Impact of the arachidonic acid/leukotriene signaling pathway for vasculogenesis and leukocyte differentiation of mouse embryonic stem cells

Inaugural dissertation submitted to the
Faculty of Medicine
in partial fulfillment of the doctoral degree in human biology
Justus-Liebig-University Giessen-Germany

Submitted by

Huang, Yu-Han
From
Tainan, Taiwan

Giessen 2015

From the Institute of Physiology

Faculty of Medicine at the Justus-Liebig-University Giessen, Germany

Director of the Institute: Prof. Dr. R. Schulz

First Supervisor and Committee Member: Prof. Dr. Sauer
Second Supervisor and Committee Member: Prof. Dr. Dr. Arnhold
Examination chair and Committee Member: Frau Prof. Dr. Roeb
Committee Member: Prof. Dr. Boening

Date of Examination: 26 October 2015

Table of Contents

Table of Contents I
1. Introduction 1
1.1 Stem cells 1
1.2 Leukotriene signaling pathway 5
1.3 Vasculogenesis and angiogenesis 11
1.4 Leukocyte differentiation 14
1.5 Calcium 16
1.6 Reactive oxygen species 17
2. Aim of the study 19
3. Materials 21
3.1 Company 21
3.2 General materials 23
3.3 Chemicals and substances 24
3.4 Cell line 26
3.5 Medium 26
3.6 Buffers 27
3.7 Antibodies 28
3.8 Inhibitors 30
3.9 Substances 32
3.9.1 H_2DCF-DA 32
3.9.2 Fluo-4, AM 32
4. Methods 33
4.1 Cell culture 33
4.1.1 Mouse embryonic stem cells 33
4.1.2 Mouse embryonic fibroblasts 33
4.1.3 Embryonic stem cell culture 33
4.1.4 Passage of confluent embryonic stem cells 34
4.1.5 Differentiation of confluent embryonic stem cells 34
4.1.6 Treatment conditions 35
4.2 Immunohistochemistry 36
4.2.1 Confocal laser scanning microscopy 37
4.2.2 PECAM-1 staining 37
4.2.3 Leukocyte marker staining 38
4.3 Western blot 39
4.3.1 Protein extraction 39
4.3.2 Protein gel electrophoresis 39
4.3.3 Antibody staining and expression 39

4.4 Intracellular calcium measurement 40

4.4.1 Cell dissociation and plating 40

4.4.2 Intracellular calcium detection.. 40

4.5 Reactive oxygen species measurement.. 41

4.6 Statistics.. 41

5. Results.. 42

5.1 Effect of AA on vasculogenesis of mouse ES cells................................. 42

5.1.1 AA regulates the expression of PECAM-1 in a dose-dependent manner 42

5.1.2 Influence of AA on the expression of FLAP and VE-cadherin........................... 44

5.1.3 Evaluation of vasculogenesis upon AA treatment 46

5.2 Influence of FLAP inhibitors on vasculogenesis of mouse ES cells....... 47

5.2.1 Evaluation of FLAP expression upon FLAP inhibitor treatment 48

5.2.2 Effect of FLAP inhibitors on the expression of PECAM-1 and VE-cadherin 49

5.2.3 Impact of FLAP inhibitors on vasculogenesis 51

5.3 AA regulated vasculogenesis of mouse ES cells is FLAP-dependent 53

5.4 Effect of exogenous LTs on vasculogenesis of mouse ES cells upon FLAP inhibition 56

5.4.1 Exogenous LTB_4 restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors.. 56

5.4.2 Exogenous 12(S)-HETE restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors.. 58

5.4.3 Exogenous LTD_4 restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors.. 60

5.4.4 Effects of LTs on vasculogenesis upon treatment with FLAP inhibitors 62

5.5 Influence of the LTB_4 signaling pathway on vasculogenesis 67

5.5.1 Impact of LTB_4 receptors for the expression of PECAM-1, VE-cadherin and Flk-1 67

5.5.2 Effect of LTB_4 receptor inhibition on vascular structures 69

5.6 Impact of the CysLT inhibitor BAYu9773 on vasculogenesis of mouse ES cells.. 71

5.6.1 Effect of BAYu9773 on the expression of PECAM-1, VE-cadherin and Flk-1 .. 72

5.6.2 Effect of the CysLT receptor inhibitor BAYu9773 on the formation of vascular structures.. 73

5.7 Influence of combined LTB_4 and CysLT receptor inhibitors on vasculogenesis of mouse ES cells.. 74

5.7.1 Effect of combined treatment of EBs with LTB_4 and CysLTs receptor inhibitors on PECAM-1, VE-cadherin and Flk-1 expression...................................... 75

5.7.2 Effect of combined treatment of EBs with LTB_4 and CysLTs receptor inhibitors on vasculogenesis... 77

5.8 Effect of AA on leukocyte differentiation of mouse ES cells 78
5.9 Impact of FLAP inhibitors on leukocyte differentiation of mouse ES cells 80
5.10 Influence of AA on intracellular calcium concentration and intracellular calcium-regulated vasculogenesis of mouse ES cells............. 82
5.10.1 Impact of AA on intracellular calcium concentration 83
5.10.2 Effect of intracellular calcium on PECAM-1 and VE-cadherin expression...... 85
5.10.3 Impact of calcium for AA-mediated vasculogenesis 87
5.11 Role of ROS in AA-induced vasculogenesis of mouse ES cells........... 89
5.11.1 AA induces ROS production in a time-dependent manner.............................. 90
5.11.2 Effect of AA-induced ROS production on vasculogenesis 91
6. Discussion 95
7. Summary 105
8. Abbreviations.. 109
9. List of figures and tables 115
10. References 117
Publications 143
Erklärung zur Dissertation.. 145
Acknowledgements 146

1. Introduction

Vasculogenesis is the vascular formation process initiated from endothelial progenitor cells, which implicates in both embryonic and postnatal development (1; 2). At the beginning of the embryonic stage, vascular formation is essential for composing a functional circulatory system to support the supply of oxygen and nutrition, and the removal of metabolic wastes (3; 4). During the postnatal stage, vascular formation can be induced through mobilizing *in situ* or transplantation of endothelial progenitor cells to provide vital novel-vascular connections after ischemic diseases or tissue injuries (5; 6). Due to the importance of vascular formation in development, dysfunction or dysregulation of vasculogenesis would lead to severe pathological effects including ischemic diseases, immune disorders, and tumorigenesis (7; 8). Therefore, in this thesis we focus on studying the signaling pathways involved in regulating vasculogenesis. One of the potential candidates is the arachidonic acid (AA)/leukotriene (LT) signaling pathway that is generated by fully-differentiated immune cells. To study vasculogenesis and leukocyte differentiation, we used embryoid bodies (EBs) differentiated from embryonic stem (ES) cells as *in vitro* model.

1.1 Stem cells

Stem cells regardless of their sources are considered as undifferentiated cells with three common properties: proliferation, self-renewal and the ability to differentiate into different specialized cell types *in vitro* and *in vivo* (9). Proliferation is a consecutive procedure resulting in the physical division of the cells (10). Self-renewal, the key process to ensure the cell to remain undifferentiated over long periods of time through keeping cell cycle progression, is the key procedure to generate more stem cells (11; 12). The characteristics of stem cells to differentiate into cells of the three germ layers

make them suitable to replace defective cells or tissues. This can be used as a therapeutic strategy to cure diseases, e.g. to enhance injured rat spinal cord recovery by stem cell transplantation (13). Depending on the differentiation potential, stem cells can be subdivided into totipotent stem cells, pluripotent stem cells and multipotent stem cells (Figure 1.1).

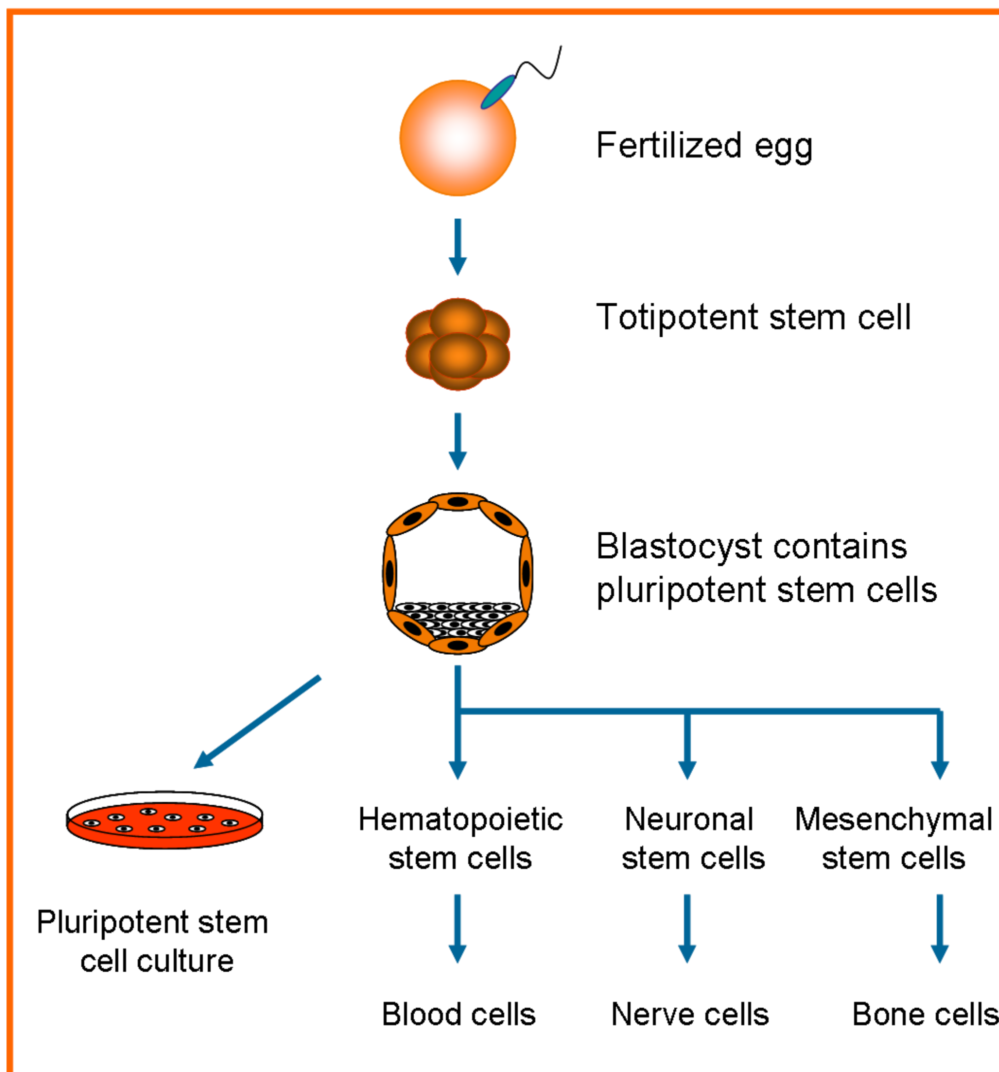


Figure 1.1 Development of ES cell. The ES cells have the potency to develop into different cell types of the body.

Totipotent stem cells are able to differentiate into any cell type that further becomes an individual. After fertilization, an egg begins to differentiate from the 2-cell

stage to the 4-cell stage and then the 8-cell stage (14). During these periods of time the fertilized eggs are totipotent cells, which then develop into blastocysts containing pluripotent stem cells (15; 16). A human fertilized egg takes typically 4 to 5 days to form a blastocyst and a mouse fertilized egg takes 3.5 days.

Pluripotent stem cells are able to differentiate into all cell types of the three germ layers, endoderm, ectoderm and mesoderm (Figure 1.2). The three germ layers continue to develop an organism. The pluripotent stem cells can further be divided into three types: ES cells, embryonic germ (EG) cells, and embryonic carcinoma (EC) stem cells (17).

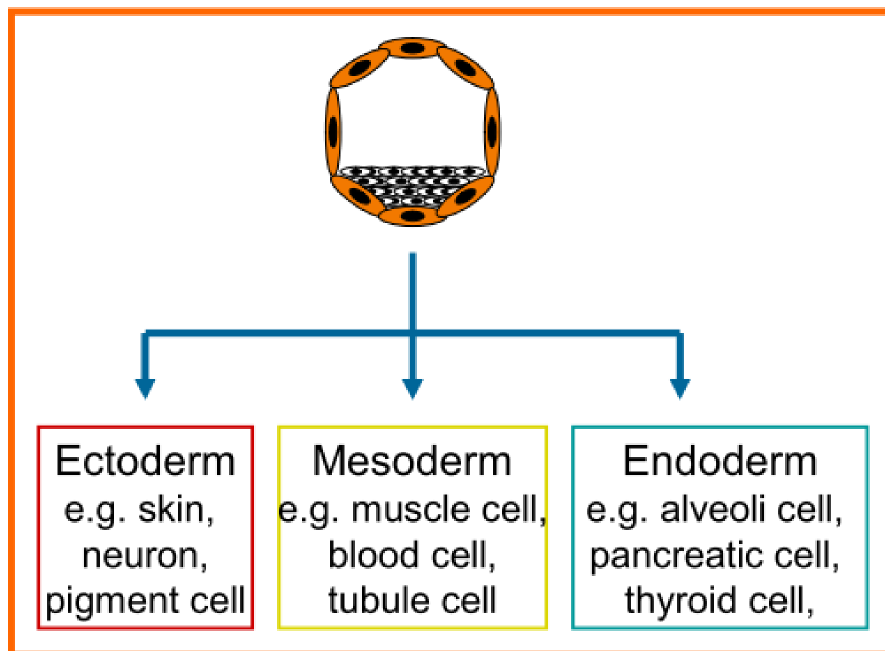


Figure 1.2 Development of pluripotent stem cells. Pluripotent stem cells can differentiate into the three germ layers.

Multipotent stem cells, also known as adult stem cells, have the same feature of self-renewal and possess the ability to differentiate into limited cell types. Adult stem cells exist in the tissues and organs inside adult individuals such as bone marrow, heart,

liver and neurons (18-20). They are capable of replacing, regenerating and repairing the injured tissues to maintain the function of the tissues and organs (21).

Embryonic stem cells

Pluripotent ES cells, which are derived from the inner cell mass of blastocysts, can be cultured for long periods of time *in vitro* (15). There are several stem cell markers expressed in ES cells such as octamer-binding transcription factor 4 (Oct 4), signal transducer and activator of transcription 3 (STAT-3), stage-specific embryonic antigen (SSEA)-1, alkaline phosphatase (AP), sex determining region Y-box 2 (Sox2), Nanog, kruppel-like factor 4 (Klf4), CD34, CD133, and ATP-binding cassette sub-family G member 2 (ABCG2) (22-27). Thus the detection of these makers in cells indicates the capabilities of self-renewal and pluripotency.

The establishment of pluripotent stem cell lines, which are obtained directly from the inner cell mass of mouse blastocysts, can be traced back to 1981 (28). In order to maintain the ES cells undifferentiated, they are either co-cultured with inactivated mouse fibroblasts or on plates coated with gelatin, and treated with medium containing the cytokine leukemia inhibitory factor (LIF) (29-31). LIF, a member of the interleukin (IL)-6 cytokine family, is considered a pivotal player for maintaining the ES cells undifferentiated (32). LIF has two isoforms, diffusible form (D-LIF) and extracellular matrix–bound form (M-LIF), with discriminative properties (33). LIF regulates the Janus kinase (JAK)/STAT-3 and the mitogen-activated protein (MAP) kinase pathway via its cell surface receptor complex composed of glycoprotein 130 (gp130) and low-affinity LIF receptor (LIFRβ). The tyrosine resident of gp130 is in charge of STAT-3 activation for maintaining ES cell self-renewal (34-36). When ES cells reach about 75% confluence, the LIF medium is removed and then the ES cells stop self-renewing and

initiate differentiation into EBs (37).

In addition to LIF other factors have been reported to maintain the pluripotency and self-renewal of ES cells, for instance, transcription factors Oct4, Sox2, and Nanog (38). Moreover, the Wnt/β-catenin signaling pathway can also determine the fate of ES cells toward either differentiation or self-renewal (39). The Wnt signaling pathway leads to opposite results through coupling of the Wnt proteins and the Frizzled family receptor (40). Some of the studies have demonstrated that the Wnt signaling pathway in ES cells promotes both mesoderm and endoderm development. On one hand, the Wnt signaling pathway induces higher expression of the mesodermal and endodermal markers, Brachyury-T, fetal liver kinase 1 (Flk-1), forkhead box protein A2 (Foxa2), LIM homeobox 1 (Lhx1), and alpha-fetoprotein (AFP) in ES cells and thus promotes differentiation (41). On the other hand, the Wnt signaling pathway participates in pluripotency maintenance by regulating the expression of Oct4, Nanog, and Sox2. Previous studies also showed that short-term treatment of Wnt or blockade of β-catenin can trigger ES cell self-renewal (38; 42).

1.2 Leukotriene signaling pathway

LTs were first discovered in 1938 in lung as "slow reacting substance" (SRS) or "slow reacting substance of anaphylaxis" (SRS-A). Later in 1979, the name of LTs was given due to their production by leukocytes (43). LTs are generated after immunological and nonimmunological stimulation from leukocytes, macrophages, and mastocytoma cells, which have effects on respiratory, immune and gastrointestinal systems (44). Moreover, LTs, which are derived from AA by 5-lipoxygenase (5-LO), are inflammatory lipid mediators. Under pathological conditions, LTs launch the proinflammatory signal

through activating cell-surface protein G-bound receptors and lead to changes of intracellular calcium, intracellular cyclic adenosine monophosphate (cAMP), or diacylglycerol levels (45). Recent studies have reported that LTs are involved in cardiovascular disease, asthma, cancer occurrence and fibrosis (46-48). For example, LTs are considered as mediators in asthma, in which the cysteinyl leukotrienes (CysLTs) regulate pathological signaling pathways of asthma through CysLT receptor 1 (CysLT1-R) (49). Therefore, LTs inhibitors are generally prescribed as medical treatment for asthma, atherosclerosis and chronic inflammatory diseases (50).

