Aus dem Institut für Kardiovaskuläre Genomik und Epigenomik
der Medizinischen Fakultät Mannheim
(Leitung: Prof. Dr. Gergana Dobreva)

The impact of mechanical forces on endothelial cell differentiation

Inauguraldissertation
zur Erlangung des medizinischen Doktorgrades
der
Medizinischen Fakultät Mannheim
der Ruprecht-Karls-Universität
zu
Heidelberg

Vorgelegt von
Patricia Laura Schäfer

aus
Mannheim
2024

Dekan: Herr Prof. Dr. med. Sergij Goerdt

Doktormutter: Frau Prof. Dr. rer. nat. Gergana Dobreva

TABLE OF CONTENTS

1 LIST OF ABBREVIATIONS

CD31	cluster of differentiation 31 (PECAM1)
CD34	cluster of differentiation 34
c-Myc	protooncogene c-Myc
CPC	cardiac progenitor cell
Ctr	control
D0/2/…	day 0/2/… after start of differentiation
DAPI	4',6-diamidino-2-phenylindol
DMEM	Dulbecco’s modified eagle medium
DMSO	dimethyl sulfoxide
cDNA	complementary DNA
EB	embryonic body
EC	endothelial cell
ECM	extracellular matrix
EHT	endothelial-to hematopoietic transition
ESC	embryonic stem cell
E14	embryonic stem cell line derived from mouse strain 12910la
FACS	fluorescence activated cell sorting (flow cytometry)
FBS	fetal bovine serum
FCS	fetal calf serum
Flk1	VEGFR2, kinase insert domain receptor
FVD	fixable viability dyes

GO	gene ontology
h	hour
HPC	hematopoietic progenitor cells
HPSC	hematopoietic progenitor and stem cells
HSC	hematopoietic stem cells
HUVEC	human umbilical vein endothelial cell
Ki-67	antigen Ki-67
KO	knock out
L-Gln	l-glutamate
LIF	leukaemia inhibitory factor
NEAA	non-essential amino acids
MEF	mouse embryonic fibroblast
mESC	mouse embryonic stem cell
min	minute(s)
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
NO	nitrogen oxide
Pa	pascal
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PECAM1	platelet endothelial cell adhesion molecule 1

Pen/Strep	penicillin/streptomycin
PE-Cy7-A	tandem fluorochrome out of R-phycoerythrin and cyanine dye 7
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RNAseq	RNA sequencing
RT	room temperature
s	second(s)
SD	standard deviation
Str	stretched
Tnnt2	gene, which encodes the cardiac muscle troponin T protein
US	unstretched
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth receptor 2 (=Flk1 in mice)
VSM	vascular smooth muscle cell
vWF	von-Willebrand-Factor
w/o	without

2 ABSTRACT

Mechanical forces are vital for the development and maintenance of endothelial cells. However, how tensile forces or extracellular matrix stiffness affect differentiation of endothelial cells during embryogenesis is largely unexplored. In my dissertation, I applied non-cyclic uniaxial stretch on differentiating mouse embryonic stem cells and used materials with different stiffness, like laminin, matrigel, gelatine and poly-l-lysine, to study the interplay between these distinct types of mechanical forces on endothelial cells. Using flow cytometry analysis, immunofluorescence stainings and quantitative expression analysis, I identified key functions of stretch and matrix stiffness in triggering gene expression program facilitating endothelial cell development and differentiation. I found that application of non-cyclic uniaxial stretch for 2 hours at early developmental stages, such as stem cells, mesodermal precursors and cardiovascular precursors, was sufficient to significantly increase endothelial cell numbers, while application of stretch at later stages was not beneficial. Differentiation of embryonic stem cells on laminin strongly promoted endothelial cell differentiation, similarly on matrigel, although to a smaller range. However, the differentiation on gelatine and poly-l-lysine did not increase endothelial cell numbers. To study the interplay between matrix stiffness and tensile forces, I analysed endothelial cell numbers in differentiating embryonic stem cells subjected to stretch at different stages and cultured on different matrices. Interestingly, I found that application of mechanical stretch on cells cultured on a soft matrix, e.g. matrigel, did not enhance endothelial cell differentiation, while application of the same stretch strength on cells cultured on a stiffer matrix, e.g. gelatine, resulted in significant increase in endothelial cells. To study the molecular mechanisms behind the enhanced endothelial cell differentiation, I performed RNA-sequencing analysis. I identified key genes involved in endothelial cell and vascular system development to be highly upregulated upon application of stretch even after a short period of time (2 hours), such as Vegfa, Fgf7, Igf1.

In summary, my work suggests a tight interplay between tensile forces and extracellular matrix stiffness during endothelial cell development and differentiation and identified critical factors involved in this process.

3 INTRODUCTION

Endothelial cells (EC) play a crucial role in the growth and survival of all vertebrates by performing tasks that are necessary for tissue formation and maintenance. All tissues depend on the steady supply of essential nutrients, oxygen and growth factors, as well as the effective removal of toxic molecules and CO_2. ECs form different types of blood vessels, ranging from the smallest capillaries to the aorta and vena cava (Bruce Alberts, 2002).

EC differentiation and vascular growth play essential roles in various crucial processes throughout an organism's lifespan. For instance, the vital process of wound healing is inseparability linked to the formation of new vessels. The complex process of sprouting and revascularization within a wound is orchestrated by a multitude of influences, including inflammatory molecules, growth factors and mechanical stresses applied to the injured tissue (Kilarski et al., 2009; Singh et al., 2022).

The phenomenon of expanding an existing vascular network finds clinical application in the therapeutic management of substantial wounds as well. Specifically, the utilization of tissue expanders beneath the skin, gradually inflated over time, induces a stretching effect on the surrounding tissue, leading to the enlargement not only of the skin but also of the associated vasculature (Heit et al., 2012; Lee et al., 1989).

Besides medical or pathology triggered incidents, physiological processes like the proliferation of vital tissue require an adapted vascular network. For instance, the augmentation of muscle tissue necessitates the concurrent expansion and proliferation of the supplying vessels. The mechanical stimuli arising from the escalating volume of muscle tissue and the consequent upregulation of blood flow initiate a cascade of events, including the remodelling, enlargement and proliferation of both the vascular network and the ECs it is composed of (Prior et al., 2004).

Getting insights into the factors and genetics of EC development holds significance not only in physiological contexts but also in clinical applications, contributing to advancements in therapeutic approaches.

3.1 Formation of the first endothelial cells and the vascular system

During embryonic development, the cardiovascular system is the first formed system in developing vertebrae (Risau and Flamme, 1995).

The formation of blood vessels starts in the developing embryo at the point of time when it is no longer possible to provide enough oxygen and nutrition to the whole embryo only trough diffusion (Sadler, 2014). There are two possible forms of gaining new blood vessels. The first form, prolonging and sprouting already existing vessels, is called angiogenesis (Risau, 1997; Rizzi et al., 2017). The second one, the complete new assembly and differentiation of ECs out of precursor cells, is called vasculogenesis (Sadler, 2014) **(Fig.1A)**. EC differentiation originates from embryonic stem cells that give rise to mesodermal progenitors in the lateral plate mesoderm. These precursors become hemangioblasts, from which endothelial precursor cells are derived. They finally differentiate into ECs, which mature and specialize over time (Tsang et al., 2017; Yamaguchi et al., 1993; Yamashita, 2004).

Vasculogenesis appears in mouse embryos between day 8 and day 10 (Goldie et al., 2008; Wood et al., 1997). At this time, cells in the lateral plate mesoderm, more specifically in the splanchnopleuric mesoderm and the yolk sac, start to form blood islands containing cells called hemangioblasts. The hemangioblasts in the centre of the blood islands differentiate into hematopoietic precursor cells, the ones at the outer layers into precursor cells of ECs, called angioblasts (Risau and Flamme, 1995). The extra-embryonic angioblasts in the yolk sac and the intra-embryonic angioblasts both proliferate under the influence of the growth factor VEGF (vascular endothelial growth factor) that is produced from the surrounding mesoderm. The extra-embryonic angioblasts start to differentiate into vascular ECs and pool to form a primary capillary plexus (Sadler, 2014). The intra-embryonic vascular ECs form tubes which give rise to the dorsal aorta and the cardiac veins **(Fig.1B)**. The primary capillary plexus remodels and consolidates with the cardiac veins and the dorsal aorta. All together, they form the vascular system of the embryo. Through the release of growth factors like PDGF (platelet-derived growth factor) they stimulate the migration of smooth muscle cells and pericytes (Rymo, 2011). Later growth of vessels happens nearly exclusively by angiogenesis with the out-sprouting from already existing vessels due to external stimuli.

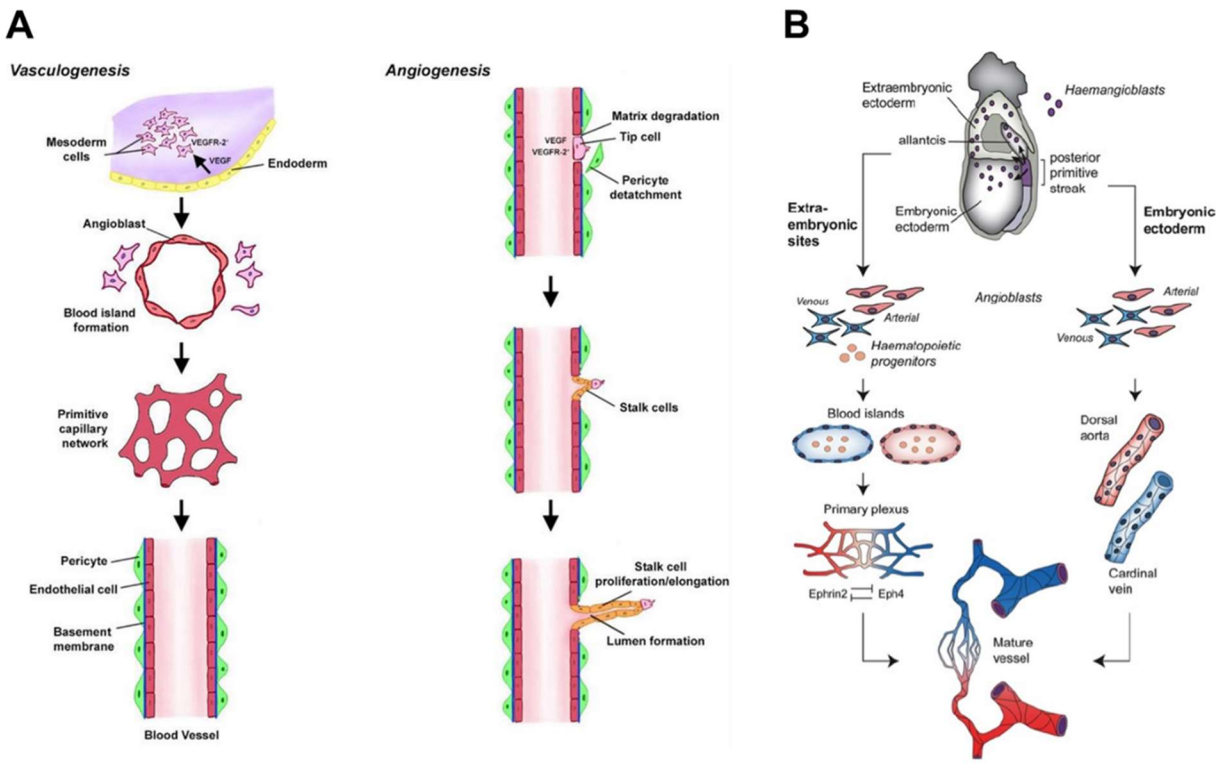

Figure 1: Vascular system development (A) Vasculogenesis versus angiogenesis (Markiewski et al., 2020) **(B)** Vessel development in the mammalian embryo (Markiewski et al., 2020; Rymo, 2011)

3.2 Mechanical forces during embryonic development and vascular development

The remodelling of the primary capillary plexus in the dorsal aorta and the cardinal veins, the origin of most other vessels, begins with the onset of the first heartbeat and the resulting hemodynamic mechanical force on the ECs (Lucitti et al., 2007; Risau, 1997). During the continued growth of the developing embryo, the hemodynamic forces of the increasing blood flow have an effect on the vascular ECs. Additionally they get affected by mechanical forces from the constantly growing surrounding tissue, transduced over cell-cell connections and the extracellular matrix (Vining and Mooney, 2017). Mechanical forces upon ECs can be categorized in two different components: shear stress, which affects the ECs in the direction of flow and stretch induced by pressure within the vessels, which affects the EC in an rectangular direction to the direction of the blood stream (Davies, 1991). Many ways how the EC detects mechanical stress have been discussed **(Fig.2)**. The cell membrane together with membrane attachments and the linked cytoskeleton is considered as a primary mechanosensor (Davies, 1995), with every single cell getting additional input over molecules from the integrin family (between the cells and the ECM (extracellular

matrix)) and cadherin molecules (cell to cell) (Fang et al., 2019). It has been shown that the integrin β3 expression on EC itself is influenced by mechanical stress. Integrin expression gets upregulated upon cells being exposed to unicyclic stretch and the adherence to their substrate as well as their resistance to mechanical forces strengths (Suzuki et al., 1997). Another option of mechanosensing in ECs are mechanosensitive channels. There are two different types. The first type are shear activated potassium and calcium channels like Kir, TRPV4 and Piezo1 (Fang et al., 2019) which get activated trough blood flow. The second type are stretch activated, cation-specific ion channels. When the cation-specific channels get activated, the calcium level inside of the cells rises (Sokabe et al., 1993).

Another possible way of mechanosensing appears to be provided by adhesion complexes. These multi-molecule complexes are forming the connection between the intracellular actin cytoskeleton and the extracellular matrix. If mechanical stress is applied to these complexes, small GTPases are activated which themselves activate Rho kinase and lead to an actomyosin contraction. The mechanical stress transmitted in this pathway from the extracellular matrix causes an elongation and an increase in the size of the ECs. (Riveline et al., 2001). One example is the junction molecule VE-cadherin. It transduces mechanical forces applied to a layer of ECs onto the cytoskeleton of the EC (Fujiwara, 2006). Together with the platelet EC adhesion molecule 1 (PECAM1) and the vascular endothelial growth receptor 2 (VEGFR2), it is considered a mechanosensory complex that transduced mechanical forces and leads to changes within the EC (Tzima et al., 2005).

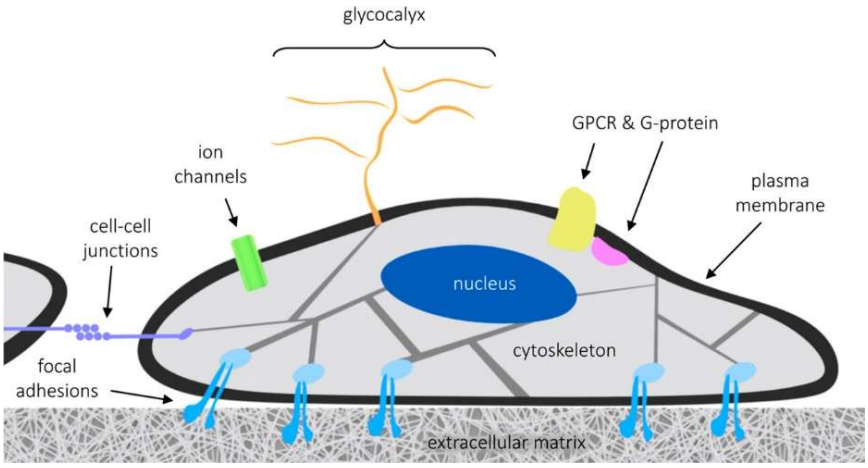

Figure 2: Cytoskeleton and molecules of an EC (James and Allen, 2018) which are linked to the mechanosensing and transduction of extracellular mechanical stress

3.3 Endothelial to hematopoietic transition and forming of the first hematopoietic stem cells

During embryonic development, the first HSC (hematopoietic stem cells) origin from the hemogenic endothelium. These ECs can be found in the dorsal aorta, umbilical artery and vitelline artery (de Bruijn et al., 2000; Kumaravelu et al., 2002; Müller et al., 1994; North et al., 1999) and undergo a process called endothelial-to-hematopoietic transition (Frame et al., 2016; Sugden and North, 2021). Within this process, VE-cadherin and Tie2 positive cells of the endothelium produce hematopoietic stem and progenitor cells (Li et al., 2006; Zovein et al., 2008) which protrude into the vessel lumen (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010) **(Fig.3)**. These progenitor and stem cells are the origin of the different erythroid, lymphoid and myeloid cell lineages that circulate in the blood vessels of the developing embryo. This blood production is called definitive wave of haematopoiesis (Sugden and North, 2021).

Prior to this event, there is another wave of haematopoiesis: the primitive wave of haematopoiesis (Gore et al., 2018). The cells forming the primitive wave of haematopoiesis origin from the lateral platelet mesoderm. It gives raise to restricted progenitors, which can either produce cells from the erythroid or the myeloid lineages. Originating from them, the first primitive erythrocytes and immune cells arise to fill the newly formed vessels (Sugden and North, 2021). The development of vessels and blood cells starts the blood circulation in the growing embryo and with it the shear stress that is applied upon the vascular endothelium. It was shown that ECs sense the still relatively low shear stress with cilia on the cell surface and that the cilial function is one of the keys to definitive haematopoiesis through EHT (endothelial-to hematopoietic transition) (Liu et al., 2019). Induced by shear stress, the activation of prostaglandin E2 synthesis (Diaz et al., 2015), the activation of protein kinase A (Kim et al., 2015) and the upregulation of adenosine receptors (Jing et al., 2015) have a positive impact on hematopoietic stem cell production via EHT. Different publications demonstrated as well that the vascular endothelium, which is exposed to shear stress, upregulates the production of NO in the endothelium, which promotes the endothelial-to hematopoietic transition and the production of HPC (hematopoietic progenitor cells) (Adamo et al., 2009; North et al., 2008; Wang et al., 2011). Therefore, it was widely

shown that shear stress promotes EHT, but it remains unclear whether the same effect applies to tensile forces like static stretch.

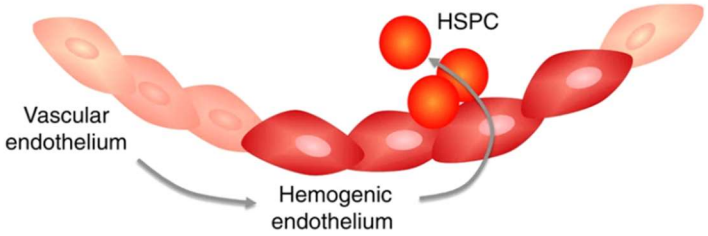

Figure 3: Hematopoietic transition, modified from (Dejana et al., 2017)

3.4 Endothlial cell differentiation

By simulating the in vivo conditions, ECs can be differentiated out of stem cells. During their differentiation, they pass through several stages, similar to the ones passed during in vitro angiogenesis **(Fig.4)**. Embryonic stem cells (ESC) start differentiating spontaneously when cultured without differentiation inhibitors like LIF (leukaemia inhibitory factor) (Vittet et al., 1996). Between day 1 and day 3 many cells in culture still show the characteristics of stem cells (Vittet et al., 1996). These cells start to differentiate into cells dedicated to a specific germ layer. Between day 3 and day 4 of differentiation, the population of cells related to the mesodermal germ layer starts to show mesodermal precursor cells (Jezierski et al., 2007; Keller et al., 1993; Kennedy et al., 2007; Lu et al., 2007; Psaltis et al., 2011; Zambidis et al., 2005).

A population of these cells differentiates into myocardial progenitor cells that mainly give rise to the myocardial cells. However, this population as well contributes to the population of vascular smooth muscle cell as well as ECs (Kattman et al., 2007).

Another population of the mesodermal precursor cells differentiates within two more days into bi-potent progenitor cells (Jezierski et al., 2007; Mora-Roldan et al., 2021), called hemangioblasts, which can give rise to angioblasts, vascular smooth muscle cells as well as hematopoietic stem cells (Choi et al., 1998; Kataoka et al., 2011; Kennedy et al., 1997). Around day 8, the angioblasts further differentiate into ECs (Jezierski et al., 2007) and the hematopoietic stem cells give rise to various cell types from the hematopoietic lineage (He et al., 2004; Wiles and Keller, 1991).

