cultivated on OP9 in the presence of DiI-Ac-LDL and APC-anti-CD45 labelled antibody while recording a movie. Part (10%) of one frame of a movie at day 8.5 (end of endothelial to blood cell transition) of ESC-differentiation is shown. Left panel: phase contrast; right panel: overlay of all images recorded in fluorescent wavelengths. The arrow points to $CD45^+$ suspension blood cells, the arrowhead to one $CD45^+$ adherent blood cell. The asterisk depicts an endothelial sheet colony expressing endothelial markers, the diamond a colony showing molecular endothelial characteristics but no typical sheet morphology. Only living adherent cells expressed endothelial markers. All $CD45^+$ cells were alive, as evidenced by their proliferation in the movie. No living and free floating cells expressing endothelial specific markers were observed.
Scale bar: 100 µm.

In these movies, there were no $CD45^+$ endothelial cells embedded into endothelial sheet colonies. Vice versa, no suspension cell expressing endothelial markers could be identified (Figure 5.10). This shows that all described markers for the distinction of endothelial and blood cells can be employed in one imaging approach.

5.4. Hemogenic endothelium generates blood: proof of cell identities by the simultaneous use of multiple markers in time lapse experiments

Since VE-Cadherin::VENUSClaudin5 ESCs, the addition of DiI-Ac-LDL and fluorescent labelled anti-CD45 antibody to living cultures were successfully employed in time-lapse movies, we combined all three molecular and functional markers into one imaging approach. We imaged the appearance of blood from endothelium derived from VE-Cadherin::VENUSClaudin5 transgenic ES cells in the presence of DiI-Ac-LDL and APC-anti-CD45 antibody.

In Figure 5.11 (and Supplementary Movie 5.3) a blood producing endothelial colony of monoclonal origin and the corresponding lineage tree are depicted. The endothelial cell adopted an endothelial morphology, expresses VE-Cadherin dependent VENUSClaudin5 fusion protein, forms tight junctions with adjacent cells and enriches DiI-Ac-LDL to detectable levels as soon as the cell is semi-adherent (Figure 5.11 first row). At this stage, it is not yet expressing the blood specific antigen CD45, thereby excluding the possibility of the existence of a bi-potent blood and endothelial precursor expressing molecules of both lineages (Figure 5.11 second row). As soon as the endothelial cell detaches from the sheet and transforms into free-floating cells, the endothelial markers VE-Cadherin::VENUSClaudin5 and DiI-Ac-LDL labelling are already undetectable (Figure 5.11 third row). Shortly after, anti-CD45 signals slowly increase on nascent blood cells and become detectable approximately three hours after cells are free-floating. These $CD45^+$ cells (Figure 5.11 fourth row) keep proliferating as soon as they detach from the endothelial sheet which ensures that the endothelium-derived free-floating cells are definitely living blood cells.

These data prove not only by morphology but also by the expression of several specific molecular markers that endothelial cells can produce blood cells.

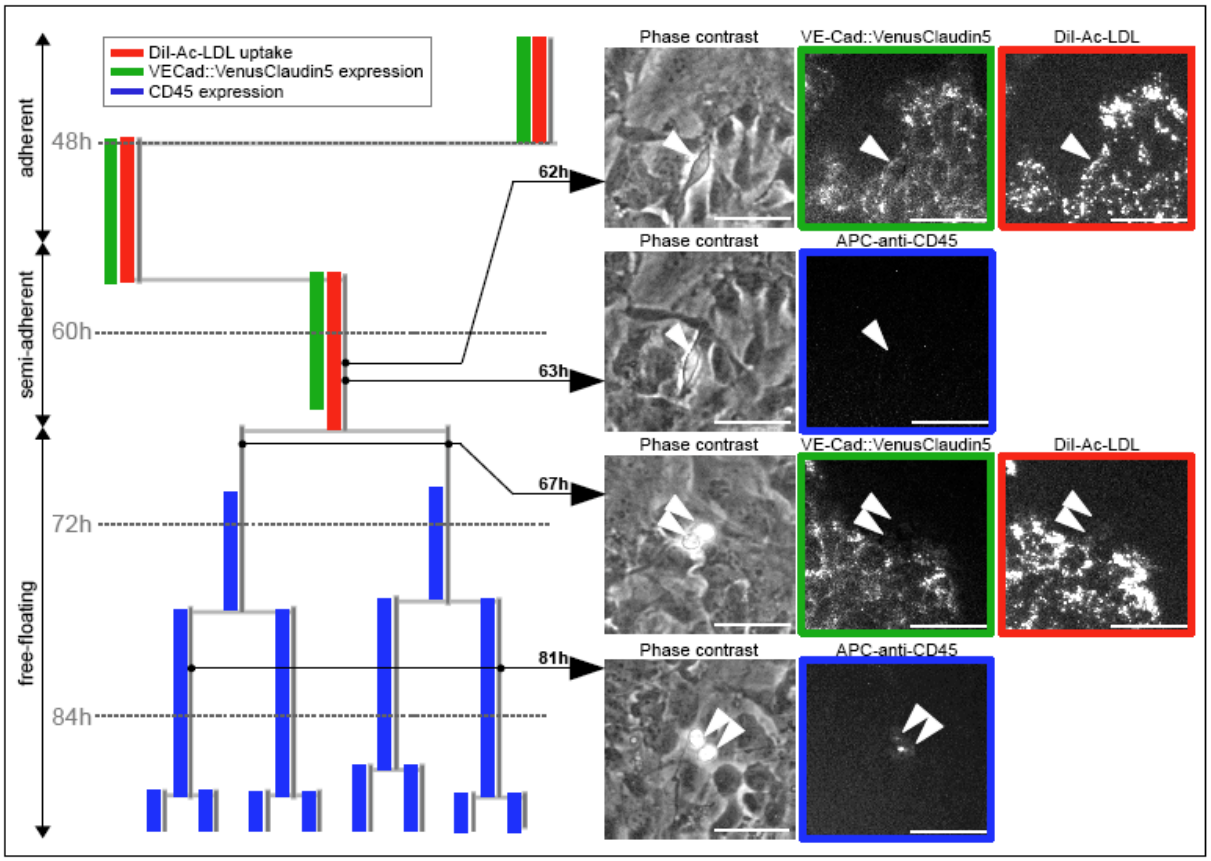

Figure 5.11: Identification of endothelial and blood cells by continuous analysis of functional and molecular markers. Part of a pedigree depicting marker expression during hemogenic transition of an EC is shown on the left side. Hours after the start of the movie at day 4 of ESC differentiation are indicated. Coloured lines to left of the tree depict marker expression. In the shown hemogenic EC, VE-Cadherin::VenusClaudin5 expression and tight junction formation was detectable 18 h, and DiI-Ac-LDL uptake 40 h after the start of the movie (not shown). A hemogenic endothelial cell expresses VE-Cadherin, incorporates VENUSClaudin5 into tight junctions and takes up DiI-Ac-LDL (62h).The endothelial cell is negative for CD45 expression (63h). As a positive control for functional anti CD45 staining, other $CD45^+$ stained cells are visible at the same time point in Supplementary Movie 5.3. Emerging blood cells lose VE-Cadherin::VENUSClaudin5 expression and DiI-Ac-LDL uptake (67h). The generated blood cells express CD45 (81h). Arrowheads indicate the cells shown in the images.
Scale bar: 50 µm.

5.5. Non-sheet endothelial colonies producing blood cells can be observed in time lapse movies

Previous studies performed by others (Hirashima et al., 1999; Matsumura et al., 2003; Hashimoto et al., 2007) have described two types of $Flk1^+$ or VE-$Cadherin^+$ colonies having distinct phase contrast morphologies: sheet-like colonies consisting of flat cells with prominent nucleus forming circular clusters and cord-like colonies, showing an elongated colony morphology consisting of densely packed cells with less unique appearance. In line with these studies the use of VE-Cadherin expression, tight junction

formation and DiI-Ac-LDL uptake in time-lapse experiments identified non-sheet endothelial colonies in this study. Most colonies express VE-Cadherin, form tight junctions and take up DiI-Ac-LDL (Table 5.3). Of these, only 29.5% are sheet-like endothelial colonies and 67.3% are of cord-like morphology. Figure 5.12 shows an image taken from a movie highlighting two VE-Cadherin::VENUSClaudin5^{+}DiI-Ac-LDL^{+} colonies from both different morphological appearances.

Table 5.3: Cells expressing VENUSClaudin5 and take up DiI-Ac-LDL can produce blood cells. Flk1^{+}E-Cadherin^{-} ESC-derived cells were differentiated on OP9 and imaged by time lapse microscopy. Monoclonal colonies were evaluated for the presence of endothelial characteristics. More than 770 colonies from 5 independent experiments were counted. The combination of all markers including clear sheet colony morphology allows the highest stringency for EC colony detection. Only colony types highlighted in grey were observed to produce blood.

Endothelial marker	**Presence (+) or Absence (-) of marker**						
VE-Cadherin::VENUSClaudin5	-	-	-	+	+	+	+
DiI-Ac-LDL	+	-	+	-	-	+	+
Sheet morphology	-	+	+	+	-	+	-
% Colonies of all colonies with endothelial characteristics	0	0	0	0.5	2.7	29.5	67.3

In our movies we only observed endothelial colonies that are VE-Cadherin::VENUSClaudin5^{+} and in addition take up DiI-Ac-LDL to produce blood cells (Table 5.3). Quantifications of 5 independently generated movies lead to the conclusion that both VE-Cadherin::VENUSClaudin5^{+}DiI-Ac-LDL^{+} colonies with sheet-like and with cord-like morphology have approximately the same ability to give rise to blood cells: 6.5% and 4.8% of sheet-like and cord-like endothelial colonies produce blood cells, respectively. We further compared both colony types to understand whether the blood cell generation is regulated by an identical mechanism.

In Figure 5.13 A and Supplementary movie 5.4 one cord-like endothelial colony is shown expressing VE-Cadherin::VENUSClaudin5 and DiI-Ac-LDL (first panel). At this time-point, no cell expresses the hematopoietic cell antigen CD45 (second panel). Upon detaching from the endothelial colony the blood cell cluster present in the surrounding area does not express endothelial markers anymore (third panel), but up-regulates CD45 shortly after (fourth panel).

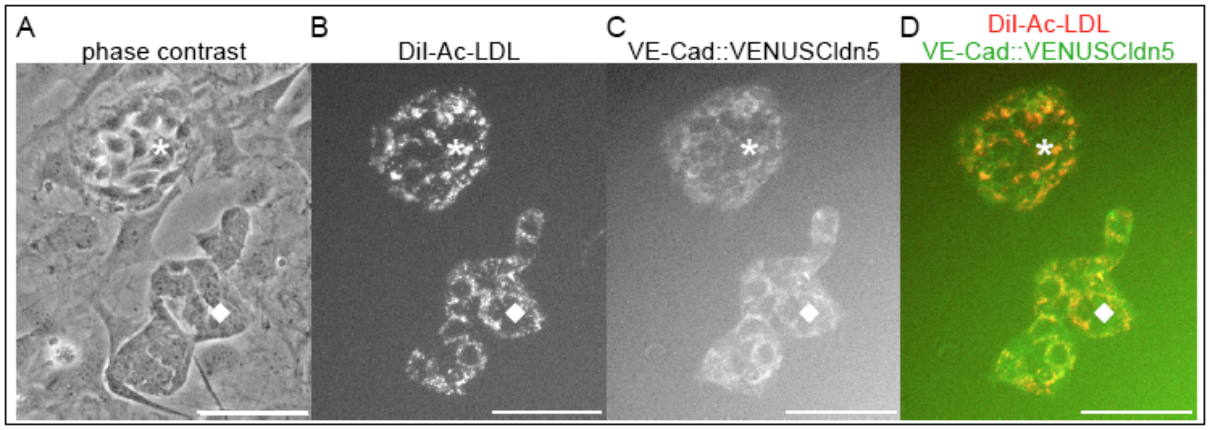

Figure 5.12 Colonies displaying molecular and functional endothelial characteristics can appear in different morphologies. VE-Cadherin::VENUSClaudin5 transgenic ESC-derived mesodermal cells were cultured on OP9 for 3 days in the presence of DiI-Ac-LDL. Two colonies of monoclonal origin are shown that take up DiI-Ac-LDL **(B)**, express VE-Cadherin and form tight junctions **(C)**. An overlay of B and C is depicted in **D**. As shown in **A**, these colonies have different morphologies: sheet-like (asterisk) and cord-like (diamond).
Scale bar: 100 µm.

The comparison of 9 endothelial to blood transitions from cord-like endothelial cells revealed that the nascent blood cells leave the endothelial colonies either as adherent or as suspension cells but neither of them is going through a semi-adherent phase. However, onset of VE-Cadherin expression, tight junctions formation, DiI-Ac-LDL incorporation as well as endothelial transition into blood (first detection of free-floating blood cells evaluated in cord-like endothelial colonies was compared to onset of semi-adherence in hemogenic sheet-like endothelial colonies; Figure 5.13 B) took place within the identical time window and same kinetics as in the sheet-like endothelial colonies. This suggests that the basic mechanism of blood cell production from endothelial cells is identical in both colony types. The lack of a semi-adherent state rather seems to be unnecessary for endothelium-derived blood cells to adopt their hematopoietic fate. Instead, semi-adherence is probably closely linked to the expression of certain surface molecules expressed on sheet-like rather than on cord-like endothelial cells.

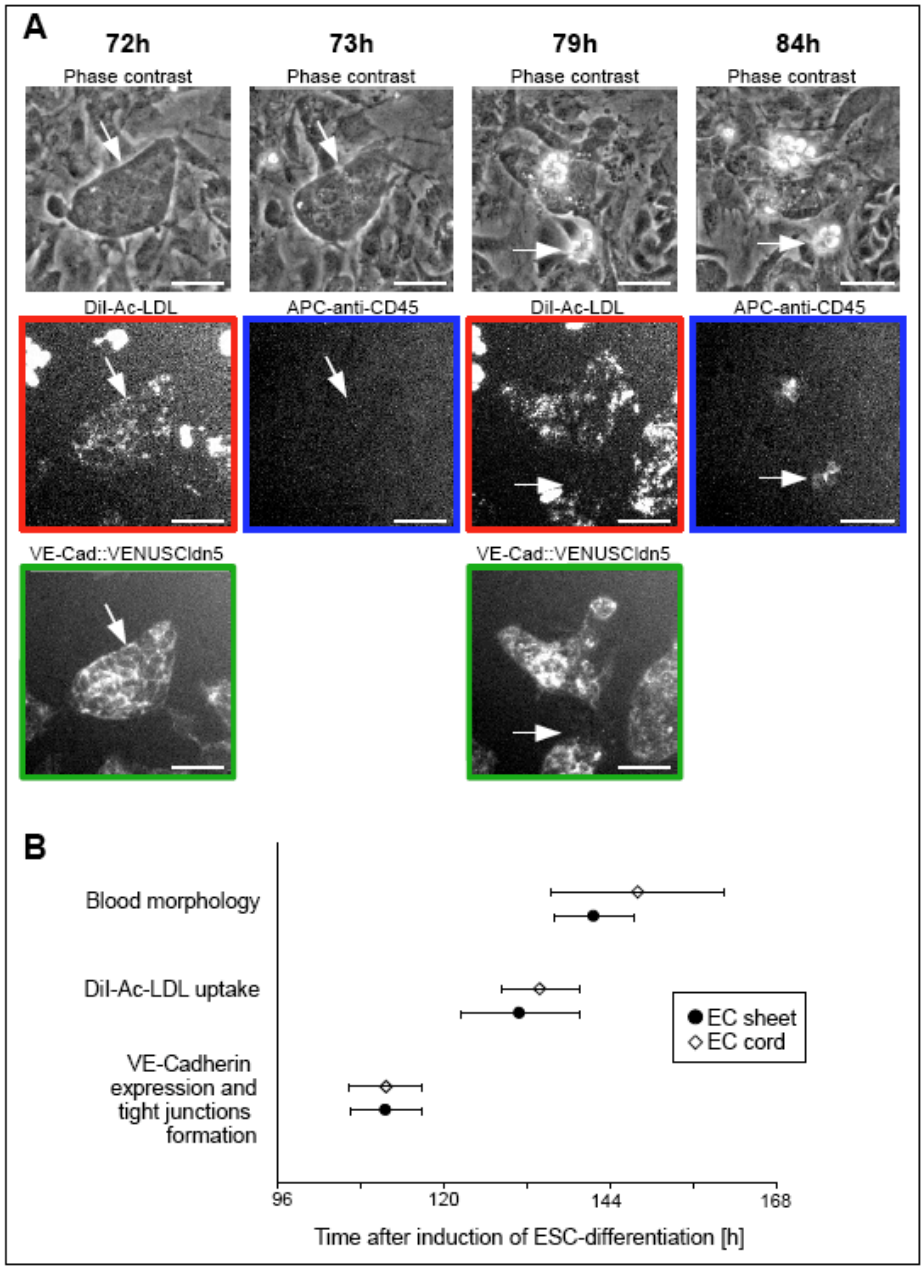

Figure 5.13: Cord-like endothelial colonies can produce hemogenic endothelial cells with identical onset of VE-Cadherin::VENUSClaudin5 expression, tight junction formation, DiI-Ac-LDL uptake and transition into the hematopoietic state. A movie of VE-Cadherin::VENUSClaudin5 transgenic ESC-derived mesodermal cells cultivated with DiI-Ac-LDL and APC-anti-CD45 antibody was recorded. **A:** Images extracted from a movie showing the production of blood cells from a cord-like endothelial colony. A cord-like endothelial colony expressing VE-Cadherin, forming tight junctions and taking up DiI-Ac-LDL (72h) does not express CD45 (73h) and produces free-floating cells that have lost the molecular and functional endothelial characteristics (79h) but have acquired CD45 expression after 84h. **B:** Range of onset of marker expression in sheet- and cord-like endothelial cells producing blood. Time after induction of ESC-differentiation is indicated. 9 cord-like and 9 sheet-like endothelial colonies from 2 independent experiments were analyzed. As "blood morphology" the detection of free-floating blood cells generated from cord-like endothelium was compared to the onset of semi-adherence in hemogenic endothelial sheet colonies. As visible, sheet-like and cord-like endothelial colonies do not distinguish significantly in respect to onset of blood morphology ($p=0.99$), DiI-Ac-LDL uptake ($p=0.20$) and VE-Cadherin::VENUSClaudin5 expression ($p=0.41$).

5.6. Characterization of hemogenic endothelial cells: hemogenic endothelial cells express CD41, c-kit and Tie2

5.6.1. Hemogenic endothelial cells and nascent blood cells express CD41

Despite its specific expression on megakaryocytes and platelets in adult mice, CD41 is also expressed on emerging blood cells in yolk sac (YS) and the aorta-gonads-mesonephros (AGM) region (Emambokus et al., 2003). Others have postulated that CD41 expression in E8.25 YS cells determines the commitment to the hematopoietic lineage and that CD41 is the first marker to define the divergence of hematopoiesis from putative hemogenic endothelium during development (Mikkola et al., 2003; Li et al., 2005). Bertrand et al. found that hematopoietic progenitors identified in the sub-aortic patches at day 10.5 of mouse embryogenesis rather express CD41 than CD45 pointing to the possibility that CD41 is present on early hematopoietic cells before CD45 becomes up-regulated (Bertrand et al., 2005).

To prove that the *in vitro* observed endothelial to blood cell transitions correspond to the *in vivo* situation, we wanted to test whether CD41 can be detected with the same expression pattern in the movies of ESC-derived differentiation cultures as for *in vivo* studies.

By imaging the differentiation of VE-Cadherin::VENUSClaudin5 ESC-derived mesodermal cells in the presence of differently labelled anti-CD41 and anti-CD45 antibodies the kinetic appearance and subsidence of marker expression was investigated in endothelial to blood cell transitions.

A blood cell producing monoclonal endothelial colony is depicted in Figure 5.14 and Supplemental Movie 5.5. Starting at a time-point in which individual cells could be identified within the endothelial sheet colony single cell tracking of one hemogenic endothelial cell was performed which is depicted in the corresponding lineage tree. We discovered that a colony of monoclonal origin acquires the typical endothelial sheet morphology after expressing of VE-Cadherin and forming tight junctions (Figure 5.14, first row). Simultaneously these cells adopt an endothelial morphology with some VE-Cadherin$^+$ tight junctions forming cells specifically binding anti-CD41 antibody (Figure 5.14, second row). Consequently, all CD41$^+$ cells become semi-adherent and down-regulate VE-Cadherin::VENUSClaudin5 (Figure 5.14, third row). CD41 expression is maintained and shortly after the cells become free-floating CD45$^+$ (Figure 5.14, fourth row).