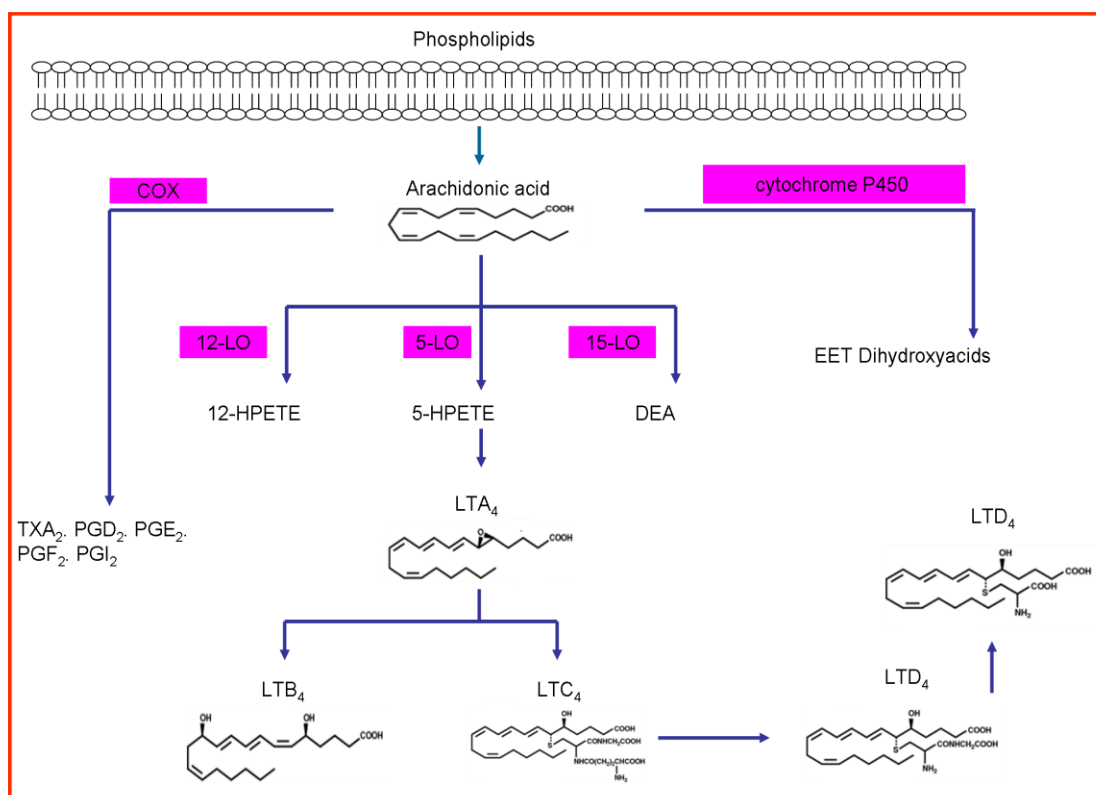


Figure 1.3 Cellular metabolism of AA. The figure is modified from https://www.caymanchem.com/app/template/landing,EIALeukotrieneResearch.vm;jsessionid=EEAEFB409423347FDE326280AABDD091

Biological effects of arachidonic acid

AA is a 20-carbon polyunsaturated omega-6 essential fatty acid, which can be

synthesized in humans from dietary linoleic acid. Inside the body, AA plays a role in cell membrane fluidity and membrane enzyme activities (51). Originally derived from membrane phospholipids through enzymatic reactions carried out by cytosolic phospholipase A_2 ($cPLA_2$), AA can be further converted to eicosanoids. The four *cis* double bonds of AA influence AA cooperation with oxygen molecules and then initiate downstream signaling pathway via the oxygenases cyclooxygenase (COX), lipoxygenase (LO), or cytochrome P450 (Figure 1.3) (52-54).

Through production of a variety of derivatives, AA involves in several inflammation-related responses. For instance, AA takes part in the innate immune system through the AA-derivatives prostaglandins, thromboxanes and LTs (48; 49). Among these AA metabolic pathways, we focused in our study on the LO signaling pathway, especially the 5-LO pathway. The 5-LO has been found to be commonly expressed in the cardiovascular system (55). In addition, products from the 5-LO signaling pathway have been reported to play a role in vasculogenesis of ES cells (56; 57).

Leukotriene synthesis

LTs can be divided into leukotriene B_4 (LTB_4) and CysLTs including leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4) and leukotriene E_4 (LTE_4) (Figure 1.3). Synthesis of LTs begins with the activation of $cPLA_2$ which hydrolyzes endogenous AA from membrane phospholipids followed by translocation of 5-LO and free AA to the nuclear membrane in response to extracellular stimuli (58). Engaged with 5-lipoxygenase activating protein (FLAP), AA can be presented to 5-LO and then be converted into 5-hydroperoxyeicosatetraenoic acid (5-HPETE), and the subsequent intermediate leukotriene A_4 (LTA_4) (59; 60). Unstable LTA_4 can further form LTB_4 by reaction catalyzed

by LTA_4 hydrolase. LTA_4 is unstable since the epoxide in C5 and C6 can be conjugated with glutathione through glutathione S-transferase -- LTC_4 synthase, to form LTC_4. LTB_4 and LTC_4 are transported to the extracellular space where LTC_4 can again rapidly be converted to LTD_4 by removing the glutamic acid through gamma-glutamyl transpeptidase: LTD_4 can be further converted to LTE_4 by glycine removal (61; 62).

5-lipoxygenase and 5-lipoxygenase-activating protein

5-LO, a key enzyme involved in the signaling pathway of LT synthesis, is one of the human lipoxygenases principally expressing in leukocytes including neutrophils, eosinophils, monocytes/macrophages, mast cells, B-lymphocytes and dendritic cells (50). 5-LO is a monomeric enzyme composed of 672 or 673 amino acids, which comprises an N-terminal β-sandwich and a C-terminal catalytic domain containing the prosthetic iron. The enzymatic activity of 5-LO is regulated through phosphorylation of serine (Ser) residues, Ser-271, Ser-633, and Ser-523, by kinases such as protein kinase A and C (63).

As an activator of 5-LO, membrane-integrated FLAP is as essential as 5-LO for LT synthesis. Although FLAP promotes LT synthesis, it itself has no enzymatic activity. Both 5-LO and FLAP are expressed in several types of cells, especially in bone-marrow-derived cells including eosinophils, neutrophils and mast cells (64). Additionally, recent studies have suggested that FLAP is more critical in CysLTs synthesis than LTB_4 in human Mono Mac 6 cells (65).

Leukotriene B_4 and receptors

LTB_4 (5 [S], 12 [R]-dihydroxy-6, 14-cis-8, 10-trans-eicosatetraenoic acid) was first recognized as a regulator of neutrophils and is synthesized under the regulation of

several inflammatory mediators such as endotoxin, tumor necrosis factor and interleukins (66). Mostly found in polymorphonuclear neutrophils (PMNs), LTA_4 is converted into LTB_4 to mediate neutrophil-dependent microvascular permeability and promotes bronchial, gastrointestinal, and vascular smooth muscle contraction *in vivo* (67; 68). Furthermore, LTB_4 can regulate the movement of leukocytes both *in vivo* and *in vitro* (62). By means of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and activator protein 1 (AP1) signaling pathway, LTB_4 induces synthesis of various proinflammatory cytokines such as IL-6, IL-8, tumor necrosis factor α (TNFα) and fibroblast growth factor (FGF) to promote inflammatory-related responses including causation of pain as well as proliferation and differentiation of stem cells (69-73). Thus, LTB_4 is considered as a chemoattractant in several inflammatory and allergic disorders such as asthma, allergic rhinitis and allergic conjunctivitis (74).

LTB_4 initiates the following signaling pathway through binding to respective receptors including peroxisome proliferator-activated receptor alpha (PPARα), LTB_4 receptor-1 (BLT1) and LTB_4 receptor-2 (BLT2). After LTB_4 binding, activated PPARα induces the signaling cascade by conjugating with PPAR responsive elements to facilitate degradation of fatty acids. Hence, activated PPARα could conversely down-regulate the lipid mediators derived from AA, i.e. LTB_4, through promoting degradation, and thus relieve inflammation (75; 76). The other two LTB_4 receptors, BLT1 and BLT2, are cell-surface G-protein coupled receptors. BLT1, which is activated by LTB_4, is predominantly expressed in leukocytes, smooth muscle cells as well as endothelial progenitor cells and has higher affinity for LTB_4. BLT2, which is activated not only by LTB_4 but also by several other hydroxyeicosotetraenoic acids (HETEs), is ubiquitously expressed and has lower affinity for LTB_4 (77; 78). Despite the different expression patterns and affinities, activation of BLT1 and BLT2 receptors by LTB_4

binding can both result in cell proliferation, differentiation and migration (79). Through binding to BLT1 or BLT2 receptors, LTB_4 initiates several intracellular signaling pathways such as intracellular calcium mobilization, extracellular signal-regulated kinase 1 and 2 (ERK1, 2) activation and inflammatory protein generation in inflammatory cells (77; 78).

CysLTs and Cys receptors

First found in leukocytes, LTA_4-derived CysLTs are converted to other downstream products including LTC_4, LTD_4 and LTE_4. The structure of LTC_4 includes cysteine and three conjugated double bonds. Within the LT synthesis pathway, LTC_4 is firstly transformed to LTD_4 and subsequently to LTE_4 the most stable CysLT that can exist for longer periods of time after synthesis (43; 80). However, LTE_4 has lower binding affinity to corresponding cell surface receptors (81). Like LTB_4, CysLTs also involve in inflammatory disorders and responses by directly or in directly inducing leukocyte movement (80).

In order to function as lipid mediators of inflammation, CysLTs bind to cell surface G-protein-coupled receptors, CysLT1-R or CysLT receptor 2 (CysLT2-R), and trigger various downstream signaling pathways depending on different cell types (82; 83). Both of the receptors are expressed on immune cells, e.g. basophils, eosinophils, mast cells, and macrophages. CysLT1-R is also commonly expressed in structural cells such as airway smooth muscle cells, epithelial, and endothelial cells. Compared to all CysLTs, LTC_4 has the higher affinity toward CysLT2-R than CysLT1-R, and LTD_4 has the highest affinity to both CysLT-Rs (49).

1.3 Vasculogenesis and angiogenesis

Blood vessel formation is an essential event during early stages of development from embryos, which establishes vessel networks to support nutrition and oxygen supply as well as waste removal. Accordingly, dysregulation of the vasculogenic process can contribute to various pathological conditions including tumorigenesis (84). There are two steps for vascular establishment: it first begins with vasculogenesis and then follows by angiogenesis, both of which require Notch signaling pathway for initiation (85-87).

Vasculogenesis

Vasculogenesis, also known as new vascular formation, refers to the process of endothelial precursor cells developing *de novo* vessels from the mesoderm (88; 89). Vasculogenesis starts with the differentiation of endothelial precursor cells specified from mesoderm, and can be regulated by FGF2 and bone morphogenetic protein (BMP) (90; 91). Depending on various genetic regulations, the heterogeneous endothelial cells can differentiate into arterial, venous, hemogenic or lymphatic vessels, respectively, during vasculogenesis. In adult, endothelial precursor cells are derived from bone marrow cells and can participate in vasculogenesis (6). To regulate vasculogenesis, several signaling pathways are involved, for instance FGF and vascular endothelial growth factor (VEGF) mediated pathways (92). To initiate vasculogenesis, FGFs first induce angioblasts from the mesoderm to differentiate into endothelial cells, and then initiate branching from the tip of the branchless endothelial cells (93). The ends of these cells continue the branching process further until forming a new vascular structure (94). On the other hand, VEGF modulates vasculogenesis through binding to one of the two high-affinity tyrosine kinase receptors, vascular endothelial growth factor receptor-1 (VEGFR1) and vascular endothelial growth factor receptor-2 (VEGFR2) (95). VEGFR2,

also named Flk-1, is one of the markers of endothelial cells, which is expressed early during differentiation and is involved in the regulation of migration, proliferation and survival of endothelial cells (87). VEGFR1 and VEGFR2 activation lead to increasing levels of Notch1 and its ligand, Delta like ligand 4 (Dll4), to establish the vessel structures (94). To further determine whether endothelial cells differentiate into arterial or venous cells, sonic hedgehog (SHH) plays an important role in mediating the expression of VEGF. While SHH-increases the expression of VEGF and prompts endothelial cells developing into the arteries, the lower expression of VEGF leads to venous development (96).

According to previous studies, vasculogenesis can occur not only intraembryonically, but also extraembryonically. These two types of vasculogenesis are independent of each other (97). Extraembryonic vasculogenesis takes place in the yolk sac blood islands while intraembryonic vasculogenesis occurs inside the developing embryo (98). The allantoic bud was discovered as a place generating vascular endothelial progenitor cells and hematopoietic progenitor cells as well as the place where vasculogenesis takes place (99). During extraembryonic vasculogenesis, endodermal cells promote mesodermal cells to initiate blood vessel formation. Hemangioblasts migrate together to form the blood islands around which the cells can further differentiate into vessels, whereas the cells inside differentiate into blood cells. The surrounding cells differentiate initially into angioblasts and then into endothelial cells (87).

Angiogenesis

Angiogenesis refers to the process that extends new vessels from existing vessels through endothelial cell sprouting triggered by shortage of nutrition and oxygen supply

(100). Higher concentrations of VEGF result from lack of nutrition and oxygen supply and promote Dll4 binding to corresponding receptors to activate Notch signaling pathway and subsequent sprouting of endothelial cells (89; 101). The sprouting process is initiated by extended filopodia of tip cells scanning the environment and then conducting the vessel formation and growth in the certain direction. The process is first initiated by migration of tip cells arising from existing blood vessels; then it is continued with the conversion of other endothelial cells directly behind the migrating tip cells into stalk cells; finally the process is completed with basement membrane establishment, during which cells behind the stalk cells differentiate into phalanx cells and form the lumen in the new vessels (89; 102; 103). As a pivotal process in new vessel formation, angiogenesis takes place in female adults during menstrual cycle or in adults during wound repair. Dysregulated angiogenesis is associated with many diseases, such as ischemia-related disorders and tumorigenesis (104).

Notch signaling pathway

Notch signaling (Figure 1.4) is a critical pathway that mediates endothelial cell differentiation to form vessel-like structures. The Notch signaling pathway consists of four receptors - Notch1, Notch2, Notch3 and Notch4 - and five ligands - Jagged1, Jagged2, Dll1, Dll3, and Dll4 (94). Among those receptors, Notch 1 and Notch 4 are specifically involved in activating the blood vessel formation process, while Dll4 is the most important Notch ligand for inducing both vasculogenesis and angiogenesis (105). Due to its significance in blood vessel formation, defects of the Notch signaling pathway would lead to insufficient vascular formation, vessel degeneration and abnormal vessel remodeling resulting in embryonic death (106; 107).

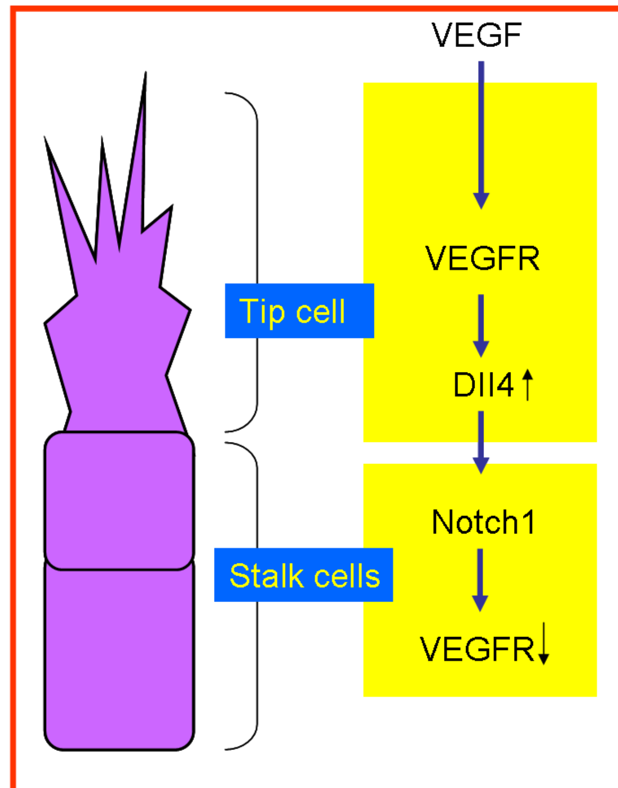


Figure 1.4 Notch signaling pathway regulates the differentiation of tip cells and stalk cells.

1.4 Leukocyte differentiation

Inflammation is a rapid pathological and physiological response to injury and pathogen infection in the cells and tissues inside the body, and induces new vascular formation (angiogenesis), immune cell proliferation as well as leukocyte migration in the influenced cells and tissues (108; 109). To accomplish the inflammatory response, proinflammatory mediators like cytokines and chemokines are generated to initiate the early response of inflammation. Moreover, cytokines and chemokines are also required for communicating immune responses to promote or reduce inflammation (110; 111). LTs are one kind of important proinflammatory mediators and are formed by leukocytes, macrophages, granulocytes and other cells at the very beginning of the inflammatory response (112; 113). Due to the high expression of 5-LO and FLAP in leukocytes, LTs

were named after the primary production origin – leukocyte – as leukotrienes (43). LT generation varies according to different types of leukocytes. For example, LTB_4 is mostly produced by neutrophils, while CysLTs are mainly produced by eosinophils, mast cells, and basophils; moreover, both of them can be produced by macrophages and dendritic cells (49).

Leukocytes participate in the anti-infectious and inflammatory responses, which can be divided into granulocytes, i.e. neutrophils, eosinophils and basophils as well as agranulocytes, i.e. lymphocytes, monocytes and macrophages (114; 115). Natural killer cells, neutrophils, eosinophils, basophils, macrophages and dendritic cells are pivotal players in the innate immune system, which acts as the first barrier and fast defensive mechanism towards foreign pathogens in an antigen-independent manner that recognizes the pathogens directly through pathogen-associated molecular patterns (116). On the other hand, B lymphocytes (B cells) and T lymphocytes (T cells) are major players in the adaptive immune system. They recognize foreign antigens through specific molecular structures such as T-cell receptors (117; 118).

The differentiation of leukocytes begins with hematopoietic stem cells derived from blastocysts, which are stored in the hematopoietic stem cell (HSC) niche within the bone marrow to maintain the undifferentiated state and self-renewing capacity (119; 120). Leukocyte differentiation can be regulated by intrinsic signals. For example, the clonal level of HSCs determines the commitment and self-renewal decisions of HSCs through intrinsic mechanisms that pre-program the behavior of HSCs, such as their size and aging (121). Additionally, leukocyte differentiation can be triggered by environmental signals. Whenever pattern-recognition receptors such as toll-like receptors recognize foreign pathogens, signals from the activated immune system can lead to differentiation

of HSCs into leukocytes, which in turn further facilitates the immune response (122).

1.5 Calcium

Calcium is a necessary mineral for human bodies, especially for maintaining the development of bones and teeth. Calcium also plays a role in blood clotting formation, muscle contraction and acts as a secondary messenger during neuronal signal transduction (123; 124). In addition, calcium intervenes in regulating proliferation, development and differentiation of neuronal cells as well as endothelial progenitor cells (125; 126). Based on the sources calcium can be divided into extracellular calcium and intracellular calcium. Extracellular calcium moderates hormone secretion through conjugating with a G protein-coupled calcium-sensing receptor, while intracellular calcium moderates hormone secretion through activating phospholipase C and PLA_2 signaling pathways (127). Recent studies have provided evidence that endothelial cell functions are closely associated with the intracellular calcium concentration. For example, intracellular calcium concentration can be induced for supporting proliferation, tubulogenesis and endothelial colony forming cells in response to VEGF (128). The two main stores of intracellular calcium are the endoplasmic reticulum and the mitochondria (129).

Previous studies have also demonstrated that calcium involves in regulating the LT synthesis pathway. High concentrations of both extracellular and intracellular calcium induce PLA_2 activation and then enhance AA production (127). Furthermore, up-regulated intracellular calcium has been reported to promote 5-LO activation and translocation from the cytosol into nuclear membranes to facilitate LT synthesis (130; 131). *In vivo* experiments performed by Alric *et al.* have demonstrated that cirrhotic rats

treated with the calcium ionophore A23187 or opsonized zymosan induced massive LT secretion from macrophages. On the contrary, shortage of calcium contributed to reduced LT production in cirrhotic rats (132). Furthermore, *in vitro* study has reported that treatment with the calcium ionophore A23187 can increase LTB_4 secretion in white blood cells, while the LTC_4 production is similar compared to the untreated white blood cells isolated from the same rats. (133).

Besides of interfering with LT synthesis, calcium levels can be reversely regulated by the LT signaling pathway. For instance, LTB_4 is able to modulate calcium levels in human polymorphonuclear leukocytes and differentiated HL-60 cells in a LTB_4-receptor-dependent manner (134). Moreover, LTC_4 secretion can enhance the intracellular calcium response in the nucleus according to LTC_4 doses (135).

1.6 Reactive oxygen species

Reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide, single oxygen and hydroxyl radicals that are commonly involved in inflammatory reactions and pathological processes (136; 137). ROS are composed of unpaired electrons which are highly instable and reactive (138). Despite of being concerned as toxic and damaging sources in human bodies, ROS are also found pivotal in mediating cell signal transduction and regulating cell apoptosis (139-141). Under physiological conditions, ROS are produced through several intracellular events such as oxidative reactions carried out by NAPDH oxidases, oxidative metabolism of AA catalyzed by lipoxygenases, or the respiratory chain reaction conducted in mitochondria (142). Once produced, ROS act as specific secondary messengers in signaling cascades modulating cell proliferation and differentiation, through which ROS up-regulate certain

groups of genes related with cell growth (143).