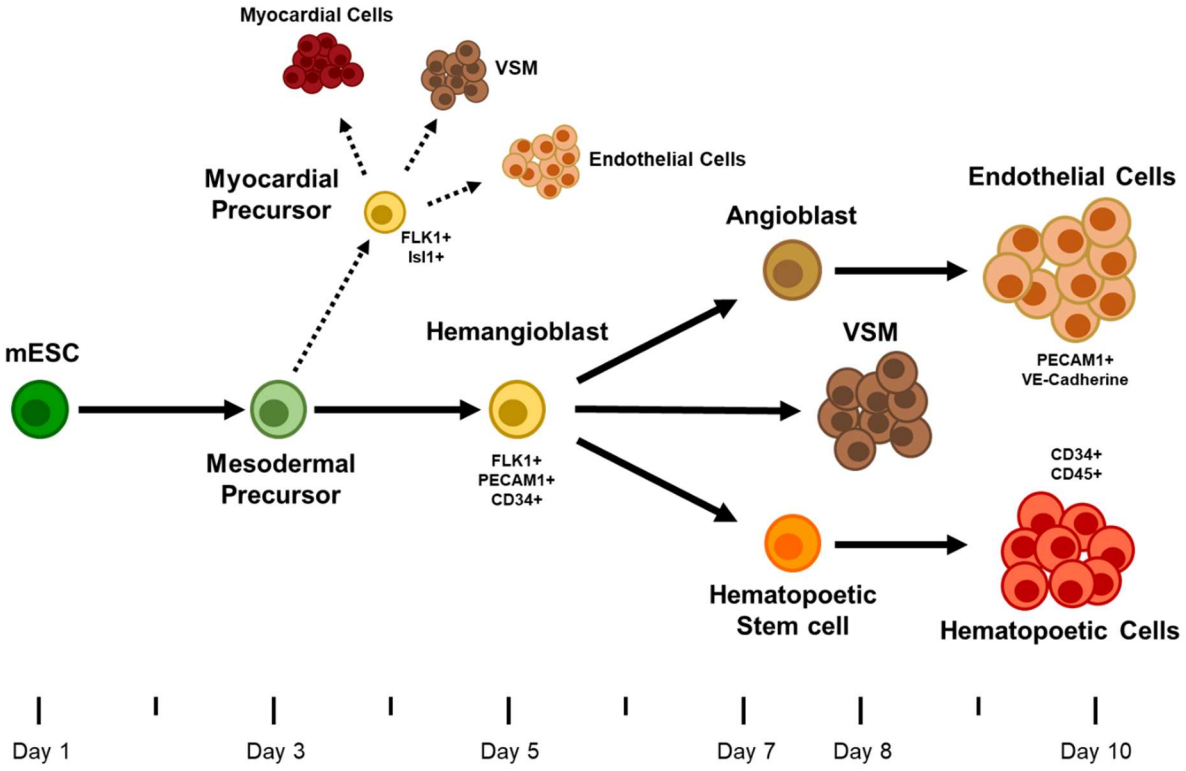

Figure 4: Differentiation stages of the different lineages during endothelial and hematopoietic differentiation

3.5 The impact of the extracellular matrix in an in vitro cell culture

It has been shown that cells in in vitro culture respond highly to the extracellular matrix they are cultured in. For example, could more differentiated phenotypes be observed in neuroblastoma or epithelial cells cultured on an ECM mimicking their in vivo surrounding. Additionally a longer surviving time of hepatocytes was shown, when they were cultured on an ECM, that is quite similar to their usual environment in vivo (Kleinman et al., 1987). Stem cells are able to detect the stiff- or softness of their surrounding micro environment over mechanosensors and change their gene expression accordingly (Smith et al., 2018). Matrigel is known to be a relatively soft ECM, with a stiffness range between 39 Pa (Alcaraz et al., 2008; Semler et al., 2000; Soofi et al., 2009; Zaman et al., 2006) to 650 Pa (Reed et al., 2009). The elastic modulus of laminin is somewhat higher, varying between 110-730 Pa (Alcaraz et al., 2008). The stiffness of gelatine hydrogels can be modified depending on their concentration, from 1kpa for a 4% solution, up to 12kPa for an 8% solution (Sun et al., 2019). The ECM poly-l-lysin has a high reported stiffness ranging from 3-400 kPa (Ren et al., 2008) up to 20-800kPa (Richert et al., 2004).

Earlier studies showed that the stiffness of the extracellular matrix has an impact on EC proliferation and behaviour. So displayed ECs grown on a relative soft matrix and subjected to shear stress, increased cell elongation and tighter EC junctions (Kohn et al., 2015). The authors concluded that a matrix, which mimics the elasticity of young and healthy vessels, is protective against pro-atherosclerotic influences. Another study suggested that an increase of the stiffness of the ECM during differentiation had a positive impact on the proliferation and sprouting rate of the EC (Chen et al., 2012).

3.6 Influence of mechanical stress on cell differentiation

As earlier studies showed, mechanical stress applied in vitro, affects cell fate morphology and proliferation of ECs and endothelial progenitor cells.

So far, most studies focused on the influence of shear stress on ECs. ECs which were stimulated via a cyclic flow in an in vitro circulatory system changed their microtubule structure, elongated and aligned themselves following the blood flow (Galbraith et al., 1998; Hastings et al., 2007; Ives et al., 1986), presumably to lower their resistance against the flow (Sumagin et al., 2008). Another paper published in 2005 showed that in case the cells were treated with shear stress while differentiating, an increase of Flk1+ cells could be observed (Yamamoto et al., 2005). Additionally, a higher number of cells was in an active state of the cell cycle and the shear stress increased the expression of vascular EC markers like Flk1, VE-cadherin and PECAM1. Furthermore, shear stress treated cells showed an enhanced tube formation in further 3D culture (Yamamoto et al., 2005). In the same year, another research team published results that mesenchymal progenitor cells, which were differentiated under the influence of shear stress, develop a higher level on EC markers like PECAM1 or VE-cadherin and showed an increased formation of capillary like networks. Further findings were the upregulation of vascular growth factors and the downregulation of growth factors for smooth muscle cells (Wang et al., 2005).

While the positive impact of shear stress on EC differentiation is well recognized, there are not many studies about the impact of tensile forces upon EC differentiation and proliferation. Mechanical strain is found to prevent the apoptosis of vascular ECs (Liu et al., 2003). Other studies indicated that mechanical stress increased the proliferation rate of differentiated vascular ECs (Burke and Kelly, 2016; Upchurch et al., 1998). A

study using HUVECs (human umbilical vein endothelial cell) revealed that uniaxial cyclic stretch together with growth factors (VEGF and hepatocyte growth factor) lead, in coherence to the previously mentioned studies (Galbraith et al., 1998; Hastings et al., 2007; Ives et al., 1986), to an alignment of the cells. Moreover, they were able to direct the growth of the EC and new sprouting into the strain direction. Interestingly, the applied uniaxial cyclic stretch inhibited the branching of the developing vascular structures but let to a thickening of the EC (Matsumoto et al., 2007). Another experiment cultured bovine aortic ECs on flexible cell culture dishes and applied unidirectional cyclic stretch. It was found, that the cells showed an higher rate of cell division and an increased DNA synthesis (Sumpio et al., 1987). In vivo experiments in rats found, that tensile forces applied upon the ear of a living rat led to an increase in vessel diameter. They as well detected an increase in the area covered by PECAM1 positive ECs, when analysing section cuttings from the rats ears (Pietramaggiori et al., 2007).

4 AIMS OF THE THESIS

In my research, I studied the influence of static, non-cyclic and uniaxial stretch on the differentiation of ECs derived from mouse embryonic stem cells. I established a protocol to achieve efficient stem cell differentiation within a specialized stretching chamber, to serve as the basis for this project.

Employing a multifaceted approach, I used flow cytometry analysis (FACS), immunofluorescence staining and quantitative expression analysis to identify key functions of stretch and matrix stiffness in triggering the gene expression program, facilitating EC development and differentiation. A key point of the investigation involved the assessment of different durations of uniaxial stretch on EC numbers. Additionally, I explored how the developmental stage, at which cells are exposed to stretch, influences EC differentiation. Additionally, my research evaluated the impact of extracellular matrix on EC differentiation. By using materials with varying stiffness, including laminin, matrigel, gelatine and poly-l-lysine, I gained insights into the intricate interplay between tensile forces and extracellular matrix components in influencing EC differentiation. Furthermore, my research aimed to unravel the molecular mechanisms underlying the potential effects of mechanical stimulus on EC development. This was achieved through the identification of key genes using RNA-sequencing analysis.

With the knowledge gained, I hope to support the research aiming to understand the influence of biomechanical factors towards EC development, as well as contributing towards the research for clinical usage in wound healing and vascular regeneration.

5 MATERIALS AND METHODS

Standard laboratory equipment was used to perform the experiments. The following is a complete list of all instruments, reagents and detailed descriptions of the methods used.

5.1 Instruments

Table 1: List of instruments.

Type of Instrument	**Name of Instruments (Manufacturer, city)**
Liquid Nitrogen Tank	Biosafe-Control 220 (Cryotherm GmbH. & Co. AG, Kirchen)
Incubator	Heracell™ 150i CO2 Incubator, 150 L, Electropolished Stainless Steel (ThermoFisher SCIENTIFIC, Waltham Massachusetts)
Clean Working Bench	Thermo Electron Corporation HERAsafe KS 18 Class II, Type A2 6' Biological Safety Cabinet (ThermoFisher SCIENTIFIC, Waltham Massachusetts)
Confocal Fluorescence Microscope	ZEISS Axio Imager 2 for Life Science Research (Zeiss, Oberkochen)
Fluorescence-Lamp Microscope	X-Cite-Fluoreszenz-Lampenbeleuchtungen, X-Cite 120Q (Excelitas Technologies, München)
Microscope	Zeiss Primovert (Zeiss, Oberkochen)
Counting Chamber	Zählkammer CE, Dopp. Neubauer Impr. (Karl Hecht GmbH., Sondheim)
Centrifuge Cell Culture	Rotina 380 Centrifuge (Hettich, Tuttlingen)
Plate Centrifuge	Rotina 420R Centrifuge (Hettich, Tuttlingen)
Micro-Centrifuge	MiniStar silverline (VWR International, Radnor Pennsylvania)
Table Centrifuge	Mikro 185 Kleinzentrifuge (Hettich, Tuttlingen)
Water bath	Water bath WNB (memmert, Schwabach)

Pipet Boy	BRAND accu-jet® pro Pipet Controllers, BrandTech (VWR International, Radnor Pennsylvania)
Eppendorf pipet 0,1-2 µl/2-20 µl/20-200 µl/100-1000 µl	PIPETMAN L P10/P20/P200/P1000L, 1-10 µL/2-20 µL/ 20-200 µL/100-1000 µL, Metal Ejector (GILSON Inc. Middleton USA)
Vortex	Vortex-Genie 2 (Scientific Industries Inc.; Bohemia New York)
Axio Scanner	ZEISS Axio Scan Z1 Digital Slide Scanner (Zeiss, Oberkochen)
Autoclaver	Systec VX-150 (Systec, Linden)
Stretching Chamber	Stretch Chamber STB-CH-4W (Green Leaf Scientific, Dublin)
Stretching Instrument	Manual Cell Stretching System STB-100 (Green Leaf Scientific, Dublin)
Thermic cycler	C1000 Touch Thermal Cycler (Bio-Rad Laboratories GmbH., Feldkirchen)
qPCR machine	StepOnePlus™ Real-Time PCR System (ThermoFisher SCIENTIFIC, Waltham Massachusetts)
Freezing Container	Cryo freezing container, Mr Frosty (ThermoFisher SCIENTIFIC, Waltham Massachusetts)
FACS Canto	BD FACSCanto™II Clinical Flow Cytometry System (BD Biosciences, Heidelberg)

5.2 Materials

Table 2: List of materials.

Short Name	**Name and Unit (manufacturer, city)**
Falcon tubes 15ml, 50ml Falcon tubes	15ml and 50ml Falcon tubes (SIGMA-Aldrich, St. Louis Missouri)
PCR stripes 8-well single cap PCR strips	PCR SingleCap 8er-SoftStrips 0.1 ml and 0.2ml, clear (Biozym Scientific GmbH Hessisch Oldendorf)
PCR plates 384 well PCR plates	MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL (ThermoFisher SCIENTIFIC, Waltham Massachusetts)
6/12/48/96-well-plates	Cellculture multiwellplate, 6 well, 12, well, 48, well, 96 well, PS, transp. (VWR International, Radnor Pennsylvania)
Pipet tips 10 µl, 200 µl, 1000 µl tips	epT.I.P.S. Standard 0.1-10/2-200/50-1000 (Eppendorf AG, Jülich)
Filter tips 10 µl, 200 µl, 1000 µl	Filterspitzen 10 µl, 200 µl, 1000 µl, natur Universal, graduiert, steril, im Rack (Greiner Bio-One, Kremsmünster)
Reaction tubes 1.5 ml, 2 ml tubes	Reagiergefäß 1,5ml/ 2ml (Sarstedt AG & Co. Nümbrecht)
Cell culture plates adherent 5cm, 10cm, 15cm	ZELLKULTUR SCHALE, PS, 60/15mm / 100/20mm / 145/20mm, NOCKEN, CELLSTAR® (Greiner Bio-One, Kremsmünster)
Petri dishes 10cm	Petrischale für Gewebekulturen, 100x20mm (Greiner Bio-One, Kremsmünster)

5.3 Reagents

Table 3: List of reagents, peptides and recombinant proteins.

Name	**Characteristics (article number, manufacturer)**
Gelatin from bovine skin, Typ B	Cat# G9391, SIGMA-Aldrich
Laminin	Cat# 354232, Corning
Chloroform	Cat#3313.4, Carl-Roth GmbH &Co. KG
Dimethylsulfoxid for molecular biology	Cat# D8418, SIGMA-Aldrich
N-2™ Supplement (100X)	Cat# 17502-048, Gibco
B-27™ Supplement (50x), serumfrei	Cat# 17504044, Gibco
B-27™ Supplement (50x), minus Vitamin A	Cat# 12587010, Gibco
MEM Non-Essential Amino Acids Solution	Cat# 11140035, Gibco
L-Glutamine (200mm)	Cat# 25030123, Thermo Fisher Scientific
ESGRO® Recombinant Mouse LIF Protein	Cat# ESG1107, Merck Millipore
Recombinant Human/Mouse/Rat Activin A Protein	Cat# 338-AC-010, R&D Systems
Recombinant Human BMP-4 Protein	Cat# 314-BP-010, R&D Systems
Recombinant Human VEGF 165 Protein	Cat# 293-VE-010, R&D Systems
Recombinant Human FGF basic/FGF2/bFGF (146 aa) Protein	Cat# 233-FB-010, R&D Systems
Recombinant Human FGF-10 Protein	Cat# 345-FG-025, R&D Systems
2-Mercaptoethanol for Molecular Biology	Cat# M3148, Merck KGaA

Sodium Pyruvate (100mm)	Cat# 11360039, Thermo Fisher Scientific
Penicillin Streptomycin (10,000 U/mL)	Cat# 15140122, Thermo Fisher Scientific
DMEM, high glucose, NEAA, no glutamine	Cat# 10938025, Gibco
DMEM/F-12, no glutamine	Cat# 21331020, Gibco
Neurobasal™ Medium	Cat# 21103049, Gibco
Ham's F-12 Nutrient Mix	Cat# 11765054, Gibco
Fetal Bovine Serum, qualified	Cat# 10270106, Gibco
KnockOut™ Serum Replacement	Cat# 10828028, Gibco
KnockOut™ DMEM	Cat# 10829018, Gibco
TRIzol Reagent	Cat# 15596018, Invitrogen
Mitomycin C from Streptmyces caespitosus	Cat# M4287, SIGMA-Aldrich
Trypsin EDTA (0.05%), phenol red	Cat# 25300054, Thermo Fisher Scientific
Trypsin EDTA (0.25%), phenol red	Cat# 25200056, Thermo Fisher Scientific
StemPro Accutase Cell Dissociation Reagent	Cat# A1110501, Thermo Fisher Scientific
Mowiol® 4-88, 50 g	Cat# 0713.1, Carl-Roth GmbH &Co. KG

Table 4: List of commercial kits.

Commercial Kits	
High-Capacity cDNA Reverse Transcription Kit	Cat# 4368813, Applied Biosystems, Foster City California
qPCRBIO SyGreen Blue Mix Hi-ROX (50 x 1 mL)	Cat# PB20.16-51, Nippon Genetics Europe, Düren
BD Cytofix/Cytoperm Kit	Cat# 554714, Becton Dickinso, Heidelberg
RNeasy Plus Universal Mini Kit	Cat# 73404, QIAGEN, Hilden

5.4 Antibodies

Table 5: List of antibodies.

Antibodies	
Fixable Viability Dye (FVD) eFluor 450	Cat# 65-0863-14, ThermoFisher SCIENTIFIC, Waltham Massachusetts
CD31 (PECAM1) Antibody, PE Cyanine7 (390), eBiosc	Cat# 25-0311-82, ThermoFisher SCIENTIFIC, Waltham Massachusetts
Rat IgG2a kappa Isotype Control, PE Cyanine7, eBio	Cat# 25-4321-81, ThermoFisher SCIENTIFIC, Waltham Massachusetts
CD309 (Flk1) Antibody, APC (Avas12a1), eBioscience	Cat# 17-5821-81, ThermoFisher SCIENTIFIC, Waltham Massachusetts
Rat IgG2a kappa Isotype Control, APC, eBioscience	Cat# 17-4321-81, ThermoFisher SCIENTIFIC, Waltham Massachusetts
Cardiac Troponin T Alexafluor ® 647, 50 µg	Cat# 565744, Becton Dickinso, Heidelberg
Mouse IgG1, (Alexa Fluor 647) 0.5 mL	Cat# PA557732, ThermoFisher SCIENTIFIC, Waltham Massachusetts
Human/Mouse/Rat CD31/PECAM-1 Antibody Antigen Affinity-purified Polyclonal Goat IgG	Cat#AF3628, R&D Systems, Minneapolis
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 555	Cat# A21432, Invitrogen, Carlsbad
DAPI	Cat#4083S, Cell Signaling, Danvers Massachusetts

5.5 Primers

Table 6: List of primers.

Primer Name	**Sequence 5´->3´**	**Company**	**Gene name**
qPecam1_for	AACAGAAACCCGTGGAGATG	Sigma-Aldrich	PEACM1
qPecam1_rev	GGCTTCCACACTAGGCTCAG		
m18S-f	ACCGCAGCTAGGAATAATGGA	Sigma-Aldrich	18S RNA
m18S-r	CAAATGCTTTCGCTCTGGTC		
qTnnT2_for	TTAAAGCTCTCCCCATGCCC	Sigma-Aldrich	Tnnt2
qTnnT2_rev	CTCGGCTCTCCCTCTGAACA		
CD34 fw	CTGGGTAGCTCTCTGCCTGAT	Sigma-Aldrich	CD34
CD34 rv	TGGTAGGAACTGATGGGGATATT		

5.6 Mediums

Table 7: Complete MEF medium.

Volume 600ml	Final con.	composition	Stock conc.
500ml		DMEM high glucose (4.5g/l)	
6ml	1x	MEM NEAA	100x
6ml	1x	L-Glutamine	100x
6ml	1x	Pen/Strep	100x
6ml	1x	Sodium pyruvate	100x
8µl		B-Mercaptoethanol	
55ml	10%	FCS	

Table 8: ES cell freezing medium.

Volume 50ml	Final con.	composition	Stock conc.
40ml		Complete ES cell medium	
5ml	20%	FCS	
5ml	20%	DMSO	

Table 9: Complete DMEM-ES cell medium.

Volume 600ml	Final con.	composition	Stock conc.
500ml		DMEM high glucose (4.5g/l)	
6ml	1x	MEM NEAA	100x
6ml	1x	L-Glutamine	100x
6ml	1x	Pen/Strep	100x
6ml	1x	Sodium pyruvate	100x
8µl		B-Mercaptoethanol	
90ml	15%	FCS	

Table 10: Complete KO-medium.

Volume 600ml	Final con.	composition	Stock conc.
500ml		Knockout DMEM	
6ml	1x	MEM NEAA	100x
6ml	1x	L-Glutamine	100x
6ml	1x	Pen/Strep	100x
6ml	1x	Sodium pyruvate	100x
8µl		B-Mercaptoethanol	
55ml	10%	FCS	
Add fresh to an 45ml aliquot			
5ml	10%	Serum Replacement	
5µl	1x (co-culture with feeders)	LIF	7x
10µl	2x (culture without feeders)	LIF	7x

Table 11: Master Mix for qPCR.

Composition	Volume (µl)
SYBR Green Master Mix	5
Primer Mix (10 µM each, forward + reverse)	1
H_2O	2

Table 12: Time and Temperature for qPCR.

Replication no.	Process	Duration	Temperature
1x	Initial Denaturation	10 min	95°C
40x	Denaturation	15 sec	95°C
	Annealing	60 sec	60°C
	Elongation	1 sec	72°C
1x	Final Elongation	10 sec	40°C

5.7 Software and Algorithms

Table 13: List of used software and algorithms.

Image J 1.47v	Image J; https://imagej.nih.gov/ij/download.html
FACSDiva™ software	BD BIOSCIENCES; https://www.bdbiosciences.com/en-us/instruments/research-instruments/research-software/flow-cytometry-acquisition/facsdiva-software
FCS Express™ 7.18.0025.	De Novo by Dotmatics; https://denovosoftware.com/full-access/download-landing/
GraphPad Prism 9.5.1	GraphPad; https://www.graphpad.com/
Leica Application Suite X 3.7.5.24914	Leica Microsystems; https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/downloads/

5.8 Methods

5.8.1 Cell culture and differentiation

5.8.1.1 Mouse embryonic fibroblast culturing

MEFs (mouse embryonic fibroblast) from E13.5 wild-type mouse embryos were thawed in complete ESC medium before being centrifuged (1200gx5min) to remove the toxic DMSO (dimethyl sulfoxide) contained in the freezing medium. After centrifugation, cells were suspended in complete MEF medium and seeded onto adherent cell culture plates. Feeder cells were allowed to grow and spread in the incubator at 37°C and 5% CO2 for 2-4 days until cells reached a confluence of 80% on the plates. Then, they were washed with PBS (phosphate-buffered saline) and trypsinized using 0.25% Trypsin to obtain single cells. The cells were then centrifuged and resuspended in fresh, complete MEF medium before being split at a 1:2 ratio. This process was repeated with further splits at ratios of 1:4, 1:8 and so on, until the desired number of cells was reached.