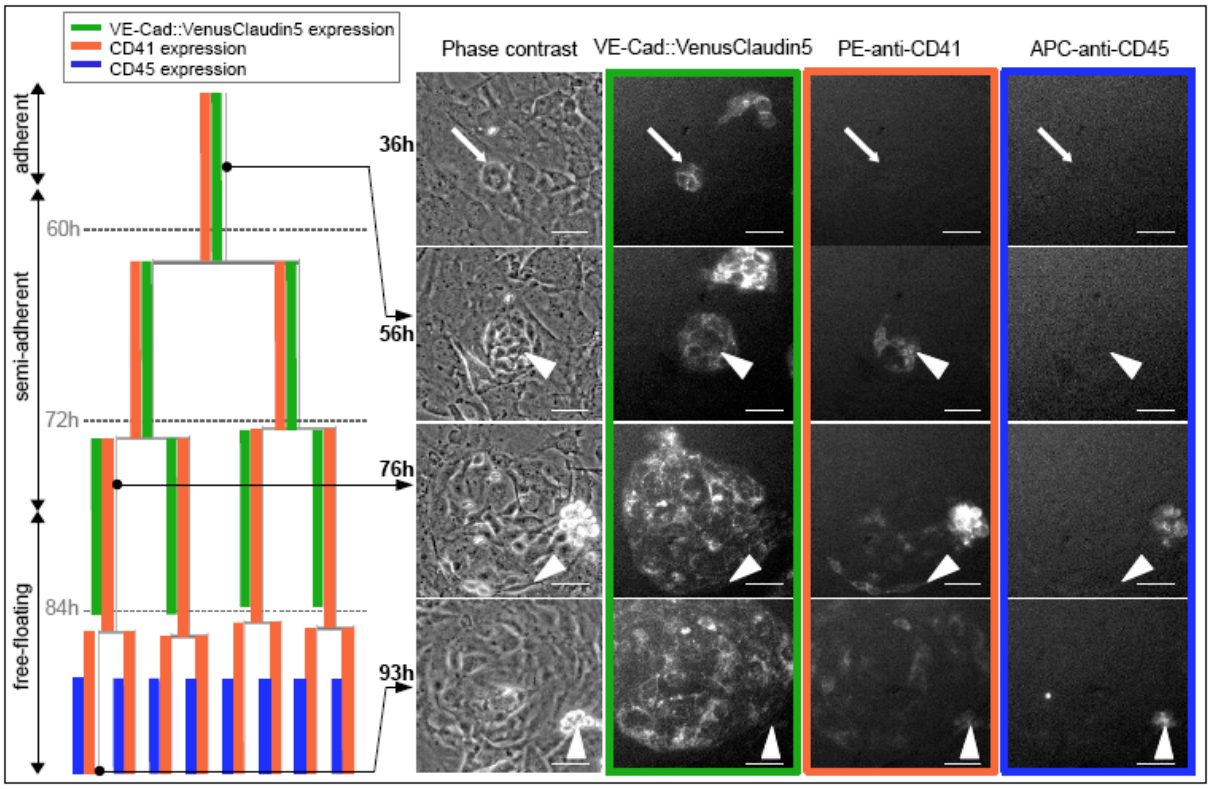

Figure 5.14: Nascent blood cells generated by endothelial cells express CD41 before CD45. Part of a pedigree depicting marker expression during hemogenic transition of an EC is shown (compare Supplementary Video 5.5). A monoclonal endothelial colony expressing VE-Cadherin::VENUSClaudin5, but not the hematopoietic cell markers CD41 or CD45 was observed in the movie (36 h, arrow). Single cell tracking was started at the time-point when individual cells with sheet EC morphology could be identified. Some VE-Cadherin::VENUSClaudin5$^+$ cells with sheet EC morphology start expressing CD41 (56 h). All CD41$^+$ cells develop into blood cells (76 h) as identified by CD45 staining after they have become free-floating (93 h).
Scale bar: 50 µm.

Our results show at the single cell level that the expression of CD41 is identical to the results obtained in *in vivo* studies. Clearly, the CD41 antibody only labelled endothelial cells that were already committed to the hematopoietic lineage whereas endothelial cells devoid of hemogenic potential never expressed CD41 antigen. It could therefore be confirmed that CD41 is a very early hematopoietic marker being detectable on nascent blood cells and on endothelial cells having committed to the hematopoietic fate already. In addition, we could show that CD41 bridges the gap between the hemogenic endothelial state and the CD45$^+$ blood cell state at the single cell level as it was suggested in previous studies (Bertrand et al., 2005).

5.6.2. Hemogenic endothelial cells express c-kit and Tie2

In order to more clearly define the hemogenic endothelial cells, their expression of c-kit and Tie2 was analyzed in time lapse experiments. In addition to adult hematopoietic stem cells and progenitors, c-kit is expressed on embryonic blood cells (Okada et al., 1993; Bernex et al., 1996) and functions as the receptor for SCF (Lyman et al., 1998). In addition to its blood-specific expression, c-kit was identified on some endothelial cells lining hematopoietic cell clusters in the omphalomesenteric and umbilical arteries of E9.5 embryos – locations where endothelial cell to blood cell transitions might occur (Yoshida et al., 1998). Similarly, a crucial role for Tie2 was postulated for the quiescence of adult HSCs (Arai et al., 2004). Identical to c-kit, Tie2 was discovered on hematopoietic and endothelial cells in the omphalomesenteric artery of E9.5 embryos as well as in the vascular network of E8.25 yolk sacs (Takakura et al., 1998; Li et al., 2005; Ema et al., 2006). Therefore, c-kit and Tie2 might have a role in the hematopoietic transition of endothelial cells. Consequently, their expression was analyzed during the transition process.

Although initial experiments showed that, in contrast to Tie2, the c-kit antibody is only detectable very weakly in the living culture, requiring a higher antibody concentration as usual, the anti-c-kit antibody could be successfully employed in the living culture without manipulating it. Tie2 was detected very brightly and seemed to be strongest in intracellular vesicles containing the engulfed receptor and antibody.

As shown in Figure 5.15 (first row of images) and in Supplementary Movie 5.6, $Tie2^+$ cells are observed from day 4.5 of ESC-differentiation. The anti-Tie2 antibody brightly labels all cells contained in the hemogenic endothelial sheet colony. Hemogenic endothelial cells become semi-adherent, maintain the Tie2 expression and weakly started to express c-kit (Figure 5.15 middle row). In contrast, non-hemogenic endothelial cells are c-kit negative. While c-kit expression increased on hemogenic endothelial and nascent blood cells during the transition, Tie2 was either down-regulated on several free-floating blood cells (Figure 5.15 last row) or expressed further on.

The level of Tie2 expression was the same in ten hemogenic endothelial colonies observed in three independent experiments. Since Tie2 is detectable in constant amounts on hemogenic, non-hemogenic and eventually on nascent blood cells, this suggests that Tie2 is not a critical regulator of endothelial to blood cell transitions. In contrast, c-kit was only detected on hemogenic endothelial and nascent blood cells. Thus, c-kit marks hemogenic commitment but also does not regulate endothelial cell to

blood cell transitions, since its expression begins as soon as the endothelial cell has already committed to the hemogenic fate. However, it is possible that c-kit is expressed at lower levels in a larger population or at different time points since the anti-c-kit antibody is only detected very weakly and potentially under the detection level.

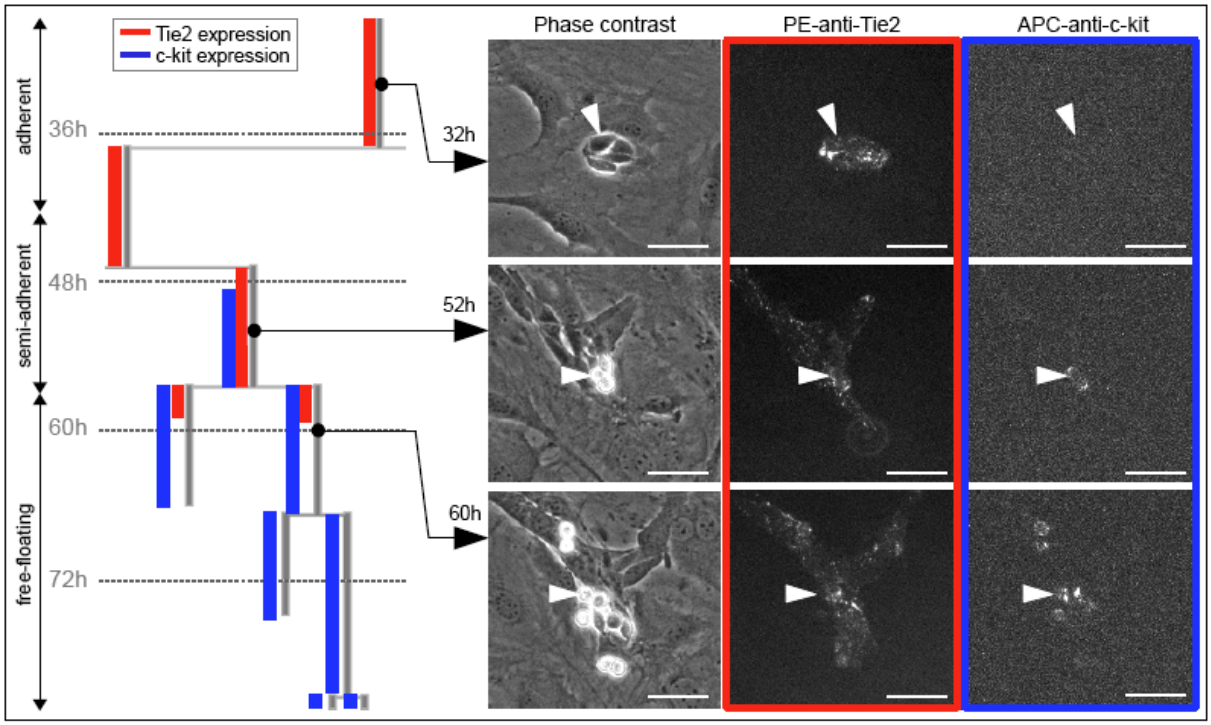

Figure 5.15: Characterization of hemogenic endothelial cells by the expression of the surface markers c-kit and Tie2. A part of a pedigree is shown on the left side depicting marker expression during hemogenic transition of an EC. Hours after start of the movie at day 4 of ESC-differentiation are indicated. Coloured lines to the left of the tree highlight marker expression. In the shown colony of monoclonal origin an endothelial cell expressing Tie2 surface marker is depicted (32h, arrowhead). Its daughter gives rise to a semi-adherent hemogenic endothelial cell that expresses Tie2 and c-kit (52h, arrowhead). Emerging blood cells are strongly positive for c-kit and partly down-regulate Tie2 (60h, arrowhead).
Scale bar: 50 µm.

5.7. Endothelium-derived blood cells can express the myeloid blood cell marker Mac1

The use of an additional blood cell specific marker not only confirms the hematopoietic identity of the CD45$^+$ suspension cells but also proves that endothelial cells have the capacity to produce lineage restricted blood cells. Although lymphoid cells develop from ES cells in the OP9 co-culture they are not detectable until day 14 of ESC-differentiation (Nakano et al., 1994). In contrast, myeloid cells expressing Mac1 can be detected from day 7 on (Fraser et al., 2002a). Thus, Mac1 is one of the first lineage markers to be

detectable on blood cells in ESC-derived cultures. Therefore, we tested whether blood cells produced by endothelium can express Mac1.

A time-lapse experiment of a differentiation culture containing a mixture of differently labelled anti-Mac1, anti-CD41 and anti-CD45 antibodies was performed and analyzed at the single cell level. An endothelial colony producing blood is depicted in Figure 5.16 and Supplementary Movie 5.7.

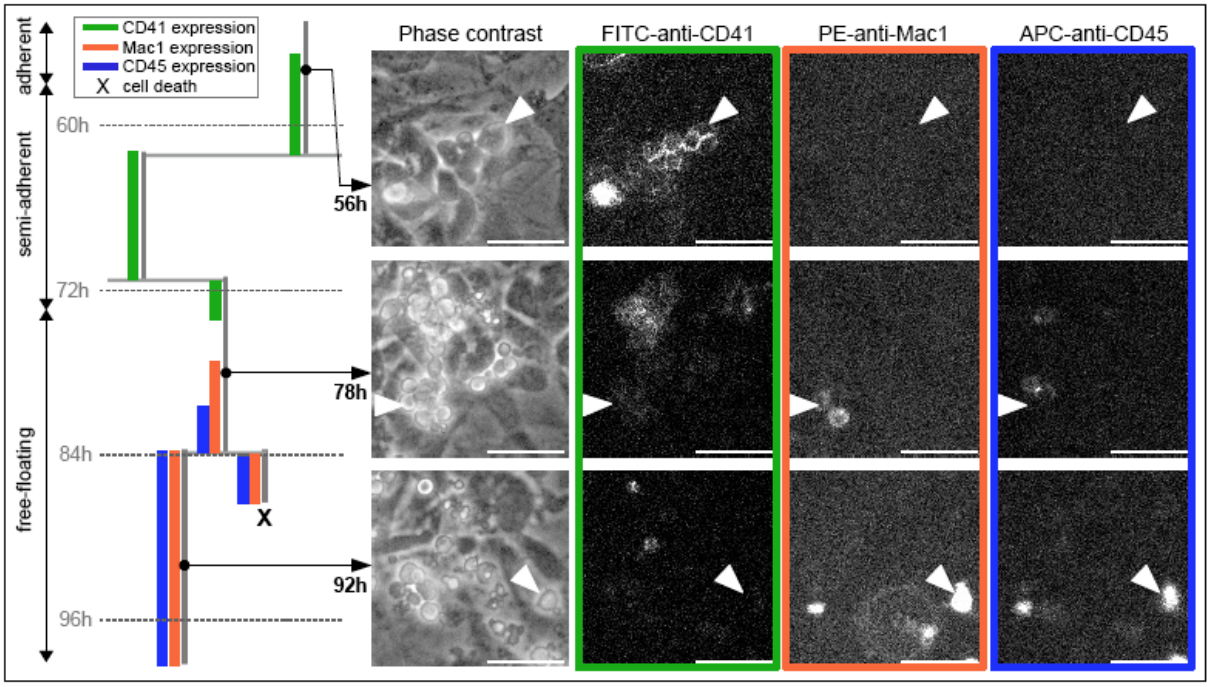

Figure 5.16: Hemogenic endothelial cells can give rise to Mac1$^+$ cells. ESC-derived mesodermal cells were cultivated in the presence of FITC-anti-CD41, APC-anti-CD45 and PE-anti-Mac1 antibodies. Time after start of the movie is indicated. Arrowheads point to the cell depicted in the pedigree on the left. One endothelium-derived CD41$^+$ blood cell does not yet express CD45 and Mac1 (56h). One of its daughters up-regulates Mac1 (78h) shortly before CD45 is detectable. 92h after start of the movie CD45 and Mac1 but not CD41 are expressed on its descendant.
Scale bar: 50 µm.

Initially, one CD41 expressing nascent blood cell develops from endothelium (Fig 5.16 first row). As its daughter becomes semi-adherent, CD41 is down-regulated as it is no longer detectable by antibody staining anymore. Shortly after, Mac1 expression starts with CD45 soon after (Fig 5.16 second and Fig 5.16 third row, respectively).

This leads to the conclusion that some blood cells co-express CD45 and the myeloid marker Mac1. Comparison of five endothelial to blood cell transitions obtained in two independent experiments revealed that the temporal onset of Mac1 and CD45 expression on individual cells is the same when both are co-expressed. Furthermore, only a portion of all blood cells derived from one endothelial colony expresses Mac1 and therefore belong to the granulocytic-monocytic lineage (Springer, 1971). Thus, one endothelial

colony can give rise to blood cells of the granulocytic-monocytic lineage in addition to hematopoietic cells of different lineages.

5.8. Hemogenic endothelial cells cannot give rise to smooth muscle cells

Although hemogenic endothelial cells expressed several endothelial markers including the morphology, the tight integration into an endothelial sheet, VE-Cadherin expression and DiI-Ac-LDL uptake, it remained possible that they are not endothelium but a multi-potent mesodermal precursor. We therefore tested whether hemogenic endothelial cells are capable of generating cells of mesodermal lineages other than blood and endothelium. As $Flk1^+$ cells can give rise to mural cells (vascular smooth muscle and pericytes) in the OP9 co-culture (Yamashita, J. et al., 2000; Schroeder et al., 2003) we tested whether smooth muscle cells can be produced by hemogenic endothelial cells.

After imaging ESC-derived mesodermal cells with constitutive nuclear label for four days, the culture was fixed and stained with an anti- -smooth muscle actin (α-SMA) antibody (Figure 5.17). In the videos cell fates of all cells within hemogenic endothelial colonies were analyzed at the single cell level (last image of the movie is shown in Figure 5.17 A, left panel) and compared to the end-point staining against α-SMA. Although α-SMA^+ cells were observed in the culture (Figure 5.17 B), their relationship to hemogenic endothelial cells was excluded (Figure 5.17 A, right panel). α-SMA^+ cells were never generated from any non-hemogenic cell within a hemogenic endothelial colony. These findings could be verified in 12 endothelial to blood cell transitions obtained from three independent experiments. In summary this means that hemogenic endothelial colonies do not have the potential to produce smooth muscle cells.

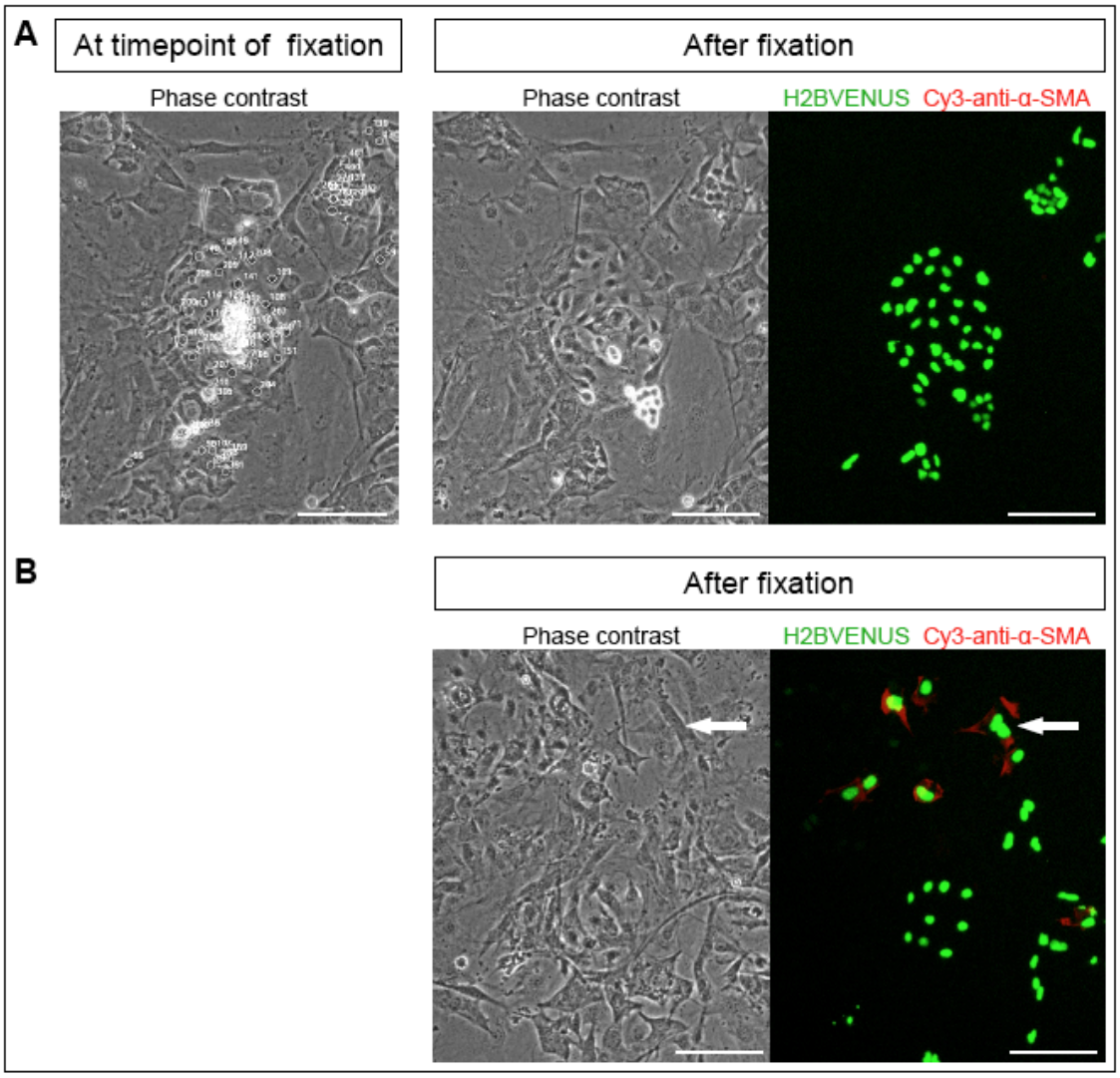

Figure 5.17: ESC-derived hemogenic endothelial cells do not produce smooth muscle cells. A time lapse experiment with ESC-derived H2BVENUS+ mesodermal cells seeded on OP9 was performed. After 4 days the culture was fixed and the existence of smooth muscle cells (α-smooth muscle actin, α-SMA) was verified by immunostaining. While suspension cells can change position, morphology and position of adherent cells is preserved during fixation. Single cell tracking of the hemogenic colony identified all adherent cells derived from the shown colony (A, left panel). Neither of them expresses α-SMA (a, right panel). The presence of α-SMA – positive cells in an adjacent position in the same culture is shown as positive control for smooth muscle cell generation and α-SMA detection under the used experimental conditions (B, arrow).