Oxidative metabolism of AA not only generates ROS as by-products during the oxidative reaction, but also produces unstable metabolites or intermediates that trigger even more ROS production by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (144-147). Previous studies have revealed that fibroblasts and neutrophils treated with LTB_4 showed increased ROS production as well as NADPH oxidase activation, which can mediate LTB_4-induced chemotaxis (148; 149). Moreover, activation of CysLT1-R promotes ROS generation to regulate the pathological process of asthma (150). *In vitro* study has also proposed that ROS production increased after LTC_4 treatment in cardiomyocytes (151). Conversely, Luchtefeld *et al.* found that inhibition of 5-LO not only abolished LTB_4 formation, but also attenuated ROS production (152). Similarly inhibition of CysLT1-R can lower ROS generation in vessels to reduce atherosclerotic plaque production (151; 153). Therefore, ROS should be carefully taken into account when studying AA/LT metabolic pathways and the resulting physiological events.

2. Aim of the study

Previous studies have already shown that LTs are pivotal players during the inflammatory response that contributes significantly to local vascular formation (154; 155). Moreover, the inflammatory response is also accompanied by massive leukocyte accumulation (156). However, it remains elusive whether LTs have any influence on vasculogenesis or leukocyte differentiation of ES cells in response to inflammation. Therefore we used the ES cell line CCE S103, which is a well-established stem cell model, to uncover the mechanisms of how LTs participate in vasculogenesis and leukocyte differentiation.

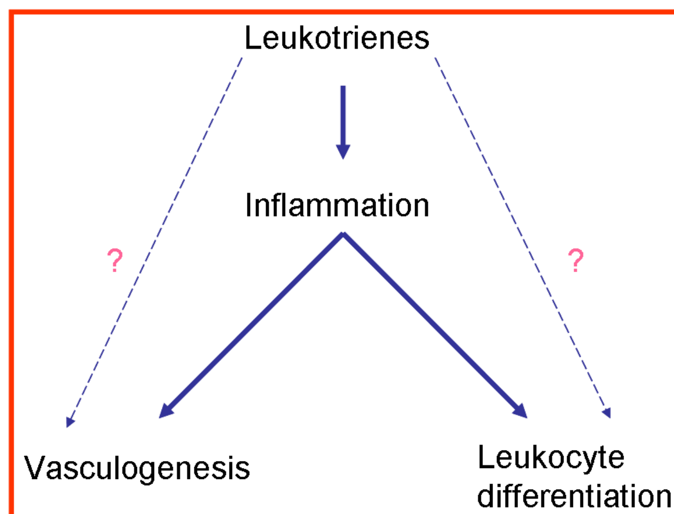


Figure 2.1 Aim of the study.

To accomplish the goal of this project, we carried out experiments regarding the following aims (Figure 2.1):

1) we investigated whether AA, the initial source of LT signaling pathway, regulates vasculogenesis and leukocyte differentiation.

2) we verified the influence of FLAP, the key enhancer of LT signaling pathway, on vasculogenesis and leukocyte differentiation.

3) to further confirm the effect of LTs on vasculogenesis, we blocked the LT signaling pathway by FLAP inhibitors and then examined whether exogenous AA or LTs would restore vasculogenesis.

4) we determined whether LT receptors play any role directly in vasculogenesis.

5) we investigate the relationship between vasculogenesis and the AA-induced calcium response and ROS generation.

3. Materials

3.1 Company

Abcam Limited	Cambridge CB OTP, Cambridgeshire, UK
Abd Serotec	Puchheim, Germany
Alexis	Grünberg, Germany
AppliChem GmbH	Darmstadt, Germany
BD Biosciences GmbH	Heidelberg, Germany
Bio-Rad Laboratories, Inc.	Hercules, California , USA
Biocat	Heidelberg, Germany
BioLegend	San Diego, California, USA
Biochrom	Berlin, Germany
Biometra	Göttingen, Germany
Biomol	Lörrach, Germany
BioVision	Milpitas, California, USA
Cell Systems	Cambridge, Massachusetts, USA
Cayman	Tallinn, Estonia
Cell Signaling	Beverly, Massachusetts, USA
Chemicon International	Hampshire, UK
Dunn Labortechnik	Asbach, Germany
Eppendorf	Hamburg, Germany
Greiner Bio-One GmbH	Eberstadt, Germany
Hanna Instruments	Kehl am Rhein, Germany
Heidolph Elektro GmbH	Kehlheim, Germany
Heraeus Instruments	Hanau, Germany
Hirschmann® Laborgeräte	Eberstadt, Germany

IKA	Staufen, Germany
Imgenex	San Diego, California, USA
Integra Biosciences	Fernwald, Germany
Invitrogen	Karlsruhe, Germany
Kendro Laboratory Products	Langenselbold, Germany
Leica	Bensheim, Germany
Merck	Darmstadt, Germany
Merck Millipore	Schwalbach/Ts., Germany
Mettler Toledo	Giessen, Germany
Molecular Devices, LLC.	Sunnyvale, California, USA
Molecular Probes	Eugene, Oregon, USA
Oelikon	Köln, Germany
PAA	Coelbe, Germany
Panmira Pharmaceuticals, LLC.	San Diego, California, USA
Roche Diagnostics GmbH	Mannheim, Germany
Roth	Karlsruhe, Germany
Santa Cruz Biotechnology Inc.	Santa Cruz, California, USA
Sartorius	Göttingen, Germany
Sigma – Aldrich	Taufkirchen, Germany
Thermo	Erembodegem, Belgium
Vasopharm	Wuerzburg, Germany
Whatman GmbH	Dassel, Germany
Carl Zeiss	Jena, Germany

3.2 General materials

5 ml polystyrene round bottom tube	BD
6 well cell culture plate	Greiner
Cell spin system	Integra
Cell strainer	BD
Confocal microscopy	Leica
Centrifuge	5417C Eppendorf
	Laborfuge 300 Heraeus
	Biofuge 15R Heraeus
Cover slip	Roth
Eppendorf tubes	Eppendorf
Glass pipettes	Roth
Gel electrophoresis chamber	Invitrogen
Gel electrophoresis power supply	Invitrogen
Homogenizer	Sigma
Incubator HERAcell® 240	Kendro
Laminar air hood	Heraeus
Magnetic stirrer	IKA
Microscope slides	Roth
Nitrocellulose membrane	Whatman
NuPAGE® 4-12% Bis-Tris Gel	Invitrogen
Optical microscope	Carl Zeiss
Petri perm plates	Greiner
pH-meter	Hanna Instruments
Pipette tips	Eppendorf

Pipettor	Hirschmann/Eppendorf
Plastic pipettes	Greiner
Reaction tubes (15 & 50 ml)	Greiner
Semi-micro balance	Mettler Toledo
Spinner flask	Integra
Tissue culture dish	BD
Vortex	Heidolph
Vacuum pump	Oelikon
Water bath	Hirschmann

3.3 Chemicals and substances

β-mercaptoethanol	Sigma
BSA	Sigma
Collagenase B	Roche
Di-sodium hydrogen phosphate dihydrate	Roche
DMEM	Sigma
DMSO	Calbiochem (Merck millipore)
Dulbecco's PBS without Ca & Mg	PAA
EDTA	Roth
ESGRO® (LIF)	Millipore
FCS	Sigma
Fluo-4, AM	Molecular Probes
Gelatine	Cell Systems
Glycerol	Sigma

H_2DCF-DA	Molecular Probes
Hydrogen peroxide	Sigma
IMDM	Biochrom
LDS sample buffer (4X)	Invitrogen
L-Glutamin 200 mM (100x)	PAA
Luminol	Sigma
Methanol	Merk
Mitomycin C	Sigma
MOPS buffer	Invitrogen
NEA	Biochrom
Nonfat dried milk power	AppliChem
Nonidet P-40	AppliChem
NuPAGE® MOPS SDS running buffer (20x)	Invitrogen
NuPAGE® sample reducing agent (10x)	Invitrogen
Novex® sharp pre-stained proteinstandard	Invitrogen
p-Coumaric acid	Sigma
Paraformaldehyde	Roth
Penicillin/streptomycin (100x)	PAA
PL pro	Invitrogen
Ponceau S	AppliChem
Potassium chloride	Roth
Potassium dihydrogen phosphate	Roth
Protease inhibitor cocktail	Biocat
SDS	Roth
Sigmacote	Sigma

Sodium chloride	Sigma
Sodium pyruvate (100 mM)	Biochrom
Triton X-100	Sigma
Tris	Roth
Tris-HCl solution	BioVision
Trypsin/EDTA	Invitrogen
Tween-20	Sigma
VAS2870	Vasopharm
Western blot stripping buffer	Thermo

3.4 Cell line

CCE S103 Mouse embryonic stem cell line isolated from embryos of mouse strain 129/sv (157).

3.5 Medium

Cultivation medium	Iscove's medium (IMDM)
(Differentiation medium)	15.6 % FCS
	0.1 mM β-mercaptoethanol
	1 mM Sodium pyruvate
	2 mM L-Glutamin
	0.1 mM non essential amino acids (NEA)
	1 % Penicillin/Streptomycin
EMFI medium	Dulbecco's medium (DMEM)
	2 mM L-Glutamin

	0.1 mM NEA
	1 % Penicillin/Streptomycin
	10 % Heat-inactivated FCS
Medium for LIF medium	Iscove's medium (IMDM)
	7.5 % FCS
	7.5 % Heat-inactivated FCS
	0.1 mM β-mercaptoethanol
	2 mM Sodium pyruvate
	2 mM L-Glutamin
	0.1 mM NEA
LIF medium	Medium for LIF medium
	1000 U/ml ESGRO LIF
	5 μg/ml PL pro

3.6 Buffers

10x PBS	1.4 M NaCl	
	64.6 mM Na_2KPO_4 x $2H_2O$	
	26.8 mM KCl	
	14.7 mM KH_2PO_4	in 1L H_2O
1x PBS	100 ml 10x PBS and 900 ml H_2O	
	Adjust pH to 7.4	
1% PBST	100 ml 1x PBS and 1 ml Triton X-100	
0.01% PBST	100 ml 1x PBS and 100 μl Triton X-100	
0.01% PBS-Tween 20	100 ml 1x PBS and 100 μl Tween 20	

10x TBS	50 mM Tris	
	150 mM NaCl	in 1 L H_2O
	Adjust pH to 7.54 (with HCl)	
1x TBS	100 ml 10x TBS and 900 ml H_2O	
0.1% TBST	100 ml 1x TBS and 1 ml Tween 20	
RIPA-Lysis buffer	150 mM NaCl	
	0.5 % Deoxycholic acid	
	1 % Nonidet P-40	
	0.5 % SDS	
	50 mM Tris	in H_2O
	Adjust pH to 7.4 (with HCl)	
Transfer buffer	1x transfer buffer	
	20% methanol	
	0.1% antioxidant	in H_2O
ECL buffer	100 mM Tris-HCL (pH 8.5)	
	0.225 mM p-Coumaric acid	
	1.25 mM Luminol	
	0.009 % (v/v) H_2O_2	

3.7 Antibodies

Blocking antibody

Purified Rat Anti-Mouse CD16/CD32 (553141)	BD Pharmingen

Primary antibody

Monoclonal rat anti-mouse CD31 (clone 390) (CBL1337)	Chemicon
Monoclonal rat anti-mouse CD144 (562243)	BD Pharmingen
Monoclonal rat anti-mouse CD18 (101409)	BioLegend
Monoclonal rabbit anti-mouse Flk-1 (#2479)	Cell Signaling
Monoclonal mouse anti-vinculin (clone hVIN-1) (V9131)	Sigma
Polyclonal goat anti-mouse PECAM-1 (sc-1506)	Santa Cruz Biotechnology, inc.
Polyclonal rabbit anti-mouse GAPDH (ab22555)	Abcam
Polyclonal goat anti-mouse FLAP/ALOX5AP (IMG-3160)	Imgenex
Polyclonal rabbit anti-mouse VE-Cadherin (ab33168)	Abcam
Rabbit anti-mouse β-actin (13E5) (4970)	Cell Signaling
Monoclonal rat anti-mouse CD45 (05-1416)	Millipore
Monoclonal rat anti-mouse CD68 (MCA1957)	Abd Serotec
DRAQ5 (4084S)	Cell Signaling

Secondary antibody

Alexa Fluor® 488 donkey anti-rat IgG (A-21208)	Invitrogen
Alexa Fluor® 488 donkey anti-rabbit IgG (A-21206)	Invitrogen
Cy5-conjugated goat anti-rat IgG (AP124S)	Chemicon
Horseradish peroxidase-conjugated horse anti-mouse IgG (7076S)	Cell Signaling
Horseradish peroxidase-conjugated goat anti-rabbit IgG (7074S)	Cell Signaling
Horseradish peroxidase-conjugated goat anti-rat IgG (7077S)	Cell Signaling

Horseradish peroxidase-conjugated rabbit anti-goat IgG (AP106P) Millipore

3.8 Inhibitors

AM643 (Panmira Pharmaceuticals, LLC. AM643 was a generous gift.)

AM643 (syn: sodium 3-{3-tert-Butylsulfanyl-1-[4-(5-methoxy-pyrimidin-2-yl)-benzyl]-5-(5-methyl-pyridin-2-ylmethoxy)-1H-indol-2-yl]-2,2-dimethyl-propionic acid) is a FLAP inhibitor that can reduce the ^{3}H-ligand binding to FLAP membranes. Based on both *in vitro* and *in vivo* studies, AM643 can significantly decrease the expression of LTB4 and CysLTs (158).

REV5901 (Biomol)

REV5901 (syn: α-Pentyl-3-(2-quinolinylmethoxy)-benzene-methanol) is a FLAP inhibitor, which also has dual roles as CysLT1 receptor inhibitor and a 5-LO inhibitor (159; 160). The compound containing a (2-quinolinylmethoxy) phenyl moiety is a highly specific peptidoleukotriene antagonist (161).

U75302 (Cayman)

U75302 (syn: 6-(6-(3R-hydroxy-1E, 5Z-undecadien-1-zl)-2-pyridinyl)-1,5S-hexanediol) is a specific antagonist for BLT1 receptor (162).

LY255283 (Cayman)

LY255283 (syn: 1-[5-ethyl-2-hydroxy-4-[[6-methyl-6-(1H-tetrazol-5-yl)heptzl]oxz] phenyl]-ethanone)) is a competitive antagonist for BLT2 receptor, which can also reduce LTB_4 production (163; 164).

BAY-u9773 (Cayman)

BAY-u9773 (syn: 4-[[(1R,2E,4E,6Z,9Z)-1-[(1S)-4-carboxy-1-hydroxybutyl]-2,4,6,9-pentadecatetraen-1-yl]thio]-Benzoic acid) is a LTE_4 analogue and a selective antagonist for CysLT receptors. It is the only antagonist for CysLT2-R (165). *In vivo*, BAY-u9773 is a powerful competitive inhibitor of LTC_4 binding to CysLT2-R (166).

BAPTA-AM (Calbiochem (Merck millipore))

BAPTA-AM (syn: 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester) is a non membrane-permeable form of BAPTA, which is a highly selective calcium chelator. Previous studies have showed that BAPTA-AM consumes vitamin D_3-induced unregulated intracellular calcium. However, BAPTA-AM only binds the intracellular calcium and has no effect on extracellular calcium (167).

VAS2870 (Vasopharm)

VAS2870 (syn: 3-benzyl-7-(benzoxazolyl)thio-1,2,3-triazolo[4,5]pyrimidine) is a cell- permeable thiotriazolopyrimidine compound and a specific NADPH oxidase inhibitor. *In vitro*, VAS2870 completely prevents production of ROS and incorporation of thymidine (168).

NMPG (Sigma)

NMPG (syn: N-(2-mercaptopropionyl)glycine), a diffusible antioxidant, is a free-radical scavenger of oxygen radicals and a reducing reagent of the oxidized form of Glutathione. Furthermore, NMPG can act as scavengers for vitamin C, vitamin E, vitamin A and coenzyme Q10 (169; 170).

Trolox (Aldrich)

Trolox (syn: 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) is an antioxidant. Trolox is a water-soluble analog of Vitamin E, which can reduce production of ROS and protect H_2O_2-induced toxicity in cells (171).

3.9 Substances

3.9.1 H_2DCF-DA

H_2DCF-DA (syn: 2',7'-dichlorodihydrofluorescein diacetate) is a cell-permeable non-polar reagent, which is used as an indicator for hydroxyl, peroxyl and other ROS activity. After H_2DCF-DA being transferred into the cells, esterase converts H_2DCF-DA into dichlorodihydrofluorescein (H_2DCF) via cutting the acetate groups. H_2DCF is non-cell-permeable, stays in the cells and then is oxidized to dichlorofluorescein (DCF) by ROS (172). Excitation wavelengths of the oxidized form are between 492-495 nm; fluorescence is detected at emission wavelengths of 517-527 nm. The fluorescence was detected by confocal laser scanning microscopy.

3.9.2 Fluo-4, AM

Fluo-4, AM (syn: 1-[2-Amino-5-(2,7-difluoro-6-hydroxy-3-oxo-9-xanthenyl) phenoxy]-2-(2-amino-5-methyl-phenoxy)ethane-N,N,N',N'-tetraacetic acid, penta-acetoxymethyl ester) is a common, bright and photostable indicator of intracellular calcium in living cells (173). Excitation is performed at 488 nm using an argon ion laser and emission is recorded at > 515 nm (174).

4. Methods

4.1 Cell culture

4.1.1 Mouse embryonic stem cells

In this project, the ES cell line, CCE S103, derived from 129/Sv mouse strain was used (37; 157). ES cells are pluripotent cells and able to differentiate into different types of cells such as endothelial cells and leukocytes. To keep the ES cells undifferentiated, ES cells were co-cultured with confluent, mitotically inactivated feeder layers of primary murine fibroblasts in medium containing the cytokine LIF (30).

4.1.2 Mouse embryonic fibroblasts

To prepare mitotically inactivated feeder layers, frozen feeder cells were thawed at 37°C in a water bath and then mixed with 20 ml warm EMFI medium. The feeder cells were then centrifuged at 200 *g* for 5 min at room temperature (RT). After centrifugation, the supernatant was removed and the pellet was resuspended in EMFI medium. Resuspended cells were plated in 5 ml EMFI medium in tissue culture plates and cultured for 24 h until 70% confluence. After 24 h, mitomycin C (10 μg/ml) was applied to inactivate the feeder cells at 37°C for 3 h. Three h later, the feeder cells were washed with 3 ml EMFI medium for 3 times, then cultured in 5 ml fresh EMFI medium. The inactivated feeder layers can be only maintained for one week. Medium change with fresh EMFI medium should be performed everyday.

4.1.3 Embryonic stem cell culture

The ES cells were thawed in the 37°C water bath with 20 ml warm LIF medium and centrifuged at 200 *g* for 5 min. After removing the medium, ES cells were resuspended with the LIF medium and cultured on mitotically inactivated feeder layers (Figure 4.1).

The ES cells were then cultivated at 37°C, with 5% CO_2 and 95% humidity.

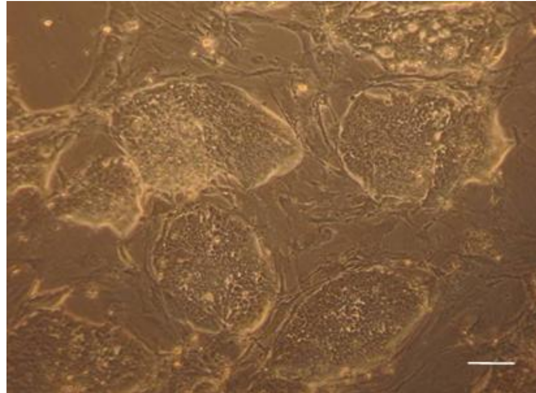


Figure 4.1 ES cells co-cultured with mitotically inactivated feeder layers. The scale bar represents 100 μm.