5.8.1.2 Feeder creation

Feeder cells were produced by inhibiting proliferation through dissolving one bottle of Mitomycin C in 2ml of PBS and filtering with a sterile filter. 150 µl of Mitomycin C were added per 15ml MEF medium. The cells were thoroughly washed with PBS and Mitomycin containing medium was introduced for 2 hours and removed afterwards. The cells were then washed three times with PBS and treated with 0.05% Trypsin. Upon thawing, one 15cm dish will suffice to fill two 10cm dishes with feeder cells. Cells were centrifuged, resuspended in freezing medium, filled into the cryo tubes, frozen using the freezing container at -80°C and transferred to the liquid nitrogen tank the following day.

5.8.1.3 Culturing embryonic stem cells

Murine E14-Nkx2.5-EmGFP ESCs (Hsiao et al., 2008) were used and in the following referred as mESC (mouse embryonic stem cell) . Feeder cells were thawed the day prior to seeding the ES cells onto adherent cell culture dishes. They were allowed to spread and attach for several hours, but preferably overnight. mESC were then thawed

in complete ESC-Medium, following the same procedure as for MEF culturing, but substituting complete KO-medium and 1xLIF to prevent differentiation instead of using complete MEF medium.

5.8.1.4 Spontaneous Differentiation

For Spontaneous Differentiation, the ESCs were thawed and cultured until D0 using the same procedure as described for Kattman Differentiation. The cells were washed, trypsinized in the same manner and counted using a counting chamber.

Adherent cell culture dishes were coated with 0.2% gelatine for at least 20 minutes. Afterwards, the gelatine solution was removed from the dishes and the cells were seeded. Specifically, 100,000 cells were reaggregated in complete ES cell medium without LIF and seeded per well on the stretching chambers. Subsequently, they were placed in an incubator at 37°C with 5% CO2.

The cells were observed regularly under a microscope and the medium was added or changed when needed. Cells proliferated and differentiated over the next few days.

5.8.2 Stretching

The cells were manually stretched using the Stretch Chambers and Manuel Stretching System, manufactured by Strax (Green Leaf Scientific). Before starting the stretching process, the stretching chambers and system had to be prepared. The chambers were autoclaved and subsequently dried in a drying chamber. The cells were cultured and prepared following the described method according to the chosen differentiation protocol. The chambers were coated with a coating material, such as gelatine or matrigel. For the differentiation process, the cells were seeded onto stretching chambers instead of traditional dishes and the procedure continued as usual. To stretch the cells, the stretching chambers were inserted into the manual stretching system. The length of the chamber in its unstretched condition was measured and the adjustment screw was turned until the chamber reached the predetermined length required for the experiment. 15% of stretch was used in the experiments described in this thesis. The stretching system, together with the stretched chamber, was positioned in a 15cm petri dish and incubated until reaching the desired stretch duration. To

release the chambers, the screw was turned in the opposite direction, causing the stretching chamber to return to its original size.

5.8.3 Coatings

5.8.3.1 Gelatine

Non-Adherent Petri dishes were coated with 2mg/ml = 0.2% gelatine by adding the solution to slightly cover the bottom of the dishes. The dishes were then placed in the incubator. After a minimum of 20 minutes the gelatine was removed.

5.8.3.2 Laminin

To coat dishes/chambers with laminin, the stock concentration was diluted to 10 µg/ml. The minimum amount required to cover the surface was added and left to incubate for 1 hour in the incubator. The supernatant was subsequently removed.

5.8.3.3 Poly-L-Lysine

To coat dishes/chambers with poly-l-lysine, the stock concentration was diluted to 50µg/ml. The liquid solution was then pipetted onto the surface, covering it adequately. The coated surface was then incubated for at least 1 hour at room temperature before being removed.

5.8.3.4 Matrigel

To apply matrigel to dishes or chambers, an aliquot of matrigel was gradually thawed on ice and then diluted to a concentration of 0.3mg/ml = 0.03%. This was achieved by using ice-cold PBS or sterile water. 500µl of this solution was used per well to coat the stretching chambers. The matrigel was left to incubate for 1 hour at room temperature inside a sterile working environment before being removed.

5.8.4 FACS

Cells were permitted to grow according to the designated protocol until the desired time point for measurement was reached. The differentiation medium was removed and the cells were carefully washed with PBS. Accutase was used to disrupt

intercellular junctions. This was followed by incubation in an incubator at 37°C for 5 minutes. The reaction was stopped by adding FBS (fetal bovine serum) containing medium. The cells were detached by gentle up and down pipetting. After centrifugation, the supernatant was discarded. The subsequent steps were performed on ice.

FACS Buffer was prepared by filtering a sterile solution of 5% FBS into PBS. Each sample was resuspended in 200 µl of FACS Buffer and distributed to different wells of a 96 U-bottom-well plate. Centrifugation was conducted at 500xg/2100rpm for 5 minutes and the supernatant was quickly removed by inverting the plate. Cell washing was repeated twice by adding 200 µl of PBS to the wells, followed by centrifugation and removal of the supernatant.

For staining, 1 µl of FVD (fixable viability dyes) was diluted in 0.8 ml of FACS-Buffer for each sample, with adjustments made according to the sample number. One hundred microliters of the FVD dilution were added to each sample, except for the single cell stainings used to set voltages and gating parameters for obtaining accurate fluorescence signal. Samples were mixed by pipetting up and down and incubated for 30 minutes at 2-8°C in a light-protected environment. Afterwards, cells were washed twice with PBS.

CD31-PE/Cyanine7 with mouse specificity (1:500), the IgG2a kappa PE/Cyanine 7-Isotype control (1:500) and Fixable Viability Dye (FVD) eFluor 450 (1:800) were used for FACS staining.

For extracellular staining, antibodies were diluted to the appropriate concentration using FACS buffer. Cells were then suspended in 25 µl of the antibody mixture and incubated for 30 minutes at 4°C in the dark. To prevent cell drying, unstained controls were used with FACS buffer. After incubation, cells were washed twice using PBS.

For FACS measurement, the cells were resuspended in 200 µl of FACS-Buffer, transferred to FACS tubes and kept in darkness on ice until undergoing FACS analysis.

Results were analysed using the software FCS Express™ by “DE NOVO”. Cells were gated as demonstrated in **Fig.5 A**. Gates were set by using cells without any staining and single stainings (**Fig.5 B**).

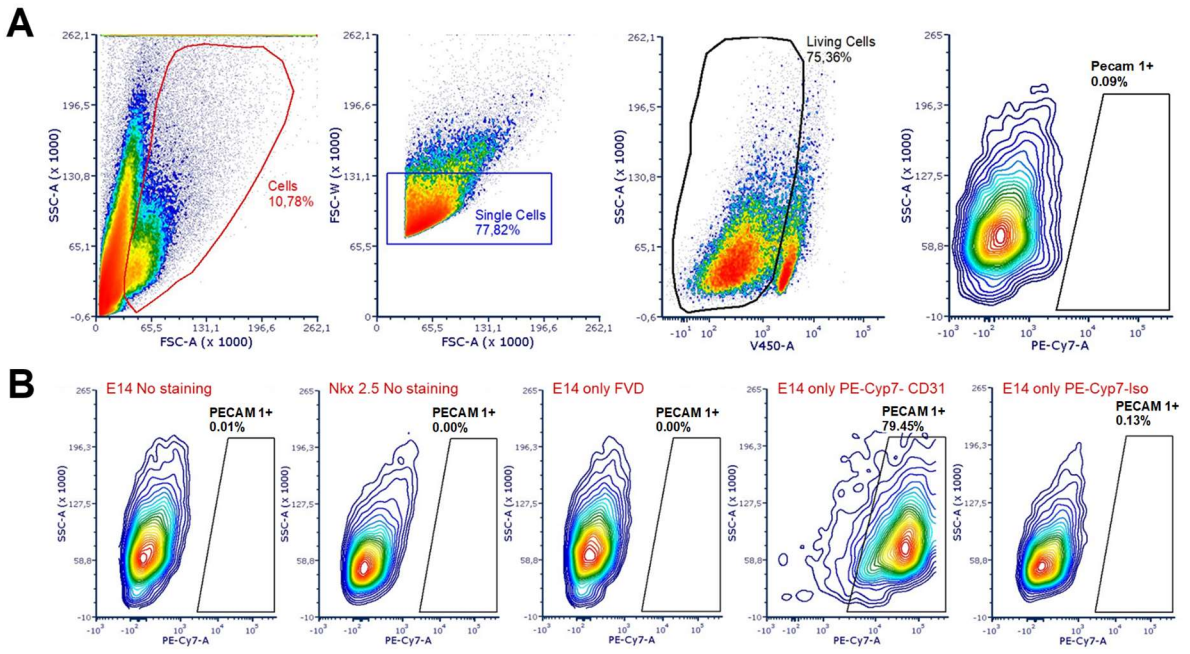

Figure 5: (A) Gate settings in FCS Express™ for the gates “cells”, “single cells”, “living cells” and the “PECAM1+” **(B) Single cell stainings** for volt and gate setting, E14 as negative control, Nkx2.5 as cells without staining but cell specific signal, only FVD, PE-Cy7-CD31 and PE-Cy7-CD31 for gating the single stainings

5.8.5 RNA-isolation

Cells were washed with PBS and lysed by adding 1ml of trizol to the samples. They were vortexed until the samples were completely dissolved in the trizol. Storing the samples is possible at -20°C until continuing with the next step.

5.8.5.1 RNA isolation using trizol

For RNA extraction, samples were thawed and 200 µl of chloroform was added to 1 ml of trizol. After vortexing for 15 seconds, the samples were incubated at RT for 15 minutes. The samples were then centrifuged at 12,000g for 15 minutes at 4°C to separate the mixture into a red layer, a cloudy middle phase and an upper aqueous phase. Fresh Eppendorf tubes containing 500 µl isopropanol and 1 µl RNase-free glycogen were prepared. The aqueous phase was transferred to the prepared tube using a pipette, taking care to avoid contamination from the middle or bottom phase. The tube was vortexed briefly and incubated for 10 minutes at room temperature. The tube was then centrifuged at 12,000g for 10 minutes at 4°C to precipitate the RNA as a white pellet at the bottom of the tube. To resuspend the pellet, 1 ml of 75% ethanol was added to each 1 ml of trizol previously used. Brief vortexing was followed by

centrifugation at 7500g for 5 minutes at 4°C. The supernatant was discarded and the previous steps were repeated. The supernatant was discarded again and the pellet was air dried for 5-10 minutes. After drying, the isolated RNA was dissolved by resuspending in 20 µl of RNase-free water and the sample was immediately placed on ice.

5.8.5.2 RNA isolation using columns

For RNA-sequencing, the RNeasy Plus Universal Mini Kit from QUIAGEN was used for RNA isolation and purification. The stored samples, lysed in qiazol, were removed from the fridge at 4°C. At the same time, the centrifuge was cooled to 4°C. One hundred microliters of gDNA Elimination Solution were added to each sample and vortexed for 15 seconds. The samples were treated with 200 µl of chloroform and vortexed again. The mixture was incubated on a rack for 15 minutes at RT. The samples were centrifuged at 12,000g/13,000 rpm for 15 minutes at 4°C. Separation occurred, yielding an aqueous phase and a red phase. The aqueous phase was carefully transferred to a new Eppendorf tube and 500 µl of isopropanol was added. The samples were incubated for 30 minutes at RT. While the samples were incubating, the RNA columns were prepared by wetting each with 200 µl of RWT buffer and incubating for 5 minutes before centrifugation. Simultaneously, 500 µl of RWT buffer was added to the samples and new Eppendorf tubes were prepared for collection. The samples, 500 µl at a time, were transferred to the prepared columns, centrifuged at 8500g for 5 minutes and these steps were repeated until the sample was completely used up. The columns were placed in the previously prepared new tubes and 500 µl of RWT buffer was added. The tubes containing the columns were centrifuged for 15 seconds at maximum speed and 4°C. The columns were then placed in new tubes and the flow-through was retained for the next step. This flow-through was added back to the column and centrifugation was repeated for 15 seconds at maximum speed and 4°C. The old tubes were discarded together with the new flow-through. Five hundred microliters of RPE buffer were added to the top of the columns and centrifugation was repeated for 15 seconds at maximum speed and 4°C. The flow-through was discarded and the last two steps were repeated to wash the sample with RPE buffer. The columns were dried by centrifugation for 2 minutes at maximum speed and 4°C. Tubes for the final collection of the isolated RNA were labelled and the columns were placed in them.

Thirty-five microliters of RNase-free H_2O was added to the top of the columns. This was incubated for 30 seconds. Centrifugation was performed for 1 minute at maximum speed and 4°C. The samples were immediately placed on ice.

5.8.6 cDNA-Synthesis

cDNA-synthesis (complementary DNA-synthesis) was performed using the High-Capacity sDNA Reverse Transcription Kit from applied biosystems following the instructions (Pub. No. 4375222).

5.8.7 qPCR

qPCR (quantitative polymerase chain reaction) was performed using the qPCRBIO SyGreen Blue Mix Hi-ROX from Nippon Genetics, following the product manual (PB20.16) and the cDNA synthesized according to point 2.8.5. For pipetting, a beforehand prepared master mix (**Tab.11**), 2 µl of cDNA and water samples were pipetted into the wells of a 96-well amplifier plate. qPCR was run on a cycler following the program described in **Tab.12**. Ribosomal 18s was used to normalize the cycle numbers.

5.8.8 Immunofluorescence microscopy

Cells were cultured on the stretch chambers as described above (2.8.2). First, the cells were carefully washed once with PBS to remove any remaining cell culture medium. The cells were fixed by pipetting 3.7% formaldehyde into each chamber and incubating for 10 minutes at RT. They were then washed three times with PBS. To permeabilize and block the cells, they were incubated in a 0.1% PBS-TritonX buffer for 1 hour at RT. The surface the cells were cultured on was divided into squares using a Pap pen, creating single compartments for different stainings. For primary antibody staining, anti-CD31 antibody was diluted 1:100 in 0.1% PBS-TritonX buffer and incubated for 1 hour at RT. The cells were then washed three times with PBS. For secondary antibody staining, a 1:400 dilution of anti-goat antibody in 0.1% PBS-TritonX was pipetted onto the cells and left for 1 hour at RT. After incubation, the cells were washed three times with PBS. DAPI (4',6-diamidino-2-phenylindol) staining was performed by diluting the antibody 1:1000 in 0.1% PBS-TritonX and allowed to stand for five minutes at RT. The

cells were then washed once with PBS. For transfer to coverslips, the bottom of the chamber was carefully cut out with a scalpel and transferred to the top of a microscope slide. For mounting, mowiol was applied to the cells, sealed to the slide with a coverslip and left overnight at 4°C. Before imaging the slides, the edges of the coverslips were sealed with histo lack to prevent them from being dispersed by the drop of distilled water used for microscopy. Images were captured using a Leica DMi8 microscope and viewed using Leica Application Suite X software and ImageJ.

5.8.9 mRNA-Seq

Samples were prepared as descripted in point 2.8.5.2 for RNA isolation using columns. A TECAN spectrophotometer was used to check the purity of the samples at a wavelength of 230nm, 260nm and 280nm before sending them for sequencing.

5.8.10 Statistics

All statistics and graphs were performed and plotted using the GraphPad Prism 9.5.1 software. Significance was calculated in most cases using a multiple unpaired Students t-test. Only for the comparison within the group of cells cultured on gelatine and stretched between day 1 and day 3 an Anova multiple comparison test was used as well to determine significant changes between the stretching durations in this subgroup. A result was considered significant with a p-value < 0.05.

6 RESULTS

6.1 Impact of extracellular matrix on endothelial cell differentiation

After determination of the appropriate culturing method, I tested how the matrix stiffness of different extracellular matrices affects the transmission of mechanical stretch onto the cells. Experimental matrices included matrigel, laminin, gelatine and poly-l-lysine. Matrigel was the softest ECM tested with stiffness ranging between 39 Pa (Alcaraz et al., 2008; Semler et al., 2000; Soofi et al., 2009; Zaman et al., 2006) to 650 Pa (Reed et al., 2009), laminin varies between 110-730 Pa (Alcaraz et al., 2008), gelatine depending on the concentration used, from 1kpa up to 12kPa (Sun et al., 2019). poly-l-lysine was the stiffest matrix used, with a stiffness ranging from 3-400 kPa (Ren et al., 2008) up to 20-800kPa (Richert et al., 2004).

Overall, this comparison off the different extracellular matrixes showed two results. Firstly, cells cultured in relatively soft mediums differentiated more into PECAM1 positive cells. Matrigel cultured cells showed a significant higher level in PECAM1 positive cells when compared to a matrix with intermediate high stiffness (gelatine) but not such a high level as cells cultured on laminin. However, the differentiation on the stiffer ECMs gelatine and poly-l-lysine did not increase EC numbers, with no difference in PECAM1+ cell numbers between gelatine and the stiffer poly-l-lysine **(Fig.6 A,B)**.

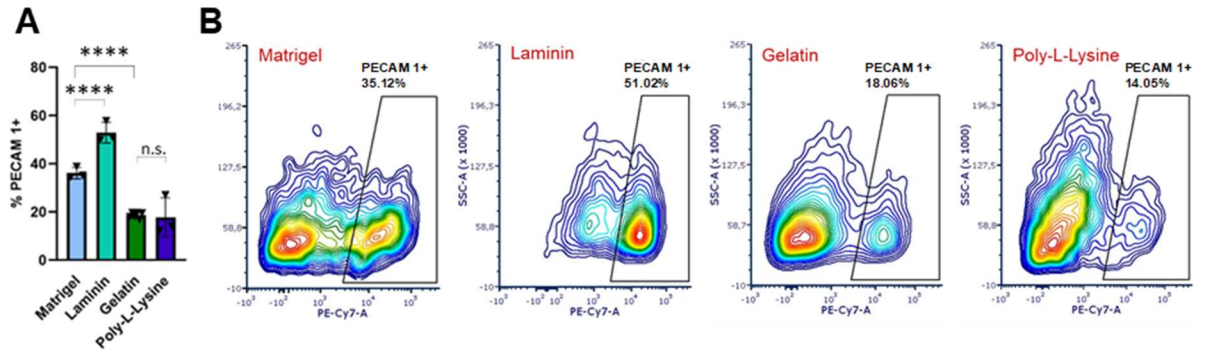

Figure 6: Comparison of FACS analysis of cells cultured on different extracellular matrices. (A) Bar chart displaying the percentage of PECAM1 positive stained cells, staining with CD-PE Cyanine7 (n=3 and n=6 for cells cultured in gelatine). **(B)** Representative flow cytometry analysis for unstretched cells cultured on matrigel (D10), laminin (D13), gelatine (D10) and poly-l-lysine (D10). Error bars represent mean ±SD.

Secondly, I found that application of mechanical stretch on cells cultured on a soft matrix, e.g. matrigel, did not enhance EC differentiation, while application of the same stretch strength on cells cultured on the stiff matrix gelatine, resulted in a significant increase in ECs when stretched at an early timepoint, compared to the PECAM1+ cell numbers in cells cultured without mechanical stimulus on the same matrices. Cell cultures which used the stiffest tested matrix pol-l-lysine, showed a decrease in EC numbers when compared to cells cultured without stretch. Laminin enhances the EC differentiation in all conditions tested and due to this strong promoting effect, eventual effects of the applied stretch might have been masked **(Fig. 7 A,B)**.