5.9. Purified mesodermal cells from E7.5 dpc embryos can give rise to hemogenic endothelium

In movies of ESC-derived cultures it was visible that a limited number of endothelial cells can produce blood cells within a clearly defined time window. This transition process includes a phase in which the hemogenic endothelial cells are semi-adherent and stick to the endothelial cells underneath. These results recapitulate *in vitro*, what has been observed *in vivo*: only during a short time frame during embryogenesis hematopoietic clusters stick to the endothelial layer at hematopoietic sites. As was shown here *in vitro*, also *in vivo* CD41 is observed as the first hematopoietic marker expressed before CD45 can be detected (Bertrand et al., 2005). Same holds true for c-kit (Okada et al., 1993; Bernex et al., 1996; Yoshida et al., 1998) and Tie2 (Takakura et al., 1998; Li et al., 2005; Ema et al., 2006). It is therefore very likely that the *in vitro* observed endothelial to blood cell transitions also occur *in vivo*. However, as a final proof for the existence of hemogenic endothelial cells, *ex vivo* time-lapse imaging and single cell tracking of purified embryonic cells was necessary.

Initially, we tested which sorted embryo-derived population could give rise to endothelial and blood cells when cultured on OP9. As described for the ESC-derived cultures, cell identities were evaluated by the formation of sheet colonies and DiI-Ac-LDL uptake (endothelium) as well as suspension cells expressing CD41 (blood). Different embryo-derived populations were tested with respect to this potential. According to previous publications $Flk1^{+}$VE-$Cadherin^{-}$ mesodermal or $Flk1^{+}$VE-$Cadherin^{+}$ endothelial cells may fulfil these criteria (Fraser et al., 2002b). In order to prevent contamination of these cultures with previously generated blood cells which can also express Flk1 (Choi et al., 1998; Yoshida et al., 1998; Drake et al., 2000; Li et al., 2005), $Flk1^{+}CD41^{-}$ cells were sorted and analyzed.

De novo hematopoietic activity is detectable in mouse embryos between E6.5 and E11.5. It is known that hemogenic cells in E6.5 embryos are extremely few (Kinder et al., 1999) which prompted us to use embryos of an older age. Whole embryos of E7.5, E8.5 and E9.5 wild-type mice were sorted by FACS and cultured on OP9 in the presence of SCF.

In four independent experiments it was confirmed that only $CD41^{-}Flk1^{+}$VE-$Cadherin^{-}$ cells from E7.5 embryos were able to produce endothelial sheet colonies and hematopoietic cells within 10 days of culture (data not shown). Even if these are cultivated for longer time, cells from E8.5 and E9.5 embryos were not able to generate endothelial sheet colonies, although free-floating and adherent blood cells as well as DiI-Ac-LDL^{+} cell

clusters were observed in each case. Therefore, only cells from E7.5 embryos have the potential to from endothelial sheets. Since sorted CD41$^-$Flk1$^+$VE-Cadherin$^+$ cells were only occasionally able to produce endothelial sheet colonies (approx. 3 cells per 25000 cells seeded had the potential to form sheet colonies) this population was not used for time-lapse experiments either.

In addition, culturing of sorted cells revealed that the colony forming potential of embryo-derived cells was much lower than compared to ESC-derived cells, probably due to increased apoptotic events of the sensitive embryonic-derived cells. Thus, more cells were seeded onto confluent OP9 cells in the following experiments.

Identical to ESC-derived cultures CD41$^-$Flk1$^+$VE-Cadherin$^-$ cells were sorted into CO_2-enriched ESC-differentiating medium, seeded onto OP9 in the presence of DiI-Ac-LDL as well as FITC-anti-CD41 antibody, and incubated to enrich medium and gas phase ad 5% CO_2 for 1h. After sealing the tissue culture flask tightly the time-lapse video was started.

As depicted in Figure 5.18 and Supplementary Movie 5.8, a cell cluster of monoclonal origin still negative for the markers DiI-Ac-LDL and CD41 (22h) gives rise to an endothelial sheet of which all cells have taken up DiI-Ac-LDL (38h). Some of the cells adopt a semi-adherent morphology and express the hematopoietic marker CD41. The semi-adherent cells detach after some time, down-regulate the DiI-Ac-LDL uptake and are free-floating in the supernatant. Only free-floating cells then brightly express CD41 with the adherent endothelial cell sheet remaining DiI-Ac-LDL$^+$ and CD41$^-$ (55h).

Altogether this shows that *ex vivo* cultured mesodermal cells purified from E7.5 dpc embryos can differentiate into hemogenic endothelium. It is thus very likely that within the developing embryo reside hemogenic endothelial cells.

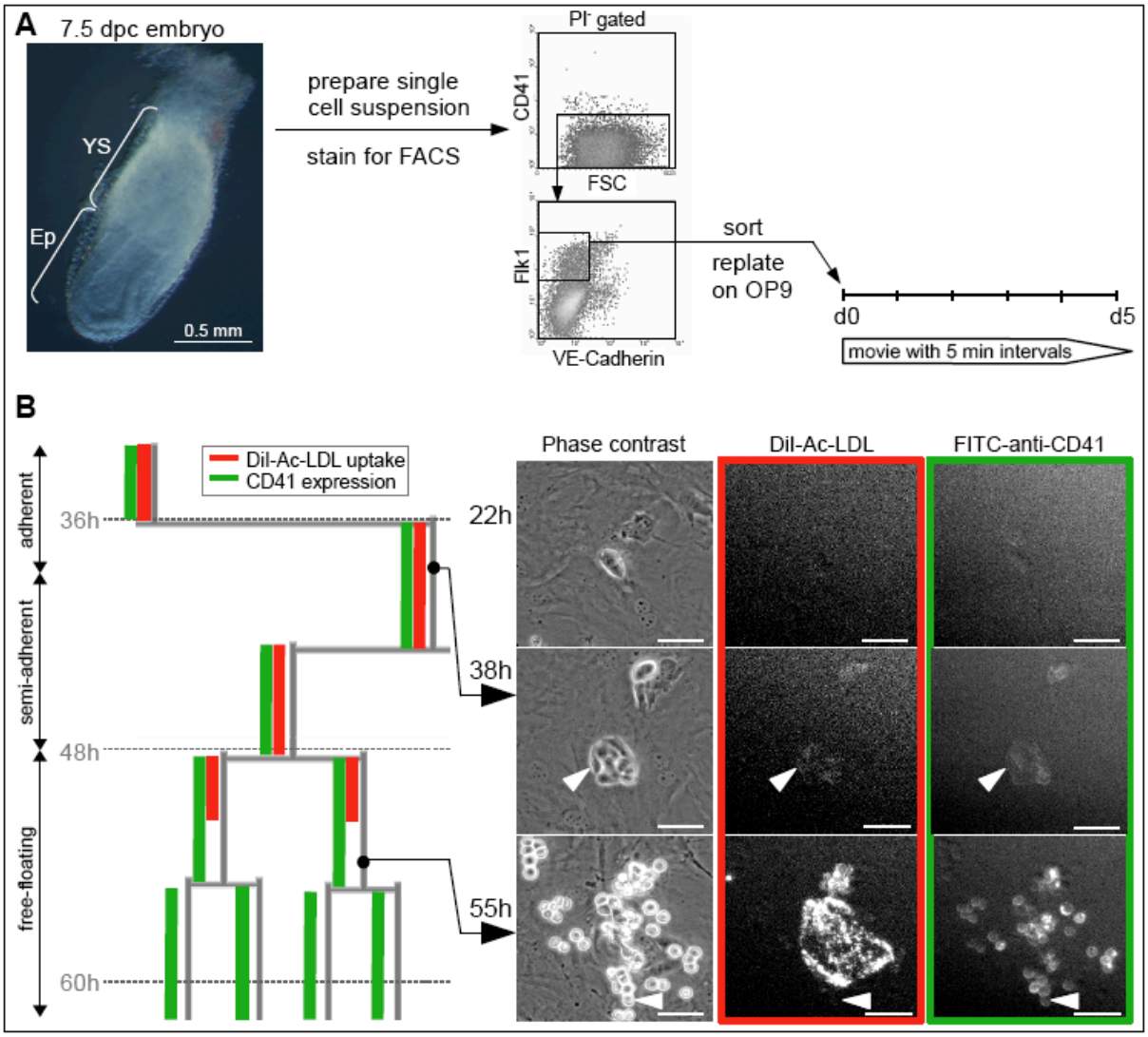

Figure 5.18: Mesodermal cells purified from E7.5 dpc mouse embryos differentiate into blood via an endothelial intermediate. Images extracted from Supplementary Movie 5.8. **A:** E7.5 embryos are dissociated and CD41$^-$Flk1$^+$VE-Cadherin$^-$ mesodermal cells are purified by FACS before they are re-plated on OP9 and recorded by time lapse imaging. Ep: embryo proper, YS yolk sac. **B:** Single cell tracking was started at the time point when individual cells with sheet EC morphology could be identified. Part of a pedigree depicting marker expression during hemogenic transition of an EC is shown. Time after start of the movie is given. A mesodermal colony that not yet expresses endothelial or hematopoietic marker (22h) differentiates into an endothelial colony that takes up DiI-Ac-LDL. Some cells with sheet EC morphology take up DiI-Ac-LDL and start expressing CD41 (38 h). Upon their differentiation into suspension cells they strongly express the hematopoietic cell marker CD41$^+$.
Scale bar: 50 □m if not indicated differently.

5.10. Establishment of an embryonic stem cell-line expressing tamoxifen inducible Cre

After proving the existence of hemogenic endothelial cells, the molecular mechanisms regulating their determination was investigated. Several transcription factors as Runx1, Notch1, GATA-2, SCL/tal1, c-myb and others are hypothesised to play a role in embryonic hematopoiesis and might be important for the specification of hemogenic endothelium (Swiers et al., 2006; Loose et al., 2007). Previous studies have addressed this issue using ESC differentiation systems with overexpression or knocked-outs of transcription factors to elucidate their role in embryonic hematopoiesis (Tsai et al., 1997; Persons et al., 1999; Yokomizo et al., 2001; Endoh et al., 2002; Kitajima et al., 2002; Lacaud et al., 2002; Kitajima et al., 2003; Schroeder et al., 2003; Lacaud et al., 2004; D'Souza et al., 2005; Dai et al., 2006). However, the continuous overexpression or complete loss of transcription factors can cause a multitude of effects during all differentiation steps that lead to unspecific read-outs. To analyze hemogenic endothelium which is a rare, transient population of cells, an inducible system is needed to manipulate transcription factor expression only at the time point of its specification and existence.

Although regulatory elements could not be identified within my thesis, an ESC cell line was prepared that expresses the Cre recombinase when exposed to hydroxytamoxifen (OHT).

Cre is a protein isolated from the bacteriophage P1 that catalyzes the site-specific recombination of two loxP carrying DNA strands. The loxP sites are two 34 bp attachment sites that are recognized, cleaved and recombined by the Cre protein which leads to the excision of the loxP-flanked DNA. Similarly, the FLP recombinase from *Saccharomyces cerevisiae* recognizes and recombines two frt-sites in DNA (Lewandoski, 2001).

As published by Zhang and Verrou, inducible control of Cre protein was achieved by fusing the Cre reading frame to two domains encoding a mutated form of the human estrogen receptor ligand-binding domain (ER^{T2}-Cre, TBDCreTBD) that responds to tamoxifen (Zhang, Y. et al., 1996; Verrou et al., 1999). In the absence of tamoxifen the fusion protein of ER^{T2}-Cre resides in the cytoplasm of the cell bound to the heat-shock protein 90 (hsp90). Upon its addition, tamoxifen uncouples the hsp90 from the ER^{T2}-Cre protein thereby allowing the transport of the fusion into the nucleus where it implements

recombination of the loxP flanked DNA segments (Figure 5.19)(Vallier et al., 2001; Guo, C. et al., 2002).

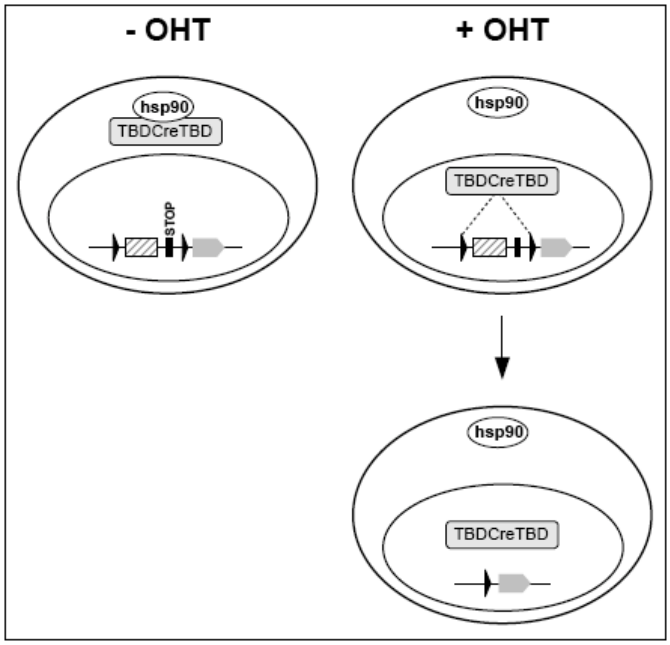

Figure 5.19: Recombination by tamoxifen inducible Cre. In the absence of tamoxifen (OHT) the TBDCreTBD is inactive because it is bound by hsp90. By OHT addition the TBDCreTBD is released from the hsp90 and translocates into the nucleus. Here, it catalyzes the recombination of two loxP sites (triangles) flanked DNA. Contains the cleaved DNA a transcriptional STOP-cassette, gene expression of the 3' regions is initiated by recombination. Figure modified from Guo et al., 2002.

To activate gene expression the reading frame is located 3' of the loxP sites which flank a STOP cassette that induces a transcriptional arrest at this specific location. Upon recombination the STOP cassette is deleted and the open reading frame is transcribed.

To produce an ES cell line expressing tamoxifen-inducible Cre, wild-type EB3 ESCs were first transfected with a linearized plasmid carrying the fusion protein of ER^{T2}-Cre driven by the chicken beta actin (CAG) promotor and a cytomegalovirus (CMV) enhancer (Figure 5.20 A). As this plasmid encodes a puromycin resistance cassette, all stable transfected cells could be selected by puromycin. Resistant ESC colonies were picked and several monoclonal ES cell lines were established. These were transiently transfected with the plasmid pCAGGS-FLP^e encoding the enhanced FLP recombinase (Schaft et al., 2001) thereby removing the puromycin resistance cassette that is flanked by two frt-sites. Successful recombination was tested in a parallel approach with or without puromycin to select the clones that are sensitive to puromycin. Although the efficiency of FLP catalyzed recombination is supposed to be 70% in undifferentiated ESCs (Schaft et al., 2001), only 2.2% (11 of 492 expanded monoclonal ESC lines) of all analyzed clones were successfully deleted of the resistance gene in this study.

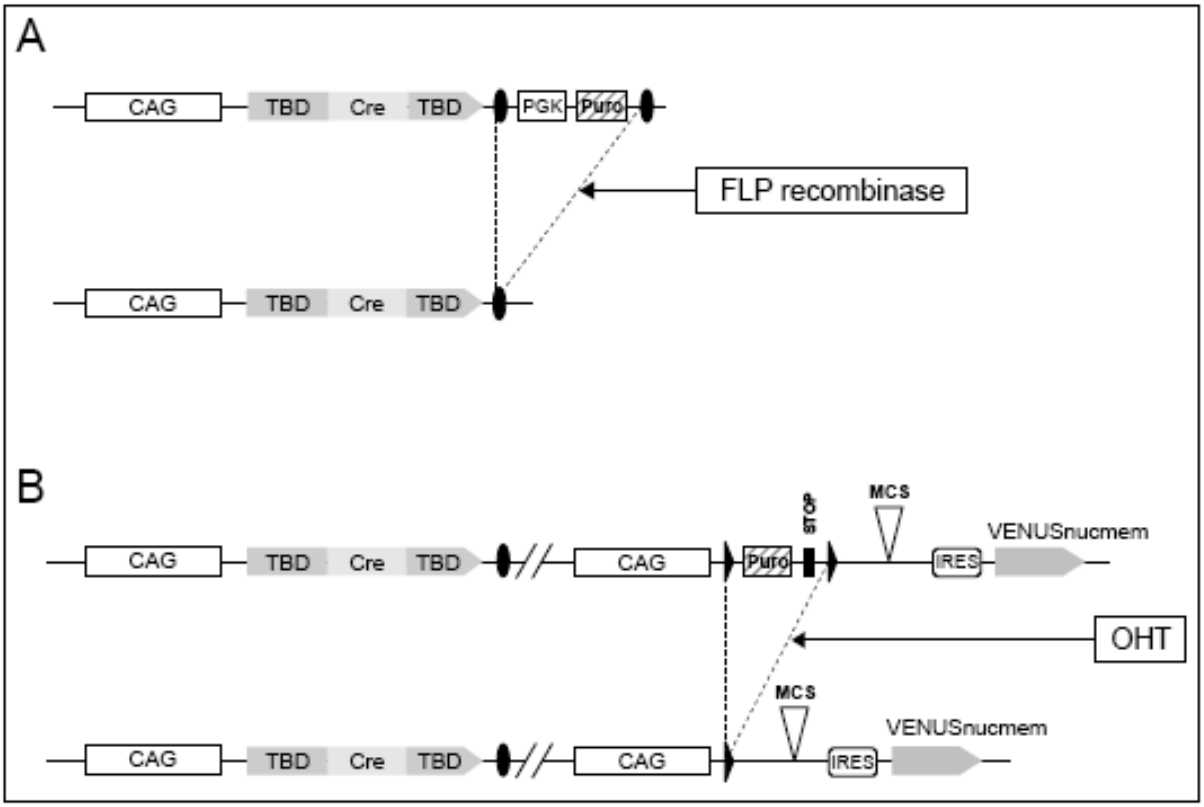

Figure 5.20: Generation of ESCs stably expressing OHT-inducible Cre and the empty reporter vector *pCAG-loxP-Puro-STOP-loxP-MCS-IRES-VENUSnucmem*. A: Monoclonal ESCs stably expressing OHT-inducible Cre were produced by transfecting *pCAG-TBDCreTBD-frt-PGK-Puro-frt* and selecting puromycin (puro) resistant clones. The puro resistance gene was removed afterwards by transient transfection with a plasmid encoding the FLP-recombinase which recognizes frt sites (black ellipses). **B:** Resulting monoclonal ESC lines were stable transfected with the reporter plasmid *pCAG-loxP-Puro-STOP-loxP-MCS-IRES-VENUSnucmem*. Transfectants were selected by puromycin. Upon OHT-addition the loxP (black triangles) flanked DNA is recombined, the STOP cassette is removed and VENUSnucmem is produced. Figure modified from Vailler et al., 2001 and Zhang et al., 1996.