4.1.4 Passage of confluent embryonic stem cells

Every 2 or 3 days, the ES cells were at about 70% confluence. In order to maintain the quality and undifferentiation of the ES cells, we passaged the cells to fresh culture plates containing monolayers of mitotically inactivated feeder cells. To passage the cells, it is mandatory to wash them once with 2 ml trypsin and then incubate cells with 2 ml trypsin for 30 s at 37°C.

4.1.5 Differentiation of confluent embryonic stem cells

For differentiation, the ES cells mentioned above were trypsinized. For enzymatic dissociation, cells were washed once with 2 ml trypsin and incubated with 2 ml trypsin for 30 s at 37°C. Afterwards, the reaction of trypsin was terminated by adding 6 ml cultivation medium. The suspended ES cells were transferred into siliconized spinner flasks which contained 125 ml cultivation medium (Figure 4.2). The second day, 125 ml cultivation medium was added to feed the cells. Starting from the next day, half of the medium was renewed everyday. On day 3, various substrates as indicated were applied

on the cells (Figure 4.3).

Figure 4.2 Silicon-coated spinner flask for ES cell differentiation.

4.1.6 Treatment conditions

To investigate the impact of AA and LTs on EBs, the EBs were treated with AA, FLAP inhibitors, LTs, LT receptor inhibitors, calcium chelator or ROS antagonists from day 3 to day 10 of differentiation to monitor their effects on vasculogenesis. On the other hand, the EBs were treated with AA and FLAP inhibitors from day 3 to day 14 of differentiation to measure their effects on leukocyte differentiation (Table 4.1). On day 3, the EBs were transferred from spinner flasks to bacteriological culture plates, and the medium of the plates was changed everyday.

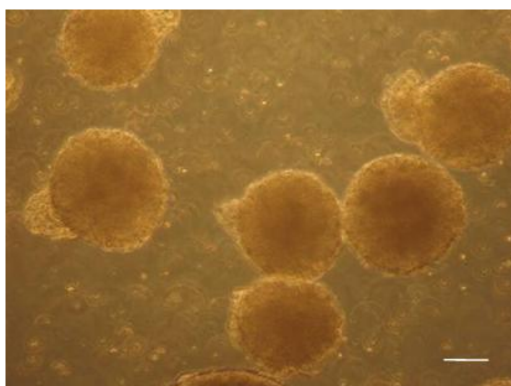


Figure 4.3 EBs in suspension at day 3 of cell culture. The scale bar represents 100 μm.

Substance	**Company**	**Concentration**
AA	Sigma	100 μM
AM643	Panmira Pharmaceuticals, LLC.	5 μM
REV5901 (L-655,238)	Biomol	2 μM
U75302	Cayman	1 μM
LY255283	Cayman	1 μM
BAYu9773	Cayman	1μM
LTB_4	Cayman	50 nM
LTD_4	Biomol	50 nM
12(S)-HETE	Cayman	50 nM
BAPTA-AM	Calbiochem (Merck millipore)	10 μM
NMPG	Sigma	100 μM
Trolox	Sigma	100 μM
VAS2870	Vasopharm	50 μM

Table 4.1 Final concentration and source of substances used in the experiments.

4.2 Immunohistochemistry

Immunohistochemistry (IHC) is a technique to directly visualize protein localization

in the tissue. The protein of the cells serves as an antigen binding to the primary antibody, which can then be recognized by a fluorophore-conjugated secondary antibody. The fluorophore is able to emit fluorescence after laser excitation which can be detected by photo-multipliers.

4.2.1 Confocal laser scanning microscopy

Confocal laser scanning microscopy was established based on a conventional light microscope, but a laser is used for the light source instead of a lamp (175). A computer is assembled to control and display the image scanning and collection. The laser line passes through a dichroic mirror to a pinhole. Fluorescence emission is recorded by photo multiplier tubes. The microscopy images in this thesis were taken using a Leica LCSM TCS-SP2 confocal laser scanning microscope equipped with the lasers: 458/488 nm Argon, 543 nm Helium/Neon (HeNe), and 633 nm HeNe. The analyses of the images were carried out with Leica confoca software LCS lite (Leica) or MetaMorph microscopy automation and image analysis software (Molecular Devices, LLC.).

4.2.2 PECAM-1 staining

On day 10, the differentiated whole-mount EBs were collected for PECAM-1 staining to evaluate vasculogenesis. The EBs were washed with phosphate-buffered saline (PBS) once and then fixed in ice-cold methanol for 20 min at -20°C. After removal from the methanol the EBs were washed in 0.01% PBST for 3 times. For permeabilization, the EBs were incubated in 1% PBST for 15 min at RT. Then EBs were washed with 0.01% PBST once to remove the remaining 1% PBST solution, and then 10% milk prepared with 0.01% PBST was used for blocking unspecific binding. Following the 1 h blocking procedure, EBs were then incubated with primary antibody, monoclonal rat anti-PECAM-1 (conc. 1:100), overnight at 4°C. The next day EBs were

first washed with 0.01% PBST for 3 times before incubation with the secondary antibody, Cy5-conjugated goat anti-rat IgG (conc. 1:100), for 1 h. 1 h later, the EBs were washed 3 times with 0.01% PBST. To quantify the vascularization, vascular structures of the EBs were recorded by the confocal laser scanning microscope. The images were composed of eight full-frame images which recorded optical slices separated by distance of 8 μm in z-direction. The images are overlay images of the EBs. From the images, branching points of vascular structures were counted. The number of branching points were scored and divided by the cross section of the EBs (μM^2) for normalization.

4.2.3 Leukocyte marker staining

For leukocyte marker staining, the EBs were plated on cover slips on day 4 and then collected on day 14. First, the cells were washed once with PBS and incubated in 4% paraformaldehyde (PFA) at 4°C. After 45 min of incubation, PFA was removed and the EBs were washed 3 times with 0.01% PBS-Tween 20, followed by blocking the EBs with 10% milk in PBS-Tween 20 for 1 h. Primary antibodies including monoclonal rat anti-mouse CD18, monoclonal rat anti-mouse CD45, and monoclonal rat anti-mouse CD68 (conc. 1:100) were incubated with EBs overnight at 4°C. On the next day, EBs were washed 3 times with 0.01% PBS-Tween 20 before application of the secondary antibody, Alexa Fluor® 488 donkey anti-rat IgG (conc. 1:100), for 1 h at RT in the dark. After 3 times washing with 0.01% PBS-Tween 20, the EBs were incubated with DRAQ5 (conc. 1:1000) for 10 min at RT in dark for nuclear staining. Finally, the EBs were washed 3 times with PBS and then fixed on the slides for further use. For analyzing the results, the MetaMorph microscopy automation and image analysis software was used. The positive cells were divided by the total number of nuclei to show the percentage of cells positive for leukocyte markers.

4.3 Western blot

4.3.1 Protein extraction

To extract proteins, EBs were harvested in Eppendorf tubes and then washed once in cold PBS. Cells were homogenized with 100 μl complete RIPA-lysis buffer and incubated on ice for 20 min. Cell lysates were then centrifuged at 12,000 *g* for 10 min to obtain the supernatant, which was stored at -80 °C until further use. Protein concentration was accessed by Lowry assay.

4.3.2 Protein gel electrophoresis

Protein samples were separated by gel electrophoresis. The extraction protein samples were mixed with proportional LDS sample buffer and reducing agent at 70° C for 10 min before loaded on the NuPAGE® SDS-PAGE gel (Invitrogen). Electrophoresis was performed with NuPAGE® MOPS SDS running buffer to separate proteins by molecular weight. The separated proteins were then transferred to a nitrocellulose transfer membrane with NuPAGE® transfer buffer (1x transfer buffer, 20% methanol, and 0.1% antioxidant) at 200 V and 60-125 mA for 1.5 h in a transfer tank. Ponceau prestaining (0.5 g Ponceau S, 1 % (v/v) acetic acid and H_2O) was performed to confirm the successful transfer.

4.3.3 Antibody staining and expression

In succession, the membranes were blocked in 5% non-fat milk in 0.1% TBST (100 ml 1x TBS and 1 ml Tween 20) for 1 h at RT after transfer. Then, the membranes were probed with indicated primary antibody diluted in 5% bovine serum albumin (BSA) (in 0.1% TBST) over night at 4°C. On the next day, the membranes were washed 3 times with 0.1% TBST and then incubated with corresponding secondary peroxidase-coupled anti-IgG antibodies diluted in 5% BSA (in 0.1% TBST) 1 h at RT. The membranes were

then washed 3 times with 0.1% TBST to remove the unbound secondary antibody. ECL buffer was used to visualize the signals from bound secondary antibodies. For analysis of the protein expression levels, the software ImageJ (National Institutes of Health (USA)) was used.

4.4 Intracellular calcium measurement

4.4.1 Cell dissociation and plating

For detecting intracellular calcium by Leica LCSM TCS-SP2 confocal laser scanning microscopy, EBs were enzymatically dissociated into single cells. On one day prior to the experiment, EBs were collected and then washed once before incubating with warm collagenase in PBS at 37° C for 5-10 min until EBs were dissociated into single cells. The isolated cells were then plated on cover slips for analysis. The intracellular calcium concentration was detected on day 2, day 4, day 6 and day 8 of differentiation.

4.4.2 Intracellular calcium detection

On the day of intracellular calcium concentration detection, plated single cells from EBs were incubated for 30 min with fluo-4, AM with or without BAPTA-AM in serum-free medium, and then changed back to normal fresh medium. As a control, intracellular calcium concentration of cells was first detected with confocal laser scanning microscopy for the initial 100-120 s without any treatment. Subsequently EBs were treated with AA for detecting AA-induced changes of intracellular calcium concentration. The whole reaction was monitored for 1000 s.

$$[Ca^{2+}]=K_d[F-F_{min}]/[F_{max}-F]$$ (176)

Concentration of intracellular calcium was defined according to the equation above. F is fluorescence measured from the experiment. K_d (the dissociation constant for indicator) of Fluo-4, AM is 345 nM. F_{min} is 2 and F_{max} is 220.

4.5 Reactive oxygen species measurement

ROS measurement is performed by detecting the strength of fluorescence after staining the EBs with the ROS fluorescence indicator H_2DCF-DA. On day 4 of differentiation, EBs were treated with AA for 1, 2, 4, 6, and 20 h. Afterwards, the EBs were treated with H_2DCF-DA for 30 min and then transferred into fresh medium. The strength of fluorescence from tested samples was detected by confocal laser scanning microscopy (Leica LCSM TCS-SP2) using the 488 nm line of the argon ion laser. Fluorescence emission was recorded at > 515 nm.

4.6 Statistics

All the experiments were repeated for least 3 times. The results are shown as mean value ± standard deviation (SD). Statistical significance was determined either by Student's t-test or ANOVA as appropriate. The *p* value of less than 0.05 was considered to be significant (*.#).

5. Results

5.1 Effect of AA on vasculogenesis of mouse ES cells

Previous studies have shown that vascular formation and inflammation are codependent events in several inflammation-related diseases (177). LTs, converted from AA, are inflammatory factors that can trigger the production of acute phase proteins to regulate the inflammatory responses (110). Additionally, previous studies demonstrated that the 5-LO signaling pathway can regulate vasculogenesis in ES cells (56). Based on these studies, we undertook to investigate whether the direct provision of AA for the LT signaling pathway could influence ES cell differentiation.

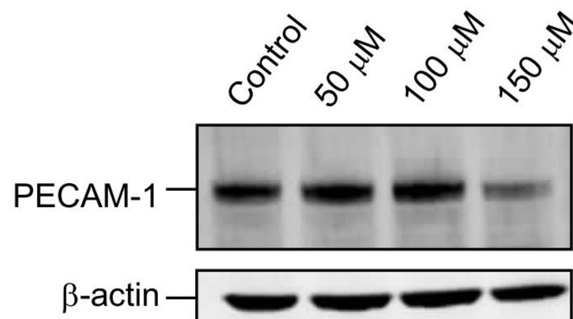
After stimuli, the membrane-released AA is initially converted to LTA_4 by 5-LO and the enhancer FLAP; LTA_4 can either form LTB_4 by the reaction catalyzed by LTA_4 hydrolase, or from CysLTs by the reaction of LTC_4 synthase. Thus, we firstly investigated the endothelial cell markers, platelet endothelial cell adhesion molecule (PECAM-1) and vascular endothelial cadherin (VE-cadherin) after AA treatment to verify whether the ES cells differentiate to vascular cells upon AA treatment. To further confirm ES cell vascular differentiation, the vascular branching structures of EBs were examined to assess new vessels are formed.

5.1.1 AA regulates the expression of PECAM-1 in a dose-dependent manner

To obtain the best working conditions, the first step is to optimize the working concentration of AA. After three days of cell culture, EBs were transferred to culture plates and treated from day 3 to day 10 with different concentrations of AA (50 μM, 100 μM, and 150 μM). On day 10, EBs were collected for detecting protein expression of PECAM-1. PECAM-1 a common endothelial cell marker is expressed in endothelial

intercellular junctions both *in vivo* and *in vitro* (178-181). The data showed that protein expression of PECAM-1 in EBs was dose-dependent increased upon treatment with 50 μM and 100 μM AA (Figure 5.1). However, upon treatment of EBs with 150 μM AA a decrease of PECAM-1 expression was observed. Based on these experiments the concentration of 100 μM AA was selected as a suitable working concentration for further experiments.

A)



B)

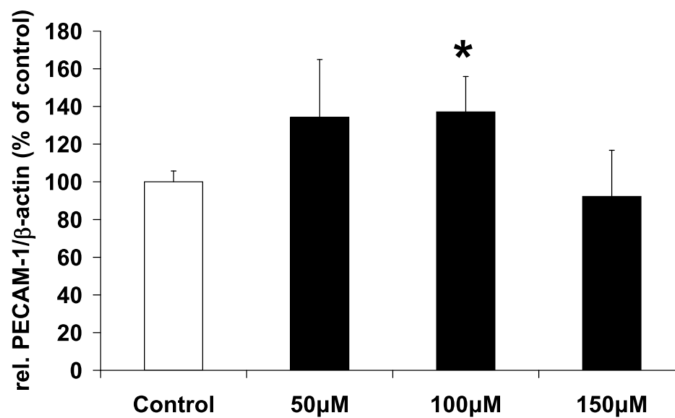


Figure 5.1 AA regulates the expression of PECAM-1 in a dose-dependent manner. Three days after differentiation, EBs were transferred to culture plates and then treated with AA (50 μM, 100 μM and 150 μM) from day 3 to day 10 of differentiation. Then, the EBs were collected for western blot. A) The expression of PECAM-1 after AA treatment increased at 50 μM and 100 μM, but decreased at 150 μM compared with the untreated control. B) The bar chart showed the means ± SD of 4 independent experiments. (*p <

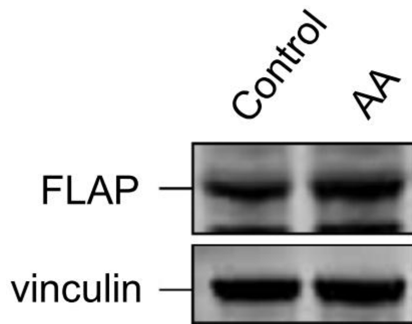
0.05, significantly different from the untreated control).

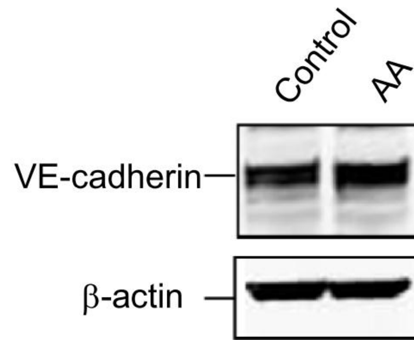
5.1.2 Influence of AA on the expression of FLAP and VE-cadherin

According to previous studies, FLAP plays a key role in the LT signaling pathway not only for transferring AA but also enhancing AA binding to 5-LO (64). Furthermore, in the results presented in chapter 5.1.1, we demonstrated that 50-100 μM AA was able to increase PECAM-1 expression. However, it is still unknown whether AA can regulate the expression of its own metabolic pathway enhancer FLAP. To investigate the regulation of FLAP by AA, FLAP expression was analyzed by western blot. After 3-days of cell culture, EBs were transferred to culture plates and treated with 100 μM AA from day 3 to day 10 of differentiation. On day 10, EBs were harvested for protein extraction and the expression of FLAP was examined. In comparison to the untreated control EBs, FLAP expression was significantly up-regulated after AA treatment (Figure 5.2A).

To further verify whether vasculogenesis can be enhanced by AA, we also determined VE-cadherin expression by western blot. Comparably to PECAM-1, VE-cadherin is a marker for endothelial cells and is expressed in all vascular structures (182). As one isoform of cadherin, VE-cadherin is an endothelial-specific transmembrane protein functioning in cell-to-cell adherens junctions, and transfers intracellular signals for vascular stabilization (183). For VE-cadherin detection, ES cells were induced to differentiate and then transferred to culture plates and treated with 100 μM AA from day 3 to day 10. On day 10, EBs were harvested for protein extraction and western blot was performed for VE-cadherin detection. Compared with the untreated control cells, the expression of VE-cadherin was up-regulated after AA treatment (Figure 5.2B).

A) B)





C)

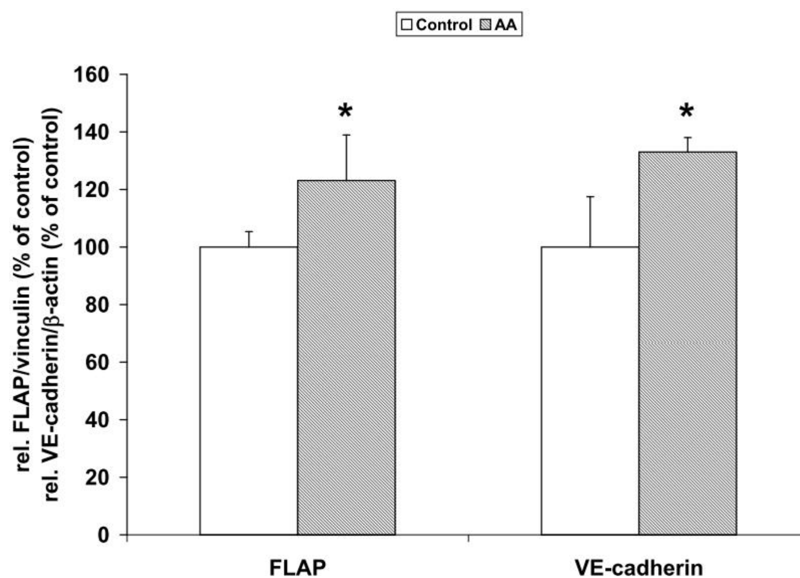
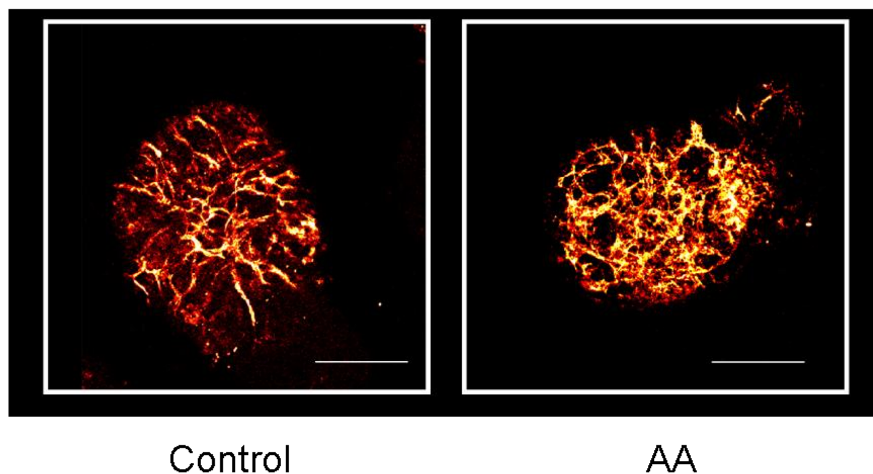


Figure 5.2 Influence of AA on the expression of FLAP and VE-cadherin. EBs were treated with AA (100 μM) after differentiation induction from day 3 to day 10 of differentiation. Protein was collected to analyze endothelial marker and FLAP expression. A) The representative blot showed that the expression of FLAP was increased in AA-treated cells compared with the untreated control. B) The representative blot showed that VE-cadherin protein level was elevated in AA-treated cells compared with the untreated control cells. C) The bar chart showed the quantitative analysis of FLAP and VE-cadherin expression in relation to the house-keeping proteins vinculin for FLAP and β-actin for VE-cadherin (n=4 in the case of FLAP; n=3 in the case of VE-cadherin, *$p < 0.05$, significantly different from the untreated control).