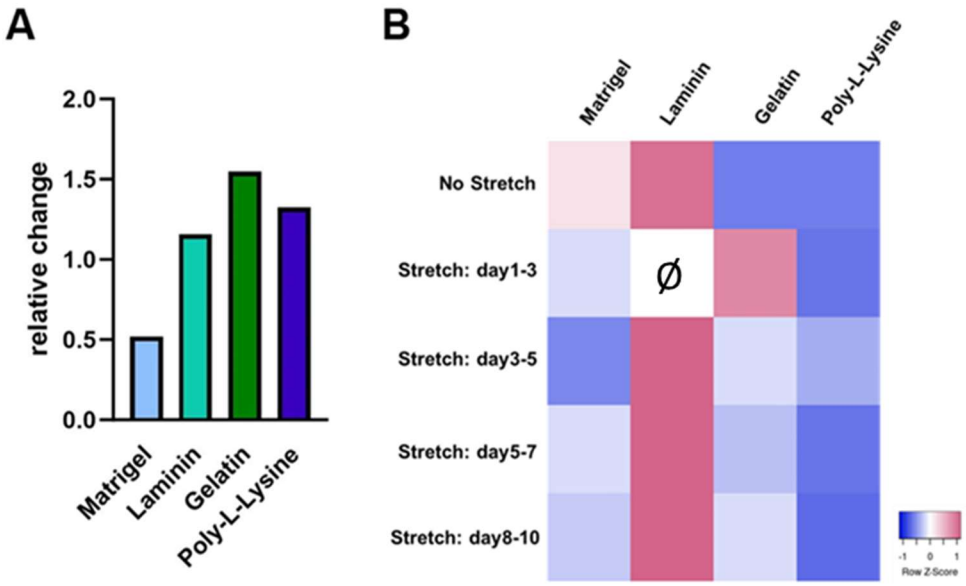

Figure 7: Relative change of the number of PECAM1+ cells in FACS analysis (A) Bar chart with relative change of the number of PECAM1+ cells cultured on different extracellular matrices and stretched for 48h between day 3-5 (germ layer specified cells), compared to the control group "No Stretch" cultured on the same medium. **(B)** Heat map visualizing increase and decrease in the number of PECAM1+ cells in quantitative FACS analysis from cells cultured on different matrices, Ø no data.

6.2 Effect of stretch on cells cultured on laminin

Since laminin was shown to promote EC differentiation in earlier studies, I firstly tested if this effect could be even more increased by the application of mechanical stretch. The cells were seeded onto stretching chambers at day 0 of the differentiation and stretched for 48 hours at various time points, representing different stages of ESC differentiation. Harvesting of the cells finally occurred on day 13 after the onset of differentiation **(Fig.8 C)**. As expected, the experiment showed a strong promoting effect of laminin upon EC differentiation in all tested conditions. The flow cytometry

data demonstrated that in the unstretched control group, an average of 52.88% of viable cells expressed PECAM1+ **(Fig.8 A,B,D)**. Cells stretched on day 3, already specified to a specific germ layer, expressed a mean of 61.18%, cells stretched from day 5 (progenitor cells) expressed a mean of 69.03% and cells stretched from day 8 (differentiated progeny cells) showed a mean of 60.50% PCAM1+ cells **(Fig.8 B,D)**. Stretched cell populations displayed a trend to increased PECAM1+ cell count but no significance when compared to the unstretched control group. Given the robust inductive effect of laminin, potential effects of stretching may have been masked.

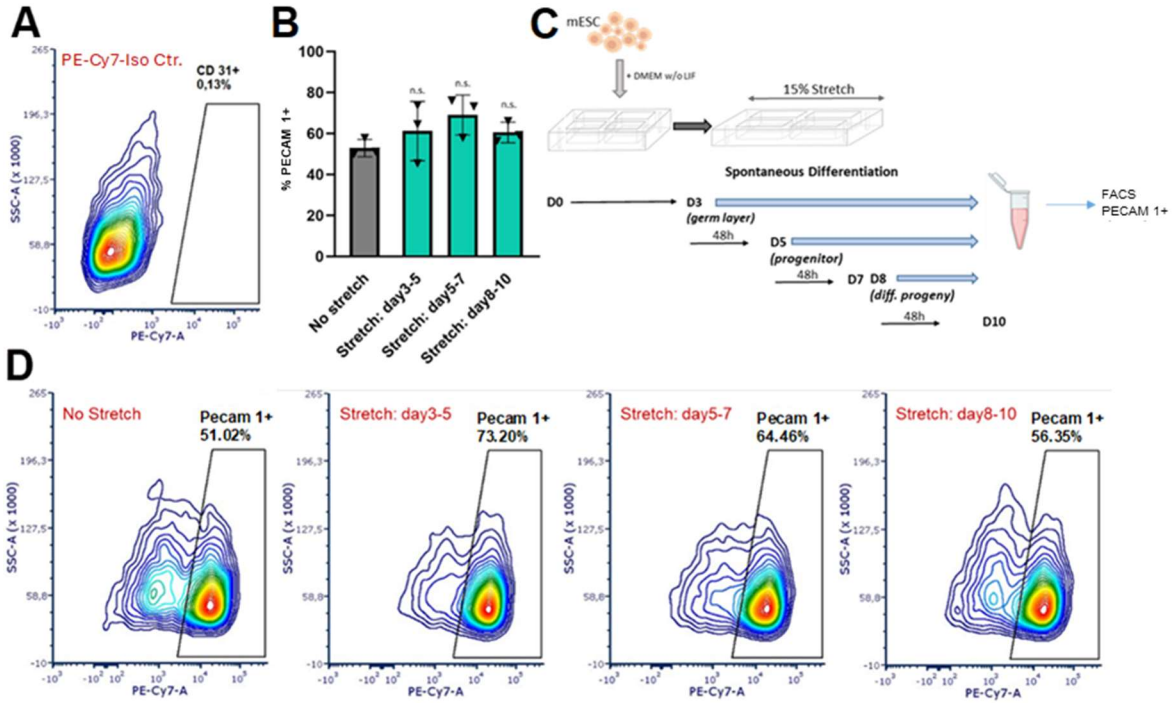

Figure 8: FACS analysis of cells cultured on laminin until day 13 (D13). (A) Negative control using the PE-Cy7-iso Ctr. **(B)** Bar chart displaying the percentage of PECAM1 positive stained cells, staining with CD31-PE Cyanine7 (n=3). **(C)** Scheme of the experimental setup including the different stretching time points **(D)** Representative flow cytometry analysis for cell populations cultured without stretch, stretched for 48h between day 3-5, day 5-7 and day 8-10. Error bars represent mean ±SD. n.s. non-significant.

6.3 Effect of stretch on cells cultured on matrigel

After finding laminin having the strongly promoting effect upon differentiation, I was interested how a soft ECM without strong EC differentiation promoting effects, would affect the transmission of stretch to the differentiating cells.

Next, I applied a matrigel coating to my chambers. Matrigel is a relatively soft ECM (Alcaraz et al., 2008; Soofi et al., 2009) which is composed of laminin, collagen IV and nidogen-1/entactin as well as proteoglycans and growth factors (Kastana et al., 2019; Mahdieh et al., 2022). I sought to determine if this extracellular matrix with an even lower stiffness, then on if its components laminin, would have any influence on the stretching outcomes.

Samples were stretched for 48h each and at day 10 the cells were dissociated, stained for PECAM1 and subjected to FACS analysis **(Fig.9 C)**. The unstretched control group, cultured without stretch, had a mean number of 36.12% PECAM1+ cells **(Fig.9 A,B,D)**. In contrast, cells stretched during differentiation manifested significantly lower levels of PECAM1+ cells **(Fig.9 B,D)** with a mean of 25.68% for cells stretched starting from day 1, 18.80% for cells stretched from day 3, 29.93% for cells stretched from day 5 and 24.75% PECAM1+ positivity for cells stretched starting from day 8.

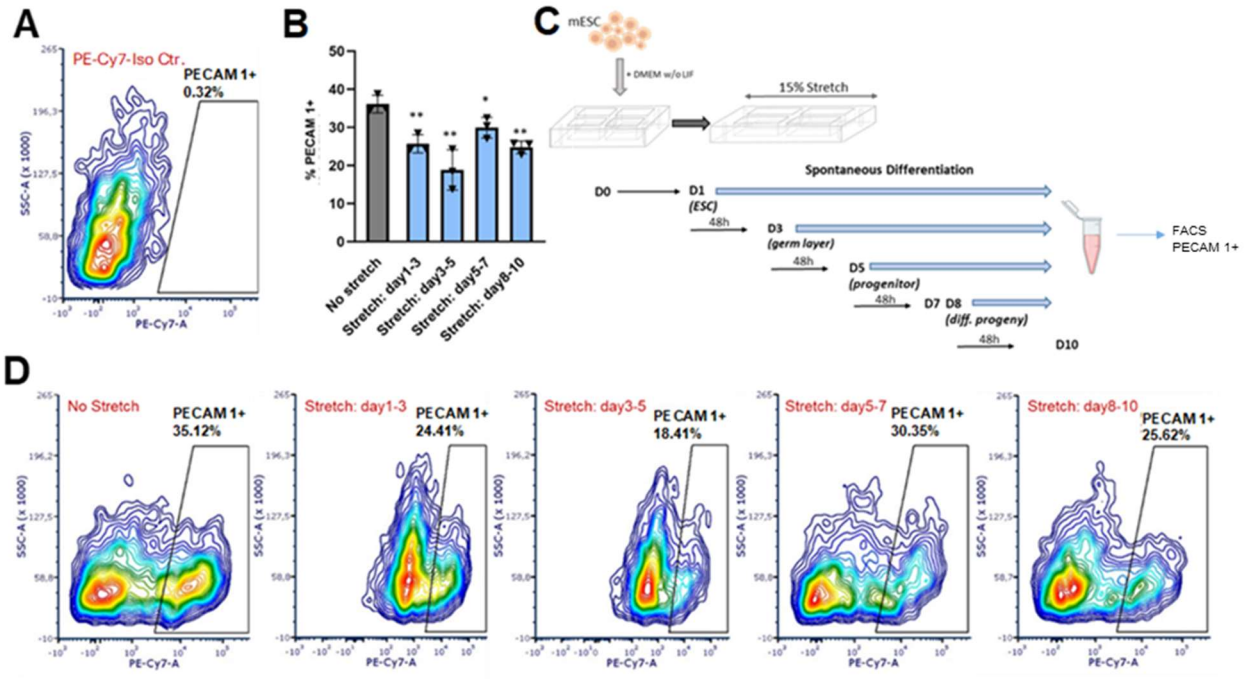

Figure 9: FACS analysis of cells cultured on matrigel until day 10 (D10) (A) Negative control using the PE-Cy7-iso Ctr. **(B)** Bar chart displaying the percentage of PECAM1 positive stained cells, staining with CD31-PE Cyanine7 (n=3). **(C)** Scheme of the experimental setup including the different stretching time points **(D)** Representative flow cytometry analysis for cell populations cultured without stretch, stretched for 48h between day 1-3, day 3-5, day 5-7 and day 8-10. Error bars represent mean ±SD. *p<0.05, **p<0.01 n.s. non-significant.

6.4 Effect of stretch on cells cultured on poly-l-lysine

In contrast to the low stiffness of the matrigel coating, poly-l-lysine coating is significantly stiffer and the cells adhere strongly to the matrix. Cells were stretched for 48 hours at each time point. Microscopic observations during differentiation showed that the distribution of cells on the matrix surface within the chambers was poor, resulting in only a small increase in the area covered and a low number of viable cells at day 10. Subsequently, cells were collected, stained for PECAM1 and analysed via FACS **(Fig.10 C)**. The number of ECs in the unstretched control group was lower than that of the other tested coatings (matrigel, laminin and gelatine), with an average of only 18.61% **(Fig.10 A,B,D)**. When cells were stretched from days 1 (mean 16.60%), 3 (mean 23.64%), 5 (mean 15.10%) and 8 (mean 13.88%) of differentiation, there was no significant change in the number of PECAM1+ cells compared to the untreated control group **(Fig.10 B,D)**.

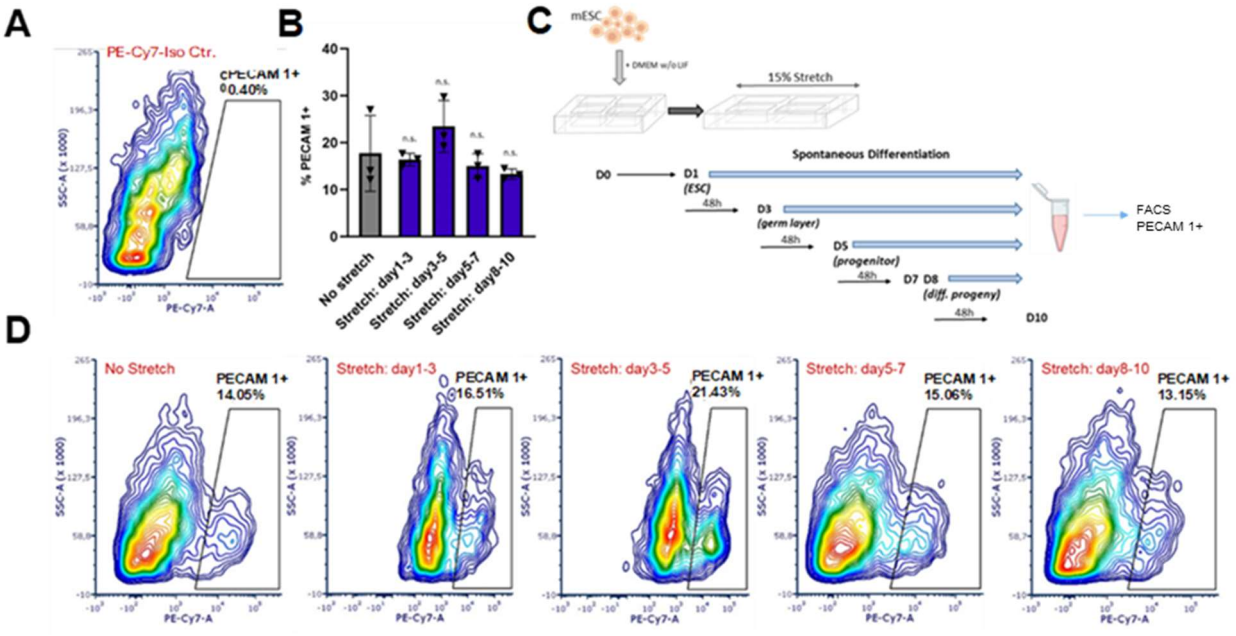

Figure 10: FACS analysis of cells cultured on poly-l-lysine until day 10 (D10). (A) Negative control using the PE-Cy7-iso Ctr. **(B)** Bar chart displaying the percentage of PECAM1 positive stained cells, staining with CD31-PE Cyanine7 (n=3). **(C)** Scheme of the experimental setup including the different stretching time points **(D)** Representative flow cytometry analysis for cell populations cultured without stretch, stretched for 48h between day 1-3, day 3-5, day 5-7 and day 8-10. Error bars represent mean ±SD. n.s. non-significant.

6.5 Effect of stretch on cells cultured on gelatine

I used a coating of 0.2% gelatine, with its medium high stiffness and non-promoting properties, to see its effect on the cultured cells and for possible effects of the

application of mechanical stretch to the EC differentiation. Stretching was performed at varying time points with 48 hours of stretch applied each time. The unstretched control group displayed an intermediate high level of PECAM1+ cells, with a mean of 18.55% **(Fig.11 A,B,D)**. The FACS analysis revealed that the cells stretched for 48h from day 1 to 3 (mean 44.16%), from 3 to 5 (mean 28.71%) and from 5 to 7 (mean 25.64%) had a highly significant increase in the number of PECAM1+ cells **(Fig.11 B,D)**. Furthermore, the samples that were stretched beginning on day 1 demonstrated more than twice the amount of PECAM1+ cells in comparison to the unstretched control group. The qPCR results showed consistency with this discovery: PECAM1 expression was increased in the samples stretched from day 1 to 3 (ESC stage) and 3 to 5 (germ layer specified cells). However, the qPCR results also showed a decrease in the number of PECAM1 mRNA (messenger ribonucleic acid) expression for the samples stretched between day 5 to 7 (progenitor cells) (**Fig.11 C).**

For cells stretched between day 8 and 10 (differentiated progeny) for 48 hours, there was no significant change in the FACS analysis for PECAM1 (mean 27.24%) as demonstrated in **Fig.11 B,D**.

To validate the FACS findings, I performed qPCR analysis to check the mRNA level of PECAM1. Since mechanical forces are known to induce EHT, I additionally checked the CD34 mRNA expression level, to differentiate if the rise in PECAM1+ cells resulted from an increase in ECs or cells undergoing EHT. Both ECs and cells undergoing EHT, are positive for PECAM1. But cells undergoing EHT are as well positive for CD34.

Corresponding to the FACS analysis, I found a significant increase in PECAM1 mRNA expression in the cell population stretched for 48h between day 1 and 3 but at the same time there was no increase in the mRNA expression for CD34 detected **(Fig. 11 C)**. The cell populations stretched between day 3 and 5 displayed a significant increase as well in PECAM1 expression but also no significant increase in the CD34 expression **(Fig.11 C)**. However, the qPCR analysis for the cell stretched between day 5 to 7 at progenitor stage indicated a substantial reduction in PECAM1 mRNA expression for cells stretched for 48h and no significant change for CD34 mRNA expression **(Fig.11 C)**.

The mRNA expression for PECAM1 and CD34 both showed no significant change for the samples stretched for 48 hours **(Fig.11 C).**

Since there was no significant increase in the CD34 expression but a highly significant increase in the PECAM1 expression for the samples stretched between day 1 to 3 and day 3 to 5, the cells positive for PECAM1 and therefore responsible for the increase in PECAM1 staining and expression, can be considered as ECs.

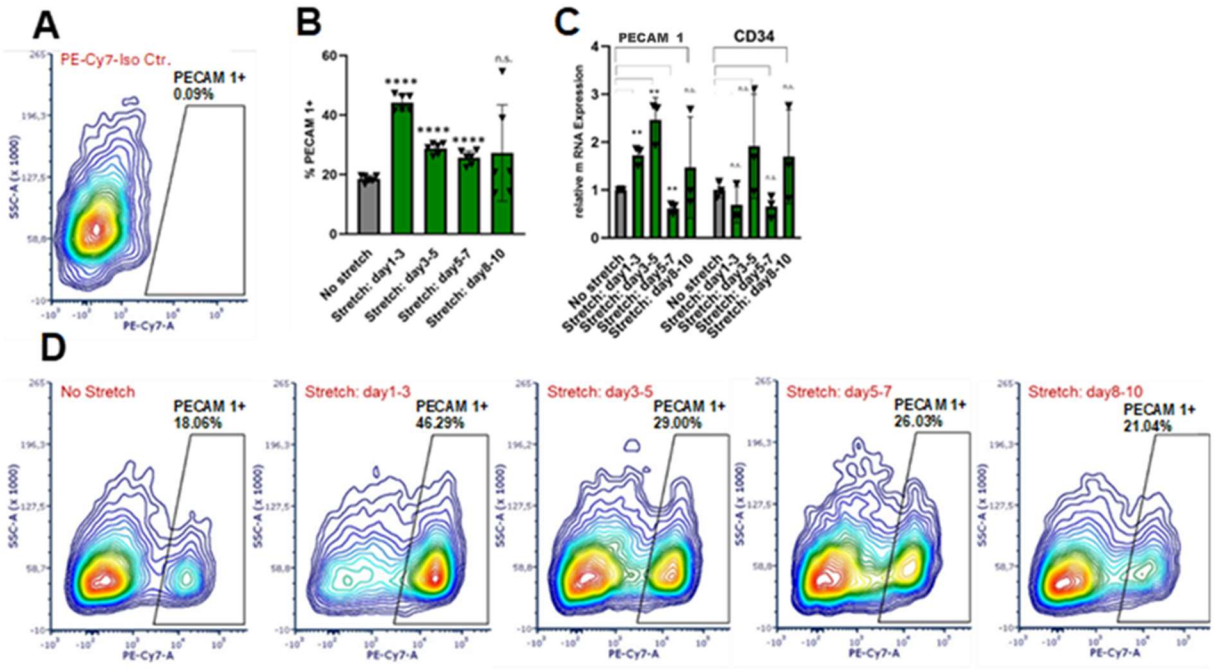

Figure 11: FACS analysis of cells cultured on gelatine until day 10 (D10). (A) Negative control using the PE-Cy7-iso Ctr. **(B)** Bar chart displaying the percentage of PECAM1 positive stained cells, staining with CD31-PE Cyanine7 (n=6). **(C)** qPCR analysis for relative mRNA expression of PECAM1 and CD34 of cells stretched for different time periods compared to the unstretched controls (n=3) **(D)** Representative flow cytometry analysis for cell populations cultured without stretch, stretched for 48h between day 1-3, day 3-5, day 5-7 and day 8-10. Error bars represent mean ±SD. ****p<0.0001, n.s. non-significant.

6.6 2 hours of stretch is sufficient to increase endothelial cell numbers

Since I observed major increase in EC numbers, when I applied stretch on ESC cultured on gelatine, I decided to use gelatine as coating when testing whether short stretching durations would be sufficient to increase EC numbers. To gain a better understanding of the impact that the stretching duration has on the number of PECAM1+ cells, I stretched cells for 2 and 6 hours at the time points already used: day

1 (ESC stage), day 3 (germ layer specific stage), day 5 (progenitor stage) and day 8 (differentiated progeny stage) of differentiation **(Fig.12)**.