Inducible gene expression of transcription factors is performed by transfecting a plasmid encoding the gene of interest followed by an internal ribosomal entry site (IRES) and a fluorescent reporter gene such as VENUSnucmem (Okita et al., 2004). 5' of the gene of interest, the loxP cassette including a puromycin resistance gene as well as the STOP cassette and the CAG promoter are localized. To verify the kinetics of gene expression the empty vector (without a gene encoding the transcription factor) was stably transfected into the OHT-inducible-Cre ESC line. Selection of puromycin resistant monoclonal colonies resulted in stable transfected ESC-lines.

Clones were selected after each step for (i) good mesodermal, endothelial and hematopoietic differentiation of ESCs on OP9, (ii) OHT-dependent expression of VENUSnucmem after transient transfection with the pCAG-loxP-Puro-STOP-loxP-VENUSnucmem reporter plasmid (iii) strong expression of the reporter reflecting a high

Cre activity in more than 80% of all cells of each clone and (iv) no expression of the VENUSnucmem reporter plasmid when cultured without OHT (leakiness).

To test the efficiency and the kinetics of the stably transfected tamoxifen-inducible Cre an empty vector reporter construct containing Cre inducible VENUSnucmem expression vector was electroporated into the OHT-inducible-Cre ESC lines (Figure 5.20 B). Plasmid-carrying ESC clones were selected by puromycin. In addition to their capacity to differentiate into mesoderm, blood and endothelium (data not shown) the established ESC lines were tested in respect to VENUSnucmem expression after exposure to OHT.

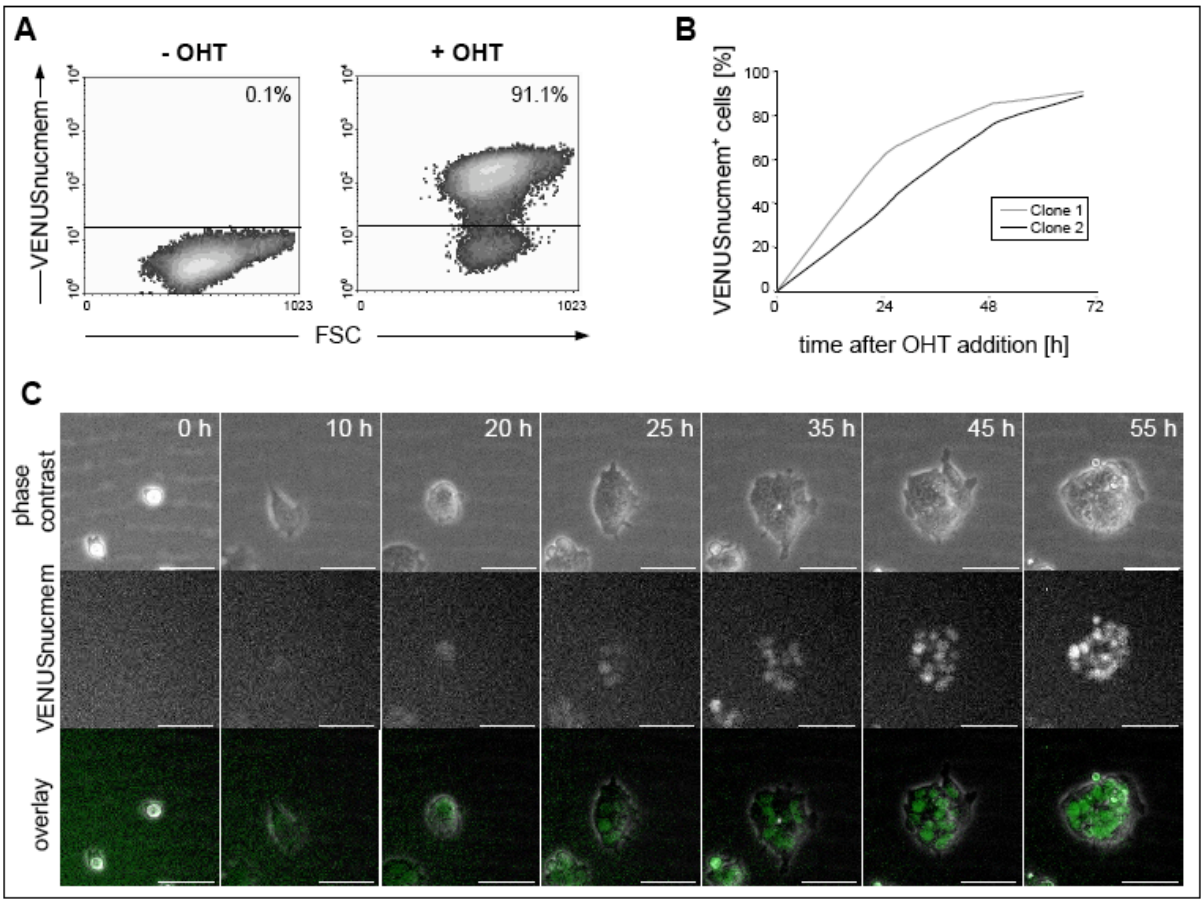

Figure 5.21: Cre activity in the established undifferentiated ESC line indicated by VENUSnucmem expression. Analysis of one (Clone EB3x230-22x407ax309-2 (Clone 1); A,C) and two (Clones EB3x230-22x407ax309-2 (Clone 1) and EB3x230-22x407ax309-8; (Clone 2) B) ESC lines that stable expresses OHT-inducible Cre and *pCAG-loxP-Puro-STOP-loxP-MCS-IRES-VENUSnucmem* is shown. **A:** FACS analysis of undifferentiated monoclonal ESCs gated for living (PI^-) cells of the right size and granularity (not shown). All cells are $VENUSnucmem^-$ when cultured without OHT (left panel). 91.1% of cells express VENUSnucmem after 3 days of OHT exposure (right panel). **B:** Two different clones were culture with OHT up to 3 days and analyzed by FACS at different time points. Approximately 90% of VENUSnucmem up-regulation can be achieved in both tested clones after 72h of OHT exposure. **C:** Images extracted from a time-lapse experiment depicting the onset of VENUSnucmem expression within 55h in one ESC clone. Although being of monoclonal origin cells in the colony vary in their expression of VENUSnucmem.
Scale bar: 50 µm.

Undifferentiated ESC lines were cultivated with or without OHT and analyzed by FACS for the expression of VENUSnucmem. One example is shown in Figure 5.21 A. In the absence of tamoxifen no VENUSnucmem expression is detectable demonstrating that the Cre activity is tightly controlled. Upon addition of tamoxifen for 3 days 91.1% of analyzed cells up-regulated VENUSnucmem, whereas 8.9% are VENUSnucmem$^-$ cells that failed to recombine and initiate gene and/or protein expression. Similar results were obtained from other clones tested. As previously reported, the efficiency of Cre mediated recombination and target gene activation is dependent on the ESC clones selected and can be a mosaic of recombined (VENUSnucmem$^+$) and not recombined (VENUSnucmem$^-$) cells (Zhang, Y. et al., 1996; Lewandoski, 2001; Vallier et al., 2001).

To study detailed kinetics of the onset of gene expression after addition of OHT, FACS analysis was performed at different time points after the addition of OHT (Figure 5.21 B). Two different ESC-clones are depicted that up-regulate VENUSnucmem expression to about 90% within 3 days of OHT induction. This shows firstly that the kinetics of Cre activity are very similar in the two tested clones and second that it takes 3 days for complete recombination. These results are similar to published data by Zhang, Vallier and others who showed that a maximal Cre recombination in undifferentiated ES cells can be achieved 75 hours after OHT induction (Zhang, Y. et al., 1996; Vallier et al., 2001).

In an additional approach, the applicability of tamoxifen-mediated Cre recombination in time-lapse movies was tested. In Figure 5.21 A images from a time lapse experiment show the sequential activation of the VENUSnucmem gene in undifferentiated ES cells. Again not all cells contained in the colony exhibit the same start and intensity of VENUSnucmem expression. All cells shown in Figure 5.21 A derive from a monoclonal origin. However, variances in protein expression can be explained by the slow activity of tamoxifen-inducible Cre recombinase which results in recombination after the starting cell has divided and thus induces gene expression in the progeny at different time-points and with different efficiency.

In summary we shown that (i) the Cre recombinase is slowly acting and (ii) that not all transgenic cells exposed to OHT express Cre and/or recombine the loxP-flanked DNA sequence which is consistent with previous publications. Although these results were generated in undifferentiated ESCs and these experiments would have to be repeated

with differentiated cells, we don't think that the Cre expression and recombination is faster in cells that have a slower generation time, e.g. mesoderm-derived cells observed in time lapse analysis.

Even though the Cre-loxP system is routinely used in basic research, our analysis highlights certain disadvantages that might impair the use of this cell line for the analysis of hemogenic endothelium considering that hemogenic endothelial cells are a very small population that is only very transiently present in the culture. As the Cre is active during three days after induction, the Cre recombinase could be active before or after the time of interest. Since in addition only a portion of cells express Cre or recombine at all the potentially analysable cell population is even smaller than it already is. Thus, if the OHT-inducible Cre is employed to gain knowledge about hemogenic endothelium it should be considered that the OHT-inducible Cre can hardly be controlled in time.

6. Discussion

6.1. Endothelial cells can act as direct precursors of blood cells

We report here that endothelial cells can give rise to blood cells. Time lapse imaging of ESC-derived cells allowed the observation of single endothelial cells arising from mesoderm during their differentiation into blood. Beside their morphological identification, cell identities of endothelial and blood cells were proven by simultaneous detection of specific molecular and functional markers. Hemogenic endothelial cells express the surface antigens that can also be detected in intraembryonic hemogenic sites in the embryo. In addition, hemogenic endothelial cells can give rise to blood cells of different types, among them being cells of monocytic-granulocytic lineage. We showed that hemogenic endothelial cells and its relatives only produce hematopoietic or endothelial cells, thus, the precursor of the hemogenic endothelial colony is bi-potent but not multi-potent. Finally, the existence of hemogenic endothelial cells in the developing embryo was verified by imaging mesodermal cells purified from E7.5 embryos to differentiate into blood via an endothelial intermediate *ex vivo*. In summary this proves, that hemogenic endothelial cells do exist during embryonic development.

Tracing more than 200 single hemogenic endothelial cells within more than 100 hemogenic endothelial colonies of monoclonal origin showed that only during a short time window of differentiation a very limited number of endothelial cells acquires a hemogenic fate and differentiates into blood. The hemogenic endothelial cells were observed to loosen the connection to the adherent endothelial cell layer and kept contact to the endothelium for approximately one day before being released to the supernatant. Irrespective of the ESC lines used, all observed endothelial to blood cell transitions were highly similar in respect to (i) the frequency of events within the culture, (ii) the number of hemogenic endothelial cells within the endothelial sheet colony and (iii) the time window in that blood cells are produced from endothelial cells. The identical kinetics point to the existence of a complex developmental process that is strongly controlled on the cellular and molecular level.

Although strong similarities between *in vitro* ESC-differentiation systems and embryonic development *in vivo* have been demonstrated in many previous studies (Weiss et al., 1994; Porcher et al., 1996; Nishikawa et al., 1998a; Yamashita, J. et al., 2000; Lacaud et al., 2002; Mitjavila-Garcia et al., 2002; Mikkola et al., 2003; Lacaud et al., 2004; Hashimoto et al., 2007), the presence of hemogenic endothelial cells *in vivo* remained questionable. Thus, we also visualized *ex vivo* cultured mesodermal cells purified from E7.5 embryos and monitored their differentiation into blood thereby recapitulating the *in vivo* situation. Like ESC-derived cells, embryonic mesodermal cells differentiated into endothelial sheet colonies containing cells that adopt a hematopoietic morphology. Interestingly, marker expression, morphological changes and kinetics were identical to endothelial cell to blood cell transitions observed in ESC-differentiations. This proves that ESC-derived mesodermal cells differentiating into endothelium and blood directly copies embryogenesis. Both systems in addition resemble *in vivo* hematopoiesis in several features:

Firstly, de novo hematopoiesis in the mammalian embryo is restricted to a very short time frame within development: the YS produces blood between E6.5 and E10.5, the AGM region between E9.5 and E12.5 and the placenta between E10.5 to E14.5 (Palis et al., 1999; Baron, 2003; Godin et al., 2005; Ottersbach et al., 2005; Rhodes et al., 2008). The same holds true for the *in vitro* and *ex vivo* observed endothelial to blood cell transitions which were only observed to occur between day 6 and day 7.5 in ESC-differentiations or between day 1 and 2 in primary cell culture but never later.

Secondly, few hemogenic cells exist in the developing embryo: few cells having hemogenic potential exist in the YS (Kinder et al., 1999), in placenta (Ottersbach et al., 2005) and AGM region (Taoudi et al., 2007) could be hemogenic. Similarly, only about 2 % percent of endothelial cells present in the *in vitro* or *ex vivo* cultures acquire hemogenic potential.

Thirdly, hematopoietic clusters visible in the lumen of the dorsal aorta, the umbilical, vitelline and omphalomesenteric arteries at day 11.5 dpc appear to stick to the vessel wall (Taoudi et al., 2007). Consistent with this, in *in vitro* and *ex vivo* time lapse experiments blood emerging from endothelium forms clumps, as well, and maintains the contact to the endothelial cells approximately 1 day.

Fourthly, endothelial and blood cell markers analysed in this study, e.g. CD41, are expressed with identical kinetics compared to *in vivo* analysis in previous publications (Mikkola et al., 2003; Bertrand et al., 2005; Li et al., 2005). It was suggested in these

studies, since CD41 is detectable as first hematopoietic molecule before CD45, it shows the hematopoietic identity already at the endothelial level (Mikkola et al., 2003). We observed the onset of CD41 antigen at a time-point when endothelial cells still express VE-Cadherin::VENUSClaudin5 and form tight junctions with neighbouring cells. Throughout the transition process CD41 is strongly detectable on developing blood cells but CD45 can be detected first when blood cells are free-floating. This shows again a strong similarity to the *in vivo* situation.

Based on the similarities between *in vitro*, *ex vivo* and *in vivo* hematopoiesis an identical transition process is very likely to exist *in vivo* as well.

6.2. Identification of endothelial cells

The continuous observation of differentiating ESC- or embryo-derived mesodermal cells provided evidence for the existence of hemogenic endothelial cells that are morphologically, molecularly and functionally undistinguishable from non-hemogenic endothelial cells: all cells share identical morphology, organization in sheets, formation of tight junctions that incorporate Claudin5, expression of VE-Cadherin and uptake of Ac-LDL – features that do not hold true for other cell types in our cultures. Although blood cells never exhibited these endothelial characteristics here, previous studies could show the presence of endothelial markers on nascent embryonic blood cells. Tagging chicken embryonic endothelial cells *in ovo* by DiI-Ac-LDL injection resulted in DiI-Ac-LDL$^+$ hematopoietic cells (Jaffredo et al., 1998; Jaffredo et al., 2000). Similarly, Sugiyama could identify DiI-Ac-LDL$^+$ erythrocytes and multi-potent precursors in E10.5 mouse embryos that were pulsed with DiI-Ac-LDL beforehand (Sugiyama et al., 2003). In addition, a low number of VE-Cadherin$^+$CD45$^+$ double positive cells were identified in E9.5 or E11.5 AGM regions by others (Fraser et al., 2003; Taoudi et al., 2005) and Kim found foetal liver HSCs expressing VE-Cadherin between E13.5 and E16.5 (Kim et al., 2005). Since the specificity of VE-Cadherin and DiI-Ac-LDL uptake was proven in my thesis and by others (Voyta et al., 1984; Breier et al., 1996), it is likely that the blood cells still expressed a detectable amount of endothelial markers as a consequence of their endothelial origin. Here, it is possible that free-floating blood cells are shortly positive for the endothelial markers but that the intensity is beneath our detection level. Both markers localize in the cytoplasm as soon as suspension cells have developed: DiI-Ac-LDL and VE-Cadherin::VENUSClaudin5 which we expect to be internalized and degraded in the cytoplasm after separating from the adherent endothelial sheet. As free-

floating blood cells usually contain a large nucleus, the cytoplasmatic compartment is very small. This could explain why we did not detect any staining quickly after cells detached from the adherent layer. In contrast, all studies mentioned above proved the endothelial markers on hematopoietic cells by FACS analysis, a method that is more sensitive compared to the microscope used for the detection of the in culture immunostainings.

However, the used combination of VE-Cadherin expression, DiI-Ac-LDL uptake, tight junction formation and morphology allows the clear identification of endothelial cells.

6.3. The bi-potent mother of hemogenic and non-hemogenic endothelial cells

Hemogenic and non-hemogenic endothelial cells derive from one precursor cell. By single cell tracking of all its descendants the precursor cell was shown to exclusively produce hemogenic and non-hemogenic endothelial cells but not cells of a different lineage, e.g. cardiomyocytes. As an additional control its bi-potent character was confirmed by looking at smooth muscle cells that are known to develop from the same starting population as endothelial cells. Without any exception smooth muscle cells differentiated from a origin distinct of hemogenic endothelial cells. Thus, the mother cell of hemogenic and non-hemogenic endothelial cells is only bi-potential.

To clarify the identity of the bi-potential founder cells, their expression of all hemogenic and endothelial markers employed in the analysis was evaluated. Interestingly the common progenitor of hemogenic and non-hemogenic endothelial cells and its mother cell was shown to form tight junctions and express VE-Cadherin and Tie2 beginning day 4.5 of ESC-differentiation. Considering that Tie2, VE-Cadherin and Claudin5 incorporating tight junctions are endothelial cell markers, this suggests that the bi-potent mother cell displays endothelial characteristics. This interpretation is also supported by the analysis of PECAM-1 (data no shown) in embryo-derived cultures. PECAM-1 was also shown to be up-regulated at day 0.5 after plating embryonic mesodermal cells. Tie2 and PECAM-1 are surface molecules that are in addition to their endothelial expression present on embryonic blood cells. But blood cell identity could be excluded by the absence of other hematopoietic markers. This clearly shows the endothelial character of the bi-potential mother cell.

The typical endothelial sheet morphology and the incorporation of DiI-Ac-LDL were observed to start shortly before hemogenic endothelial cells became semi-adherent. The

development of the sheet morphology as well as the uptake of Ac-LDL probably requires the activity of a multitude of molecules the expression of which might be delayed in contrast to the up-regulation of single molecules as VE-Cadherin, Tie2 and PECAM-1 expression. The incorporation of Ac-LDL into the membrane of endosomes additionally suggests that the dye has to accumulate to reach detectable levels which might also delay the detection.

However, the existence of a bi-potent cell producing one cell with exclusive hemogenic and one with solely angiogenic potential, raises the question if an asymmetric cell division occurs to generate daughters with different developmental fates. A cell divides asymmetrically when cues present during the cell division lead to asymmetric fates of the daughters, e.g. asymmetric influence of the niche or protein segregation (Yamashita, Y. M. et al., 2003; Faubert et al., 2004). This requires that the asymmetric fate is determined during the division process itself and should be detectable at defined time-points during generation time. Here, we observed that hemogenic characteristics as e.g. the onset of semi-adherence, start at distinct time-points in different hemogenic cells (data not shown), which suggests that the decision leading to an hemogenic and non-hemogenic daughter is not correlated to the cell division.

6.4. Specification of hemogenic endothelial cells

Cell fate decisions can occur as a result of intrinsic or extrinsic cues. Intrinsic signals, for example the activity of transcription factors, can modulate gene expression to either induce or inhibit a cell fate. Signals influencing the cell fate from outside can be soluble factors such as cytokines, growth factors, hormones, secreted proteins or ligands being present on neighbouring cells or on surrounding matrix.