5.1.3 Evaluation of vasculogenesis upon AA treatment

During ES cell differentiation, new vessel-like structures are formed. In order to further confirm whether vascular structure formation can be regulated by AA, EBs were examined after immunohistochemical staining of endothelial cells using an antibody against PECAM-1. Three days after induction of cell differentiation, EBs were treated with AA (100 μM) until day 10 of differentiation and vessel-like structures were analyzed by confocal microscopy. The branching points in AA-treated EBs were significantly increased as compared with the untreated control (Figure 5.3).

Taken together, these data demonstrated that AA can improve vasculogenesis of EBs possibly by up-regulation of the endothelial markers, PECAM-1 and VE-cadherin. Moreover, AA increased the level of FLAP expression thereby promoting the LT signaling pathway.



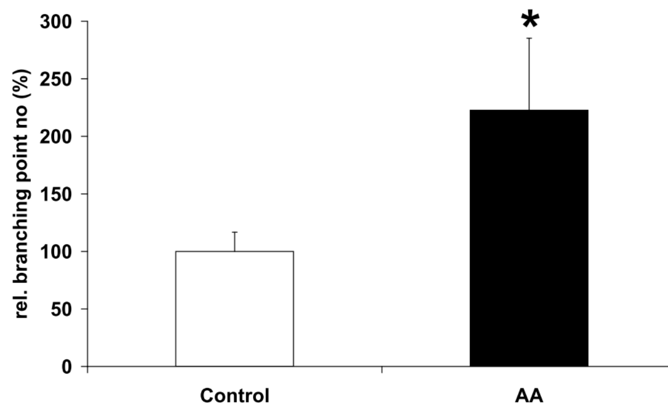


Figure 5.3 Evaluation of vasculogenesis upon AA treatment. After transfer to culture plates, 3-day-old EBs were treated with AA (100 μM) until day 10. Whole EBs were collected to measure branching points. The representative images showed that the branching points of AA-treated EBs were increased in comparison to the untreated EBs. The bar chart showed that the branching points of AA-treated EBs were significantly increased compared to the untreated control. The upper panel shows representative images of EBs labeled with anti PECAM-1 antibody. The scale bar represents 300 μm. The bar chart represents mean ± SD of 3 separate experiments in which at least 30 individual EBs were analyzed. (*$p < 0.05$, significantly different from the untreated control).

5.2 Influence of FLAP inhibitors on vasculogenesis of mouse ES cells

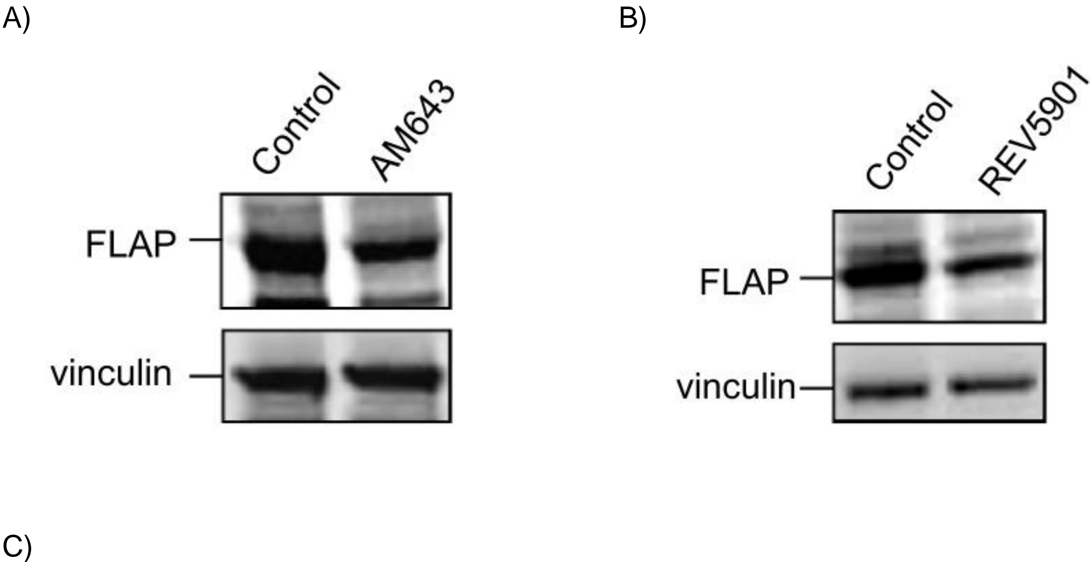
To initiate AA conversion, FLAP is required for enhancing AA binding to 5-LO (59; 64). Furthermore, it has been recently demonstrated that PECAM-1 expression is down-regulated in FLAP knockout cells (56). Therefore we assumed that FLAP might directly regulate ES cell differentiation due to its influences on AA conversion and PECAM-1 expression. Hence we evaluated the effect of FLAP on ES cell differentiation by blocking FLAP activity with two different FLAP inhibitors, AM643 and REV5901.

The endothelial cell markers PECAM-1 and VE-cadherin were examined after treatment with the FLAP inhibitors AM643 and REV5901 to verify whether vascular differentiation of ES cells was affected. Furthermore, branching points of vascular

structures in EBs were measured as indicators of vasculogenesis. For this experiment, ES cells were induced for differentiation and then treated with either AM643 (5 μM) or REV5901 (2 μM), respectively, from day 3 to day 10 of differentiation. Whole EBs were analyzed for vasculogenesis by IHC while the extracted proteins of EBs were subjected to western blot for analysis of PECAM-1 and VE-cadherin expression.

5.2.1 Evaluation of FLAP expression upon FLAP inhibitor treatment

To confirm the function of the FLAP inhibitors AM643 and REV5901, we examined FLAP expression by western blot after AM643 and REV5901 treatment. The western blot results revealed that FLAP expression was down-regulated by both inhibitors, AM643 and REV5901, as compared with the untreated control cells (Figure 5.4).



C)

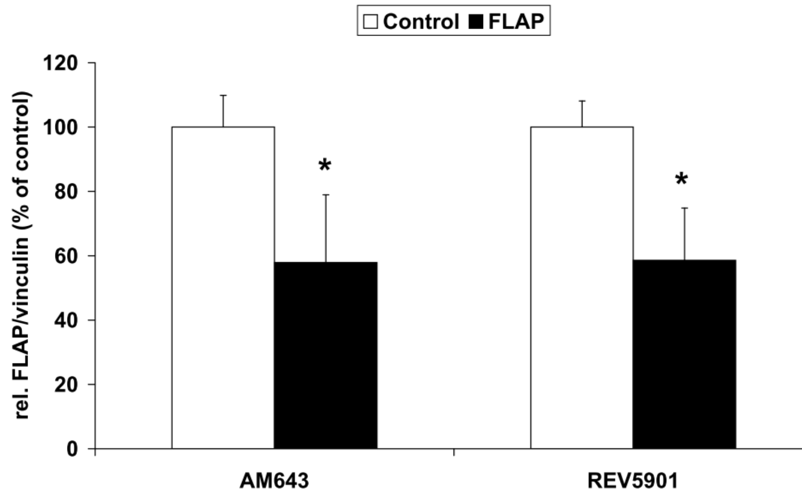
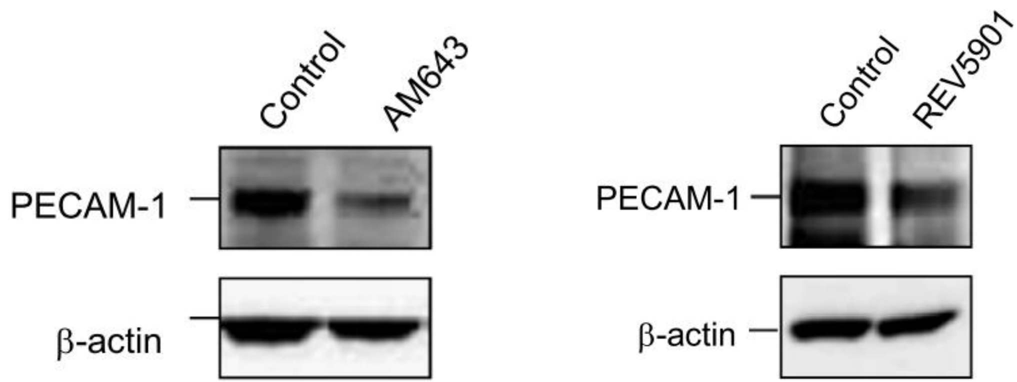


Figure 5.4 Evaluation of FLAP expression upon FLAP inhibitor treatment. EBs were treated form day 3 until day 10 with the FLAP inhibitors, AM643 (5 μM) and REV5901 (2 μM), respectively. Proteins were collected and detected on day 10. The representative pictures of western blots showed that expression of FLAP was decreased after A) AM643 and B) REV5901 treatment compared with the untreated control cells. C) The quantification results of the western blots showed the statistically significant decrease of FLAP expression in the cells treated with AM643 or REV5901 compared to the untreated control. Each value represents the mean ± SD of 3 separate experiments. *$p < 0.05$, significantly different from the untreated control.

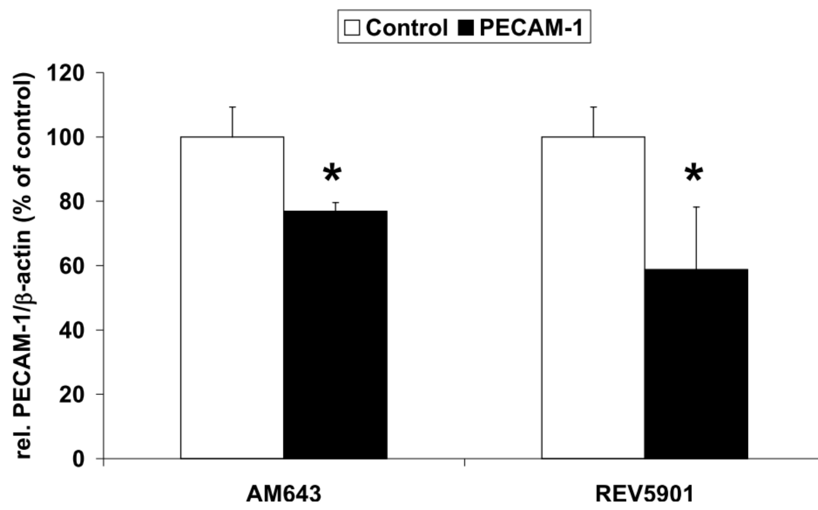
5.2.2 Effect of FLAP inhibitors on the expression of PECAM-1 and VE-cadherin

After confirming the efficacy of FLAP inhibitors on FLAP expression, the level of vasculogenesis in response to treatment with AM643 and REV5901 was investigated. After induction for differentiation for 3 days, EBs were transferred into culture plates and treated with either AM643 (5 μM) or REV5901 (2 μM) until day 10. On day 10, proteins were collected and the levels of the vascular markers PECAM-1 and VE-cadherin were examined by western blot. The results showed that PECAM-1 and VE-cadherin were both down-regulated following treatment with AM643 and REV5901, respectively (Figure 5.5).

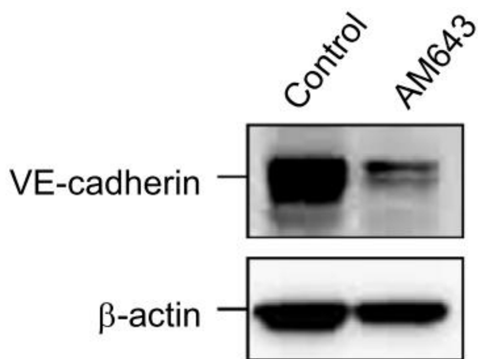
A) B)



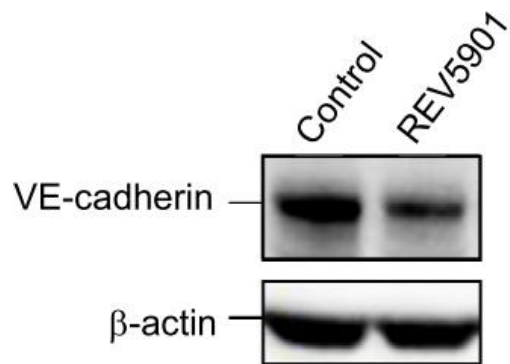
C)



D)



E)



F)

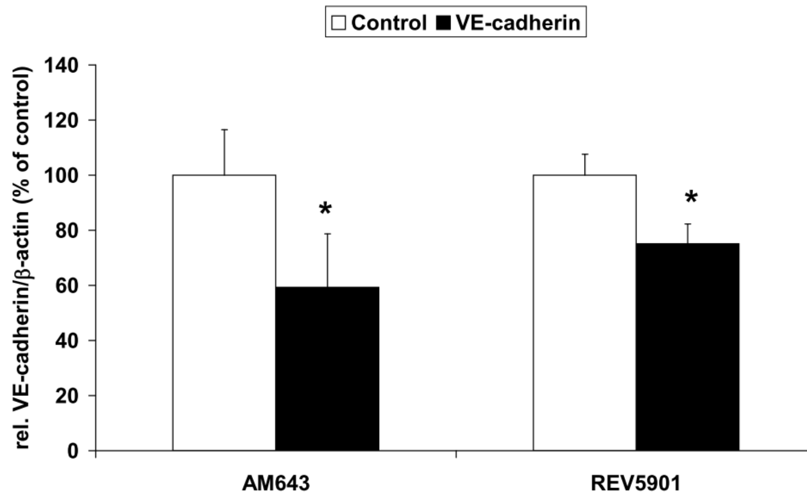


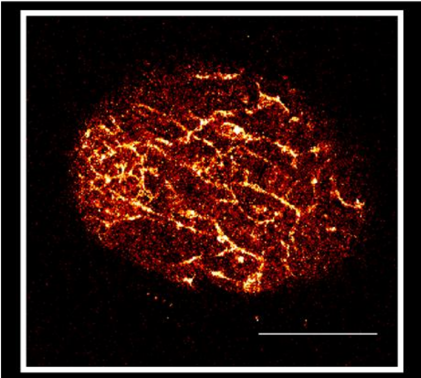
Figure 5.5 Effect of FLAP inhibitors on the expression of PECAM-1 and VE-cadherin. After differentiation for 3 days, EBs were treated with either AM643 (5 μM) or REV5901 (2 μM) until day 10. A) The representative blot showed that PECAM-1 expression of EBs treated with AM643 (5 μM) decreased compared with the untreated control cells. B) The representative blot showed similar reduction of PECAM-1 expression in EBs treated with REV5901 (2 μM) in comparison to the untreated control cells. C) The bar charts corresponding to western blots presented the down-regulation of PECAM-1 expression in the cells treated with either AM643 or REV5901 compared to the untreated control. (n=3 in the case of AM643; n=4 in the case of REV5901, *$p < 0.05$, significantly different to the untreated control) D) The representative blot revealed that VE-cadherin expression of EBs treated with AM643 (5 μM) was reduced compared to untreated control EBs. E) The representative blot showed similar reduction of VE-cadherin expression in EBs that were treated with REV5901 (2 μM) compared to the untreated control cells. F) The bar charts corresponding to western blots demonstrated significant reduction of VE-cadherin expression in the cells treated with either AM643 or REV5901 compared to the untreated control (n=4 in the case of AM643; n=3 in the case of REV5901, *$p < 0.05$, significantly different to the untreated control).

5.2.3 Impact of FLAP inhibitors on vasculogenesis

To further confirm the impact of FLAP inhibitors AM643 and REV5901 on vasculogenesis, we examined branching points of PECAM-1 positive vascular structures in whole EBs using IHC. From day 3 of differentiation, EBs were treated with either AM643 (5 μM) or REV5901 (2 μM) until day 10 and subjected to IHC for PECAM-1. The results indicated that branching points of vascular structures in EBs

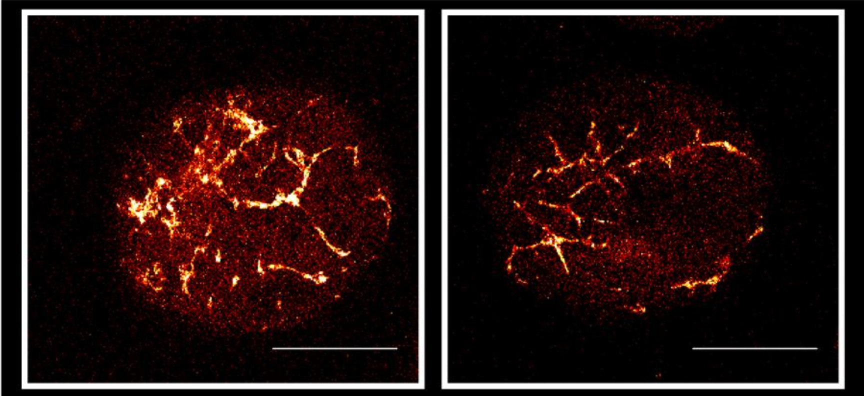
were decreased following FLAP inhibition compared to the untreated control cells (Figure 5.6). Taken together, the results in 5.2 suggest that inhibition of FLAP expression attenuates vasculogenesis by down-regulating the protein expression of PECAM-1 and VE-cadherin and decreasing vascular structure formation.