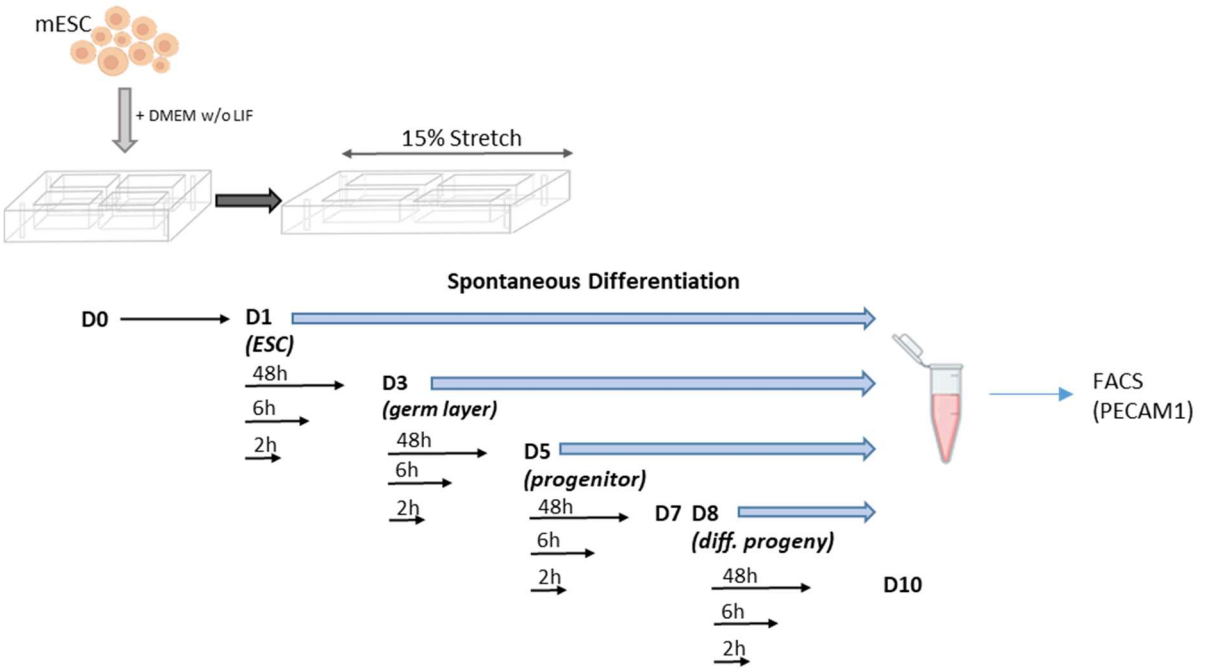

Figure 12: Scheme of the experimental setup including the different stretching time points The stretch was released after 2h or 6h and the stretching chambers transferred back to the incubator and left until day 10 after start of differentiation. Cells stretched for 48h were accordingly stretched until day 3 after start of differentiation and then left until day 10

In the FACS analysis, all samples stretched from day 1 after the start of differentiation **(Fig. 13 A)** showed a higher PECAM1+ cell count than the unstretched control group (mean 18.55%) **(Fig. 13 B,C)**. Cells stretched for two hours had a mean PECAM1+ cell count of 32.83%, while cells stretched for six hours had a mean count of 29.81% **(Fig. 13 B,C)**. The highest PECAM1+ cell count was observed in cells stretched for 48 hours, with a mean of 44.16% **(Fig.13 B,C)**. In addition to the Student-t test performed for all results, I additionally performed an Anova multiple comparison test between the different treatment groups. The Anova showed a p-value <0.0001, so it is highly unlikely that the differences observed within this group, are due to a random sampling.

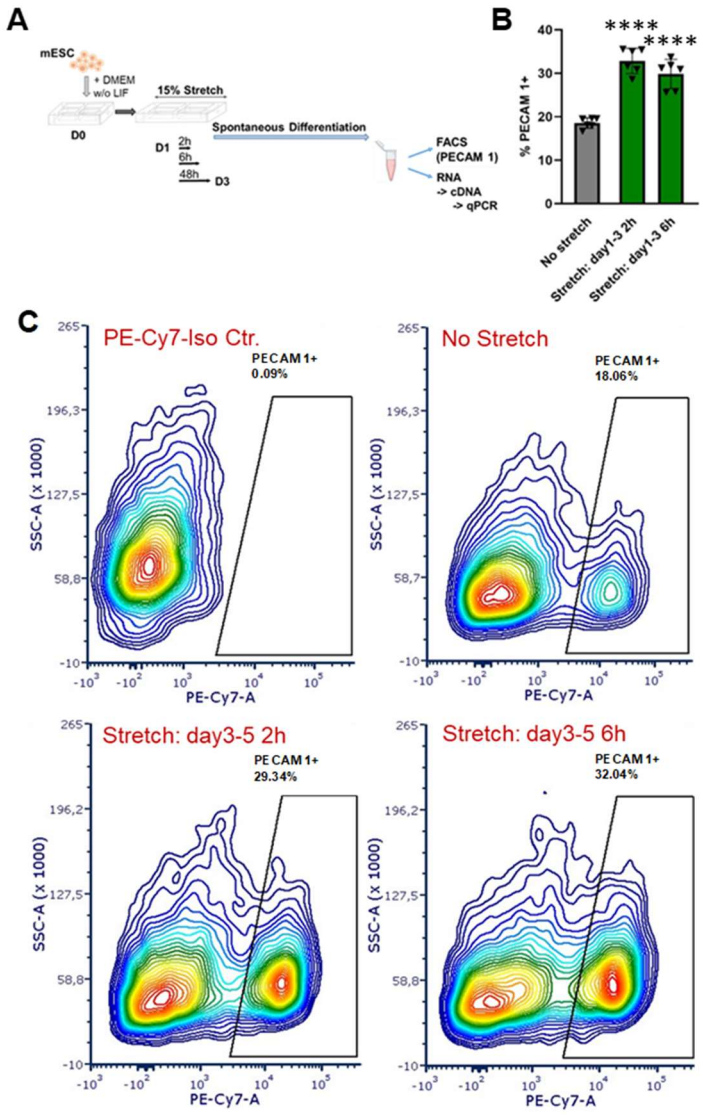

Figure 13: FACS and qPCR analysis of cells cultured on gelatine until day 10 (D10) and stretched from day 1 to 3 (A) Scheme of the experimental design including the stretching time points between day 1 and day 3. **(B)** Bar chart displaying the percentage of PECAM1 positive stained cells, staining with CD31-PE Cyanine7 (n=6). **(C)** Representative flow cytometry analysis for the unstretched control group and the cell populations stretched for 2h, 6h and 48h starting from day 1 stained with CD31-PE Cyanine7. Error bars represent mean ±SD. **p<0.01, ****p<0.0001, n.s. non-significant.

The second group of cells received the same treatment as described previously, with the exception that stretching was applied on day 3 after differentiation was initiated. Following the stretching protocol, cells were left until day 10 before being harvested for FACS analysis. The cells were stained for PECAM1 **(Fig.14 A)**.

In this group, all cell populations exhibited a remarkably greater proportion of PECAM1+ cells as compared to the unstretched control group, with a mean of 18.55% **(Fig.14 B,C)**. The samples stretched for 2 hours displayed an average of 31.33% of

PECAM1+ cells, those stretched for 6 hours showed an average of 33.70%, while the cells stretched for 48 hours showed an average of 28.71% **(Fig.14 B,C)**.

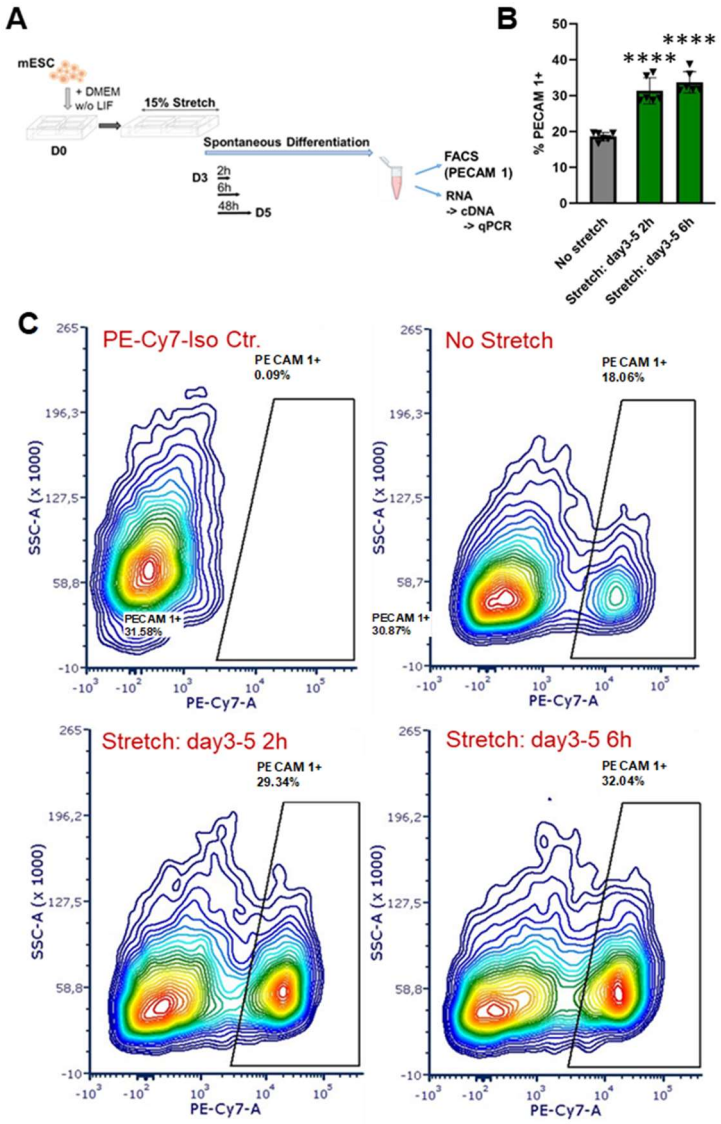

Figure 14: FACS and qPCR analysis of cells cultured on gelatine until day 10 (D10) and stretched from day 3 to 5 (A) Scheme of the experimental design including the stretching time points between day 1 and day 3. **(B)** Bar chart displaying the percentage of PECAM1 positive stained cells, staining with CD31-PE Cyanine7 (n=6). **(C)** Representative flow cytometry analysis for the unstretched control group and the cell populations stretched for 2h, 6h and 48h starting from day 1 stained with CD31-PE Cyanine7. Error bars represent mean ±SD. ****p<0.0001, n.s. non-significant.

The third group was cultured as described above except that the cells were stretched between day 5 and day 7 after the onset of differentiation **(Fig.15 A)**.

The cells that were stretched between day 5 and day 7 after the start of differentiation showed differential trends in the number of PECAM1+ cells compared to the untreated

control group (mean 18.55%) **(Fig.15 B,C)**. The cell cultures stretched for 2h and 6h showed a significant decrease in PECAM1+ cells with a mean of 13.92% in cells stretched for 2h and a mean of 14.91% in cells stretched for 6h. In contrast, cells expanded for 48 hours from day 5, showed after differentiation a significant increase in the number of PECAM1+ cells with a mean of 25.64% **(Fig.15 B,C)**.

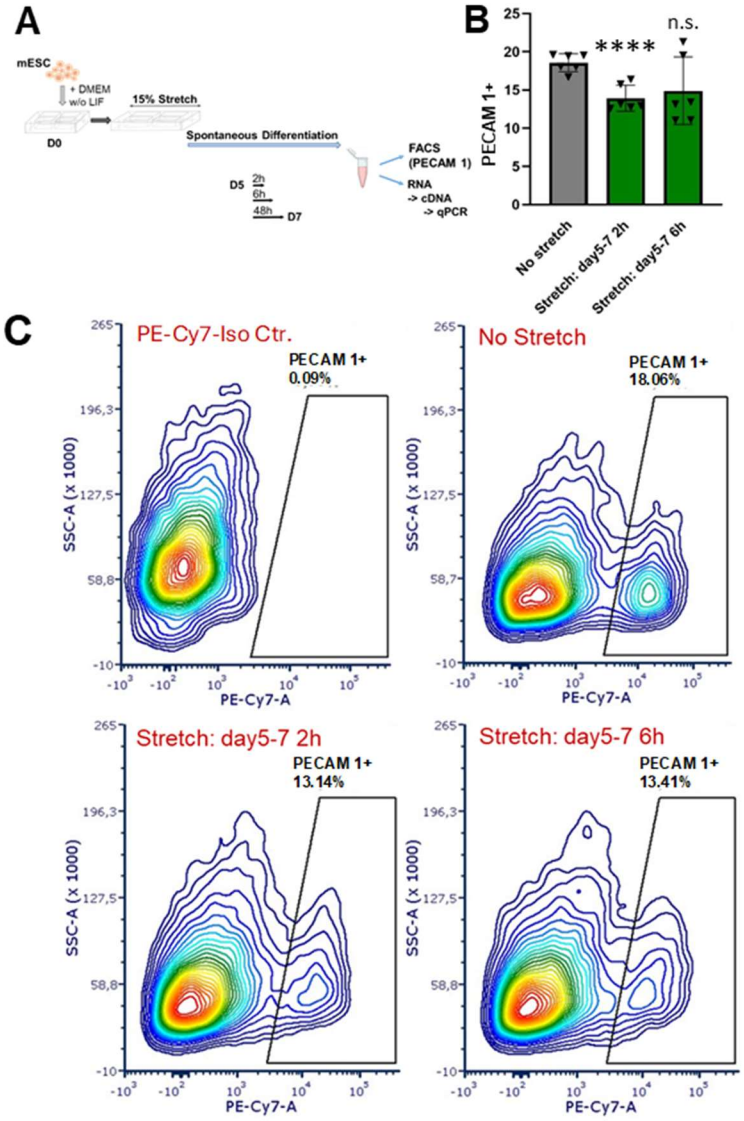

Figure 15: FACS and qPCR analysis of cells cultured on gelatine until day 10 (D10) and stretched from day 5 to 7 (A) Scheme of the experimental design including the stretching time points between day 1 and day 3. **(B)** Bar chart displaying the percentage of PECAM1 positive stained cells, staining with CD31-PE Cyanine7 (n=6). **(C)** Representative flow cytometry analysis for the unstretched control group and the cell populations stretched for 2h, 6h and 48h starting from day 1 stained with CD31-PE Cyanine7. Error bars represent mean ±SD. **p<0.01, ***p<0.001, ****p<0.0001, n.s. non-significant.

The cells in the final group received the same treatment as the others, but stretching was applied from day 8 **(Fig.16 A)**.

Samples where mechanical stretching was applied starting on day 8 after the start of differentiation showed a significant decrease in the number of PECAM1+ cells or no significant change compared to the untreated control group (mean 18.55%) **(Fig.16 B,C)**. The samples stretched for 2h decreased to a mean of 13.77% and the cells stretched for 6h to a mean of 8.91% of PECAM1+ cells within the cell population **(Fig.16 B,C)**. The samples stretched for 48 hours from day 8 (mean 27.24%) did not display a significant change when compared to the control group.

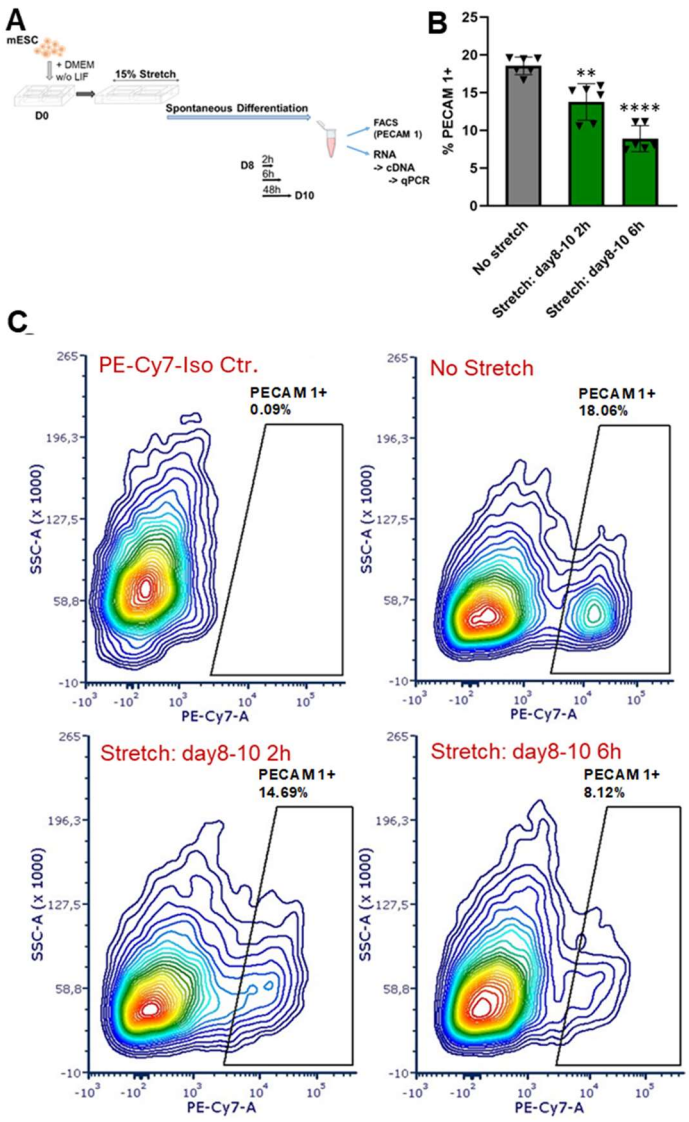

Figure 16: FACS and qPCR analysis of cells cultured on gelatine until day 10 (D10) and stretched from day 8 to 10 (A) Scheme of the experimental design including the stretching time points between day 1 and day 3. **(B)** Bar chart displaying the percentage of PECAM1 positive stained cells, staining with CD31-PE Cyanine7 (n=6). **(C)** Representative flow cytometry analysis for the unstretched control group and the cell populations stretched for 2h, 6h and 48h starting from day 1 stained with CD31-PE Cyanine7. Error bars represent mean ±SD. *p<0.05, **p<0.01, ****p<0.0001, n.s. non-significant.

Over the course of all the single experiments I found that application of non-cyclic uniaxial stretch for at least 2 hours at early developmental stages, such as stem cells, mesodermal precursors and cardiovascular precursors, was sufficient to significantly increase EC numbers, while application of stretch at later stages was not beneficial. The stretch at this earliest time point for 48h resulted in the highest measured increase.

To support and control these findings, I conducted immunostaining for PECAM1 to verify the increase in PECAM1+ cells treated with mechanical stretch while differentiating. Staining for PECAM1 and DAPI was performed on the cells (section 2.8.9) and images were acquired using the Leica MDi8 microscope. I observed an increase in PECAM1 positive (red) stained cells at day 10, in cells that I stretched for 2 hours, 6 hours and 48 hours compared to those from the unstretched control group **(Fig.17)**. The number of cells stained positive with PECAM1 increased with the duration of applied stretch. The cells stretched for 48 hours showed the highest number of cells stained positively for PECAM1 what is consistent to the results found in the FACS.

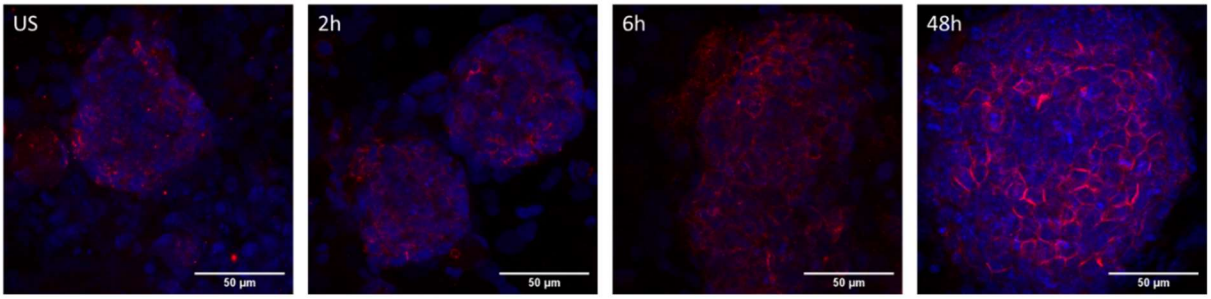

Figure 17: Immunostaining for PECAM1, Immunofluorescence microscopy for unstretched control and cultures stretched for 2h,6h and 48h starting from day 1 after start of differentiation, CD31 (red) for ECs and DAPI (blue) for nuclei, scale bar 50 µm.

6.7 Stretch induces gene expression program inducing endothelial cell development

To understand which effect the stretching has on the molecular mechanisms within the cells that might lead to the found results, I decided to perform mRNAseq. Since significant changes in the EC number already appeared at a stretching duration of 2h, I decided to use samples stretched for 2h and 6h for mRNAseq to study primary transcriptional response. To investigate whether stretching elicits changes in gene expression, I selected samples that were stretched for 2 and 6 hours, in addition to an

unstretched control group. Samples were collected immediately after the release of stretch.

Both, cells stretched for 2h (730 genes) and cells stretched for 6h (606 genes), showed a higher number of significantly upregulated genes than downregulated genes **(Fig.18 A,C)**. Both stretching time points showed overexpression of genes related to GO (gene ontology) pathways for EC proliferation, vascular endothelial growth factor production and vascular processes in the circulatory system **(Fig.18 B,D).** Even after only a short period of stretching (2h), I found key genes like Vegfa, Fgf7, Igf1 and Egfr to be highly upregulated.

However, the GO analysis for the cells stretched for only 2h showed significant downregulation in genes like Ttn, Fgr or Igf2, which are linked to pathways like muscle cell differentiation or myofibril assembly **(Fig.18 B)**.