While the analysis of VE-Cadherin, Tie2 and PECAM-1 clearly shows that the mother cell already displays endothelial characteristics before giving rise to two daughters with different fate, it also points to the dispensability of these molecules for the endothelial to blood cell transition itself. If Tie2, PECAM-1 or VE-Cadherin would be important for the specification of hemogenic endothelium or the endothelial cell to differentiate into blood we would expect the expression to be limited to a subset of cells. As this was not observed in the time lapse movies of ESC or embryo-derived mesodermal cells differentiating into blood, a significant role for these molecules to influence the transition process is very unlikely. This is in accordance with previous publications that show a broad expression pattern of Tie2 and PECAM-1 on most endothelial and blood cells

(Dumont et al., 1995; Drake et al., 2000; Ema et al., 2006; Takahashi et al., 2007). Altogether, the ubiquitous expression of Tie2 and PECAM-1 leads to the conclusion that these markers doe not have a specific function in the specification of hemogenic endothelium.

Apart from surface markers expressed on embryonic endothelial and hematopoietic cells, many transcription factors have been shown to be indispensable for the initiation and regulation of early hematopoesis *in vivo* and *in vitro*, e.g. Runx1, SCL/tal1, GATA-2, c-myb, Notch1 and others (Kallianpur et al., 1994; Minegishi et al., 1999; North et al., 2002; Kumano et al., 2003; Ling et al., 2004). Hence, it is not yet exactly clear at which cellular differentiation level transcription or growth factors act. In particular, it is basically unknown whether the listed transcription factors or any cytokines influence the appearance of hemogenic endothelium or its transition into blood cells at all or whether they function in the proliferation of lateral plate mesoderm, endothelial cells or nascent blood cells. To analyse these questions the importance of the transcription factors could be analysed by over-expressing or down-regulating them using RNAi and observe the effects on the hemogenic transition of endothelial cells at the single cell level.

It was not possible to elucidate the role of transcription factors within the bounds of my thesis, but we took a look at external other cues that might lead to the determination of a hemogenic fate of endothelial cells.

6.5. Direct interactions with neighbouring OP9

The frequency of hemogenic endothelial cells in ESC- or embryo-derived differentiation cultures is very low although about 50% of seeded mesodermal cells gave rise to endothelial colonies. Previously it was seen that ESCs differentiated on OP9 stroma can generate venous, arterial and lymphatic endothelium (Kono et al., 2006; Yurugi-Kobayashi et al., 2006). Since the hematopoietic clusters are only found in the arteries of the embryo, we only expected arterial endothelium to produce blood cells in our cultures. This could explain why not all endothelial colonies gave rise to a hemogenic subset. However, we looked for cues that induce the hemogenic fate of some endothelial cells but not of others.

Initially, we compared hemogenic and non-hemogenic endothelial cells according to their behaviour, motility and localization and looked for extrinsic stimuli that might initiate hemogenic specification. Accordingly, the importance of direct interactions between

immature embryonic and OP9 cells to induce differentiation into blood was shown previously: culture of whole E6.5 to E7.5 epiblast mouse tissue on OP9 cells failed to give rise to blood cells whereas dissociation of the tissue led to blood formation suggesting the necessity of direct interaction of putative hemogenic cells with OP9 (Kanatsu et al., 1996).

Addressing the localization of individual cells within the mesoderm-derived colony before day 6.0 of ESC-differentiation is difficult as cells form 3 dimensional aggregates. However, this same clump formation was observed for hemogenic and non-hemogenic endothelial colonies. Taking a closer look at the behaviour of individual cells, hemogenic endothelial cells in sheet colonies neither preferred to localize at edge (facing OP9) or in the centre (facing other endothelial cells) of the colony. In conclusion there was no obvious difference in behaviour between hemogenic and non-hemogenic endothelial cells or colonies.

It is known from other studies that the surface marker expression of cells can be influenced by shear forces. Endothelial cells exposed to shear stress by constant blood flow or vascular remodelling can differ in the expression of surface markers as PECAM-1, Flk1 and others (Abumiya et al., 2002; Albuquerque et al., 2002; Fujiwara, 2006). Furthermore, vascular remodelling of mouse yolk sacs was shown to be affected by shear forces (Lucitti et al., 2007). Since it is known that the appearance of hematopoietic clusters in the developing aorta is strongly coordinated with the fusion of the initially paired aortae (Pardanaud et al., 1996; Pouget et al., 2006), suggests that the vascular remodelling induces the formation of intraaortic clusters. Thus, we wanted to know whether the hemogenic capacity of endothelium is induced by neighbouring dividing OP9 cells. Cell divisions of adherent cells are accompanied by stretching of the cell body and strong rearrangements of the cytoskleton thereby producing shear forces that directly act on surrounding cells. Although it is possible that surface marker expression might be altered by shear forces, this could not be proven for endothelial cells to adopt a hemogenic fate.

6.5.1. Cytokines, growth factors and secreted proteins

Although the differentiation cultures performed within this study strongly rely on suitable serum as no cytokines apart from SCF were added to the culture, the importance of OP9 cells is crucial, too. Notably, the quality of OP9 cells was discovered to be one of the

most critical factors for the observation of endothelial to blood cell transitions in this study. OP9 cells more than 25 passages old or an OP9 layer with a very high amount of adipocytes were not sufficient to support the generation of blood cells from endothelium. Even though, whether this leads to the loss of cytokine secretion, to the increased production of inhibiting signals or to defective cell-cell interactions is unclear.

In the literature, the OP9 differentiation system was mostly employed as a preparation for *in vivo* studies or to identify intrinsic differentiation mechanisms. As OP9 cells themselves were never analysed in greater detail, little is known about their ability to produce cytokines, secrete proteins or express surface molecules themselves. The analysis of the biology of OP9 cells is difficult because they are polyclonal, consist of a variety of cell types (stroma cells, mesenchymal cells, adipocytes) and easily loose their supportive capacity. Although the importance of the stroma for hematopoietic differentiation is known, its precise role in the establishment of the hematopoietic system is currently unclear.

Recent publications analysed the conditions in the dorsal aorta and tried to identify factors that promote the formation of hematopoietic clusters (signalling from ventral, the gut and sub-aortic patches). Consequently, IL-3 was discovered to enhance the production or expansion of HSCs in the AGM region (Robin et al., 2006) as well as BMP4, Activin A, bFGF and VEGF (Durand et al., 2007). While this in summary explains that VEGF, IL-3, BMP4, Activin A and bFGF contribute to the establishment of mouse hematopoiesis it remains unclear at which cellular differentiation state these factors act: whether they induce the hemogenic fate of endothelial cells, induce proliferation of the existing hemogenic endothelial or hematopoietic cell pool cannot be answered yet.

We observed endothelial cells to produce blood cells in a culture that is supplemented with 10% FCS containing a multitude of soluble factors which influence the differentiation process: growth factors, cytokines and secreted proteins that were postulated to induce hematopoietic fates in mouse, chicken or ESC differentiation studies previously (Johansson et al., 1995; Kanatsu et al., 1996; Pardanaud et al., 1996; North et al., 2007). The importance of serum was recently analysed by Era et al. who showed that sorted VE-Cadherin^{+} ESC-derived cells plated on OP9 stroma could give rise to hematopoietic cells only when cultivated in serum-containing media but not under serum-free conditions (Era et al., 2008). Along the same line, ESC-derived VE-Cadherin^{+}Ter119^{-}CD45^{-} cells sorted at day 5 of differentiation can give rise to blood cells

on type IV collagen coated dishes, a culture in which the mesodermal differentiation occurs in the absence of OP9, but only if cultivated in the presence of serum and growth factors including SCF, VEGF, IL-3, erythropoietin and G-CSF (Nishikawa et al., 1998a). In contrast, Hashimoto failed to induce hematopoiesis from VE-Cadherin^{+}CD41^{-} ESC-derived cells isolated at day 5 of differentiation cultured with serum and IL-3, SCF, G-CSF and erythropoietin on collagen IV coated dishes whereas the same population was able to generate blood cells if cultured on OP9 stroma (Hashimoto et al., 2007). Some evidence of OP9 cells to promote endothelial differentiation by secretion of Angiopoietin 1 (Ang1) and VEGF was proven by Matsumara and co-workers (Matsumura et al., 2003). In addition, OP9 cells were shown to secrete interferon-γ (IFN-γ), interleukin-3 (IL-3) and -6 (IL-6) (Groger et al., 2004).

VEGF, which might be the inducer of the hemogenic potential of endothelial cells, is the ligand of the surface receptors tyrosine kinases VEGFR-1 (=Flt1), VEGFR-2 (=Flk1) and VEGFR-3 (=Flt4), all of them acting in concert to modulate vasculogenesis, angiogenesis and hematopoiesis (Hamada et al., 2000). Accordingly, an important role of VEGF signalling for the determination of hemogenic fate was suggested previously in quails (Eichmann et al., 1997), chicken (Pardanaud et al., 1999) but also in ESC-differentiation systems where hemangioblasts differentiated into committed hematopoietic precursors in a serum-free culture containing VEGF (Park et al., 2004; Pearson et al., 2008). However, whether this shows that VEGF instructs a hemogenic endothelial fate instead of increasing proliferation and expansion of present angioblasts or putative hematopoietic endothelial cells in favour of hematopoietic cells is unclear and would have to be analysed at the single cell level.

In summary the differentiation of mesoderm into hemogenic endothelium probably relies on a multitude of factors that are either provided by the serum or by the OP9 cells. In future studies, OP9 cells sould be manipulated by viral transduction to over-express or down-regulate putatively involved proteins in combination with a reporter protein, e.g. VENUS. These cells could be mixed with un-manipulated cells to induce local patches of OP9 cells with normal or enhanced/decreased protein expression. By time lapse microscopy it could be evaluated whether hemogenic endothelial cells preferentially arise in close proximity to transduced OP9 cells.

6.5.2. Down-regulation of tight junctions

While adherent endothelial cells transform into free-floating blood cells, tight junctions were observed to be loosened. This raises the question whether the down-regulation of tight junctions actively induces the differentiation of endothelium into hematopoietic cells. Whether blocking tight junctions, e.g. by using blocking antibodies, enhances the production of blood cells was not tested within this study, but is unlikely as tight junctions are still present when the hemogenic fate of single endothelial cells becomes obvious: Claudin5 incorporating tight junctions can be observed when semi-adherent cells up-regulate $CD41^+$ and c-kit^+, the first hematopoietic markers to be expressed. This proves that the hemogenic fate of endothelial cells is decided prior to the down-regulation of tight junctions and consistent with previous results showing that tight junctions between endothelial and blood cells exist in the intra-aortic hematopoietic clusters (Bollerot et al., 2005). Similarly, Matsumura showed that the addition of a blocking antibody against VE-Cadherin antibody, a molecule that is stabilizing tight junctions in endothelial cells, to the living culture leads to dispersion of endothelial sheets but not to detaching of cells (Matsumura et al., 2003). In addition VE-Cadherin knockout-mice still establish multi-lineage yolk-sac hematopoiesis with normal but not increased numbers of blood cells (Rampon et al., 2003).

Thus, although present, tight junctions are dispensable for the establishment of the hematopoietic system and play no role in cell specification.

6.6. Endothelial cell identity and different types of endothelial colonies

To judge whether developing blood cells derive from cells with a unequivocal endothelial identity, the endothelial character was verified by the combination of morphological, molecular and functional criteria. The sheet-like morphology was considered to be one of the most reliable characteristics because this morphology was shown in several previous studies to be typical for clonally grown ESC-derived endothelial cells on OP9 stroma (Hirashima et al., 1999; Yamashita et al., 2000; Matsumura et al., 2003; Schroeder et al., 2003). Consistent with this, our read-out has proven to be very stringent as all sheet colonies expressed VE-Cadherin, formed tight junctions and incorporated DiI-Ac-LDL when analysed in the movies.

Additionally, colonies expressing functional and molecular markers specific for endothelium (DiI-Ac-LDL^+, VE-$Cadherin^+$ and presence of tight junctions) but lacking the

typical sheet morphology were observed. A few recent studies described cord-like endothelial colonies derived from $Flk1^+$ ESC-derived cells cultured on OP9 or on collagen IV (Hirashima et al., 1999; Matsumura et al., 2003; Kono et al., 2006; Hashimoto et al., 2007). Matsumura and collaborators deduced that cord-like endothelial colonies result from VEGF induced signalling through VEGFR-3 leading to dispersed colony morphology. Although no exogenous VEGF was added in the cultures in this study, VEGF is produced in small amounts by OP9 cells which might induce the formation of cord-like endothelial colonies (Matsumura et al., 2003). As OP9 cells are a polyclonal cell line it is possible that VEGF is produced unevenly, leading to local patches and accumulation of VEGF where cord-like endothelial colonies are induced.

The biological nature of the cord-like endothelial colonies in comparison to the sheet-like endothelial colonies is at the moment unclear because they were never compared in detail. OP9 cells support differentiation of ESCs into various types of endothelial cells as venous, arterial and lymphatic endothelial cells (Kono et al., 2006; Yurugi-Kobayashi et al., 2006). Although unlikely, a different subtype of endothelial cells could explain the different morphologies. We showed that cord-like and sheet-like endothelial colonies give rise to blood with about the same frequency pointing to strong similarities between both types. Interestingly, blood production from cord-like endothelial cells followed the same sequential pattern but lacked a semi-adherent intermediate phase. This suggests that semi-adherence is not a part of the mechanism or needed for the blood cells to develop but instead is mediated by surface molecules expressed on endothelial sheet but not on endothelial cord-like colonies.

In addition to emerging blood cells sticking to the sheet-like endothelial colony, blood cells were observed to stick to one another forming hematopoietic clusters in the movies. Although an endothelial-hematopoietic interaction is missing in cord-like endothelial colonies producing blood, nascent hematopoietic cells formed clusters just like blood cells derived from endothelial sheets. This suggests that the molecules involved in both kinds of interaction (endothelial-to-blood and blood-to-blood) are different.

6.7. Identification of hematopoietic cells

Apart from their free-floating morphology emerging blood cells were identified by in culture immunostaining. The presence of fluorescently labelled antibodies in the culture while a movie is recorded allows not only the detection of living blood cells without manipulating the culture but also shows the expression of transiently present antigens.

Consequently, typical hematopoietic markers such as CD45, CD41 and Mac1 were employed in time lapse experiments. Consistent with previous publications, CD41 was transiently expressed on nascent endothelium-derived blood cells, and, shortly overlapping with CD41, most free-floating blood cells up-regulated the pan-hematopoietic antigen CD45, some of which also stained for the monocytic-granulocytic marker Mac1. Whereas the use of CD45, CD41 and Mac1 clearly distinguished blood cells from cells of other lineages, it leaves room for speculations about the identity of the hematopoietic cells.

6.7.1. Are hematopoietic stem cells generated from hemogenic endothelium?

$Mac1^+$ cells were shown to arise from hemogenic endothelium. As Mac1 is expressed on monocytic and granulocytic cells (Springer, 1971) we concluded that endothelial cells can generate blood cells of the myeloid lineage. In addition, Mac1 is detectable on foetal HSCs, which can be purified from E14.5 embryos by the surface marker profile $Thy1^{low}Sca1^+lineage^-Mac1^+$ (Bertrand et al., 2005; Kim et al., 2005). However, the possibility that HSCs are produced from ESC-derived endothelium in this study is unlikely for two reasons: embryonic HSCs were shown not to express CD45 purified from E10.5 AGM regions (Bertrand et al., 2005). Here, Mac1 was expressed by the same cells that also stained brightly for CD45. This proves that $Mac1^+$ exclusively stains monocytes/macrophages and granulocytes in this culture. Second, *in vitro* differentiation of ES cells into HSCs that are able to reconstitute adult recipients was never performed successfully without genetic manipulation of the ES cells (Wang et al., 2005), thus HSCs probably do not arise in this culture or cannot be maintained. Alternatively, putatively generated HSCs need to be transferred into the proper environment to maintain or develop their stem cell characteristics, e.g. the fetal liver of newborn mice that has the capacity to maintain foetal HSCs.

6.7.2. Living blood cells not expressing CD45

In addition to $CD45^+$ and $Mac1^+$ cells, we also observed suspension cells negative for both markers arising from endothelium. CD45 is a pan-hematopoietic marker that is at least transiently expressed on all nascent blood cells including erythrocytes, which are the only lineage that down-regulate CD45 after maturation (Kina et al., 2000). However, very big free-floating $CD41^{hi}$ blood cells that did not show CD45 expression were

observed in the movies at day 8 of ESC-differentiation. According to their large morphology and their expression of CD41 these cells are probably megakaryocytes which normally express CD45. This means that not all cells expected could be stained by in culture immunostaining, but whether this results from the inability of cells of certain lineages to bind the antibody under these conditions is unclear at the moment. In the future, this should be tested by incubating differentiation cultures in the presence of labelled antibodies until mature blood cells are present. All cells from the culture should be flushed off, immunostained with various antibodies reliably identifying certain lineages and FACS analysed.

6.8. Does hemogenic endothelium produce primitive or definitive type blood cells?

Definitive and primitive type blood cells co-exist in the developing embryo. Whether hemogenic endothelium produces either or both of them is currently unknown.

Results found by others rather suggest a distinct origin of both. Analysing the haemoglobin-chain of suspension cells, Nakano and co-workers characterized the kinetic appearance of erythrocytes and found that, identical to the embryo, erythrocytes arise in two waves in ESC-differentiations. Primitive erythrocytes were generated predominantly before day 7 of ESC-differentiation and definitive erythrocytes did not arise until day 8 after induction of ESC-differentiation (Nakano et al., 1996). In addition to the different timing, only primitive but not definitive erythrocytes can still be generated after blocking signalling through c-kit, thereby pointing to distinct origin (Ogawa et al., 1993; Nakano et al., 1996). This view is supported by a different study in which VE-Cadherin$^+$ cells purified from mouse embryos only had a minor capacity to produce primitive type erythrocytes (Fraser et al., 2002b) whereas these cells were able to give rise to multi-lineage blood of the definitive type. This hints towards an endothelial origin of definitive type blood and to a non-endothelial origin of primitive hematopoietic cells.

Here, in addition to blood cells arising from endothelium, hematopoietic cells produced from a non-endothelial source were observed in time lapse movies. Quickly dividing free-floating blood cells of non-endothelial origin were generated from very few (2 or 3 mesoderm-derived cells) adherent cells that were not forming a colony. The free-floating cells were observed to all die by day 8 pointing to their primitive character. Since primitive erythrocytes decrease until day 8 of ESC-differentiation in the study explained before (Nakano et al., 1996), it is very likely that this early wave of hematopoietic cells

represents primitive type blood whereas definitive type blood arises from endothelial colonies around day 8 of differentiation.
Besides primitive erythrocytes also primitive megacaryocytes and monocytes exist in the embryo (Palis et al., 1999; Tober et al., 2007). Whether the latter two are generated from ESCs is questionable and rather difficult to analyse as these cells are poorly characterized and few markers are known to unequivocally distinguish definitive and primitive blood lineages. A potential endothelial origin of primitive megacaryocytes, macrophages and monocytes can therefore not be excluded.
Thus, the only molecular marker clearly distinguishing primitive from definitive type blood so far is embryonic haemoglobin that is expressed by primitive erythocytes but not by definitive erythrocytes. As haemoglobin is an intracellular molecule, the addition of labelled antibody detecting foetal haemoglobin can not be detected in living cultures. To address this question, an transgenic ESC or mouse line expressing a fluorescent reporter protein controlled by the regulatory sequences for foetal haemoglobin should be imaged and analyzed at the single cell level.

6.9. Correlations to extra- and intraembryonic hematopoeisis

Hemogenic endothelial cells potentially exist in all hemogenic places, the yolk sac, the AGM region or the placenta. ESCs differentiated on OP9 produce primitive hematopoietic cells on one hand and lymphoid cells on the other (Nakano et al., 1994; 1996). This suggests that ESC-differentiation systems share features of YS hematopoiesis, which is the only location that produces primitive blood (Palis et al., 1999), and hematopoiesis of AGM region or the placenta, since only these locations have the intrinsic potential to produce lymphoid cells (Cumano et al., 1996; Rhodes et al., 2008).

Convincing proof for the production of blood cells from endothelium either in the YS or in intraembryonic sites requires time lapse microscopy of living embryos. Due to the inaccessibility of living embryonic hematopoietic tissues these experiments cannot be performed yet. The primary cultures established from dissected E7.5 embryos in this study included a mixture of cells from YS and embryo proper. At this stage the P-Sp/AGM region has not yet developed which points to the YS as the origin of mesodermal cells giving rise to hemogenic endothelium, consequently. However, in E7.5 to E8.0 embryos, the para-aortic splanchnopleura has intrinsic potential to generate hematopoietic cells after *ex vivo* culture (Cumano et al., 2001). This leaves the possibility

open that E7.5 embryos contain intra-embryonic mesodermal cells that produce hemogenic endothelial cells. We thus cannot conclude whether the hemogenic endothelium maturing from E7.5 purified mesodermal cells observed in this study derived from intra-or extra-embryonic origin. To answer this question, the YS should be manually separated from the embryo proper during the dissection procedure and imaged in a different culture from cells deriving from the embryo proper.