Aa)



Control

Ab)



AM643 REV5901

B)

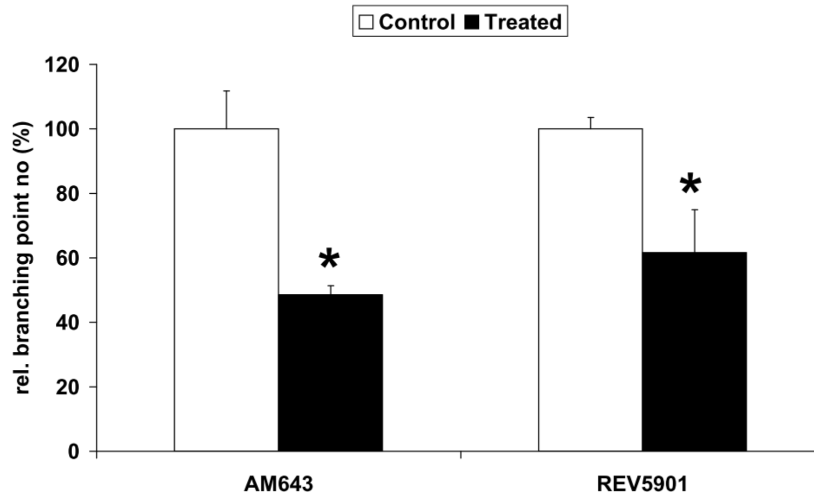


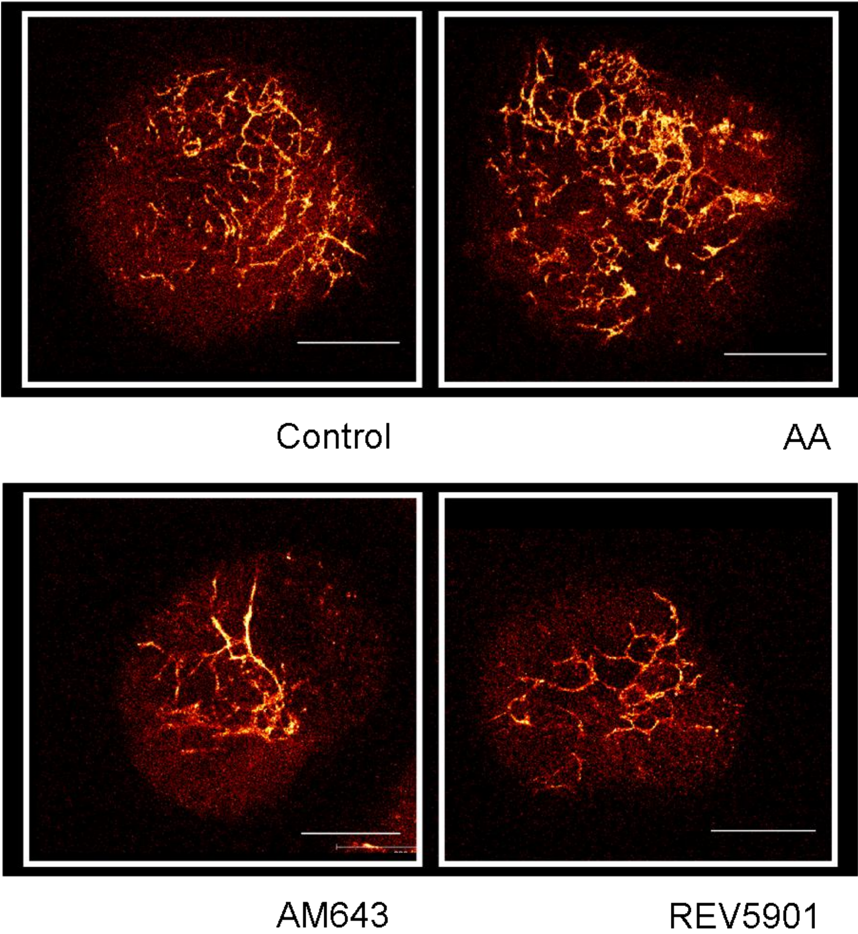
Figure 5.6 Impact of FLAP inhibitors on vasculogenesis. For detecting vasculogenesis of the EBs, they were treated with either AM643 (5 μM) or REV5901 (2 μM) from day 3 to day 10 of differentiation. Branching points were examined in whole EBs on day 10. A) Treatment of EBs with either AM643 or REV5901 (Ab) reduced vascular differentiation compared to the untreated control (Aa). B) The bar charts showed that the branching points of EBs treated with FLAP inhibitors were significantly decreased compared with the untreated control EBs. The upper panel shows representative images of EBs labeled with anti-PECAM-1 antibody. The scale bar represents 300 μm. The bar chart represents means ± SD of 3 separate experiments in which at least 30 individual EBs were analyzed. *$p < 0.05$, significantly different from the untreated control.

5.3 AA regulated vasculogenesis of mouse ES cells is FLAP-dependent

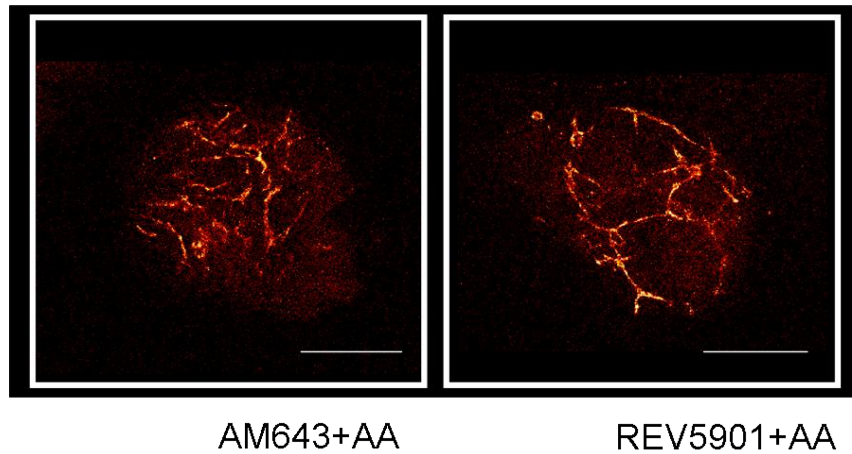
The results 5.1 and 5.2 showed that AA (result 5.1) and FLAP (result 5.2) play roles in regulating vasculogenesis. However, AA transforming enhancer FLAP only involves in one of the possible AA metabolic pathways (184; 185). Therefore, we investigated whether FLAP is essential for AA-induced vasculogenesis. To achieve this aim 3-day-old EBs were treated with the FLAP inhibitors AM643 (5 μM) or REV5901 (2 μM) and AA (100 μM) individually, or first pre-treated with FLAP inhibitors AM643 (5 μM) or REV5901 (2 μM) for 1 h and then followed by treatment with AA (100 μM) until day 10. On day 10, whole EBs were harvested to examine vasculogenesis by IHC. The results

showed that branching points of FLAP inhibitor-treated cells were decreased while vasculogenensis of AA-treated cells was increased compared to the untreated control EBs (Figure 5.7A). As expected, pre-treatment with FLAP inhibitors AM643 or REV5901 before AA application significantly reduced vasculogenesis of EBs in comparison with AA-treated EBs (Figure 5.7A and 5.7B). These results indicate that AA requires FLAP-dependent metabolic pathways for mediating vasculogenesis.

Aa)



Ab)



B)

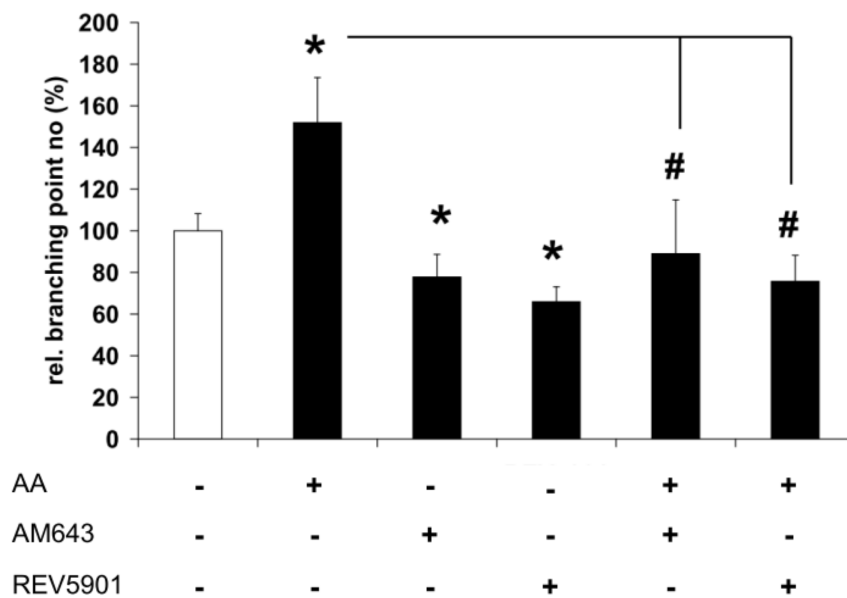


Figure 5.7 AA regulated vasculogenesis of mouse ES cells is FLAP-dependent. 3-day-old EBs were treated either with FLAP inhibitors AM643 (5 μM) or REV5901 (2 μM) or AA (100 μM) individually, or were pre-treated first with FLAP inhibitors before AA application from day 3 until day 10 of differentiation. EBs were collected on day 10 for analysis of branching points by IHC. The IHC shown in A images showed that both FLAP inhibitors reduced vasculogenesis while AA up-regulated vasculogenesis compared to the untreated control. EBs pre-treated with both FLAP inhibitors, AM643 and REV5901, before AA treatment showed significantly decreased branching points as compared to EBs treated with AA alone. B) The bar chart showed the effects of AA, AM643 and REV5901 on vasculogenesis of ES cells. The upper panel shows representative images of EBs labeled with anti PECAM-1 antibody. The scale bar

represents 300 μm. The bar chart represents mean ± SD of 3 separate experiments in which at least 30 individual EBs were analyzed. *$p < 0.05$, significantly different from the untreated control group. #$p < 0.05$, significantly different from the AA-treated group.

5.4 Effect of exogenous LTs on vasculogenesis of mouse ES cells upon FLAP inhibition

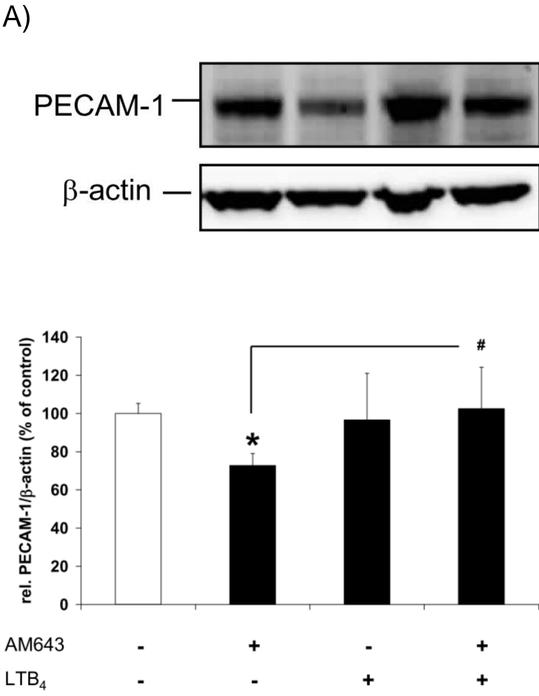
Our data showed that AA-regulated vasculogenesis is dependent on FLAP-dependent metabolic signaling (see 5.3). Additionally, BLT1 and BLT2 have been found to regulate endothelial cell proliferation, differentiation and migration (57). Hence, we speculated that LTs, the AA derivatives from FLAP, may involve in vasculogenesis. To verify our hypothesis, EBs were co-treated with FLAP inhibitors i.e. AM643 and REV5901, and LTs including LTB_4, LTD_4 and a ligand of BLT1 and BLT2, 12S-hydroxy-5Z, 8Z, 10E, 14Z- eicosatetraenoic acid (12(S)-HETE), from day 3 to day 10 of differentiation (57). On each day, EBs were pre-treated with AM643 (5 μM) or REV5901 (2 μM) for 1 h before treatment with LTB_4 (50 nM), LTD_4 (50 nM), and 12(S)-HETE (50 nM), respectively. The effect of LTs on vasculogenesis was determined by protein expression of the endothelial markers PECAM-1 and VE-cadherin expression as well as branching points of vascular structures.

5.4.1 Exogenous LTB_4 restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors

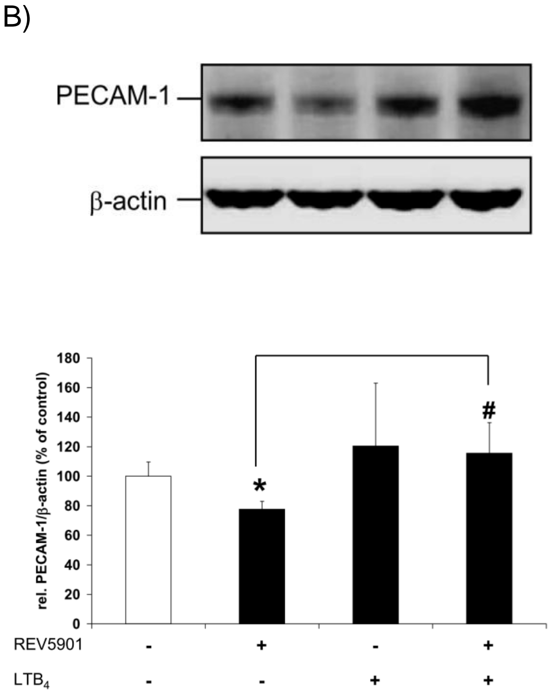
In order to investigate whether LTB_4 regulates vasculogenesis, we inhibited FLAP using AM643 and REV5901 either in the absence or presence of exogenous LTB_4 and assessed protein expression of PECAM-1 and VE-cadherin. Western blot experiments on day 10 of differentiation demonstrated that PECAM-1 expression of AM643- (5 μM) -and REV5901-(2 μM) treated EBs was down-regulated compared to the untreated control. PECAM-1 expression of EBs treated with LTB_4 (50 nM) was slightly increased in

comparison to the untreated control cells. Remarkably exogenous addition of LTB_4 (50 nM) significantly reversed the down-regulation of PECAM-1 expression observed upon pretreatment with AM643 or REV5901 (Figure 5.8A and 5.8B). Likewise the down-regulation of VE-cadherin expression of EBs upon pre-treatment with FLAP inhibitors was restored compared to EBs that were treated with inhibitors alone (Figure 5.8C and 5.8D).

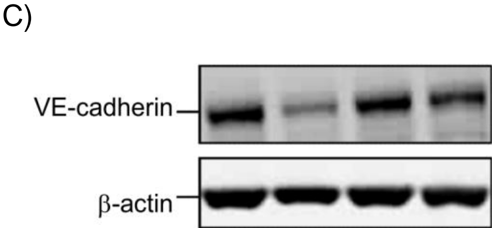
A)



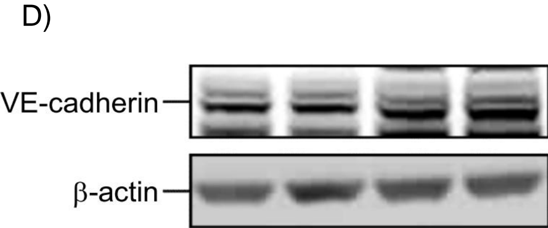
B)



C)



D)



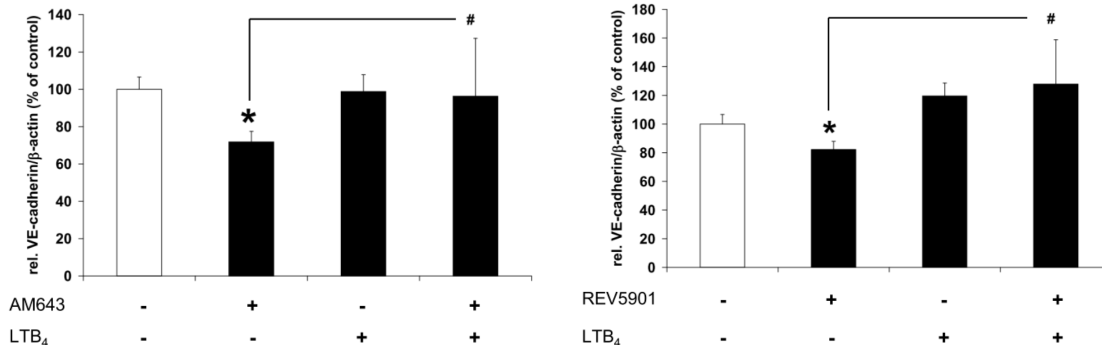
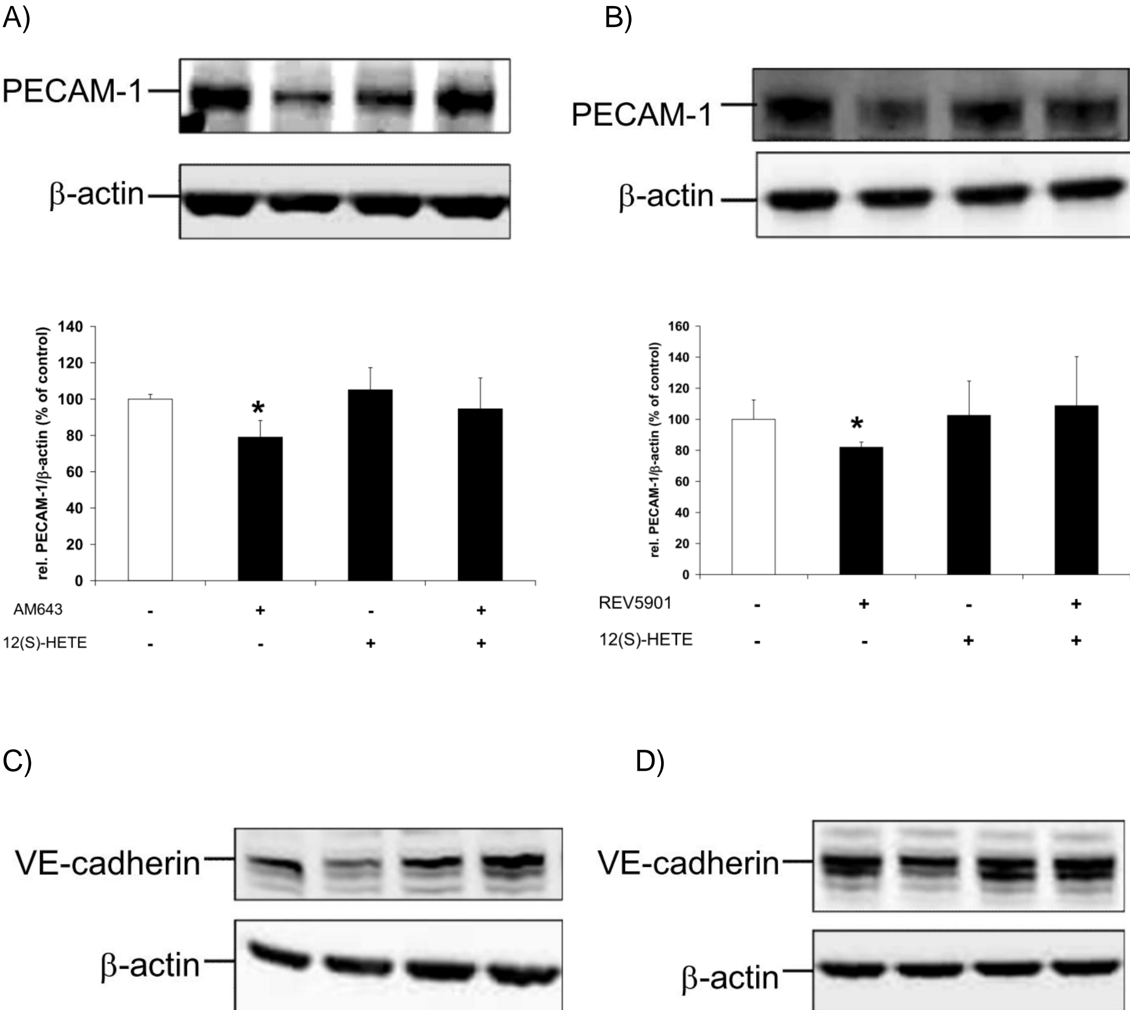


Figure 5.8 Exogenous LTB_4 restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors. 3-day-old EBs were treated either with the FLAP inhibitors AM643 (5 μM) or REV5901 (2 μM) either in the absence or presence of exogenous LTB_4 (50 nM). On day 10, these EBs were harvested for analysis by western blot. A,B) The representative blots showed that PECAM-1 expression was decreased after AM643 or REV5901 treatment compared to the untreated control cells. The treatment of LTB_4 did not significantly change PECAM-1 expression in comparison to the untreated control EBs. The decrease in PECAM-1 expression upon FLAP inhibitor treatment was reversed upon treatment with exogenous LTB_4 as compared to EBs treated with FLAP inhibitors alone. The bar charts corresponding to western blots demonstrated that the significant reduction of PECAM-1 expression after the treatment with either AM643 or REV5901 was reversed upon treatment with exogenous LTB_4 compared to the untreated control (n=4). C,D) Similar results were observed for VE-cadherin expression under same treatment conditions. The bar charts corresponding to western blots demonstrated that the significant reduction of VE-cadherin expression after treatment with either AM643 or REV5901 was reversed upon treatment with exogenous LTB_4 compared to the untreated control (n=4 in the case of AM643; n=3 in the case of REV5901). *$p < 0.05$ compared to the untreated control. #$p < 0.05$ compared to FLAP inhibitor-treated alone cells.