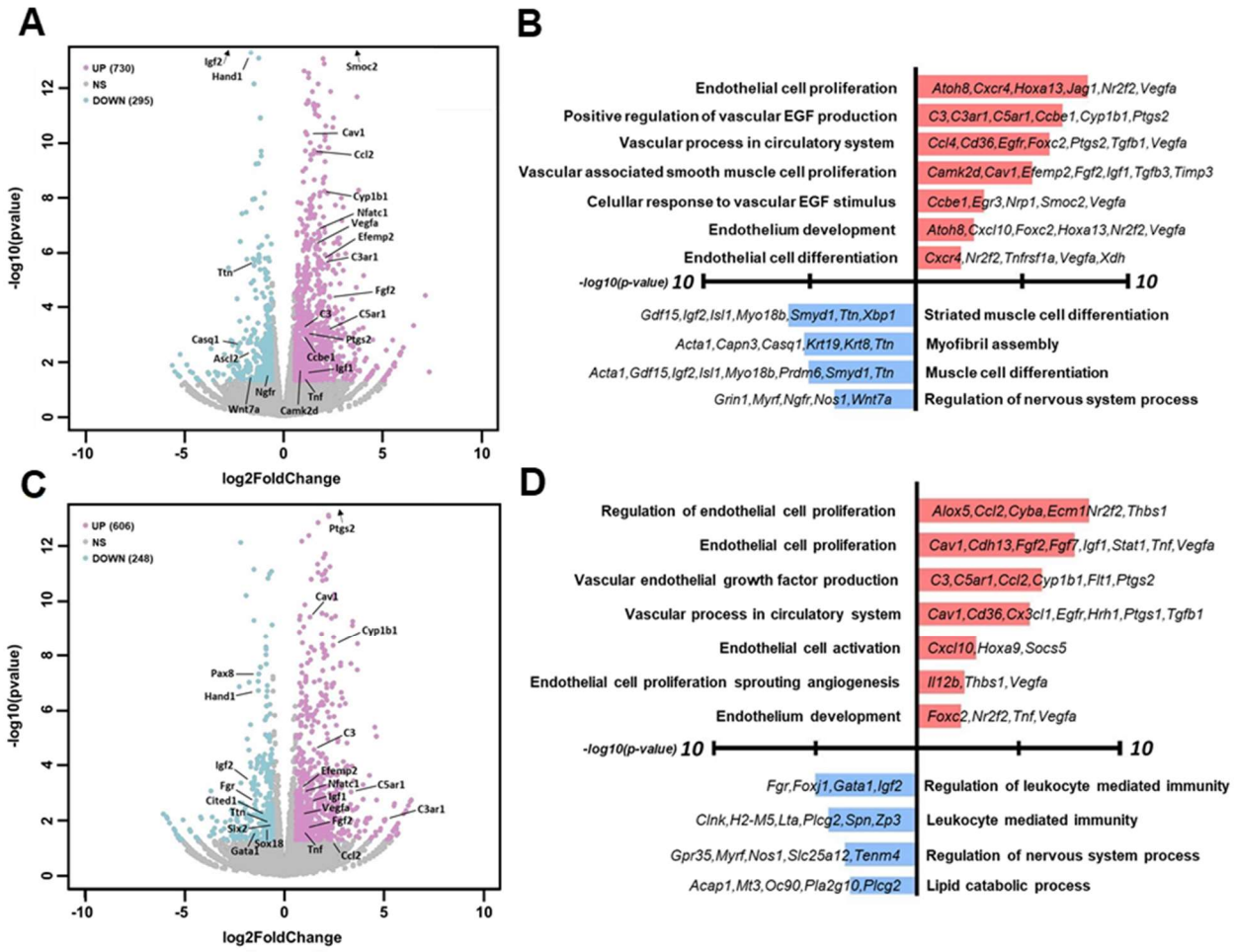

Figure 18: mRNAseq for cells stretched for 2h or 6h from day 1. (A,C) Volcano plot showing the distribution of differentially expressed genes in samples stretched for **(A**) 2h or, **(C)** 6h from day 1 versus unstretched control samples, representative up- (purple) and downregulated (blue) genes are highlighted **(B,D)** Gene ontology pathways for cells stretched **(B)** for 2h and **(D)** cells stretched for 6h, (n=4; Log2(FC) ≤ -0.58, ≥0.58; p-value < 0.05).

Looking at an overlap of all deregulated genes in the samples stretched for 2h or 6h, I found 509 genes which are significantly differentially expressed in both stretching conditions when compared to the unstretched control samples **(Fig.19 A)**. In the cluster for genes, which are upregulated in 2h- as well in the samples of 6h-stretched cells ,I found genes which are linked to GO pathways like EC proliferation, positive regulation of VEGF production, endothelium development and vascular process in circulator system **(Fig.19 B)**.

In the cluster which consists of genes that are downregulated in both stretching conditions, I found genes linked to GO pathways as placenta development and regulation of nervous system process **(Fig.19 B)**. Interestingly, there are only two genes which are significantly differentially expressed in opposite directions: Trim7 expression is upregulated in cells stretched for 2h and downregulated in cells stretched for 6h. Moreover, Prdm6, which has recently been identified as an important epigenetic regulator for neuronal crest cells in cardiac progenitor development (Hong et al., 2022), is downregulated in the cells stretched for 2h but upregulated in the cells stretched for 6h.

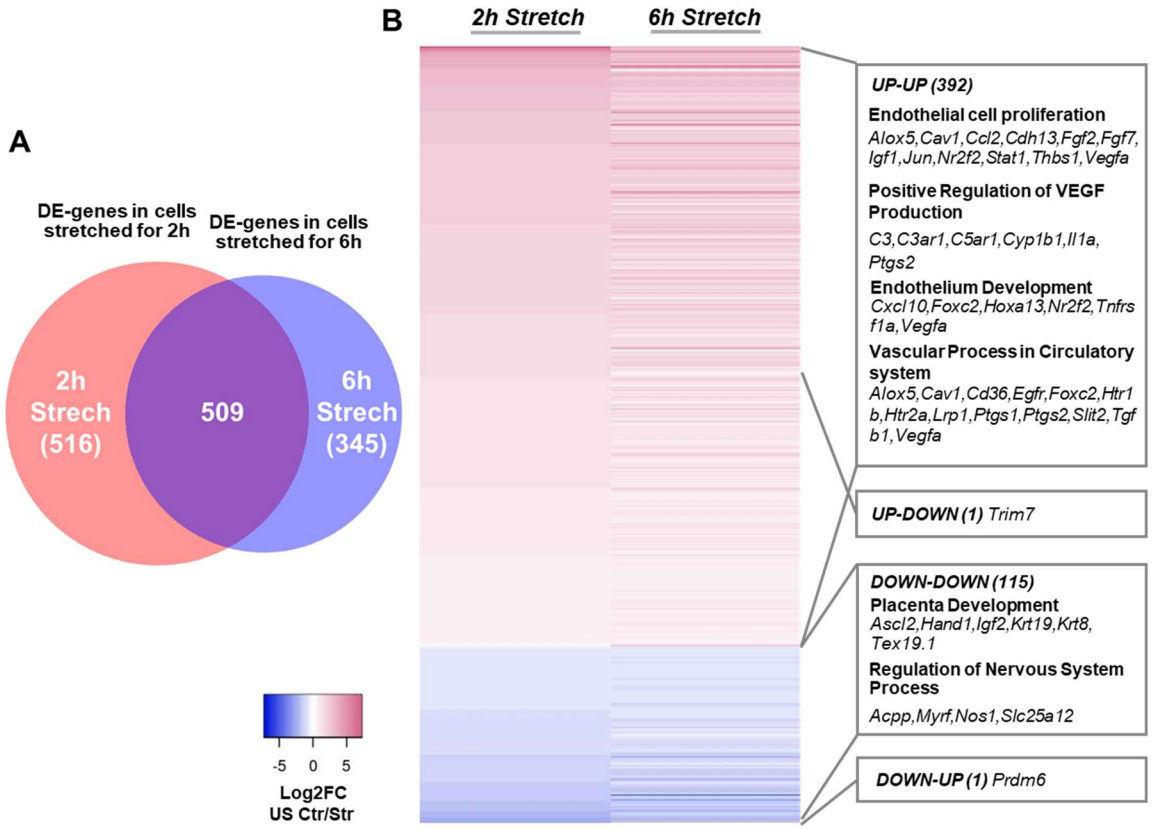

Figure 19: Deregulated genes in the mRNAseq analysis (A) Deregulated gene overlap between 2h and 6h **(B)** Clusters of up- and downregulation in single genes and related GO pathways for 2h and 6h of applied stretch (n=4; Log2(FC) ≤ -0.58, ≥0.58; p-value < 0.05).

Taken together, I could demonstrate that culturing ESC on laminin strongly promotes EC differentiation. The same effect appears in ESC differentiated on matrigel but in a smaller range. However, culturing ESC on gelatine and poly-l-lysine isn't promoting EC differentiation. The stiffness of the extracellular matrix the cells are cultured on, plays an important role when stretch is applied on developing ESC. A soft matrix like matrigel isn't enhancing EC differentiation whereas stretch applied on cell cultured on a stiffer matrix like gelatine increased the number of EC. Furthermore, I found that a continuous, uniaxial stretch of 2 hours is sufficient to increase EC numbers. On the molecular level, I showed that key genes for EC and vascular system development, such as Vegfa, Fgf7 and Igf1, were highly upregulated even after a short stretching duration of only 2 hours.

7 DISCUSSION

ECs are crucial for the function of all vessels in the mammalian body. Understanding how their development is influenced by the mechanical forces they experience and which role extracellular matrix stiffness plays, is widely unknown. There are many studies about shear stress but not many insights how tensile forces affect EC differentiation. Therefore, I studied how non-cyclic, uniaxial stretch applied on differentiating mouse embryonic stem cells and the use of ECM with different stiffnesses affect EC differentiation.

7.1 Non-cyclic, uniaxial stretch at early differentiation stages enhances endothelial cell development

I was able to show that cyclic, uniaxial stretch applied on mESC during differentiation forced the ESC into the EC lineage and resulted in a much higher percentage of ECs.

Especially the cells that I stretched at early time points during differentiation between day 1 to 3 and day 3 to 5, corresponding the stages of ESC and germ layer specified cells, showed higher increased EC numbers and increased expression of genes linked to vascular and endothelial growth. A group in 2013 found similar results, when they applied shear stress and cyclic stretch together with the administration of growth factors on adipose-derived mesenchymal stem cells and demonstrated an increase of the expression of EC marker genes Flk1,vWF and VE-cadherin (Shojaei et al., 2013).

Another study applying stretch on stem cells outlined that mesenchymal stem cells (MSC), when treated with a mixture of pressure, radial distention and shear stress, aligned like differentiated EC according to the flow and demonstrated a morphology similar to the one of EC (O'Cearbhaill et al., 2008). Trying to mimic in vivo settings, a research team (Kim et al., 2016) cultured MSCs in an in vitro system that simulated vessels and exposed the cells to shear stress and circumferential stretch. This led to an increase in EC typical markers at protein level like VE-cadherin and vWF. Their results are in line with the results of the experiments I conducted for this thesis: They found the number of cells positive for EC markers (here Flk1+ and vWF) to be increased in the flow cytometry analysis after a stretch of 5% (Kim et al., 2016). My

research adds upon their results, showing that the application of only uniaxial and non-cyclic stretch is already enough to increase EC numbers. It should be noted that I applied 15% of stretch instead of the 5% stretch used in this study (Kim et al., 2016). The main difference between this thesis and former research is the use of embryonic stem cells instead of mesenchymal stem cells. The use MSCs prevent them from being able to observe effects on stem cells, which are not specified to a lineage choice yet. Since mechanical stretch on differentiating ESC might lead to an increased production of growth factors that promote endothelial and vascular development, cells in earlier development stages might benefit more from this factor. For example, was it shown, that the vascular endothelial growth factor (VEGF) is a quite important growth factor for EC differentiation at earliest developmental stage (Bikfalvi and Bicknell, 2002; Coultas et al., 2005). I found Vegfa to be upregulated in my mRNAseq results.

In my experiments, a stretching start until progenitor stage (day 5 after start of differentiation) still led to an increase in ECs in the final cell population at day 10. Static, uniaxial stretch seems to force and guide cells at different time points of differentiation into the EC lineage. But the efficiency decreases, the later the differentiation stage is, they cells get exposed to mechanical stimuli.

7.2 Non-cyclic, uniaxial stretch at late differentiation stages decreases endothelial cell numbers

To investigate the effect of stretch in late EC development, I stretched cells at day 8 to 10, when they are already considered differentiated progenies. I found a decrease in EC number as well as a decrease in the mRNA expression of PECAM1. Cells at this time of differentiation are already differentiated into the EC lineage. Stretch at this time points seems to be too late to support EC lineage choices. This would be supported by the findings of a study which showed that EC markers constantly rise during differentiation until day 7 of differentiation (Di Bernardini et al., 2014). So, a stretching start at day 8 falls into a time when the lineage choice might be already in the past. Following this point, there has also been intensive research about the influence of mechanical forces on already differentiated ECs besides the research of stretched stem cells. Mostly, they indicate a positive influence rather than the inhibition which I observed in the cells, that I stretched for only short durations during the stage of differentiated progenies. These studies investigated the influence of mechanical stimuli

using different kinds of mechanical forces: pressure, unicyclic stretch and shear stress or the combinations of these. So led static and cyclic stretch of coronary microvascular ECs both to an increase in the VEGFR2 protein and an increase in cell proliferation and tube formation (Zheng et al., 2008). Others showed that HUVEC exposed to pressure increased in proliferation (Schwartz et al., 1999), the same was observed for bovine aortic EC (Sumpio et al., 1994). Another research project evaluated the influence of different kinds of mechanical forces on ECs: laminar shear stress applied to the cells led to a significant upregulation of proliferative genes, the loss of shear stress to a downregulation. Cyclic uniaxial stretch let to a remodelling of the EC and acted protective against apoptosis (Chien, 2006) but they also showed the opposite effect for cyclic biaxial stretch instead of cyclic uniaxial stretch. Additionally they demonstrated that persisting laminar shear stress downregulates proliferative genes as well as upregulated genes, that inhibit EC growth (Chien, 2006). It was demonstrated, that 20% stretch on HUVECs activated pathologic responses like inflammatory pathways, inhibited cell proliferation and the formation of tubes (Ma et al., 2022). Ma et al.‘s results lead to the conclusion that the stretch I applied during my research might have been too strong. The already differentiated cells in the EC lineage could have been damaged and the further EC differentiation could have been inhibited through too strong stretch. Interestingly the 15% of stretch didn’t have a negative effect on the cells in earlier stages of differentiation. Based on my findings and previously published research, it appears that excessive or undirected stretching of ECs leads to inhibition of EC differentiation and endothelial damage, whereas a physiological level of mechanical stress is required to maintain a healthy endothelium and induce EC differentiation.

7.3 Stretch induced gene expression changes towards vascular pathways

Supporting my finding that stretch at early stages increases EC numbers, I found several pathways related with vascular processes and EC proliferation to be upregulated in my stretched samples. Overall, both sample groups contained more upregulated genes then downregulated genes. GO pathways enriched in upregulated genes included EC proliferation (CCl2, Cdh13, Fgf2, Fgf7, Igf1, Vegfa), positive regulation of VEGF (C3, C5ar1, Ptgs2,), endothelium development (Cxcl, Hoxa13) and vascular process in circulatory system (Cav1, CD36, Egfr,Fox2c, Vegfa). Previous

studies showed VEGFA to be upregulated in various cells which had been exposed to mechanical forces. For example in ECs derived from porcine vessels (Conklin et al., 2002), mesenchymal stem cells (Maul et al., 2011) or murine embryonic mesenchymal progenitor cells (Wang et al., 2005). My data revealed a high consistency of the gene regulation between samples stretched for 2h and samples stretched for 6h. Genes which were upregulated in the samples stretched for 2h, stayed as well upregulated in the samples stretched for 6h. Additionally, genes which were downregulated in the 2h group were as well downregulated in the 6h group. This suggests that the molecular mechanisms induced by continuous stretching, which led to gene deregulation, were still active after 6 hours of continuous mechanical stimulus, indicating that the cells had not yet adapted to the stretch. My data shows that mechanical stretch applied on mESCs leads to activation of gene expression program and promotes EC differentiation.

7.4 Tensile forces don't promote endothelial to hematopoietic transition

It was demonstrated in earlier studies, that shear stress promotes HPSC (hematopoietic progenitor and stem cells) development (Adamo et al., 2009; North et al., 2008) but it remained unclear whether tensile forces might have the same effect on the endothelial-to-hematopoietic transition. In my studies, I couldn't find evidence for EHT upon application of static, uniaxial stretch. The number of PECAM1+ cells was significantly increased, at the same time the level of mRNA expression of CD34 as a hematopoietic marker was not elevated. Therefore, I conclude that tensile forces do not promote EHT.

7.5 Laminin increases endothelial cell numbers

My experiments showed that the usage of a laminin coating as growing medium itself already strongly promotes the differentiation of ECs. The cell populations seeded in these chambers exhibited, even without stretching, a nearly three times higher percentage of ECs compared to the ones seeded in gelatine or poly-l-lysine. Already in 1993, a research group found out that the usage of biologically active fragments of laminin increased the binding of EC to the ECM. They used matrigel which contained these specific laminin fragments as ECM and found changes in the morphology and the induction of cell migration in EC (Schnaper et al., 1993). Two other studies revealed

that parts of laminin promote the tube formation of ECs in vitro. They found that a RGD-containing sequence on the A chain of laminin induced EC attachment to the ECM through binding to an integrin receptor in the EC while a part of the laminin B chain induced cell-cell interactions and therefore tube formation (Grant et al., 1989). The molecular fragment laminin-411 was found to differentiate MPCs into EPCs with a purity from >95% compared to an EC directed differentiation on matrigel with a rate of <10% EPCs (Ohta et al., 2016). These findings are supported as well from a study showing that laminin 411 supported the differentiation of EC out of induced pluripotent stem cells (Hall et al., 2022). The cells cultured in matrigel showed a higher level of PECAM1+ cells even if not as high as the ones cultured in laminin. A possible explanation is that matrigel contains a mixture of different proteins including laminin, which have a promoting effect but that the mixture doesn't has such a strong effect as pure laminin.

7.6 Different extracellular matrix specifications strongly influence endothelial cell development

When trying different extracellular matrices as a suitable coating for the stretching experiments, I also used matrigel. Analysing the FACS results for PECAM1 from the non-stretched control groups grown on different coatings, it appeared that matrigel enhanced EC differentiation compared to a fairly neutral coating such as gelatine, although not to the same extent as a laminin coating. As earlier studies showed that matrigel (and the laminin fragments it contains) (Grant et al., 1989) promotes EC differentiation (Baatout and Cheţa, 1996), this is not an unexpected finding. The strong promoting effect is explained by the mix of basement membrane proteins and growth factors included in the matrigel (Baatout and Cheţa, 1996; Taub et al., 1990). The application of stretch induces growth factor production as well. Therefore, it is likely that the lack of response from cells which were cultured under the application of stretch on matrigel could be caused by the presence of growth factors already included in the ECM matrigel.

Furthermore, matrigel induces the formation of tubes and the forming of capillaries out of ECs (Baatout, 1997; Haralabopoulos et al., 1994; Kobayashi et al., 2004). In the cell populations grown on matrigel but stretched during their differentiation, I nevertheless found a decrease in the number of ECs compared to the untreated control group grown

on matrigel. This could be explained by the softness of matrigel as an extracellular matrix. Matrigel is reported with a stiffness of 450 Pa (Soofi et al., 2009) to 650 Pa (Reed et al., 2009) or even lower in earlier studies with values ranging between 39 Pa and 120 Pa ((Alcaraz et al., 2008; Semler et al., 2000; Zaman et al., 2006). The reduction in PECAM1+ cells could be caused by the lack of mechanical stimulation and interaction with the proteins in the extracellular matrix since matrigel is highly complaint due to its low stiffness and therefore not transducing the mechanical forces applied onto it. This is coherent to earlier studies which outlined the importance of mechanical stimulation and sufficient stiffness for cell proliferation and differentiation (Pelham and Wang, 1997; Ren et al., 2008), also especially for ECs and smooth muscle cells (Richert et al., 2004).

For trying a coating with higher stiffness I used poly-l-lysine which is reported with a high stiffness ranging from 3-400 kPa (Ren et al., 2008) to a stiffness up to 20-800kPa (Richert et al., 2004). The cells which were seeded on this coating showed a decrease in the number of ECs in the untreated control group and these decreased numbers didn't show an effect upon mechanical stretch. Furthermore, three observations could be made: the cells didn't form a layer on the coated chambers over time, the cells didn't spread over the term of differentiation and I observed a higher number of floating dead cells than in experiments with other coatings. These findings can be explained with the high stiffness and the strong adherence of the cells to the chamber coating promoted by poly-l-lysine (Choi et al., 2015; Mazia et al., 1975). Since cell rounding (Lancaster et al., 2013; Taubenberger et al., 2020; Théry and Bornens, 2008) and spreading are necessary for cell division and proliferation (Martz and Steinberg, 1972; Schnyder et al., 2020; Schutz and Mora, 1968), inhibiting them could have prevented ESC proliferation and differentiation.