6.10. Interests for post-natal hematopoiesis

Here, we have shown that nascent endothelial cells can have a hemogenic potential within a short time window of the differentiation. As pointed out previously this is in accordance with the findings obtained from *in vivo* studies that suggested a very transient existence of hemogenic cells. Consistent with this, markers that are initially expressed on endothelial and on blood cells are down-regulated on one or the other cell type, e.g. Runx1 or SCL/tal1 which are restricted to blood cells in postnatal life. Even though a bi-potential precursor cell of hematopoietic and endothelial cells in postnatal life was suggested in recent publications.

Wu et al showed that a $CD34^+CD133^+$ population from human umbilical cord blood can gave rise to endothelial and to hematopoietic cells (Wu et al., 2007). Bailey et al could transplant adult hematopoietic stem cells that contributed to the vasculature in the recipient animal (Bailey et al., 2004). Moreover, upon isolation of $CD34^+Flk1^+$ cells from human bone marrow and cord-blood could give rise to endothelial and hematopoietic cells (Pelosi et al., 2002). It was thus claimed that a bi-potential cell giving rise to endothelium and blood exists in post-natal life.

Within the time window we observed hemogenic endothelial cells only exist during a short time and endothelial cells loose the potential to achieve a hemogenic fate after passing this critical time window. It is very likely that unknown intrinsic factors modulate the capacity of endothelium to differentiate into blood and that the required molecules are expressed only during a certain maturation state. We in addition think that extrinsic cues are important to acquire a hemogenic fate. Probably these factors might be present at later stages of development or adult life and could thus maybe convert non-hemogenic into hemogenic cells. Thus, the complete absence of adult hemogenic endothelial cells cannot be ruled out.

The difficulty of the identification and analysis of bi-potential endothelial cells lies in the lack of knowledge about them and in the small number of hemogenic endothelial cells

that can only be analysed at the single cell level. Thus, whether $CD34^+Flk1^+$ or $CD34^+CD133^+$ cells are a homogeneous or a heterogeneous population of cells is unclear and requires single cell analysis to convincingly prove the existence of hemogenic endothelial cells in postnatal or adult life.

We propose a model (Figure 6.1) summarizing the results obtained in our study showing that mesodermal cells can differentiate into hemogenic endothelial cells before giving rise to blood cells. Using time lapse imaging and single cell analysis this study solves for the first time the controversy of the existence of hemogenic endothelium. This contributes significantly to the basic understanding of the origin and development of the mammalian hematopoietic system.

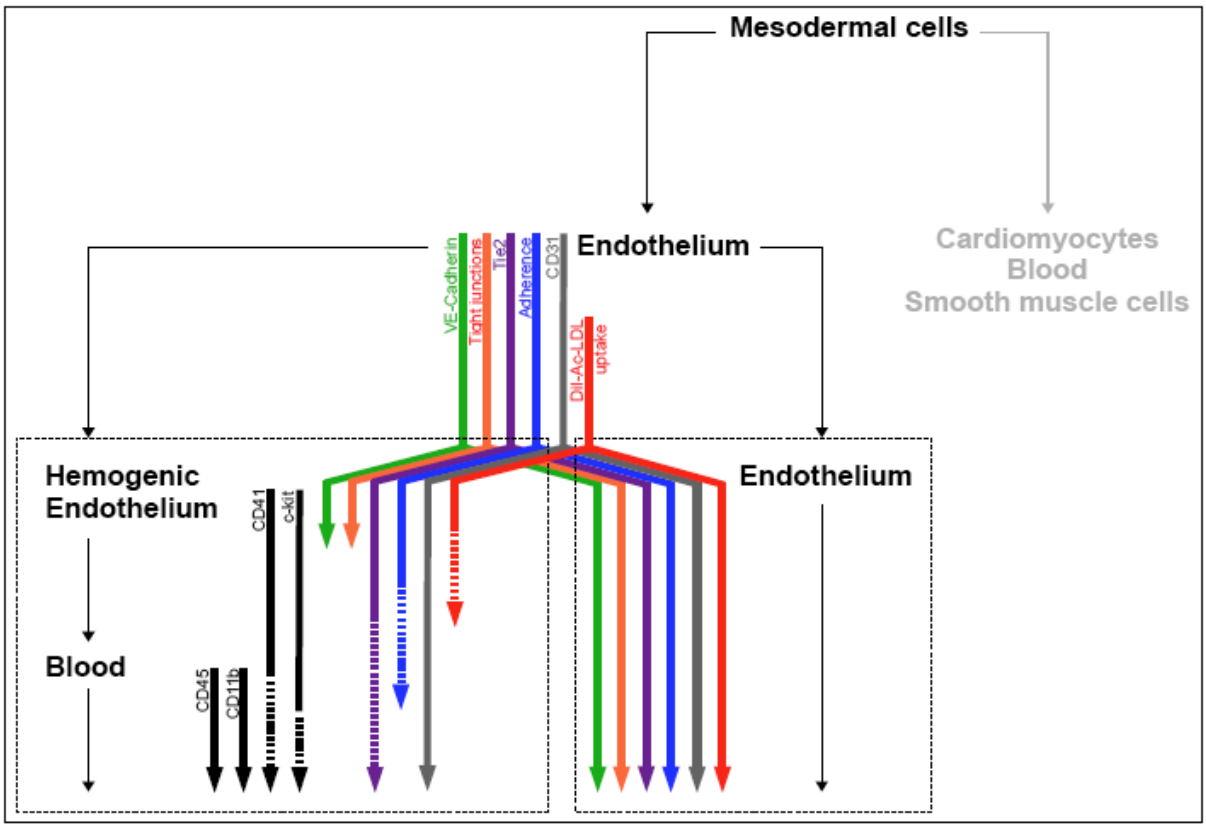

Figure 6.1: Model summarizing the results generated within this study. Mesodermal cells were shown to give rise to endothelial cells that display typical molecular and functional endothelial characteristics. They can produce daughters of different fate: non-hemogenic and hemogenic endothelial cells, both of which express identical endothelial markers. With ongoing differentiation, hemogenic endothelial cells up-regulate early hematopoietic molecules and down-regulate endothelial markers. Finally, hemogenic endothelial cells differentiate into blood cells that express several hematopoietic but no endothelial markers anymore.

7. Experimental Procedures

7.1. Molecular biology

7.1.1. Purification of plasmid DNA

High copy plasmids were purified from competent bacteria using the Qiaprep Spin Miniprep Kit or the Qiagen Plasmid Maxi Kit (cat no 27104 and 12165, respectively, Qiagen, Hilden, Germany). All DNA was solved in sterile bi-distilled water.

7.1.2. Construction of plasmid DNA

DNA was constructed using the Clone Manager software 7, 8 and 9 Professional Edition.

7.1.3. Transformation of bacteria

To transform bacteria with plasmids, heat-shock competent Escherichia coli (DH5□) were defrosted on ice for about 15 to 30 min. 25 ng plasmid DNA or the entire heat inactivated ligation cocktail were added to 50 µl bacteria. After incubating for 0.5 h on ice, cells were heated to 42°C for 45 sec before cooling them in ice for 2 min. Cells were mixed with 0.5 ml LB (LB Broth Base, cat no 12780-029; Invitrogen, Karlsruhe, Germany) medium without any antibiotics and kept on 37°C constantly shaking. 40 and 400 µl bacteria were plated on LB agar plates containing the required antibiotic, respectively, and cultured over night at 37°C.

7.1.4. Restriction digestions and ligations

Restriction digestions and ligations were carried out using enzymes and suitable buffers from NEB (New England Biolabs, Ipswich, USA) or Fermentas (St.Leon-Roth, Germany) according to the manufacturer's instructions.

7.1.5. Agarose gels

After digestion DNA fragments were separated on 0.8 to 2% agarose gels prepared with in TAE (Tris: cat no 5429.2, Roth, Karlsruhe, Germany; Acetate: cat no 1.00063.2511, Merck, Darmstadt, Germany; EDTA disodium salt dihydrate: cat no 8043.2, Roth, Karlsruhe, Germany), agarose (cat no 8700500, Biozym, Oldendorf, Germany) and

Ethidium bromide (cat no 2218.2, Roth, Karlsruhe). DNA fragments were separated with a voltage between 70 and 200 V in TAE-buffer depending on the size of gels.

7.1.6. Purification of DNA fragments

DNA fragments were cut out from agarose gels using a scalpel and purified from the agarose gel using the QIAEX II Gel Extraction Kit or the QIAQUICK Gel Extraction Kit (cat no 20021 and 28704, respectively, both Qiagen, Hilden, Germany). Purification of PCR products was performed using the QIAquick PCR Purification Kit (cat no 28104, Qiagen, Hilden, Germany).

7.1.7. Sequencing

For sequencing, DNA and primers were sent to MWG, Ebersberg, Germany.

7.2. Preparation of DNA for stable transfection

7.2.1. Phenol-Chloroform purification

Proteins and salts were removed from the DNA by Phenol-Chloroform purification. In detail, Phenol-Chloroform-Isoamylalkohl (25/14/1, cat no P2069, Sigma, Taufkirchen, Germany) was added to the reaction at the same volume and vortexed and centrifuged for 5 min at a speed of 19940 RCF. The aqueous layer was transferred into a new reaction tube and mixed with the equal volume Chloroform (cat no 1.02432.2500, Merck, Darmstadt, Germany), centrifuged and the aqueous layer was again purified with Chloroform twice. Afterwards the DNA was precipitated by Ethanol.

7.2.2. Ethanol precipitation

DNA sedimentations were carried out with Ethanol. To the solved DNA 10% of volume 3 M Sodiumacetate buffer solution (cat no S7899, Sigma, Taufkirchen, Germany) and 2 volumes of 100% Ethanol (EtOH, cat no 1.00983.2511, Merck, Darmstadt, Germany) were added and incubated 1 h at -80ºC. The sample was centrifuged for 15 min at 4ºC with 19940 RCF. The pellet was washed once with pre-cooled 70% EtOH carefully and centrifuged again before removing the supernatant. The pellet was dried on air for several minutes and solved in water.

7.3. LIF production

To produce leukemia inhibitory factor (LIF), calcium chloride ($CaCl_2$) competent bacteria (cat no 200235, Stratagene, La Jolla, USA) were transfected with the plasmid pGEX-2T-MLIF-amp encoding the LIF protein according to the manufacturer's instruction. Bacteria were plated onto 50 µg/ml ampicillin sodiumsalt (amp, cat no K039.1, Roth, Karlsruhe, Germany) containing LB agar plates, cultivated over night before a monoclonal colony was picked, transferred to 50 ml liquid LB-medium containing amp and again incubated over night.

The following day, the bacteria culture was mixed with 500 ml fresh LB medium with amp and cultivated in the presence of 0.5 ml 100 mM isopropyl-ß-D-thiogalactopyranoside (IPTG, cat no 2316.3, Roth, Karlsruhe, Germany) until the optic density of the culture was between 0.6 and 1. During the incubation, glutathione-sepharose beads (Glutathione Sepharose high Performance, cat no 17527901, Amersham Bioscience, Piscataway, USA) were prepared. 5 ml bead solution was centrifuged 2 min at 1250 RCF and washed three times with 20 ml MTPBS. The MTPBS was prepared from 150 mM NaCl (cat no S/3160/65, Fisher Scientific, Schwerte, Germany), 16 mM Na_2HPO_4 x 2 H_2O (cat no T876.2, Roth, Karlsruhe, Germany) and 4 mM NaH_2PO_4 x 2 H_2O (cat no 1.06346.1000, Merck, Darmstadt, Germany) in bi-distilled water and the pH was adjusted to 7.3 with HCl (cat no K028.1, Roth, Karlsruhe, Germany). The pellet was solved in 50% of the resulting volume MTPBS (1.5 ml MTPBS per 3 ml beads) and stored at 4 °C.

The bacteria were sedimented for 5 min at 4230 RCF at 4 °C and solved in 5 ml MTPBS. In the following, bacteria were only kept on ice. The bacterial suspension was sonificated four times for 15 sec at 90% with 20 sec pauses in between. 0.5 ml 10% Trition-X-100 (cat no 3051.2, Roth, Karlsruhe, Germany) was added before vortexing the suspension and incubating it on ice for 5 min. The LIF-containing supernatant was obtained by harvesting the solution for 5 min using 12000 RCF at 4 °C. Afterwards the supernatant was collected in a 50 ml polypropylene tube and stored on ice. 5 ml of supernatant was added to 2-2.5 ml solution of glutathione-sepharose beads and gently shaken at 4 °C for 2h. 10 ml pre-cooled 20% D(+)sucrosis (cat no 9286.1, Roth, Karlsruhe, Germany) solved in MTPBS was carefully added to the beads and sedimented for 5 min at 1250 RCF. After removing the supernatant, the beads are washed twice with 10 ml buffer 1 (1% Triton-X-100 in MTPBS) and once with 10 ml elution buffer prepared from 50 mM Tris pH8.5 (cat no 5429.2, Roth, Karlsruhe, Germany), 150 mM NaCl (cat no S/3160/65,

Fisher Scientific, Schwerte, Germany) and 2.5 mM $CaCl_2$ x 2 H_2O (cat no 5239.1, Roth, Karlsruhe, Germany). Finally the beads were re-suspended in 750 µl elution buffer. The thrombin (cat no 27-0846-01, Amersham, Bioscience, Piscataway, USA) was solved in 450 µl elution buffer and 150 µl were added to the bead solution. The mixture was incubated over night at 4 °C and shaken gently.

The next day, the beads were sedimented at 4 °C with 4300 RCF and washed with 2 ml elution buffer five times. All washing solutions were collected separately at 4 °C. 10 µl of each eluted fraction and few µl of commercially available LIF (cat no ESG1107, Millipore, Schwalbach, Germany) were mixed with 2.5 µl 1M DTT (cat no D0632, Sigma, Taufkirchen, Germany) and 12.5 µl 2 x SDS Bluebuffer separately. The Bluebuffer was was prepared from 12.5 ml 1.25 M Tris/HCl pH 6.8, 5 g SDS (cat no 2326.2, Roth, Karlsruhe, Germany), 12.5 ml 2-mercaptoethanol (cat no M6250, Sigma, Taufkirchen, Germany), 25 ml glycerine (cat no 3783.2, Roth, Karlsruhe, Germany) and some bromphenolblue (cat no 8122.0005, Merck, Darmstadt, Germany). All samples were separated by SDS-PAGE according to standard methods for 1h at 125 V. The gel was stained with Coomassie Blue using a staining solution prepared from 0.1% Coomassie Brilliantblue R250 (cat no A1092.0010, AppliChem, Darmstadt, Germany), 20% methanol (cat no 1.06007.2500, Merck, Darmstadt, Germany), 10% acetic acid (cat no K33632663 432, Merck, Darmstadt, Germany) over night at RT. To remove access staining solution, the gel was de-stained with 20% Methanol and 10% Acetic acid in water for 4h at 60 °C in the water bath. During this time, the de-staining solution was exchanged three times and the gel was shaken gently. Finally, the gel is washed with bi-distilled water for 15 min at RT while carefully shaking. Finally, the size of the LIF protein was compared to commercially available LIF. Both had to have a size of 23.4 kDa.

Whether the produced LIF can keep ESCs undifferentiated, EB3 ESCs were cultured in maintenance medium containing different concentrations of the produced LIF for more than 14 days and microscopically compared to a culture containing commercially available LIF (10^7 U/ml, cat no ESG1107, Millipore, Schwalbach, Germany) which was used at a concentration of 1500 U/ml. In following experiments, the produced LIF was used in the concentration that was suitable to keep ESCs undifferentiated.

7.4. Cell culture

All cells were cultured at 37°C, 5% CO_2 and 95% relative air humidity in a standard tissue culture incubator (Microbiological Incubator CD210; Binder; Tuttlingen, Germany).

All centrifugation steps were carried out with 200 RCF for 5 min if not indicated differently. Washing of cells was performed using sterile PBS prepared from 8 g NaCl (cat no S/3160/65, Fisher Scientific, Schwerte, Germany), 0.2 g KCl (cat no 4936.1000, Merck, Darmstadt, Germany), 0.2 g KH_2PO_4 (cat no 1.04873.1000, Merck, Darmstadt, Germany) and 1.18 g Na_2HPO_4 (cat no T876.2, Roth, Karlsruhe, Germany) in 1 l bi-distilled water and sterilized. To ensure good quality of the media, cell culture media were not stored for longer than 4 weeks after preparation.

7.4.1. OP9 cell culture

OP9 cells were cultured as described previously (Kodama et al., 1994). OP9 cells were kept in medium containing 20% of pre-tested FCS (cat no P30-3302, PAN Biotech GmbH, Aidenbach, Germany) in α-minimal essential medium (α-MEM). α-MEM was prepared from powder (cat no 25080, Gibco/Invitrogen, Karlsruhe, Germany) which was solved in a Sodium bicarbonate solution (cat no 11900-016, Gibco/Invitrogen, Karlsruhe, Germany) and sterile injection water (cat no 7202A161, Braun, Melsungen, Germany) according to manufacturer's instructions.

As OP9 cells easily loose their supportive capacity, their quality was verified continuously by their morphology: OP9 cells had to be large flat cells, not fibroblastic or transformed. The culture medium was exchanged every two days to remove dead cells and debris. OP9 cells were only kept for a maximum of 25 passages and only dissociated and diluted when 100% confluent as otherwise cells lost their supportive properties. In general, OP9 stroma was diluted every 3 to 4 days 1:4 by washing with PBS, trypsinizing with 0.05% Trypsin/EDTA (cat no 25300-054, Gibco/Invitrogen, Karlsruhe, Germany) for 2 min at 37°C. Dissociated OP9 cells were re-suspended in culture medium and mixed gently to obtain a homogenous cell solution which was diluted with medium, seeded onto fresh tissue culture dishes and moved carefully to ensure equal distribution of cells on the culture plate.

7.4.2. Embryonic stem cell maintenance culture

ESCs were maintained as described by Schroeder and co-workers (Schroeder et al., 2006). All ESCs were kept on gelatine coated dishes. 0.01% powdered gelatine (cat no G1890, Sigma, Taufkirchen, Germany) in bi-distilled was prepared and autoclaved to ensure sterility and homogeneity of the solution. Coating was performed by incubating

sterile gelatine solution in tissue culture plates for 15 min at RT. Afterwards, the supernatant was discarded.

In this study EB3 and EB5 (both different monoclonal ESC-lines derived from E14tg2a with one allele of Oct3/4 inactivated by targeted integration of the gene encoding the blasticidin resistance) ES cells, both kind gifts from Dr. Niwa (Niwa et al., 2000), were used because both showed good hematopoietic differentiation in comparison to other ESC lines (data not shown). EB3 and its transgenic offspring were cultured in 10% pre-tested FCS (cat no 2602P250915, PAN Biotech GmbH, Aidenbach, Germany), 1 mM Na-Pyruvate (cat no S8636, Sigma, Taufkirchen, Germany), 0.1 nM MEM nonessential aminoacids (cat no 11140035, Gibco/Invitrogen, Karsruhe, Germany), 10^{-4} M 2-mercaptoethanol (cat no 31350010, Gibco/Invitrogen, Karsruhe, Germany), 3 µl LIF solution per 1 ml medium (self-prepared and tested, see below) and Glasgow minimal essential medium (GMEM; cat no 21710-082, Gibco/Invirogen, Karlsruhe, Germany). EB5 and EB5-derived transgenic ESCs (VE-Cadherin::VENUSClaudin5; Guo et al., 2006) were cultured in 10% knockout serum replacement (KSR; cat no 10828-028, Gibco/Invitrogen; Karlsruhe, Germany), 1% FCS; Na-Pyruvate, nonessential aminoacids, 2-mercaptoethanol and LIF in GMEM.