5.4.2 Exogenous 12(S)-HETE restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors

Next, we investigated whether 12(S)-HETE, a ligand of BLT1 and BLT2, has the same ability as LTB_4 to restore vasculogenesis after FLAP inhibitor treatment. The expression of the endothelial markers PECAM-1 and VE-cadherin in EBs was analyzed by western blot. Western blot experiments of 10-day-old differentiated cells revealed

that the expression of PECAM-1 in AM643-(5 μM) and REV5901-(2 μM) treated EBs was down-regulated compared to the untreated control cells. PECAM-1 expression of EBs treated with 12(S)-HETE (50 nM) was comparable to the untreated control. The PECAM-1 expression of EBs pre-treated with FLAP inhibitors and subsequently with 12(S)-HETE was restored - yet without statistical significance -as compared to FLAP inhibitor-treated EBs (Figure 5.9A and B). VE-cadherin expression of co-treated EBs was also restored - yet without statistical significance - compared to FLAP inhibitor-treated EBs (Figure 5.9C and 5.9D).



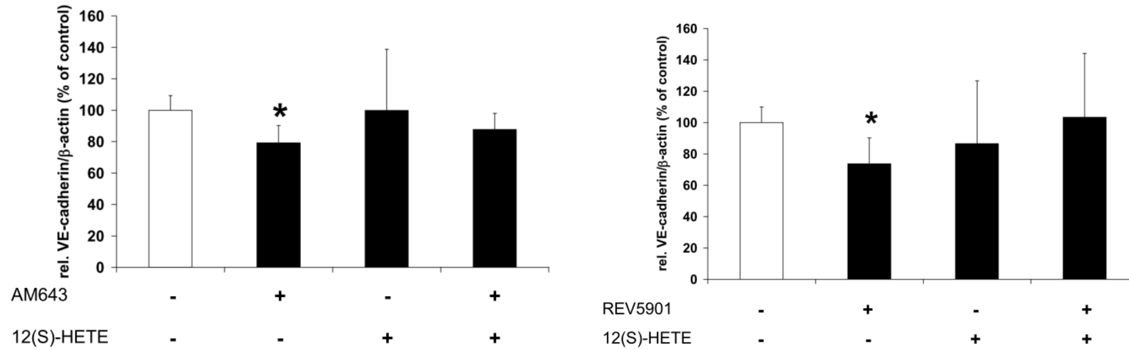
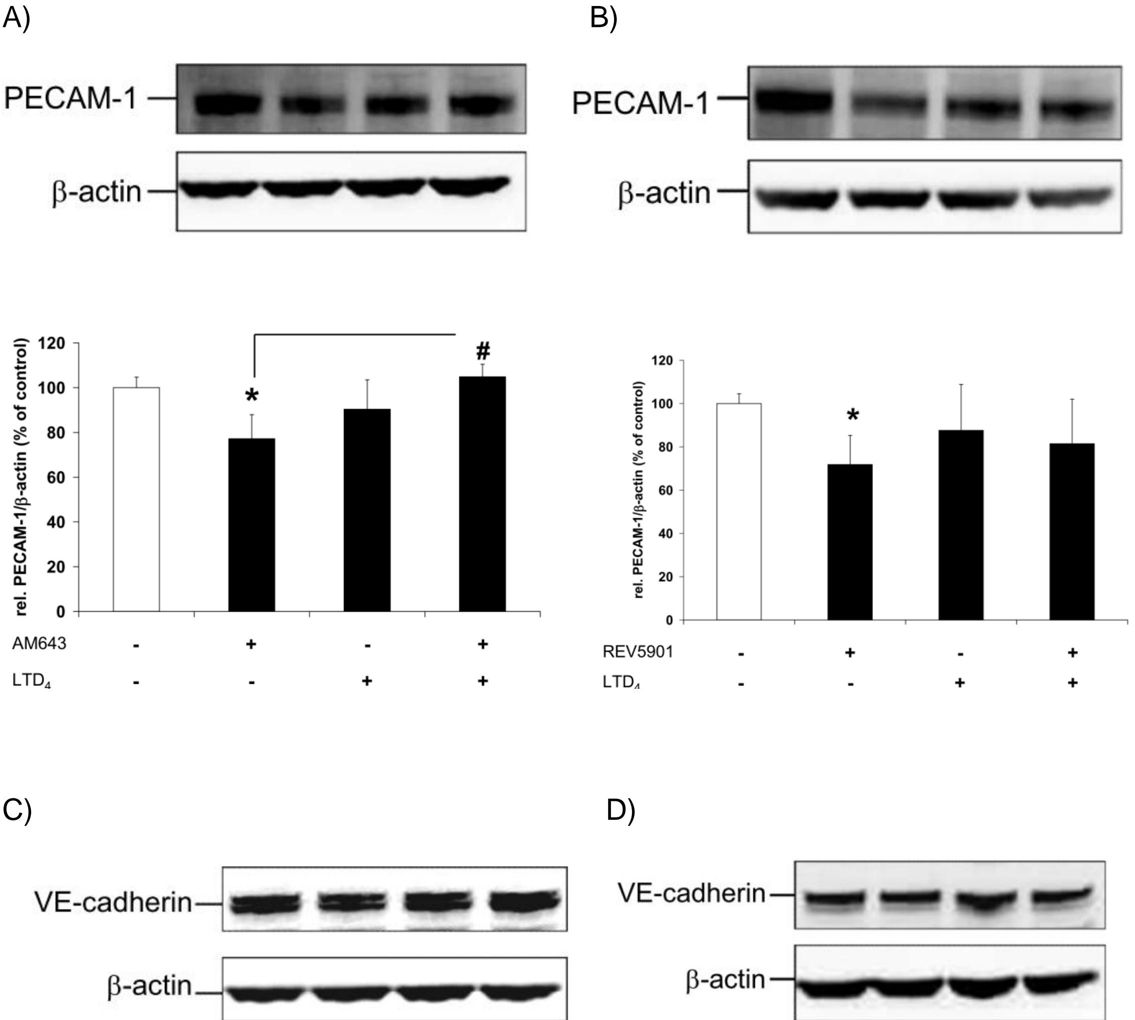


Figure 5.9 Exogenous 12(S)-HETE restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors. 3-day-old EBs were treated from day 3 until day 10 of differentiation with the FLAP inhibitors, AM643 (5 μM) or REV5901 (2 μM) either in the presence or absence of 12(S)-HETE (50 nM). On day 10, proteins were extracted and the expression of PECAM-1 and VE-cadherin was examined by western blot. A,B) Representative blots showed that PECAM-1 expression of EBs treated with AM643 or REV5901 was decreased compared to the untreated control. 12(S)-HETE alone had no effect on PECAM-1 expression in EBs compared to the untreated control. Upon pre-treatment with AM643 and then 12(S)-HETE the down-regulation of PECAM-1 expression was partially reserved in comparison to AM643- and REV5901-treated EBs. The bar charts corresponding to western blots demonstrated that the reduction of PECAM-1 expression was only partially reversed upon 12(S)-HETE treatment (n=4). C,D) Similar results were observed for VE-cadherin expression under the same treatment conditions. The bar charts corresponding to western blots suggested that the reduction of VE-cadherin expression was only partially reversed upon 12(S)-HETE treatment (n=3). *p < 0.05 compared to the untreated control. #p < 0.05 compared to FLAP inhibitor-treated cells.)

5.4.3 Exogenous LTD_4 restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors

Besides LTB_4, CysLTs are also FLAP-dependent AA metabolites. Therefore, we postulated that CysLTs might participate in FLAP-mediated vasculogenesis. To investigate whether CysLT-LTD_4 can restore the inhibition of vasculogenesis upon treatment with FLAP inhibitors, the expression of PECAM-1 and VE-cadherin in EBs was examined after exogenous addition of LTD_4. As shown in the representative blot,

PECAM-1 expression in AM643-(5 μM) and REV5901-(2 μM) treated EBs was decreased compared to the untreated control cells (Figure 5.10A and 5.10B). The expression of PECAM-1 in EBs treated with LTD_4 (50 nM) alone was comparable to the untreated control cells. The expression of PECAM-1 was restored in EBs co-treated with LTD_4 after AM643 pre-treatment compared to cells treated with AM643 alone (Figure 5.10A). However, LTD_4 did not significantly restore PECAM-1 expression in EBs pre-treated with REV5901 (Figure 5.10B). VE-cadherin expression of EBs pre-treated with AM643 first and then LTD_4 was also restored in comparison to the untreated controls (Figure 5.10C and D).



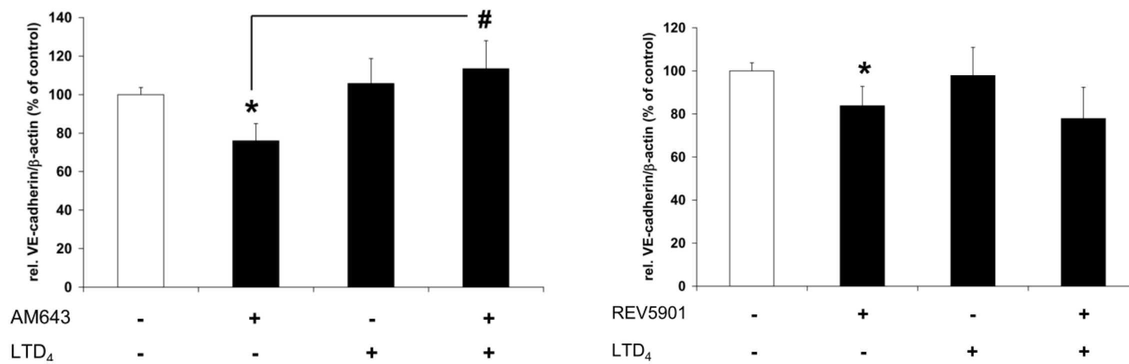


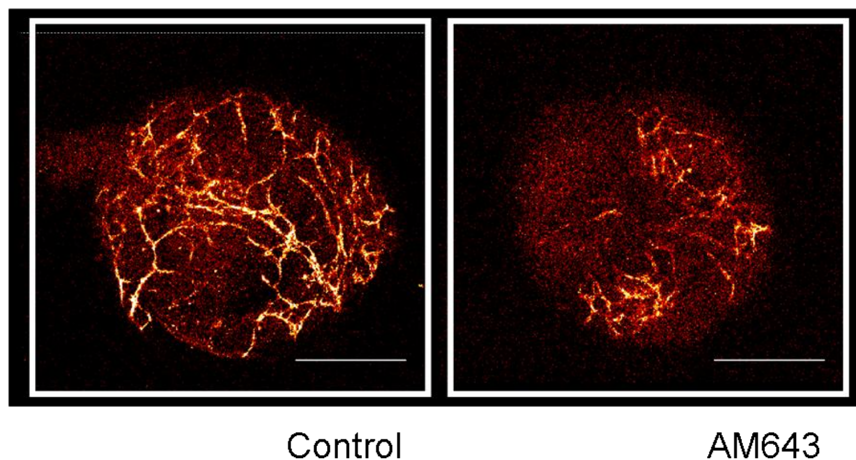
Figure 5.10 Exogenous LTD_4 restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors. 3-day-old EBs were treated with FLAP inhibitors (AM643 (5 μM) or REV5901 (2 μM)), LTD_4 (50 nM) or FLAP inhibitors together with LTD_4 until day 10, and proteins were harvested for western blot analysis. A,B) The expression of PECAM-1 in EBs treated with AM643 or REV5901 was decreased compared to the untreated controls. PECAM-1 expression in EBs treated with LTD_4 was comparable to the untreated control cells. Addition of LTD_4 (50 nM) reversed PECAM-1 expression of EBs pre-treated with 5 μM AM643 in comparison to EBs treated with AM643 alone. However, LTD_4 did not significantly reverse the down-regulated PECAM-1 expression in EBs pre-treated with REV5901 alone. The bar charts corresponding to western blots demonstrated significant reduction of PECAM-1 expression after the treatment with AM643 which was reversed upon treatment with exogenous LTD_4. This was not the case in EBs pre-treated with REV5901 and then LTD_4 (n=3). C,D) Similar results were observed for VE-cadherin expression under the same treatment conditions. The bar charts corresponding to western blots suggested that the significant reduction of VE-cadherin expression after treatment with AM643 was reversed upon co-administration of exogenous LTD_4. VE-cadherin expression of EBs pre-treated with REV5901 and then LTD_4 was not restored compared to the untreated control (n=3). *$p < 0.05$, significantly different from the untreated control. #$p < 0.05$, significantly different from FLAP inhibitor-treated cells.

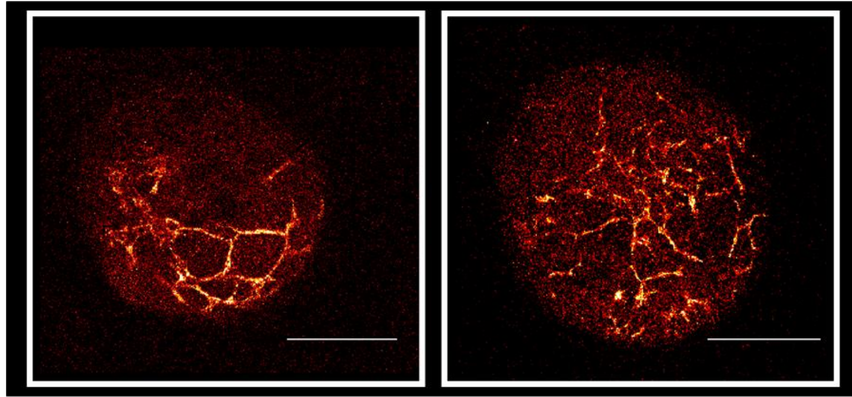
5.4.4 Effects of LTs on vasculogenesis upon treatment with FLAP inhibitors

To further confirm the physiological effects of LT supplementation on EBs treated with FLAP inhibitors, branching points were measured as indicators for vasculogenesis. As shown in figure 5.11A, branching points of EBs treated with the FLAP inhibitors, AM643 (5 μM) or REV5901 (2 μM), were decreased compared to the untreated controls (Figure 5.11A). In contrast, EBs treated with LTB_4 (50 nM), LTD_4 (50 nM) or 12(S)-HETE

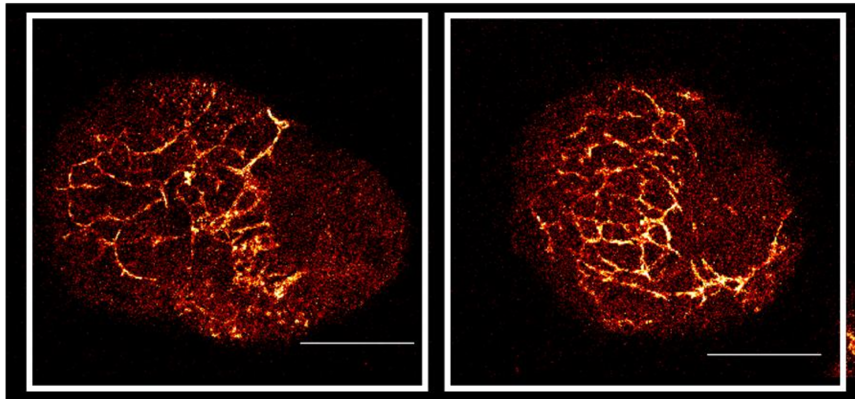
(50 nM) showed no differences in the numbers of branching points compared to the untreated control EBs (Figure 5.11A). Interestingly, we found that additional supplement of some LTs restored the branching points attenuated by FLAP inhibitors. LTB_4 restored the branching points reduced by either AM643 or REV5901 in EBs while LTD_4 only recovered the branching points upon treatment with AM643 but not with REV5901 (Figure 5.11B). In the case of 12(S)-HETE, the branching points were partially restored following co-treatment with FLAP inhibitors and 12(S)-HETE. These data suggest that vasculogenesis reduced by FLAP inhibitors can be restored upon exogenous addition of LTB_4 and 12(S)-HETE although the results of 12(S)-HETE did not reach statistical significance. The supplement of LTD_4 only restored AM643-inhibited vasculogenesis (Figure 5.11C). All together, the results from 5.4.1 to 5.4.4 provide evidence that LTB_4, LTD_4, and 12(S)-HETE participate in the LT signaling pathway to regulate vasculogenesis.

Aa)



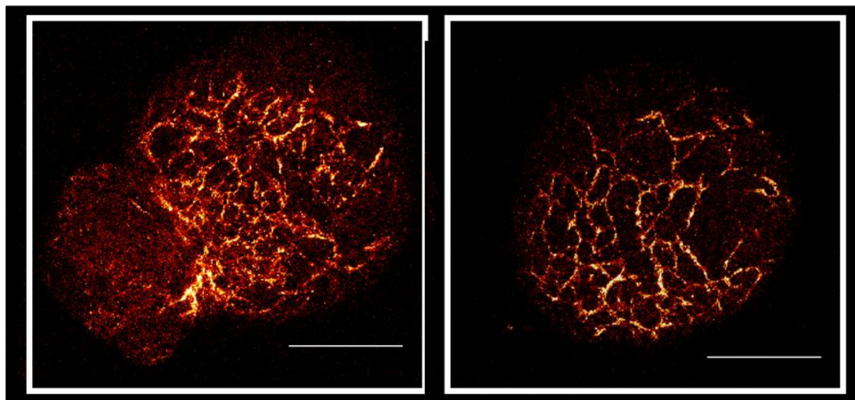


REV5901 LTB_4



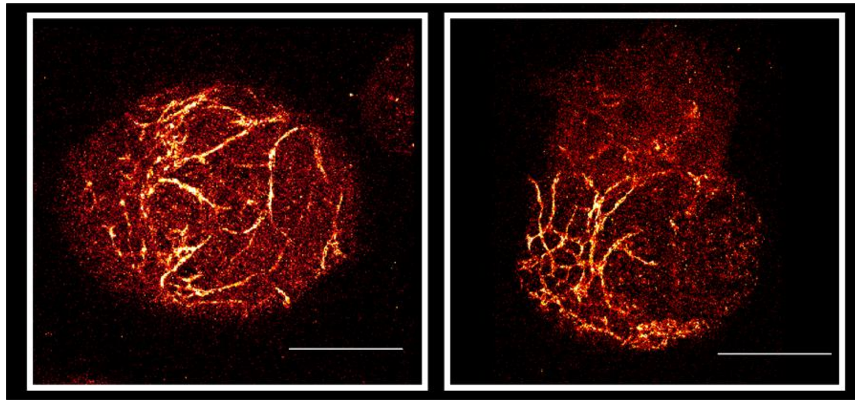
LTD_4 12(S)-HETE

Ab)