Another explanation might be that the specific features of poly-l-lysine lead to the lack of proliferation and differentiation of ECs. Poly-l-lysine has been reported to be cytotoxic in too high concentrations (Lu et al., 2009), so the concentration I used (50 µg/ml) might have been toxic for the ESC. Furthermore, poly-l-lysine was found to inhibit angiogenesis in the context of tumour cells by blocking the proliferation of the tumour cells and preventing tumour-angiogenesis (Debnath et al., 2018). Additionally, investigations revealed that poly-l-lysine lead to a significant downregulated activity of

VEGF, VEGFR2, Ki-67 and c-Myc expression (Debnath et al., 2021). To complete these finding, a poly-l-lysine dimer was found to inhibits angiogenesis and tubule formation in murine ECs (Al-Jamal et al., 2010). Taken together, it is possible that the high stiffness and cell adhesion of poly-l-lysine prevented rounding, spreading, and proliferation, or that the angiogenesis-inhibiting properties of poly-l-lysine led to a decrease in ECs and living cell numbers in general.

7.7 Limitations

Concluding, this work provides insights into the effects of tensile forces on EC differentiation and thus the development and maintenance of a healthy endothelium. However, there are certain aspects I did not cover within this work which are outlined hereafter.

At first, this thesis cannot provide insights about the proliferation of the cells or eventual changes in proliferation upon stretch within the experiments conducted. Within my work, I used methods that provide reliable information about cell numbers and cell type markers and did not cover experiments focused on the proliferation of the cells. So future studies might include immunoassays checking for proliferation proteins like Ki67 or PCNA or counting of DNA-synthesizing cells via FACS. Second, in my experiments I focused on a single EC marker, PECAM 1. I checked this marker with different methods to make my findings consistent. Including additional markers, like VE-cadherin in qPCR analysis or FACS, would have made my results even more robust. Similarly, overexpression experiments with a focus on genes I found to be expressed differentially upon stretch, could have added robustness.

Third, regarding differentiation I did neither include experiments on surface markers which are characteristic for certain differentiation stages, nor did I conduct experiments for a time span longer than 10 days. I used published literature and experience from earlier experiments to conclude in which differentiation stage the cells are and therefore which time points would be suitable for stretching. Checking for surface markers would have made it possible to be more exact about the cell's differentiation stage. Moreover, longer experimental settings exceeding the 10 days covered by my experiments would have made it possible to investigate how the stretched cells differentiate and change their morphology for example regarding tube formation.

Last but not least, I used small sample sizes focused on a certain ECM in my work as I wanted to be able to test more different ECMs. Larger sample sizes could further reduce the likelihood of statistical outliers and to strengthen the robustness of my findings.

All of this leaves room for further research in the aforementioned areas to complement the results from my research displayed in this thesis.

8 CONCLUSION AND PERSPECTIVES

In this thesis I investigated the impact of non-cyclic uniaxial stretch on cell differentiation at different developmental stages, using mouse embryonic stem cells instead of mesenchymal stem cells which have been studied before. My results revealed that applying stretch during early developmental stages significantly increases EC numbers, while such an effect cannot be observed when stretch is applied at later differentiation stages. Furthermore, the study explored the influence of different matrices on cell differentiation, demonstrating that laminin and matrigel promote EC differentiation, whereas gelatine and poly-l-lysine do not lead to the same effect. Additionally, the interplay between matrix stiffness and tensile forces was examined, revealing that mechanical stretch on cells cultured on a stiffer matrix leads to a significant increase in EC numbers compared to a softer matrix. To understand the molecular mechanisms involved in enhanced EC differentiation, I performed RNA-sequencing analysis. I identified key genes related to EC and vascular system development to be highly upregulated even after a short period of stretch application (2 hours), including Vegfa, Fgf7 and Igf1.

Although further investigation is required, my work indicates a close interplay between tensile forces and extracellular matrix stiffness during EC development and differentiation and has identified critical factors involved in this process. Still, this is a domain where much remains to be studied. The following areas seem of interest to me and are worth of further examination: In terms of further experiments, an overexpression experiment could be carried out on the genes I identified, that were dysregulated under the influence of mechanical stretch. Furthermore, it would be interesting to identify the intracellular pathways that led to the observed changes in the gene expression of stretched cells.

9 LIST OF FIGURES

10 LIST OF TABLES

11 CURRICULUM VITAE

PERSONAL DATA

Surname, Name	Schäfer, Patricia Laura
Date of Birth	06/19/1998
Place of Birth	Mannheim
Family status	Unmarried
Father	Dr. Willi Werner Schäfer
Mother	Bettina Maria Eitzen

EDUCATION

Ursulinengymnasium, Mannheim 2009 - 2017	**High School** Focus on biology and chemistry
07/03/2017	**High School diploma** Grade 1.0
Heidelberg University since 10/2017	**Medical Faculty Mannheim** Medical studies
09/11/2019	1. German State Exam Grade 2.0
2019 - 2024	Clinical Studies
09/2022 – 03/2022	Semester abroad at the Semmelweis University Budapest, Hungary

12 ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor and team leader, Prof. Dr. Gergana Dobreva, for granting me the chance and providing me with all the necessary support to write my thesis in her laboratory. I am thankful for my colleagues who taught me everything I have learned in the last few years, I know I was a pain in the ass at the beginning. I would like to thank Adel for teaching me how to keep my cells alive and how to think in research. Thank you, Linda, for all your guidance and support. Without you, I would have been lost. Thank you, Arthur, for answering my numerous questions whenever I came to you. Julio, thank you for trying to teach me bioinformatics despite often seeing only confusion on my face and helping me with the analysis! To all of you, thank you so much for the dinners and the sports sessions. I like to thank Stefanie Uhlig, FlowCore Mannheim and Institute of Transfusion Medicine and Immunology, for excellent support.

My dear friends, I have cherished our friendship for more than six years now and I could not have asked for a better time. You constantly made me feel better, laughed with me (and sometimes at me) and you still cannot believe why I did this to myself. But you all had faith in me and I appreciate you all for this. Thanks for the countless evenings in the flat share and for always being just a hop or a call away.

Fortunately, I am in a privileged position where I always have somewhere to call home: my family. Thank you for the years of encouragement and empathy. Hey Vic, I still have that little card you left next to my plate on my first day at the lab. I have kept it in my lab journal ever since. Dad, you inspired me to do all this and to not just take the easy way out. I tried my best to make it to 123 but seems you could talk longer than I. And to my mom: yes, it was a long time and it could have been shorter and easier but I didn't want it any other way. And finally, I finished it. I love you all and I always will!

Last but not least: thanks to my beloved partner Tobi. You have been with me from the beginning to the end. You never complained about being left alone or me running hours late while still being in the lab dealing with my experiments. More than once you made sure, I wouldn’t give up and you always found a solution for problems, even if I couldn’t see them. I love you, not only for this.

13 REFERENCES

Adamo, L., Naveiras, O., Wenzel, P.L., McKinney-Freeman, S., Mack, P.J., Gracia-Sancho, J., Suchy-Dicey, A., Yoshimoto, M., Lensch, M.W., Yoder, M.C., *et al.* (2009). Biomechanical forces promote embryonic haematopoiesis. Nature *459*, 1131-1135. https://doi.org/10.1038/nature08073

Al-Jamal, K.T., Al-Jamal, W.T., Akerman, S., Podesta, J.E., Yilmazer, A., Turton, J.A., Bianco, A., Vargesson, N., Kanthou, C., Florence, A.T., *et al.* (2010). Systemic antiangiogenic activity of cationic poly-L-lysine dendrimer delays tumor growth. Proc Natl Acad Sci U S A *107*, 3966-3971. https://doi.org/10.1073/pnas.0908401107

Alcaraz, J., Xu, R., Mori, H., Nelson, C.M., Mroue, R., Spencer, V.A., Brownfield, D., Radisky, D.C., Bustamante, C., and Bissell, M.J. (2008). Laminin and biomimetic extracellular elasticity enhance functional differentiation in mammary epithelia. Embo j *27*, 2829-2838. https://doi.org/10.1038/emboj.2008.206

Baatout, S. (1997). Endothelial differentiation using Matrigel (review). Anticancer Res *17*, 451-455.

Baatout, S., and Cheţa, N. (1996). Matrigel: a useful tool to study endothelial differentiation. Rom J Intern Med *34*, 263-269.

Bertrand, J.Y., Chi, N.C., Santoso, B., Teng, S., Stainier, D.Y., and Traver, D. (2010). Haematopoietic stem cells derive directly from aortic endothelium during development. Nature *464*, 108-111. https://doi.org/10.1038/nature08738

Bikfalvi, A., and Bicknell, R. (2002). Recent advances in angiogenesis, anti-angiogenesis and vascular targeting. Trends Pharmacol Sci *23*, 576-582. https://doi.org/10.1016/s0165-6147(02)02109-0

Boisset, J.C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E., and Robin, C. (2010). In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature *464*, 116-120. https://doi.org/10.1038/nature08764

Bruce Alberts, A.J., Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter (2002): Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Blood Vessels and Endothelial Cells, https://www.ncbi.nlm.nih.gov/books/NBK26848/. Retrieved 17.01.2022.

Burke, D., and Kelly, D.J. (2016). A mechanobiological model of endothelial cell migration and proliferation. Comput Methods Biomech Biomed Engin *19*, 74-83. https://doi.org/10.1080/10255842.2014.989388

Chen, P.C.Y., Herath, S., Wang, D.-A., and Asada, H. (2012). Active Manipulation of ECM Stiffness and Its Effect on Endothelial Cell Migration during Angiogenesis. Lecture Notes in Engineering and Computer Science *2197*.

Chien, S. (2006). Molecular basis of rheological modulation of endothelial functions: importance of stress direction. Biorheology *43*, 95-116.

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J.C., and Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. Development *125*, 725-732. https://doi.org/10.1242/dev.125.4.725

Choi, Y., Yagati, A.K., and Cho, S. (2015). Electrochemical Characterization of Poly-L-Lysine Coating on Indium Tin Oxide Electrode for Enhancing Cell Adhesion. Journal of nanoscience and nanotechnology *15* *10*, 7881-7885. https://doi.org/https://doi.org/10.1166/jnn.2015.11229

Conklin, B.S., Zhong, D.S., Zhao, W., Lin, P.H., and Chen, C. (2002). Shear stress regulates occludin and VEGF expression in porcine arterial endothelial cells. J Surg Res *102*, 13-21. https://doi.org/10.1006/jsre.2001.6295

Coultas, L., Chawengsaksophak, K., and Rossant, J. (2005). Endothelial cells and VEGF in vascular development. Nature *438*, 937-945. https://doi.org/10.1038/nature04479

Davies, P.F. (1991). Mechanical sensing mechanisms: shear stress and endothelial cells. Journal of Vascular Surgery *13*, 729-731. https://doi.org/https://doi.org/10.1016/0741-5214(91)90364-Z

Davies, P.F. (1995). Flow-mediated endothelial mechanotransduction. Physiol Rev *75*, 519-560. https://doi.org/10.1152/physrev.1995.75.3.519

de Bruijn, M.F., Speck, N.A., Peeters, M.C., and Dzierzak, E. (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. Embo j *19*, 2465-2474. https://doi.org/10.1093/emboj/19.11.2465

Debnath, S., Mukherjee, A., Karan, S., Debnath, M., and Chatterjee, T.K. (2018). Induction of apoptosis, anti-proliferation, tumor-angiogenic suppression and down-regulation of Dalton's Ascitic Lymphoma (DAL) induced tumorigenesis by poly-l-lysine: A mechanistic study. Biomed Pharmacother *102*, 1064-1076. https://doi.org/10.1016/j.biopha.2018.03.076

Debnath, S., Mukherjee, A., Saha, D., Dash, J., and Chatterjee, T.K. (2021). Poly-l-Lysine inhibits VEGF and c-Myc mediated tumor-angiogenesis and induces apoptosis in 2D and 3D tumor microenvironment of both MDA-MB-231 and B16F10 induced mice model. Int J Biol Macromol *183*, 528-548. https://doi.org/10.1016/j.ijbiomac.2021.04.109

Dejana, E., Hirschi, K.K., and Simons, M. (2017). The molecular basis of endothelial cell plasticity. Nat Commun *8*, 14361. https://doi.org/10.1038/ncomms14361

Di Bernardini, E., Campagnolo, P., Margariti, A., Zampetaki, A., Karamariti, E., Hu, Y., and Xu, Q. (2014). Endothelial lineage differentiation from induced pluripotent stem cells is regulated by microRNA-21 and transforming growth factor β2 (TGF-β2) pathways. J Biol Chem *289*, 3383-3393. https://doi.org/10.1074/jbc.M113.495531

Diaz, M.F., Li, N., Lee, H.J., Adamo, L., Evans, S.M., Willey, H.E., Arora, N., Torisawa, Y.S., Vickers, D.A., Morris, S.A.*, et al.* (2015). Biomechanical forces promote blood development through prostaglandin E2 and the cAMP-PKA signaling axis. J Exp Med *212*, 665-680. https://doi.org/10.1084/jem.20142235

Fang, Y., Wu, D., and Birukov, K.G. (2019). Mechanosensing and Mechanoregulation of Endothelial Cell Functions. Compr Physiol *9*, 873-904. https://doi.org/10.1002/cphy.c180020

Frame, J.M., Fegan, K.H., Conway, S.J., McGrath, K.E., and Palis, J. (2016). Definitive Hematopoiesis in the Yolk Sac Emerges from Wnt-Responsive Hemogenic Endothelium Independently of Circulation and Arterial Identity. Stem Cells *34*, 431-444. https://doi.org/10.1002/stem.2213

Fujiwara, K. (2006). Platelet endothelial cell adhesion molecule-1 and mechanotransduction in vascular endothelial cells. J Intern Med *259*, 373-380. https://doi.org/10.1111/j.1365-2796.2006.01623.x

Galbraith, C.G., Skalak, R., and Chien, S. (1998). Shear stress induces spatial reorganization of the endothelial cell cytoskeleton. Cell Motil Cytoskeleton *40*, 317-330. https://doi.org/https://doi.org/10.1002/(SICI)1097-0169(1998)40:4<317::AID-CM1>3.0.CO;2-8

Goldie, L.C., Nix, M.K., and Hirschi, K.K. (2008). Embryonic vasculogenesis and hematopoietic specification. Organogenesis *4*, 257 - 263. https://doi.org/https://doi.org/10.4161/org.4.4.7416

Gore, A.V., Pillay, L.M., Venero Galanternik, M., and Weinstein, B.M. (2018). The zebrafish: A fintastic model for hematopoietic development and disease. Wiley Interdiscip Rev Dev Biol *7*, e312. https://doi.org/10.1002/wdev.312

Grant, D.S., Tashiro, K., Segui-Real, B., Yamada, Y., Martin, G.R., and Kleinman, H.K. (1989). Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. Cell *58*, 933-943. https://doi.org/10.1016/0092-8674(89)90945-8

Hall, M.L., Givens, S., Santosh, N., Iacovino, M., Kyba, M., and Ogle, B.M. (2022). Laminin 411 mediates endothelial specification via multiple signaling axes that converge on β-catenin. Stem Cell Reports *17*, 569-583. https://doi.org/https://doi.org/10.1016/j.stemcr.2022.01.005

Haralabopoulos, G.C., Grant, D.S., Kleinman, H.K., Lelkes, P.I., Papaioannou, S.P., and Maragoudakis, M.E. (1994). Inhibitors of basement membrane collagen synthesis prevent endothelial cell alignment in matrigel in vitro and angiogenesis in vivo. Lab Invest *71*, 575-582.

Hastings, N.E., Simmers, M.B., McDonald, O.G., Wamhoff, B.R., and Blackman, B.R. (2007). Atherosclerosis-prone hemodynamics differentially regulates endothelial and smooth muscle cell phenotypes and promotes pro-inflammatory priming. Am J Physiol Cell Physiol *293*, C1824-1833. https://doi.org/10.1152/ajpcell.00385.2007

He, Z.X., Huang, S.L., Zhou, Q.F., and Li, S.N. (2004). [Generation of CD34+/Sca-1+ cells from mouse embryonic stem cells with two-step differentiation in vitro]. Zhonghua Er Ke Za Zhi *42*, 830-834.

Heit, Y.I., Lancerotto, L., Mesteri, I., Ackermann, M., Navarrete, M.F., Nguyen, C.T., Mukundan, S., Konerding, M.A., Del Vecchio, D.A., and Orgill, D.P. (2012). External Volume Expansion Increases Subcutaneous Thickness, Cell Proliferation, and

Vascular Remodeling in a Murine Model. Plastic and Reconstructive Surgery *130*, 541–547. https://doi.org/10.1097/PRS.0b013e31825dc04d

Hong, L., Li, N., Gasque, V., Mehta, S., Ye, L., Wu, Y., Li, J., Gewies, A., Ruland, J., Hirschi, K.K.*, et al.* (2022). Prdm6 controls heart development by regulating neural crest cell differentiation and migration. JCI Insight *7*. https://doi.org/10.1172/jci.insight.156046

Hsiao, E.C., Yoshinaga, Y., Nguyen, T.D., Musone, S.L., Kim, J.E., Swinton, P., Espineda, I., Manalac, C., deJong, P.J., and Conklin, B.R. (2008). Marking embryonic stem cells with a 2A self-cleaving peptide: a NKX2-5 emerald GFP BAC reporter. PLoS One *3*, e2532. https://doi.org/10.1371/journal.pone.0002532

Ives, C.L., Eskin, S.G., and McIntire, L.V. (1986). Mechanical effects on endothelial cell morphology: in vitro assessment. In Vitro Cell Dev Biol *22*, 500-507. https://doi.org/10.1007/bf02621134

James, B.D., and Allen, J.B. (2018). Vascular Endothelial Cell Behavior in Complex Mechanical Microenvironments. ACS Biomater Sci Eng *4*, 3818-3842. https://doi.org/10.1021/acsbiomaterials.8b00628

Jezierski, A., Swedani, A., and Wang, L. (2007). Development of hematopoietic and endothelial cells from human embryonic stem cells: lessons from the studies using mouse as a model. ScientificWorldJournal *7*, 1950-1964. https://doi.org/10.1100/tsw.2007.310

Jing, L., Tamplin, O.J., Chen, M.J., Deng, Q., Patterson, S., Kim, P.G., Durand, E.M., McNeil, A., Green, J.M., Matsuura, S.*, et al.* (2015). Adenosine signaling promotes hematopoietic stem and progenitor cell emergence. J Exp Med *212*, 649-663. https://doi.org/10.1084/jem.20141528

Kastana, P., Zahra, F.T., Ntenekou, D., Katraki-Pavlou, S., Beis, D., Lionakis, M.S., Mikelis, C.M., and Papadimitriou, E. (2019). Matrigel Plug Assay for In Vivo Evaluation of Angiogenesis. Methods Mol Biol *1952*, 219-232. https://doi.org/10.1007/978-1-4939-9133-4_18

Kataoka, H., Hayashi, M., Nakagawa, R., Tanaka, Y., Izumi, N., Nishikawa, S., Jakt, M.L., Tarui, H., and Nishikawa, S. (2011). Etv2/ER71 induces vascular mesoderm from Flk1+PDGFRα+ primitive mesoderm. Blood *118*, 6975-6986. https://doi.org/10.1182/blood-2011-05-352658

Kattman, S.J., Adler, E.D., and Keller, G.M. (2007). Specification of multipotential cardiovascular progenitor cells during embryonic stem cell differentiation and embryonic development. Trends Cardiovasc Med *17*, 240-246. https://doi.org/10.1016/j.tcm.2007.08.004

Keller, G., Kennedy, M., Papayannopoulou, T., and Wiles, M.V. (1993). Hematopoietic commitment during embryonic stem cell differentiation in culture. Mol Cell Biol *13*, 473-486. https://doi.org/10.1128/mcb.13.1.473-486.1993

Kennedy, M., D'Souza, S.L., Lynch-Kattman, M., Schwantz, S., and Keller, G. (2007). Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. Blood *109*, 2679-2687. https://doi.org/10.1182/blood-2006-09-047704

Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N., and Keller, G. (1997). A common precursor for primitive erythropoiesis and definitive haematopoiesis. Nature *386*, 488-493. https://doi.org/10.1038/386488a0

Kilarski, W.W., Samolov, B., Petersson, L., Kvanta, A., and Gerwins, P. (2009). Biomechanical regulation of blood vessel growth during tissue vascularization. Nat Med *15*, 657-664. https://doi.org/10.1038/nm.1985

Kim, D.H., Heo, S.J., Kang, Y.G., Shin, J.W., Park, S.H., and Shin, J.W. (2016). Shear stress and circumferential stretch by pulsatile flow direct vascular endothelial lineage commitment of mesenchymal stem cells in engineered blood vessels. J Mater Sci Mater Med *27*, 60. https://doi.org/10.1007/s10856-016-5670-0

Kim, P.G., Nakano, H., Das, P.P., Chen, M.J., Rowe, R.G., Chou, S.S., Ross, S.J., Sakamoto, K.M., Zon, L.I., Schlaeger, T.M.*, et al.* (2015). Flow-induced protein kinase A-CREB pathway acts via BMP signaling to promote HSC emergence. J Exp Med *212*, 633-648. https://doi.org/10.1084/jem.20141514

Kissa, K., and Herbomel, P. (2010). Blood stem cells emerge from aortic endothelium by a novel type of cell transition. Nature *464*, 112-115. https://doi.org/10.1038/nature08761

Kleinman, H.K., Luckenbill-Edds, L., Cannon, F.W., and Sephel, G.C. (1987). Use of extracellular matrix components for cell culture. Anal Biochem *166*, 1-13. https://doi.org/10.1016/0003-2697(87)90538-0

Kobayashi, S., Ito, E., Honma, R., Nojima, Y., Shibuya, M., Watanabe, S., and Maru, Y. (2004). Dynamic regulation of gene expression by the Flt-1 kinase and Matrigel in endothelial tubulogenesis. Genomics *84*, 185-192. https://doi.org/10.1016/j.ygeno.2004.02.009

Kohn, J.C., Zhou, D.W., Bordeleau, F., Zhou, A.L., Mason, B.N., Mitchell, M.J., King, M.R., and Reinhart-King, C.A. (2015). Cooperative effects of matrix stiffness and fluid shear stress on endothelial cell behavior. Biophys J *108*, 471-478. https://doi.org/10.1016/j.bpj.2014.12.023

Kumaravelu, P., Hook, L., Morrison, A.M., Ure, J., Zhao, S., Zuyev, S., Ansell, J., and Medvinsky, A. (2002). Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. Development *129*, 4891-4899. https://doi.org/10.1242/dev.129.21.4891

Lancaster, O.M., Le Berre, M., Dimitracopoulos, A., Bonazzi, D., Zlotek-Zlotkiewicz, E., Picone, R., Duke, T., Piel, M., and Baum, B. (2013). Mitotic rounding alters cell geometry to ensure efficient bipolar spindle formation. Developmental cell *25 3*, 270-283. https://doi.org/http://dx.doi.org/10.1016/j.devcel.2013.03.014

Lee, S.H., Hong, C., and Futrell, J.W. (1989). The influence of tissue expanders on grafted vessels. Yonsei medical journal *30 4*, 327-333.