If cells are kept too dense, ESCs spontaneously differentiated, even in the presence of LIF. Undifferentiated ESCs grow clonally and form densely packed cell clusters whereas differentiated ESCs loose the contact to each other and rather grow disperse. Thus, the cultures were checked every 1 to 2 days microscopically and controlled in regard to their density. Usually, cultures were separated every Monday, Wednesday and Friday 1:10 by washing with PBS and trypsinizing with 0.05% Trypsin/EDTA (cat no 25300-054, Gibco/Invitrogen, Karlsruhe, Germany). To maintain undifferentiated ESCs, cells were trypsinized very gently for 2 min at RT and re-suspended in culture medium as soon as the cells were observed to come off the culture plate. Afterwards cells were plated on cell culture dishes coated with gelatine beforehand.

7.4.3. Mesodermal differentiation of embryonic stem cells on OP9

The mesodermal differentiation of ESCs on OP9 was carried out as described by Fraser et al. (Fraser et al., 2002a).

To induce their differentiation, undifferentiated ESCs of passage number lower than 20 were trypinized as usual and re-suspended in ESC-differentiating medium containing 10% pre-tested FCS (cat no P30-3302, PAN Biotech GmbH, Aidenbach, Germany), $5x10^{-5}$

M 2-mercaptoethanol (cat no 31350010, Gibco/Invitrogen, Karlsruhe, Germany) and α-MEM (cat no 11900-016, Gibco/Invitrogen, Karlsruhe, Germany). For a good mesodermal differentiation process the medium was always prepared immediately before starting the differentiation. Care was taken especially of the 2-mercaptoethanol, as this agent can μonly support the differentiation if not too old. Thus, the 2-mercapoethanol solution should not be used for longer than 6 weeks after opening. After re-suspension, ESCs were counted in a Neubauer counting chamber and 80.000 cells were re-suspended in differentiation medium and carefully seeded per 60 cm^2 dish containing confluent OP9 stroma cells of a passage number lower than 25. ESCs differentiated to mesoderm within 4 days of culture without washing or changing the media (Nakano et al., 1994). To induce endothelial or hematopoietic differentiation cells were re-plated on a new layer of OP9 cells grown confluent at day 4 of differentiation. Either, ESC-derived cells and OP9 cells contained in the culture were directly transferred in a ratio of about 1:100 to a fresh confluent OP9 layer by trypsinizing the culture with 0.05% Trypsin/EDTA. After incubating for 2 min at 37°C, the reaction was stopped with differentiating medium, cells were re-suspended carefully and diluted to the amount needed with differentiating medium. Alternatively, the culture was FACS sorted (see below) at day 4 of differentiation to enrich for mesodermal cells. After dilution or purification of the culture, ESC-derived cells were plated onto fresh OP9 and cultured in ESC-differentiation medium supplemented with 100 ng/ml stem cell factor (SCF, PeproTech, Rocky Hill, USA, cat no 250-03) for the desired length.

7.4.4. Freezing and thawing of cells

To store ESCs and OP9 cells, cells were first frozen in dimethylsulfoxide (DMSO) - containing culture medium. After trypsinization, cells were re-suspended in normal culture medium and sedimented by centrifugation. The pellet was solved in culture medium containing 10% DMSO (cat no D2438, Sigma, Taufkirchen, Germany) and transferred to cryotubes and frozen in Cryo Freezing containers (cat no 5100-0001, Nalgene, Rochester, USA) at -80 °C. After one day, cells were transferred into the nitrogen tank. In the nitrogen tank, all cells were stored in the gas phase.

For thawing ESCs or OP9 cells were removed from the nitrogen tank and the lid of the cryotube was slightly opened to permit gas pressure release upon warming up. Cells were kept in a 37 °C water bath for the time the cells needed to defrost completely. After transferring them carefully into a Falcon tube, 9 ml preheated culture medium was

slowly (the suspension was dropped onto the cells within 10 min). To allow the solving of DMSO from the cells, the suspension was incubated for 10 min at RT before centrifuging it. The pellet was re-suspended carefully in preheated medium and cells were transferred into cell culture dishes and incubated. Approximately 5 x 10^5 frozen ES cells were transferred into a 10 cm^2 dish and approximately 4 x 10^5 frozen OP9 cells were plated in a 25 cm^2 dish after thawing.

7.4.5. Transfections

7.4.5.1. Transient transfections

All transient transfections were carried out using Lipofectamin2000 (cat no 11668-019, Gibco/Invitrogen, Karlsruhe, Germany) and Opti-MEM (cat no 51985, Gibco/Invitrogen, Karsruhe, Germany).

7.4.5.1.1. Embryonic stem cells

For transient transfection, ESCs were cultured to approx. 30% confluence in a 96-well-tissue culture plate. 0.16 to 0.2 µg DNA were prepared for each well of a 96 well plate and mixed with Opti-MEM to a volume of 50 µl. Separately, 5 µl Lipofectamin2000 (1 mg/ml) per each µg DNA was mixed with Opti-MEM to 50 µl volume. DNA and Lipofectamin2000 were pooled, vortexed and incubated for 20 min at RT. The supernatant of the ESCs was removed and cells were washed twice with Opti-MEM before incubating them with 100 µl Lipofectamin-DNA-mix per well for 4 h at 37ºC. Afterwards the medium was replaced by ESC maintenance medium. The analysis was carried out 24 to 48 h after transfection.

7.4.5.1.2. HEK 293T cells

To check the fluorescence signal produced from ubiquitously expressed plasmid DNA, HEK cells were transfected. Cells were prepared in a 10 cm^2 dish to be approx. 30% confluent at the day of transfection. For transfection 10 µl Lipofectamin2000 were mixed with 90 µl Opti-MEM and incubated 15 min at RT. 2 µg of DNA was added and vortexed well. After incubating another 15 min at RT the culture medium was removed and replace with new the Lipofectamin2000-DNA medium. Cells were growing in the Lipofectamin-DNA-containing medium for at least 24 h before analysis.

7.4.5.1.3. OP9 cells

To check specific localization of ubiquitously expressed tagged fluorescent proteins, OP9 cells were transiently transfected. 30% confluent OP9 grown in a 6-well dish were transfected as follows: 15 µl Lipofectamin2000 were mixed with 85 µl opti-MEM. Separately, 2 µg DNA were mixed with Opti-MEM to a final volume of 100 µl. Both were incubated for 20 min at RT, mixed and incubated for another 20 min at RT. Afterwards, the medium of OP9 cells was removed, replaced with the lipofectamin-DNA mixture and incubated for 2 days before analysis.

7.4.5.2. Stable transfections

7.4.5.2.1. Stable transfection of embryonic stem cells

For stable transfection a 60cm^2 dish containing confluent grown ESCs with a low passage number (maximum 3 passages after thawing) was prepared. Cells were trypsinized for 2 min at RT using 0.05 % Trypsin/EDTA (cat no 25300-054, Gibco/Invitrogen, Karlsruhe, Germany). The reaction was stopped by adding ESC maintenance medium and cells were centrifuged, washed with PBS, counted and sedimented again. The pellet was solved in pre-cooled PBS to a concentration of 10^7 cells per ml. 400 µl cell suspension was pored into a cuvette (4mm diameter; cat no 1652088, BioRad, Munich, Germany) and mixed gently with 40 µg DNA solved in sterile water (see above). After incubating 10 min on ice, the electroporation was performed with 240 V, capacitance of 700 µF, resistance of 300 Ω using a GenePulser II (BioRad, Munich, Germany). As a selection control, mock transfected cells were treated equally. Electroporated cells were transferred into 8 ml preheated differentiation medium immediately, transferred into gelatine-coated 60 cm^2 dishes and incubated over night. The following day, the medium, dead cells and debris was removed by washing once with PBS. Afterwards cells were cultured for another week in maintenance medium containing a selecting agent, e.g. puromycin dihydrochloride (cat no 0240.3, Roth, Karlsruhe, Germany) at a concentration of 1 µg/ml, or geniticin (G418, cat no 10131-019, Gibco/Invitrogen, Karlsruhe, Germany) at a concentration of 200 µg/ml. Selection was carried out up to 10 days before monoclonal ESCs were picked or all cells were pooled to obtain a polyclonal ESC line.

7.4.5.2.2. Stable transfection by co-transfection of plasmids

Large plasmids containing no selection cassette (pVE-mitoVENUS-IE) (Hisatsune et al., 2005) were stably brought into ESCs by co-transfection similarly as described previously

(Kitajima et al., 2002). $2x10^6$ undifferentiated ESCs of low passage number were transfected with either 4 µg of a VENUS-expressing plasmid (pUC19xIRES-VENUS) or 50 µg linearized plasmid pVE-mitoVENUS-IE, respectively, each with 3.15 µg plasmid encoding geneticin resistance (pCAG-IRES-neo) (= 1/5 molar of the target plasmid). As a selection control mock transfected cells were treated equally. The electroporation was carried out as described earlier using a GenePulserII (BioRad, Munich, Germany) programmed to 240V, 300 µF and ∞ Ω (see stable transfection of ESCs). The following day selection with 200 µg/ml geneticin (G418, cat no 10131-019, Gibco/Invitrogen, Karlsruhe, Germany) was started and performed for up to 10 days before monoclonal ESC colonies were picked and expanded.

7.5. Primary cell culture

7.5.1. Mouse lines

For the isolation of mesodermal cells from mouse embryos CD-1 wild-type mice were mated over night and checked for vaginal plugs before noon on the following day. Females which showed vaginal plugs were taken as 0.5 dpc.

7.5.2. Preparation of single cell suspensions from E7.5 to E9.5 dpc embryos

Staging and dissection of embryos was performed as described previously (Fraser et al., 2002b). Pregnant mice carrying E7.5 dpc, E8.5 dpc or E9.5 dpc embryos were sacrificed immediately before dissection. Uteri were uncovered and the uterus tissue was cut between each embryo without complete separation thereby facilitating the following dissection. Uteri were removed from the pregnant mice and washed in PBS. Importantly, all uteri, embryos and primary cells were kept in PBS on ice during the isolation if not indicated differently. Embryo-containing deciduae were released from the uterus tissue. The deciduae were opened one after another with forceps and whole embryos were removed carefully from residual decidua tissue (it is possible to first isolate all embryo containing deciduae before isolating the embryo proper; alternatively, deciduae can be dissected immediately). All extraembryonic tissue and embryo proper was used for cell preparation from E7.5 and E8.5 embryos. E9.5 embryos were dissected into yolk sac and AGM regions further on. The AGM region was extracted by cutting posterior of the heart and anterior of the vitelline and umbilical arteries. All embryos were collected in 10 ml 4°C cold PBS and stored on ice. Storing dissected embryos for longer then 2 h in PBS on ice is not recommended as cell death increases quickly. Thus, all dissections were carried

out as fast as possible. Nor more than embryos of 8 pregnant mice should be dissected by one person, otherwise the dissection will take too long.

After preparing all embryos, they were centrifuged at 300 G for 5 min and gently re-suspended in 1ml 0.05% preheated Trypsin/EDTA (cat no 25300-054, Gibco/ Invitrogen, Karlsruhe, Germany). It is of major importance that 0.05% Trypsin/EDTA instead 0.25% Trypsin/EDTA is used as the reaction is too strong with Trypsin/EDTA of a high concentration. According to the size of the embryos, the mixture was incubated for 7 min (E7.5) to 10 min (E9.5 AGMs) at 37 °C and shaken carefully to obtain a single cell solution. Embryos should not be incubated for longer even if not all tissue is solved yet as this kills the cells. Instead, the mixture eventually containing tissue residues will solve completely within the next step. The trypsinization was stopped by adding 9 ml sterile stop-solution containing 0.05 mg/ml DNAseI (cat no 11284932001, Roche, Penzberg, Germany), 0.1% 1M $CaCl_2$ x $2H_2O$ (cat no C7902, Sigma, Taufkirchen, Germany), 20% FCS in Hank's buffered salt solution (HBSS, 10x HBSS diluted with injection water; cat no 14185, Gibco/Invitrogen, Karlsruhe, Germany) at RT using a 2 ml pipette. The cells should be treated very gently avoiding heavy shaking to ensure viability of cells. Then, cells were sedimented as before, re-suspended in ESC differentiating medium and incubated in a 15 ml Falcon tube with slightly open lid for 1h in the tissue culture incubator (37 °C; 5% CO_2) to allow the cells to recover. Cells were centrifuged, washed with PBS, re-suspended in 4 to 8 ml (according to the size of the pellet) PBS and counted. From 1 pregnant mouse carrying E7.5 and E8.5 embryos on average 1.9 x 10^5 and 1.2 x 10^6 living cells were extracted, respectively. The AGM regions of all E9.5 embryos of one pregnant mouse contained on average 1.3 x 10^6, the yolk sacs 8 x 10^5 cells. All sedimented cells were re-suspended in 100 µl pure FCS and immunostaining for FACS was performed.

7.6. Protein detection

7.6.1. Flow cytometry (FACS)

7.6.1.1. Detection of fluorescent markers by flow cytometry

All cells were prepared in FACS buffer containing 20% FCS, propidium iodide (PI, P4170, Sigma, Taufkirchen, Germany) and PBS and filtered through a cell strainer (cat no 352235, BD Falcon, Franklin Lakes, USA) before analysis.

For analyzing samples by flow cytometry, a FACSCalibur System (Becton Dickinson, San Jose, USA) equipped with 488 nm and 635 nm lasers was used. FITC, Dylight488 and

VENUS signals were detected using the filter 530/30, PE and tdTOMATO signals were analysed with the 585/42 filter, PI was detected with the filter 630 LP and APC and Alexa647 signals were detected with the filter 661/16.

For cell sorting a FACSAria cell sorter (Becton Dickinson, San Jose, USA) was used. VENUS, FITC and Dylight488 signals were analyzed using the 488 nm laser and the emission filter 530/30. PE or DiI-Ac-LDL were excited with the 488 nm laser and detected using the emission filter 575/26. PE-Cy7 signals were evaluated using the 488 nm laser and the 780/60 emission filter. APC and Alexa647 were illuminated with the 633 nm laser and detected in the 660/20 emission filter. PI was excited using the 407 nm laser and detected using the 585/42 emission filter. Sorting was performed with a maximal flow rate of 4 at a high precision mode (0/16/0). All sorted cells were re-analysed to ensure a pure population of sorted cells.

Data were analysed with FACSDiVa (Becton Dickinson, San Jose, USA), CELLQuest Pro (Becton Dickinson, San Jose, USA) and WinMDI 2.8 (www.facs.scribbs.edu) softwares.

7.6.1.2. Purification of mesodermal cells by flow cytometry

7.6.1.2.1. Purification of embryonic stem cell-derived mesodermal cells

After 4 days of differentiation culture, ESC-derived cells were washed once with PBS and solved with 1 ml Hank's enzyme free dissociation buffer (cat no 13150-016, Gibco/Invitrogen, Karlsruhe, Germany) per 60 cm^2 of culture for 20-40 min at 37 °C (Trypsin/EDTA cannot be used here to dissociate the cells as the destruction of epitopes by Trypsin/EDTA prevents antibody binding). The reaction should not be carried out for longer as cells will die due to the dissociation. As the OP9 differentiation culture was very dense, cells attached strongly to one another. Even 40 min of incubation with Hank's enzyme free dissociation buffer did not completely dissociate the culture, but a single cell suspension can be obtained by washing with PBS: 9 ml of PBS was added and mixed to obtain a single cell solution. Cells were sedimented, washed once and counted before re-suspending the pellet in 100 µl pure FCS. Antibody staining was performed in FCS using Alexa647 labelled anti-E-Cadherin (clone ECCD2, a kind gift from Shin-Ichi Nishikawa, self-labelled with Alexa647 as described below) and biotinylated-anti-Flk1 (clone AVAS12a, cat no 13-5821-82, eBioscience, San Diego, USA) antibodies for 20 min at 4 °C. After washing with PBS staining with Streptavidin labelled with PE or PE-Cy7 (cat no 12-4317-87 and cat no 25-4317-82, respectively, eBioscience, San Diego, USA) in FCS was performed for another 15 min at 4 °C. After washing with PBS, the pellet was solved

in FACS buffer containing 20% FCS and PI (cat no P4170, Sigma, Germany) in PBS at a final concentration of 10^7 cells per ml. Living (PI^-) $Flk1^+$E-$Cadherin^-$ cells of the right size and granularity were sorted into ESC-differentiation medium.

7.6.1.2.2. Purification of primary mesodermal cells

Immunostaining of primary embryonic cells was carried out using an Alexa647 or Dylight488 labelled anti-VE-Cadherin (clone VECD1, a kind gift from Shin-Ichi Nishikawa) (Shirayoshi et al., 1986), a biotinylated-anti-Flk1 (clone AVAS12a, cat no 13-5821-82, eBioscience, San Diego, USA) and a PE- or FITC-labelled anti-CD41 (clone MWReg30, cat no 558040 and cat no 553848, respectively, Pharmingen, Heidelberg, Germany) antibody. After staining for 20 minutes at 4 °C, cells were washed and stained with PE or PE-Cy7-labelled Streptavidin (cat no 12-4317-87 and cat no 25-4317-82, respectively, eBioscience, San Diego, USA) in FCS and incubated for another 15 min at 4 °C. After washing cells were re-suspended in FACS buffer containing 20% FCS, PI and PBS and FACS sorting was performed. Living (PI-) cells of the right size and granularity were gated. $CD41^-Flk1^+$VE-Cadherin, $CD41^-Flk1^+$VE-$Cadherin^+$ and $CD41^+$ cells were sorted into ESC-differentiation medium enriched with 5% CO_2 beforehand. Sorted cells were counted: From one pregnant mouse carrying E7.5 embryos on average 2500 $CD41^-$ $Flk1^+$VE-$Cadherin^-$, 350 $CD41^-Flk1^+$VE-$Cadherin^+$ and 50 $CD41^+$ living cells were purified. 25.000 cells were seeded on 12.5 cm^2 confluent OP9 in ESC differentiation medium containing 100 ng/ml stem cell factor (SCF, PeproTech, Rocky Hill, USA, cat no 250-03) and incubated for 1h at 37 °C and 5% CO_2 before starting the time lapse experiment.

7.6.2. Immunhistochemistry of fixed cultures

The detection of smooth muscle cells in ESC-differentiation cultures was performed as described previously (Schroeder et al., 2003). In detail, the supernatant of the culture was removed and cells were washed once with PBS shortly. Cells were fixed for 3 min in Methanol (cat no 1.06007.2500, Merck, Darmstadt, Germany) containing 5% DMSO (cat no D2438, Sigma, Taufkirchen, Germany) on ice. The specimen was washed three times 3 min with Methanol on ice and air dried. Blocking was performed in a 5% BSA (cat no A9418, Sigma, Taufkirchen, Germany) solution prepared in water for 2 h at RT by shaking gently. Smooth muscle cells were stained at 4 °C over night with an anti-α-smooth muscle actin antibody (cat no C6198, Sigma, Taufkirchen, Germany) diluted

1:400 in blocking solution. Unbound antibody was removed by washing twice 5 min with PBS. The specimen was kept in PBS at 4 °C until analyzed by microscopy.

7.6.3. In culture immunostaining

For the detection of surface markers in the living culture antibodies directly labelled with a fluorescent dye were added to the culture. Table 7.1 lists the antibodies successfully used and their final concentration in the culture medium. PE-anti-Ter119 (cat no 553673, Pharmingen) antibody was also tested, but no specific staining was observed in ESC-differentiation cultures although erythrocytes are known to be present (Nakano et al., 1996). This means that the anti-Ter119 antibody cannot be used in in culture immunostainings.

Table 7.1: Antibodies and their fluorescent conjugates that were used for immunostaining in the living culture.

Antibody	Fluorescent dyes	Clone	Company	Cat no	Concentration in culture
anti-CD45	PE	30-F11	eBioscience	553081	20 ng/ml
anti-CD45	APC	30-F11	eBioscience	17-0451-82	20 ng/ml
anti-CD41	PE	MWReg30	Pharmingen	558040	20 ng/ml
anti-CD41	FITC	MWReg30	Pharmingen	553848	20 ng/ml
anti-Mac1	PE	M1/70	eBioscience	12-0112-081	20 ng/ml
anti-Tie2	PE	TEK4, CD202	eBioscience	12-5987-81	20 ng/ml
anti-c-kit	APC	2B8	eBioscience	17-1171-81	100 ng/ml
anti-CD31	FITC	MEC13.3	Pharmingen	553372	20 ng/ml

7.6.4. Staining of living endothelial cells by DiI-Ac-LDL

In ESC differentiations, 50 ng/ml 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate acetylated low density lipoprotein (DiI-Ac-LDL, cat no BT-902, Biomedical Technologies, Stoughton, USA) were added to the living culture beginning day 4 of ESC differentiation. In primary cell cultures, 100 ng/ml DiI-Ac-LDL was added immediately before plating FACS purified cells.