Li, Z., Chen, M.J., Stacy, T., and Speck, N.A. (2006). Runx1 function in hematopoiesis is required in cells that express Tek. Blood *107*, 106-110. https://doi.org/10.1182/blood-2005-05-1955

Liu, X.M., Ensenat, D., Wang, H., Schafer, A.I., and Durante, W. (2003). Physiologic cyclic stretch inhibits apoptosis in vascular endothelium. FEBS Lett *541*, 52-56. https://doi.org/10.1016/s0014-5793(03)00285-0

Liu, Z., Tu, H., Kang, Y., Xue, Y., Ma, D., Zhao, C., Li, H., Wang, L., and Liu, F. (2019). Primary cilia regulate hematopoietic stem and progenitor cell specification through Notch signaling in zebrafish. Nat Commun *10*, 1839. https://doi.org/10.1038/s41467-019-09403-7

Lu, H., Guo, L., Kawazoe, N., Tateishi, T., and Chen, G. (2009). Effects of poly(L-lysine), poly(acrylic acid) and poly(ethylene glycol) on the adhesion, proliferation and chondrogenic differentiation of human mesenchymal stem cells. J Biomater Sci Polym Ed *20*, 577-589. https://doi.org/10.1163/156856209x426402

Lu, S.J., Feng, Q., Caballero, S., Chen, Y., Moore, M.A., Grant, M.B., and Lanza, R. (2007). Generation of functional hemangioblasts from human embryonic stem cells. Nat Methods *4*, 501-509. https://doi.org/10.1038/nmeth1041

Lucitti, J.L., Jones, E.A., Huang, C., Chen, J., Fraser, S.E., and Dickinson, M.E. (2007). Vascular remodeling of the mouse yolk sac requires hemodynamic force. Development *134*, 3317-3326. https://doi.org/10.1242/dev.02883

Ma, H., Du, M., Hou, T., Guo, J., Liu, Y., Jia, Y., Wang, L., and An, M. (2022). HMGB1/RAGE axis accelerates the repair of HUVECs injured by pathological mechanical stretching via promoting bFGF expression. Biochem Biophys Res Commun *636*, 75-83. https://doi.org/10.1016/j.bbrc.2022.10.063

Mahdieh, Z., Cherne, M.D., Fredrikson, J.P., Sidar, B., Sanchez, H.S., Chang, C.B., Bimczok, D., and Wilking, J.N. (2022). Granular Matrigel: restructuring a trusted extracellular matrix material for improved permeability. Biomedical Materials *17*, 045020. https://doi.org/10.1088/1748-605X/ac7306

Markiewski, M.M., Daugherity, E., Reese, B., and Karbowniczek, M. (2020). The Role of Complement in Angiogenesis. Antibodies *9*, 67. https://doi.org/https://doi.org/10.3390/antib9040067

Martz, E., and Steinberg, M.S. (1972). The role of cell-cell contact in "contact" inhibition of cell division: a review and new evidence. J Cell Physiol *79*, 189-210. https://doi.org/10.1002/jcp.1040790205

Matsumoto, T., Yung, Y.C., Fischbach, C., Kong, H.J., Nakaoka, R., and Mooney, D.J. (2007). Mechanical strain regulates endothelial cell patterning in vitro. Tissue Eng *13*, 207-217. https://doi.org/10.1089/ten.2006.0058

Maul, T.M., Chew, D.W., Nieponice, A., and Vorp, D.A. (2011). Mechanical stimuli differentially control stem cell behavior: morphology, proliferation, and differentiation. Biomech Model Mechanobiol *10*, 939-953. https://doi.org/10.1007/s10237-010-0285-8

Mazia, D., Schatten, G., and Sale, W.S. (1975). Adhesion of cells to surfaces coated with polylysine. Applications to electron microscopy. The Journal of Cell Biology *66*, 198 - 200. https://doi.org/https://doi.org/10.1083/jcb.66.1.198

Mora-Roldan, G.A., Ramirez-Ramirez, D., Pelayo, R., and Gazarian, K. (2021). Assessment of the Hematopoietic Differentiation Potential of Human Pluripotent Stem Cells in 2D and 3D Culture Systems. Cells *10*. https://doi.org/10.3390/cells10112858

Müller, A.M., Medvinsky, A., Strouboulis, J., Grosveld, F., and Dzierzak, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. Immunity *1*, 291-301. https://doi.org/10.1016/1074-7613(94)90081-7

North, T., Gu, T.L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marín-Padilla, M., and Speck, N.A. (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. Development (Cambridge, England) *126*, 2563-2575. https://doi.org/10.1242/dev.126.11.2563

North, T.E., Goessling, W., Peeters, M., Li, P., Lord, A.M., Dzierzak, E., and Zon, L.I. (2008). Hematopoietic Stem Cell Development Is Dependent on Blood Flow and Nitric Oxide Signaling. Blood *112*, 728-728. https://doi.org/10.1182/blood.V112.11.728.728

O'Cearbhaill, E.D., Punchard, M.A., Murphy, M., Barry, F.P., McHugh, P.E., and Barron, V. (2008). Response of mesenchymal stem cells to the biomechanical environment of the endothelium on a flexible tubular silicone substrate. Biomaterials *29*, 1610-1619. https://doi.org/10.1016/j.biomaterials.2007.11.042

Ohta, R., Niwa, A., Taniguchi, Y., Suzuki, N.M., Toga, J., Yagi, E., Saiki, N., Nishinaka-Arai, Y., Okada, C., Watanabe, A.*, et al.* (2016). Laminin-guided highly efficient endothelial commitment from human pluripotent stem cells. Sci Rep *6*, 35680. https://doi.org/10.1038/srep35680

Pelham, R.J., Jr., and Wang, Y. (1997). Cell locomotion and focal adhesions are regulated by substrate flexibility. Proc Natl Acad Sci U S A *94*, 13661-13665. https://doi.org/10.1073/pnas.94.25.13661

Pietramaggiori, G., Liu, P.H., Scherer, S.S., Kaipainen, A., Prsa, M.J., Mayer, H.F., Newalder, J., Alperovich, M., Mentzer, S.J., Konerding, M.A.*, et al.* (2007). Tensile Forces Stimulate Vascular Remodeling and Epidermal Cell Proliferation in Living Skin. Annals of Surgery *246*, 896-902. https://doi.org/10.1097/SLA.0b013e3180caa47f

Prior, B.M., Yang, H.T., and Terjung, R.L. (2004). What makes vessels grow with exercise training? J Appl Physiol (1985) *97*, 1119-1128. https://doi.org/10.1152/japplphysiol.00035.2004

Psaltis, P.J., Harbuzariu, A., Delacroix, S., Holroyd, E.W., and Simari, R.D. (2011). Resident vascular progenitor cells--diverse origins, phenotype, and function. J Cardiovasc Transl Res *4*, 161-176. https://doi.org/10.1007/s12265-010-9248-9

Reed, J., Walczak, W.J., Petzold, O.N., and Gimzewski, J.K. (2009). In situ mechanical interferometry of matrigel films. Langmuir *25*, 36-39. https://doi.org/10.1021/la8033098

Ren, K., Crouzier, T., Roy, C., and Picart, C. (2008). Polyelectrolyte multilayer films of controlled stiffness modulate myoblast cells differentiation. Adv Funct Mater *18*, 1378-1389. https://doi.org/10.1002/adfm.200701297

Richert, L., Engler, A.J., Discher, D.E., and Picart, C. (2004). Elasticity of native and cross-linked polyelectrolyte multilayer films. Biomacromolecules *5*, 1908-1916. https://doi.org/10.1021/bm0498023

Risau, W. (1997). Mechanisms of angiogenesis. Nature *386*, 671-674. https://doi.org/10.1038/386671a0

Risau, W., and Flamme, I. (1995). Vasculogenesis. Annu Rev Cell Dev Biol *11*, 73-91. https://doi.org/10.1146/annurev.cb.11.110195.000445

Riveline, D., Zamir, E., Balaban, N.Q., Schwarz, U.S., Ishizaki, T., Narumiya, S., Kam, Z., Geiger, B., and Bershadsky, A.D. (2001). Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. J Cell Biol *153*, 1175-1186. https://doi.org/10.1083/jcb.153.6.1175

Rizzi, A., Benagiano, V., and Ribatti, D. (2017). Angiogenesis versus arteriogenesis. Rom J Morphol Embryol *58*, 15-19.

Rymo, S.F. (2011). On the stimulatory effect of microglial cells

on angiogenesis. In Medical Biochemistry and Cell Biology (Göteborg, Sweden: University of Gothenburg), pp. 12.

Sadler, T.W. (2014). Taschenlehrbuch Embryologie 12.Auflage, 12 edn (Stuttgart: Georg Thieme Verlag KG), pp. 121-123

Schnaper, H.W., Kleinman, H.K., and Grant, D.S. (1993). Role of laminin in endothelial cell recognition and differentiation. Kidney Int *43*, 20-25. https://doi.org/10.1038/ki.1993.5

Schnyder, S.K., Molina, J.J., and Yamamoto, R. (2020). Control of cell colony growth by contact inhibition. Scientific Reports *10*, 6713. https://doi.org/10.1038/s41598-020-62913-z

Schutz, L., and Mora, P.T. (1968). The need for direct cell contact in "contact" inhibition of cell division in culture. J Cell Physiol *71*, 1-6. https://doi.org/10.1002/jcp.1040710102

Schwartz, E.A., Bizios, R., Medow, M.S., and Gerritsen, M.E. (1999). Exposure of human vascular endothelial cells to sustained hydrostatic pressure stimulates proliferation. Involvement of the alphaV integrins. Circ Res *84*, 315-322. https://doi.org/10.1161/01.res.84.3.315

Semler, E.J., Ranucci, C.S., and Moghe, P.V. (2000). Mechanochemical manipulation of hepatocyte aggregation can selectively induce or repress liver-specific function. Biotechnol Bioeng *69*, 359-369. https://doi.org/10.1002/1097-0290(20000820)69:4<359::aid-bit2>3.0.co;2-q

Shojaei, S., Tafazzoli-Shahdpour, M., Shokrgozar, M.A., and Haghighipour, N. (2013). Effects of mechanical and chemical stimuli on differentiation of human adipose-derived stem cells into endothelial cells. Int J Artif Organs *36*, 663-673. https://doi.org/10.5301/ijao.5000242

Singh, M., Akkaya, S., Preuß, M., Rademacher, F., Tohidnezhad, M., Kubo, Y., Behrendt, P., Weitkamp, J.T., Wedel, T., Lucius, R.*, et al.* (2022). Platelet-Released Growth Factors Influence Wound Healing-Associated Genes in Human Keratinocytes and Ex Vivo Skin Explants. Int J Mol Sci *23*. https://doi.org/10.3390/ijms23052827

Smith, L.R., Cho, S., and Discher, D.E. (2018). Stem Cell Differentiation is Regulated by Extracellular Matrix Mechanics. Physiology (Bethesda) *33*, 16-25. https://doi.org/10.1152/physiol.00026.2017

Sokabe, M., Nunogaki, K., Naruse, K., and Soga, H. (1993). Mechanics of patch clamped and intact cell-membranes in relation to SA channel activation. Jpn J Physiol *43 Suppl 1*, S197-204.

Soofi, S.S., Last, J.A., Liliensiek, S.J., Nealey, P.F., and Murphy, C.J. (2009). The elastic modulus of Matrigel as determined by atomic force microscopy. J Struct Biol *167*, 216-219. https://doi.org/10.1016/j.jsb.2009.05.005

Sugden, W.W., and North, T.E. (2021). Making Blood from the Vessel: Extrinsic and Environmental Cues Guiding the Endothelial-to-Hematopoietic Transition. Life (Basel) *11*. https://doi.org/10.3390/life11101027

Sumagin, R., Brown, C.W., 3rd, Sarelius, I.H., and King, M.R. (2008). Microvascular endothelial cells exhibit optimal aspect ratio for minimizing flow resistance. Ann Biomed Eng *36*, 580-585. https://doi.org/10.1007/s10439-008-9467-2

Sumpio, B.E., Banes, A.J., Levin, L.G., and Johnson, G., Jr. (1987). Mechanical stress stimulates aortic endothelial cells to proliferate. J Vasc Surg *6*, 252-256. https://doi.org/https://doi.org/10.1016/0741-5214(87)90037-1

Sumpio, B.E., Widmann, M.D., Ricotta, J., Awolesi, M.A., and Watase, M. (1994). Increased ambient pressure stimulates proliferation and morphologic changes in cultured endothelial cells. J Cell Physiol *158*, 133-139. https://doi.org/10.1002/jcp.1041580117

Sun, Y., Deng, R., Ren, X., Zhang, K., and Li, J. (2019). 2D Gelatin Methacrylate Hydrogels with Tunable Stiffness for Investigating Cell Behaviors. ACS Applied Bio Materials *2*, 570-576. https://doi.org/10.1021/acsabm.8b00712

Suzuki, M., Naruse, K., Asano, Y., Okamoto, T., Nishikimi, N., Sakurai, T., Nimura, Y., and Sokabe, M. (1997). Up-regulation of integrin beta 3 expression by cyclic stretch in human umbilical endothelial cells. Biochem Biophys Res Commun *239*, 372-376. https://doi.org/10.1006/bbrc.1997.7364

Taub, M., Wang, Y., Szczesny, T.M., and Kleinman, H.K. (1990). Epidermal growth factor or transforming growth factor alpha is required for kidney tubulogenesis in matrigel cultures in serum-free medium. Proc Natl Acad Sci U S A *87*, 4002-4006. https://doi.org/10.1073/pnas.87.10.4002

Taubenberger, A.V., Baum, B., and Matthews, H.K. (2020). The Mechanics of Mitotic Cell Rounding. Frontiers in Cell and Developmental Biology *8*. https://doi.org/https://doi.org/10.3389/fcell.2020.00687

Théry, M., and Bornens, M. (2008). Get round and stiff for mitosis. HFSP Journal *2*, 65 - 71. https://doi.org/https://doi.org/10.2976/1.2895661

Tsang, K.M., Hyun, J.S., Cheng, K.T., Vargas, M., Mehta, D., Ushio-Fukai, M., Zou, L., Pajcini, K.V., Rehman, J., and Malik, A.B. (2017). Embryonic Stem Cell Differentiation to Functional Arterial Endothelial Cells through Sequential Activation of ETV2 and NOTCH1 Signaling by HIF1α. Stem Cell Reports *9*, 796-806. https://doi.org/https://doi.org/10.1016/j.stemcr.2017.07.001

Tzima, E., Irani-Tehrani, M., Kiosses, W.B., Dejana, E., Schultz, D.A., Engelhardt, B., Cao, G., DeLisser, H., and Schwartz, M.A. (2005). A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. Nature *437*, 426-431. https://doi.org/10.1038/nature03952

Upchurch, G.R., Jr., Leopold, J.A., Welch, G.N., and Loscalzo, J. (1998). Nitric Oxide Alters Human Microvascular Endothelial Cell Response to Cyclic Strain. J Cardiovasc Pharmacol Ther *3*, 135-142. https://doi.org/10.1177/107424849800300206

Vining, K.H., and Mooney, D.J. (2017). Mechanical forces direct stem cell behaviour in development and regeneration. Nat Rev Mol Cell Biol *18*, 728-742. https://doi.org/10.1038/nrm.2017.108

Vittet, D., Prandini, M.-H., Berthier, R., Schweitzer, A., Martin-Sisteron, H., Uzan, G., and Dejana, E. (1996). Embryonic Stem Cells Differentiate In Vitro to Endothelial Cells Through Successive Maturation Steps. Blood *88*, 3424-3431. https://doi.org/https://doi.org/10.1182/blood.V88.9.3424.bloodjournal8893424

Wang, H., Riha, G.M., Yan, S., Li, M., Chai, H., Yang, H., Yao, Q., and Chen, C. (2005). Shear stress induces endothelial differentiation from a murine embryonic mesenchymal progenitor cell line. Arterioscler Thromb Vasc Biol *25*, 1817-1823. https://doi.org/10.1161/01.ATV.0000175840.90510.a8

Wang, L., Zhang, P., Wei, Y., Gao, Y., Patient, R., and Liu, F. (2011). A blood flow-dependent klf2a-NO signaling cascade is required for stabilization of hematopoietic stem cell programming in zebrafish embryos. Blood *118*, 4102-4110. https://doi.org/10.1182/blood-2011-05-353235

Wiles, M.V., and Keller, G. (1991). Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development (Cambridge, England) *111*, 259-267. https://doi.org/10.1242/dev.111.2.259

Wood, H.B., May, G., Healy, L., Enver, T., and Morriss-Kay, G.M. (1997). CD34 expression patterns during early mouse development are related to modes of blood vessel formation and reveal additional sites of hematopoiesis. Blood *90*, 2300-2311. https://doi.org/https://doi.org/10.1182/blood.V90.6.2300

Yamaguchi, T.P., Dumont, D.J., Conlon, R.A., Breitman, M.L., and Rossant, J. (1993). flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. Development *118*, 489-498. https://doi.org/10.1242/dev.118.2.489

Yamamoto, K., Sokabe, T., Watabe, T., Miyazono, K., Yamashita, J.K., Obi, S., Ohura, N., Matsushita, A., Kamiya, A., and Ando, J. (2005). Fluid shear stress induces differentiation of Flk-1-positive embryonic stem cells into vascular endothelial cells in vitro. Am J Physiol Heart Circ Physiol *288*, H1915-1924. https://doi.org/10.1152/ajpheart.00956.2004

Yamashita, J.K. (2004). Differentiation and diversification of vascular cells from embryonic stem cells. Int J Hematol *80*, 1-6. https://doi.org/10.1532/ijh97.04043

Zaman, M.H., Trapani, L.M., Sieminski, A.L., Mackellar, D., Gong, H., Kamm, R.D., Wells, A., Lauffenburger, D.A., and Matsudaira, P. (2006). Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis. Proc Natl Acad Sci U S A *103*, 10889-10894. https://doi.org/10.1073/pnas.0604460103

Zambidis, E.T., Peault, B., Park, T.S., Bunz, F., and Civin, C.I. (2005). Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. Blood *106*, 860-870. https://doi.org/10.1182/blood-2004-11-4522

Zheng, W., Christensen, L.P., and Tomanek, R.J. (2008). Differential effects of cyclic and static stretch on coronary microvascular endothelial cell receptors and vasculogenic/angiogenic responses. Am J Physiol Heart Circ Physiol *295*, H794-800. https://doi.org/10.1152/ajpheart.00343.2008

Zovein, A.C., Hofmann, J.J., Lynch, M., French, W.J., Turlo, K.A., Yang, Y., Becker, M.S., Zanetta, L., Dejana, E., Gasson, J.C.*, et al.* (2008). Fate tracing reveals the endothelial origin of hematopoietic stem cells. Cell Stem Cell *3*, 625-636. https://doi.org/10.1016/j.stem.2008.09.018