7.6.5. Labelling of antibodies

The labelling of E-Cadherin (Clone ECCD2) and VE-Cadherin (Clone VECD1) (Shirayoshi et al., 1986) antibodies, both kind gifts from Dr. Shin-Ichi Nishikawa, was performed according to the manufacturer's instructions.

Both antibodies were labelled with Alexa647 (cat no A20186, Bioprobes/Invitrogen, Karlsruhe, Germany).

In addition, the VECD1 antibody was labelled with Dylight488 (cat no 46402, Thermo Scientific, Rockford, USA). 200 µg VECD1 antibody solved in 34 µl was mixed with 6.22 µl of Dylight488 solution (2 mg/ml) and gently mixed at RT for 1.5 h in the dark. Afterwards, the unbound fluorescent dye was dialysed against PBS for 1.5 days at 4 °C using a Slide-A Lyzer Mini Dialysis Unit 10000 Micro (cat no 69574, Pierce Perbio, Bonn Germany). To ensure sterility and stability, ad 0.09% NaN_3 (cat no S2002, Sigma, Karlsruhe, Germany) and ad 5 mg/ml BSA (cat no A9418, Sigma, Taufkirchen, Germany) were added to all antibodies and titrated as described below.

7.6.6. Titration of antibodies

The E-Cadherin antibody was titrated using 0.1, 0.5, 1, 5 and 10 µl labelled antibody per 10^6 undifferentiated ESCs that were solved with Hank's enzyme free dissociation buffer (cat no 13150-015, Gibco/Invitrogen, Karlsruhe, Germany) for 30 min at 37 °C before, and compared to a previous batch labelled antibody.

The VE-Cadherin antibody was titrated by staining a 7 day-ESC-differentiation culture. 10.000 undifferentiated ESCs were seeded on a 60 cm^2 dish confluent OP9 and cultivated in ESC-differentiation medium. After 7 days, cells were solved with 1 ml Hank's enzyme free-dissociation buffer (cat no 13150-015, Gibco/Invitrogen, Karlsruhe, Germany) for 30 min at 37 °C, washed with PBS and stained as described below.

Alternatively, VE-Cadherin antibody was titrated using primary endothelial cells extracted from the lungs of adult wild-type mice (Dong et al., 1997). After dissection and washing with PBS, the lungs were cut into small pieces of 1-2 mm size using a scalpel before digesting them in 10 ml collagenase I (cat no X5D8000, Worthington, Lakewood, USA) solved ad 1 mg/ml in PBS to a final activity of 199 U/ml. While incubating for 45 min at 37 °C, the solution was shaken heavily every few minutes to achieve a single cell solution. The solution was filtered through a cell strainer (pore size 70 µm; cat no 352350, BD Falcon, Franklin Lakes, USA) to remove tissue residues. Cells were

centrifuged for 10 min at 370 RCF. The pellet was washed with FACS buffer (20% FCS in PBS), counted and centrifuged for 5 min at RCF 370 before solving cells in FCS and staining for FACS.

The antibody staining was performed for 20 min at 4 °C at the concentrations 0.1, 0.5, 1, 5 and 10 µl per 10^6 cells in FCS. As a positive control pre-tested antibody, as negative control unstained sample were used, respectively. After washing, cells were solved in 20% FCS in PBS and propidium iodide and living (PI^-) cells of the right size were analyzed by FACS in respect to ratio of labelled cells and signal intensity. The antibody concentration was selected to be suitable that brightly and specifically labelled all endothelial cells compared to the control antibody.

7.7. Time lapse microscopy

7.7.1. Preparation of specimen

Initial experiments confirmed that cultures behave normal without feeding and constant gas exchange (up to 7 days tested) if the medium and the air inside the culture dish is saturated with 5% CO_2 and gas exchange is prevented. Thus, purified cells to be recorded by time lapse analysis were plated in medium already saturated with 5% CO_2. According to the following formula the time needed for enrichment of the medium with CO_2 was calculated: $t=H^2/D$ with t being the time [s], H the fill level of the media [cm] and D being the diffusion coefficient of 5% CO_2 (2.0×10^{-5} cm^2) (Dr. Marcus Hauser, Universität Magdeburg, personal communication). After plating cells the tissue culture dish was kept in the incubator for another hour to allow gas exchange. Then the flask was tightly sealed quickly inside the incubator and time-lapse analysis was started.

7.7.2. Acquisition of time lapse images

Time lapse movies were recorded using a Zeiss inverted fluorescence microscope (Axiovert 200 M, Zeiss, Hallbergmoos, Germany), a motorized stage (cat no 0431478, Märzhäuser, Wetzlar-Steindorf, Germany) and a PECON heating system to heat a XL incubator (Erbach, Germany) at 37 °C. Images were acquired using Aviovision 4.5 software up-graded with a software developed in our laboratory (Timm Schroeder, unpublished).

Phase contrast images were taken every 4 to 5 min with 5x (cat no 440321-9902-000) or 10x EC-Plan-Neofluar Ph1 (cat no 440331-9902-000) objectives, a 0.63x or 1x TV-adaptor using a AxioCam HRm camera at 1388x1040 or 2776x2080 pixel resolution (all

Zeiss, Hallbergmoos, Germany). Fluorescence images were acquired in regular intervals using a mercury (HBO 103 W/2) or a xenon (XBO 75 W/2 ORF, both Osram, Augsburg, Germany) lamp. Illumination to detect nuclear staining was performed every 12 to 15 min (150 msec excitation using filterset 46, cat no 000000-1196-681, Zeiss, or 46HE, cat no 489046-0000-000, Zeiss, for H2BVENUS signals). VE-Cadherin::VENUSClaudin5 signals (1000 msec using filterset 46HE) and all antibody stainings were detected every 45 to 60 min. For detecting specific antibody signals filterset 46HE was used for FITC, filterset 43 (cat no 000000-1114-101, Zeiss) and filterset 43HE (cat no 489043-0000-000, Zeiss) was used for PE and filtersets 26 (cat no 488026-0000-000, Zeiss) or 50 (cat no 488050-0000-000, Zeiss) were used for APC. All antibody stainings were illuminated 800-1000 msec. DiI-Ac-LDL staining was detected every 12 to 15 min (150 msec using filterset 43 or 43HE). All filtersets were purchased from Zeiss (Hallbergmoos, Germany). Images acquired were saved as jpg images compressed to 99% of the original quality.

7.7.3. Data analysis

Data were analysed using TTT (Timm's Tracking Tool, unpublished software developed in our laboratory) on Siemens Celsius R630 workstations with 4 GB of RAM and SUSE Linux operating systems with KDE desktop. Movies were analysed using all individual images acquired and by separation of phase contrast and all fluorescent wavelengths without further compression to maintain highest image quality. Image contrast was manually enhanced for optimal recognition of relevant cellular features separately for each wavelength channel. Individual cells were observed and tracked manually evaluating every time point. As a example demonstrating the analysis of the data using TTT, Supplementary Movie 7.1 is added. Each individual frame acquired is loaded and played with very low speed to follow individual cells.

All relevant properties and behaviour (division, death, position, adherence, fluorescence, morphology) of cells of interest were stored and displayed in pedigrees. All cells with questionable identity were excluded from relevant analyses.

7.7.4. Image processing

Movies were converted to greyscale, annotated and assembled to Audio Video Interleave (avi)-files using Metamorph Offline 6.1r4. To obtain QuickTime Movie (mov)-files, assembly of frames was performed by QuickTime 7.1.6 software. Still images were processed on Axiovision 4.5 software.

7.8. Statistical analysis

The statistical significance of differences was determined using the student's t-test for unpaired data.

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9. Legends to Time lapse Movies

A DVD containing all time lapse movies is inserted at the back cover.

Supplementary Movie 5.1

Time lapse movie corresponding to Figure 5.3 showing that cells with an endothelial morphology can generate blood cells. ESCs differentiated on OP9 stroma into mesodermal cells which were purified by FACS and re-plated on OP9 before starting the acquisition of time lapse images. The arrowheads and arrow inserted in the movies point to the cells displayed in Figure 5.3. The mesodermal starting cell (1st pause) founds a colony (2nd pause) that adopts an endothelial morphology (3rd pause). With ongoing differentiation, some cells detach from the endothelial sheet (4th pause) and turn semi-adherent (5th pause) before they are released into the supernatant and become hematopoietic-looking cells (6th pause).
Time scale: days – hours:minutes:seconds

Supplementary Movie 5.2

Time lapse movie corresponding to Figure 5.4 displaying phase contrast images (left panel) and H2BVENUS detecting fluorescence images (right panel) that show that cells with an endothelial morphology deriving from a monoclonal origin can generate blood cells. ESCs differentiated on OP9 stroma into mesoderm which were purified by FACS and re-plated on OP9 before starting the acquisition of the time lapse movie. The arrow is following the starting cell and one daughter after each cell division until the end of the movie. The arrowheads in the pauses depict the cells displayed in Figure 5.4. 1st pause: single mesodermal cell starting the colony. 2nd pause: all cells of the colony exhibit a clear endothelial morphology. 3rd pause in movie: blood cells detach from the endothelial sheet but keep adhering to the endothelial cells. 4th pause: blood cells detach and are free-floating.
Time scale: days – hours:minutes:seconds

Supplementary Movie 5.3

Time lapse movie corresponding to Figure 5.11: proof of cell identity by molecular and functional markers. ESCs differentiated on OP9 stroma into mesoderm which were

purified by FACS and re-plated on OP9 before starting the acquisition of the time lapse movie. Upper left panel: phase contrast, upper right panel: detection of DiI-Ac-LDL, lower left panel: detection of VE-Cadherin::VENUSClaudin5, lower right panel: detection of APC-anti-CD45 antibody. The arrow marks the hemogenic endothelial cell and follows one daughter after each cell division. During the pauses in the movie, the arrowheads point to the cells depicted in Figure 5.11. Fluorescent images were all acquired at distinct time points and not at each time point a phase contrast image was taken. A single hemogenic endothelial cell expressing VE-Cadherin, forming tight junctions and taking up DiI-Ac-LDL is depicted (1st pause). It does not express the hematopoietic marker CD45 (2nd pause). Blood cells arising from the hemogenic endothelial cell have down-regulated the endothelial cell markers VE-Cadherin::VENUSClaudin5 and DiI-Ac-LDL uptake (3rd pause) but instead express the hematopoietic cell marker CD45 (4th pause).
Time scale: days – hours:minutes:seconds

Supplementary Movie 5.4

Time lapse movie corresponding to Figure 5.13: endothelial colonies of cord-like morphology can give rise to blood cells. ESCs differentiated on OP9 stroma into mesoderm which were purified by FACS and re-plated on OP9 before starting the acquisition of the time lapse movie. Upper left panel: phase contrast, upper right panel: detection of APC-anti-CD45 antibody, lower left panel: detection of VE-Cadherin::VENUSClaudin5, lower right panel: detection of DiI-Ac-LDL. Fluorescent images were all acquired at distinct time points and not at each time point a phase contrast image was taken. The time lapse movie starts, when the cord-like endothelial colony has developed. A colony is shown that displays all molecular and functional endothelial characteristics (VE-Cadherin expression, tight junction formation and DiI-Ac-LDL uptake) but no endothelial sheet morphology (1st pause) or the blood cell marker CD45 (2nd pause). With ongoing differentiation, the endothelial colony gives rise to suspension cells that have lost the ability to express VE-Cadherin, form tight junction or take up DiI-Ac-LDL (3rd pause) but they instead express the hematopoietic antigen CD45 (4th pause).
Time scale: days – hours:minutes:seconds

Supplementary Movie 5.5

Time lapse movie analyzed in Figure 5.14: hemogenic endothelial cells express CD41 antigen. ESCs differentiated on OP9 stroma into mesoderm which were purified by FACS and re-plated on OP9 before starting the acquisition of the time lapse movie. Upper left panel: phase contrast, upper right panel: detection of VE-Cadherin::VENUSClaudin5, lower left panel: detection of PE-anti-CD41 antibody, lower right panel: detection of APC-anti-CD45 antibody. Fluorescent images were not acquired at each time point a phase contrast image was taken. The arrow marks the hemogenic endothelial cell and follows one daughter after each cell division as soon as single cells can be recognized in the movie. During the pauses in the movies, the arrowheads point to the cells depicted in Figure 5.14. The time lapse movie starts, when the endothelial colony has developed. A colony is shown that displays the molecular and functional endothelial characteristics VE-Cadherin expression and tight junction formation but not the hematopoietic markers CD41 and CD45. It adopts a sheet-like colony with single endothelial cells up-regulating CD41 (1^{st} pause). Cells expressing CD41 become semi-adherent (2^{nd} pause) and finally free-floating. These cells do not express VE-Cadherin::VENUSClaudin5 anymore but the blood cell markers CD41 and CD45 (3^{rd} pause).
Time scale: days – hours:minutes:seconds

Supplementary Movie 5.6

Time lapse movie corresponding to Figure 5.15 showing that hemogenic endothelial cells express Tie2 and c-kit antigens. ESCs differentiated on OP9 stroma into mesoderm which were purified by FACS and re-plated on OP9 before starting the acquisition of the time lapse movie. Left panel: phase contrast, middle panel: detection of PE-anti-Tie2 antibody, right panel: detection of APC-anti-c-kit antibody. Fluorescent images were not acquired at each time point a phase contrast image was taken. The movie shown is moving slightly as the specimen weakly changed position during the acquisition of the movie. The arrow marks the founder cell and follows one daughter after each cell division as soon as single cells can be recognized in the movie. During the pauses in the movies, the arrowheads point to the cells depicted in Figure 5.15. A mesodermal founder cell is shown that differentiates into an endothelial colony expressing Tie2 and displaying sheet morphology (1^{st} pause). $Tie2^{+}$c-kit^{+} turn semi-adherent (2^{nd} pause). After complete detachment, some of them have down-regulated Tie2 but still express c-kit (3^{rd} pause).
Time scale: days – hours:minutes:seconds

Supplementary Movie 5.7

Time lapse movie corresponding to Figure 5.16 showing that hemogenic endothelial cells can give rise to $Mac1^+$ blood cells. ESCs differentiated on OP9 stroma into mesoderm which were purified by FACS and re-plated on OP9 before starting the acquisition of the time lapse movie. Upper left panel: phase contrast, upper right panel: detection of FITC-anti-CD41, lower left panel: detection of APC-anti-CD45 antibody, lower right panel: detection of PE-anti-Mac1 antibody. Fluorescent images were not acquired at each time point a phase contrast image was taken. The arrow marks the hemogenic endothelial cell and follows one daughter after each cell division. During the pauses in the movies, the arrowheads point to the cells depicted in Figure 5.16. An endothelial sheet colony is shown. The semi-adherent hemogenic endothelial cell expresses CD41 (1^{st} pause). With ongoing differentiation, its daughter up-regulates Mac1 antigen on its surface (2^{nd} pause). Slightly later, it has down-regulated CD41, but expresses Mac1 and CD45 (3^{rd} pause).
Time scale: days – hours:minutes:seconds

Supplementary Movie 5.8

Time lapse movie corresponding to Figure 5.18: generation of hemogenic endothelial cells from mesodermal cells purified from E7.5 dpc mouse embryos. Mesodemal cells were purified from E7.5 dpc embryos and plated in OP9 stroma before time lapse imaging was started. Left panel: phase contrast, middle panel: detection of DiI-Ac-LDL uptake, right panel: detection of FITC-anit-CD41 antibody. Fluorescent images were all acquired at distinct time points and not at each time point a phase contrast image was taken. The arrow marks the hemogenic endothelial cell and follows one daughter after each cell division as soon as single cells can be recognized in the movie. The arrowheads point to cells depicted in Figure 5.18. At the start of the movie one single mesodermal cell is shown that gives rise to an endothelial sheet colony, that expresses DiI-Ac-LDL and partly CD41 (pause in movie). $CD41^+$ cells detach from the endothelial sheet, down-regulate DiI-Ac-LDL uptake and strongly increase the expression of CD41 antigen.
Time scale: days – hours:minutes:seconds

Supplementary Movie 5.9

Time lapse movie corresponding to Figure 5.21 showing undifferentiated ESCs that express VENUSnucmem after induction by activation of Cre recombinase with tamoxifen.

Upon plating ESCs in the presence of tamoxifen, the time lapse movie was started. Left panel: phase contrast images; middle panel: VENUSnucmem detecting fluorescence images; right panel: overlay of phase contrast and fluorescent images detecting VENUSnucmem. Undifferentiated ESCs form a colony and start the up-regulation of VENUSnucmem approx. 12 h after start of the movie. Individual cells forming the colony do not express VENUSnucmem equally as visible by the fluorescence intensity of single nuclei.
Time scale: days – hours:minutes:seconds

Supplementary Movie 7.1

This time lapse movie covering a period of about 5 hours demonstrates the temporal and optical resolution of the primary image sequence used for cell tracking. All frames acquired in 5 hours are used for the compilation of the movie. Upper left panel: phase contrast, upper right panel: detection of fluorescent H2BVENUS, lower left panel: phase contrast including the tracks for single cell tracking, lower right panel: overlay of phase contrast and fluorescent H2BVENUS images.
Time scale: days – hours:minutes:seconds

10. Abbreviations

°C	degree Celsius
µ	micro
AGM	aorta-gonads-mesonephros
AML	acute myeloid leukaemia
amp	ampicillin
APC	allophycocyanine
approx	approximately
BC	blood cell
bFGF	bovine fibroblast growth factor
BMP4	bone morphogenic protein 4
bp	base pair
BSA	bovine serum albumin
CAG	chicken beta actin
cat no	catalogue number
Clnd5	claudin5
CMV	cytomegalovirus
d	day
D	diffusion coefficient
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate
DiI-Ac-LDL	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate acetylated low density lipoprotein
DNA	deoxyribonucleic acid
DMSO	dimethyl-sulfoxide
dpc	days post coitum
E	embryonic day
EC	endothelial cell
E-Cadherin	epithelial Cadherin
EDTA	ethylene-diamin-tetraacetic acid
e.g.	example given
Ep	embryo proper
ES	embryonic stem
ESC	embryonic stem cell
EtOH	Ethanol

F	Farad
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
Fig	figure
FITC	fluoresceinisothiocyanate
Flk1	foetal liver kinase 1
Flt	fms-related tyrosine kinase
FLP	FLP recombinase
FLP^e	enhanced FLP recombinase
FSC	forward scatter
g	gram
G418	geniticin
GB	giga byte
G-CSF	granulocyte colony stimulating factor
h	hour
H	hight
H2B	histone 2B
HBSS	Hank's buffered saline solution
HSC	hematopoietic stem cell
hsp	heat shock protein
kDa	kilo Dalton
IFN	interferon
IL	interleukin
IPTG	isopropyl- β-D-thiogalactopyranoside
IRES	internal ribosomal entry site
L	litre
LB	LB broth base
LIF	leukaemia inhibitory factor
LTR-HSC	long-term repopulating hematopoietic stem cells
M	molar
m	milli
M-CSF	macrophage colony stimulating factor
MEM	minimal essential medium
min	minutes
n	nano

neo	neomycin (geniticin)
nucmem	nuclear membrane
OHT	4-hydroxy-tamoxifen
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrine
PI	propidium iodide
P-Sp	para-aortic splanchnopleura
puro	puromycin
RCF	radial central force
rpm	rounds per minute
RT	room temperature
Sca-1	stem cell antigen 1
SCF	stem cell factor
SCL	stem cell leukaemia
SDS	sodium dodecyl sulfate
sec	seconds
SMA	smooth muscle actin
SSC	side scatter
TAE	Tris acetate EDTA
tal1	T cell acute leukaemia
TBD	tamoxifen binding domain
TTT	Timm's Tracking Tool
V	Volt
VE-Cad	vascular endothelial cadherin
VE-Cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
wt	wildtype
YS	yolk sac

Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt habe. Ferner habe ich weder versucht, eine Dissertation einzureichen oder eine Doktorprüfung durchzuführen, noch wurde die Dissertation oder Teile derselben einer anderen Prüfungskommission vorgelegt.

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Hanna Eilken

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