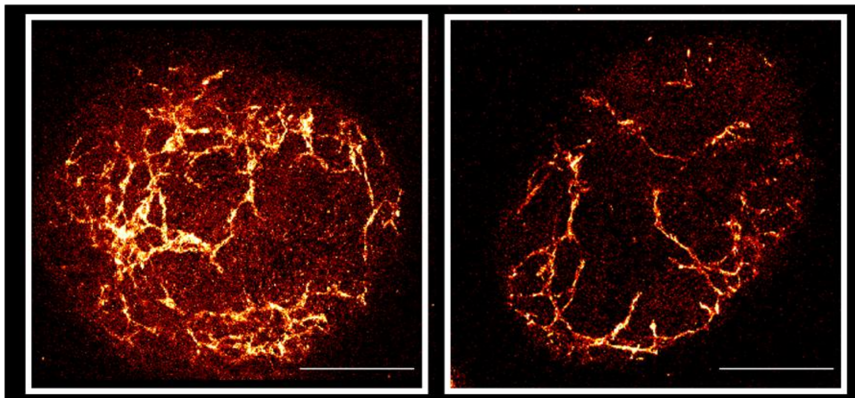


AM643+LTB_4 REV5901+LTB_4

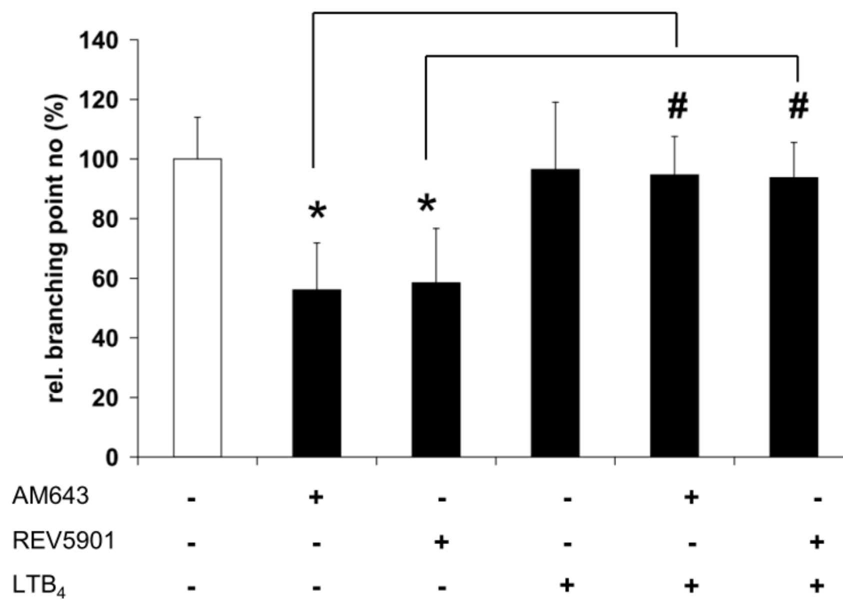
Ac)



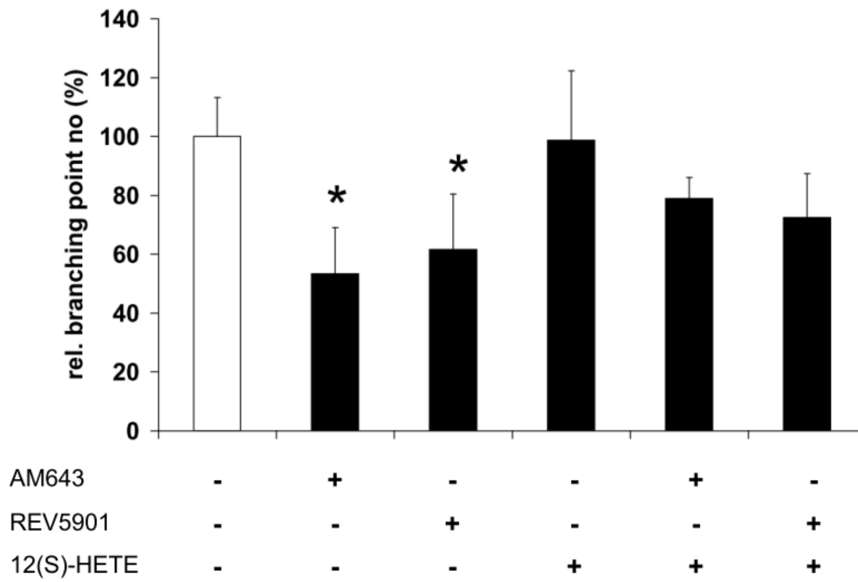
Ad)



B)



C)



D)

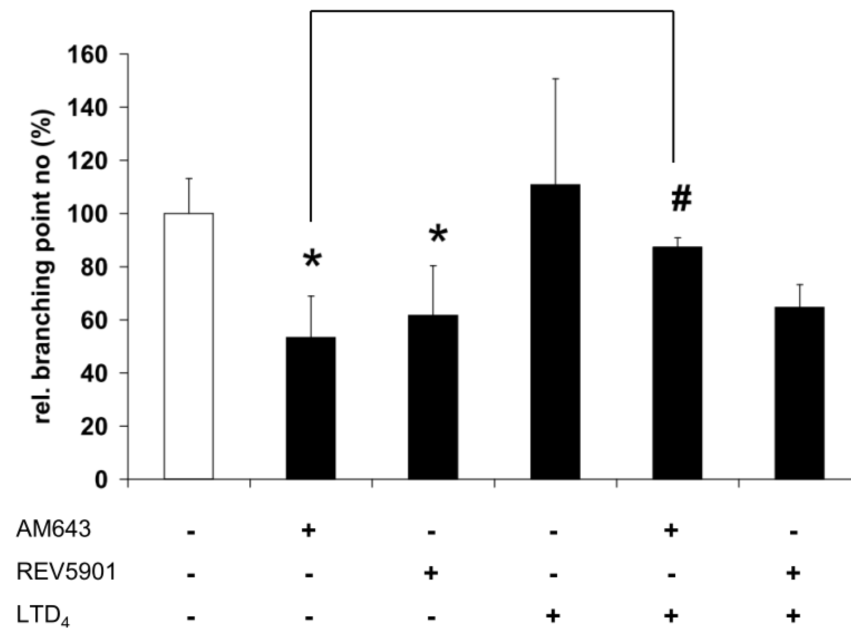


Figure 5.11 Effects of LTs on vasculogenesis upon treatment with FLAP inhibitors. From day 3 until day 10 of differentiation EBs were treated with the FLAP inhibitors AM643 (5 μM) or REV5901 (2 μM), or LTs (LTB_4 (50 nM), LTD_4 (50 nM), or 12(S)-HETE (50 nM)), or the combination of both, respectively. Aa) The representative pictures showed decreased branching points in FLAP inhibitor-treated EBs compared to the untreated control EBs. The LT-treated EBs showed nearly the same amount of branching points as the untreated EBs. Ab) The representative pictures suggested that

EBs, which were pre-treated with FLAP inhibitors, contained increased branching points after LTB_4 supplement compared to EBs treated with FLAP inhibitors alone. Ac) Addition of 12(S)-HETE partially restored the number of branching points reduced by both FLAP inhibitors. Ad) Compared to EBs treated with FLAP inhibitors alone, addition of LTD_4 increased the number of branching points in EBs pre-treated with AM643 but not with REV5901. B-D) The bar charts showed the effect of LTB_4 (B), 12(S)-HETE (C) and LTD_4 (D) on branching points (%) in either presence or absence of AM643 and REV5901. The upper panel shows representative images of EBs labeled with anti PECAM-1 antibody. The scale bar represents 300 μm. The bar chart represents mean ± SD of 3 separate experiments in which at least 30 individual EBs were analyzed. *$p < 0.05$ compared to the untreated control cells. #$p < 0.05$ compared to FLAP inhibitor-treated cells.

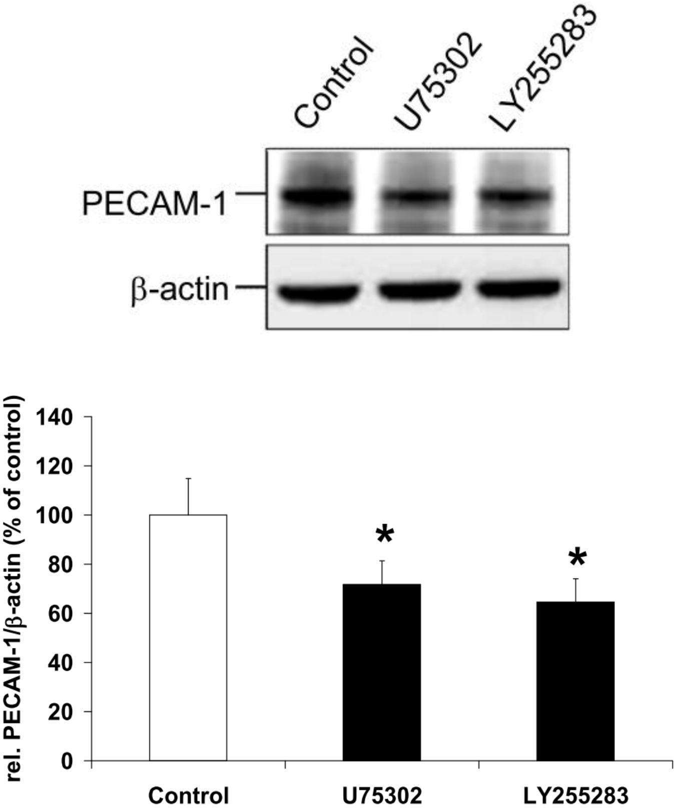
5.5 Influence of the LTB_4 signaling pathway on vasculogenesis

In result 5.4.1 we realized that LTB_4 is one of the mediators of AA-induced FLAP-dependent vasculogenesis. To further investigate how the LTB_4 signaling pathway involves in vascular differentiation, we inhibited BLT1 or BLT2 with either U75302 which is a specific inhibitor for BLT1 or LY255283 which is a BLT2 inhibitor. EBs were treated with U75302 (1 μM) or LY255283 (1 μM), respectively, from day 3 to day 10 of differentiation and then subjected to vasculogenesis examination. Branching points and expression of endothelial markers including PECAM-1, VE-cadherin and Flk-1 were assessed as indicators of vasculogenesis.

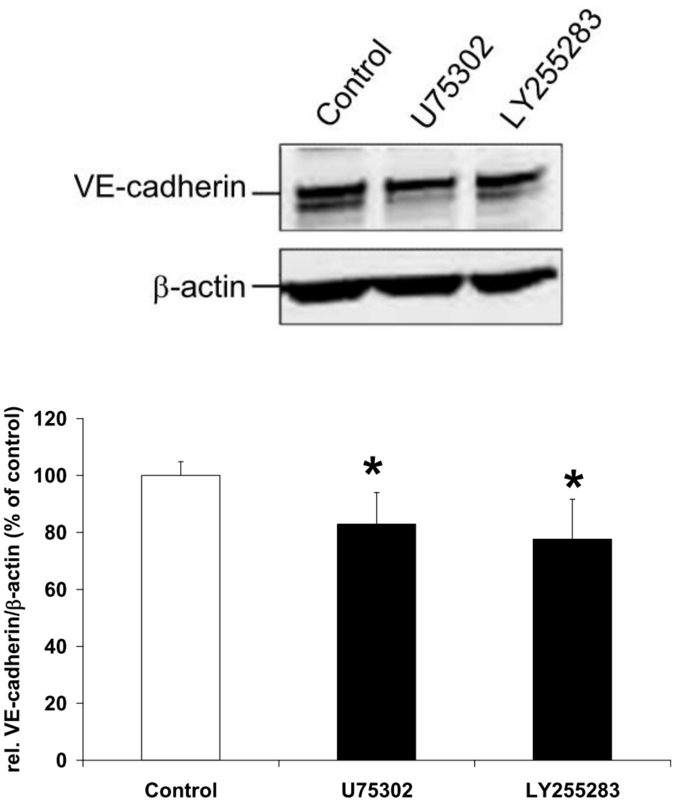
5.5.1 Impact of LTB_4 receptors for the expression of PECAM-1, VE-cadherin and Flk-1

After treatment of EBs with either U75302 (1 μM) or LY255283 (1 μM) from day 3 to day 10 of differentiation, EBs were collected for detecting the expression of the endothelial markers PECAM-1, VE-cadherin and Flk-1. Our data showed significant reduction of PECAM-1, VE-cadherin and Flk-1 expression in EBs treated with BLT1 and BLT2 antagonists in comparison to the untreated control cells (Figure 5.12).

A)



B)



C)

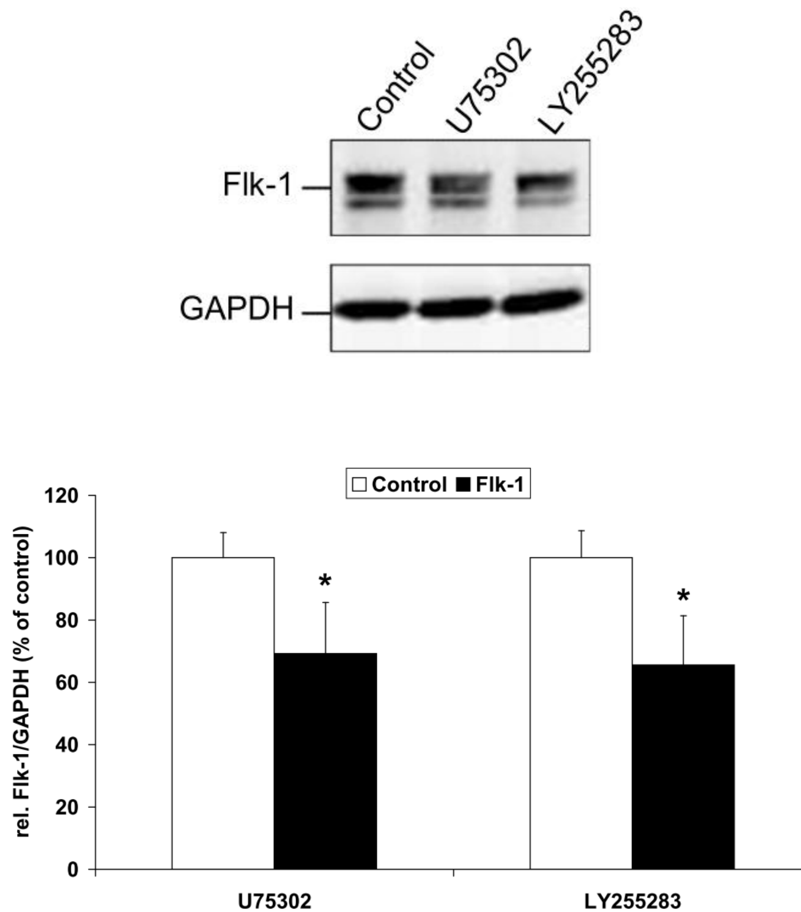


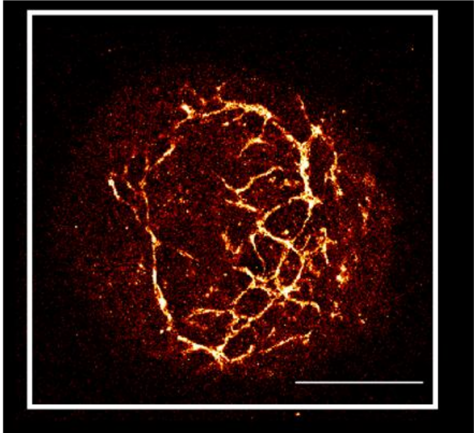
Figure 5.12 Impact of LTB_4 receptors for the expression of PECAM-1, VE-cadherin and Flk-1. EBs were treated with BLT1/2 receptor inhibitors (U75302 (1 μM) or LY255283 (1 μM)) from day 3 to day 10 of differentiation and collected on day 10. A) The representative pictures and bar charts showed that PECAM-1 expression was decreased in BLT1/2 inhibitor-treated EBs compared to the untreated control cells (n = 4). B) Expression of VE-cadherin in EBs treated with U75302 and LY255283 was also decreased in comparison to the untreated control cells both presented in the representative pictures and bar charts (n = 4). C) Likewise representative pictures and statistic analysis of Flk-1 expression showed reduction in BLT1/2 inhibitor-treated EBs compared to the untreated controls (n = 3) *$p < 0.05$, significantly different from the untreated control.

5.5.2 Effect of LTB_4 receptor inhibition on vascular structures

Analysis of PECAM-1 positive branching points was further used to confirm the effects of BLT1/2 inhibitor treatment on vascular structure formation. The experimental

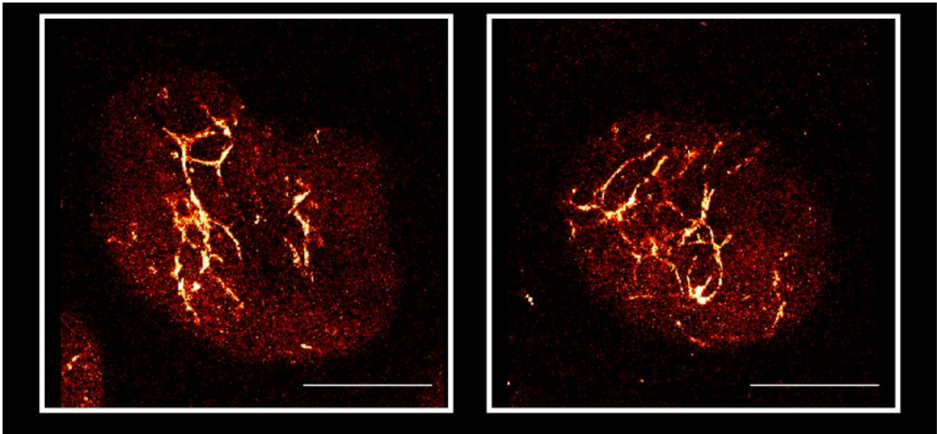
protocols were the same as described in 5.5.1. In comparison to the untreated control cells, the number of branching points in EBs treated with U75302 (1 μM) or LY255283 (1 μM) were significantly decreased (Figure 5.13). The statistical analysis of the number of branching points in EBs treated with U75302 or LY255283 also showed significant reduction of vascular structures compared to the untreated control cells. Taken together, these data suggested that LTB_4 involves in LT signaling pathway-regulated vasculogenesis via binding to its receptor BLT1/2.

Aa)



Control

Ab)



U75302 LY255283

B)

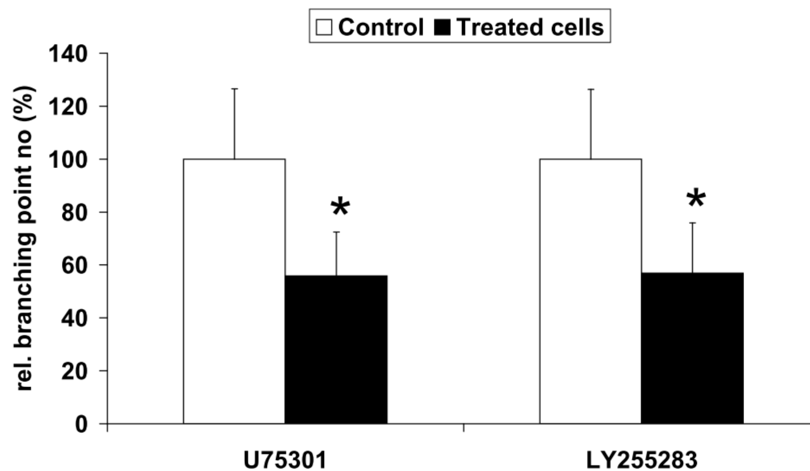


Figure 5.13 Effect of LTB_4 receptor inhibition on vascular structures. EBs were treated with either U75302 (1 μM) or LY255283 (1 μM) from day 3 to day 10 of differentiation. On day 10, EBs were collected and the branching points of EBs were examined. A) The representative pictures showed that EBs treated with U75302 and LY255283 contained reduced numbers of branching points in comparison to the untreated control cells. B) The quantified results also demonstrated that branching points of EBs treated with BLT1/2 inhibitors were significantly down-regulated in comparison to the untreated control. The upper panel shows representative images of EBs labeled with anti PECAM-1 antibody. The scale bar represents 300 μm. The bar chart represents mean ± SD of 3 separate experiments in which at least 30 individual EBs were analyzed (n = 4 in the case of LY255283; n = 5 in the case of U75302). $^{*}p < 0.05$, significantly different from the untreated control.

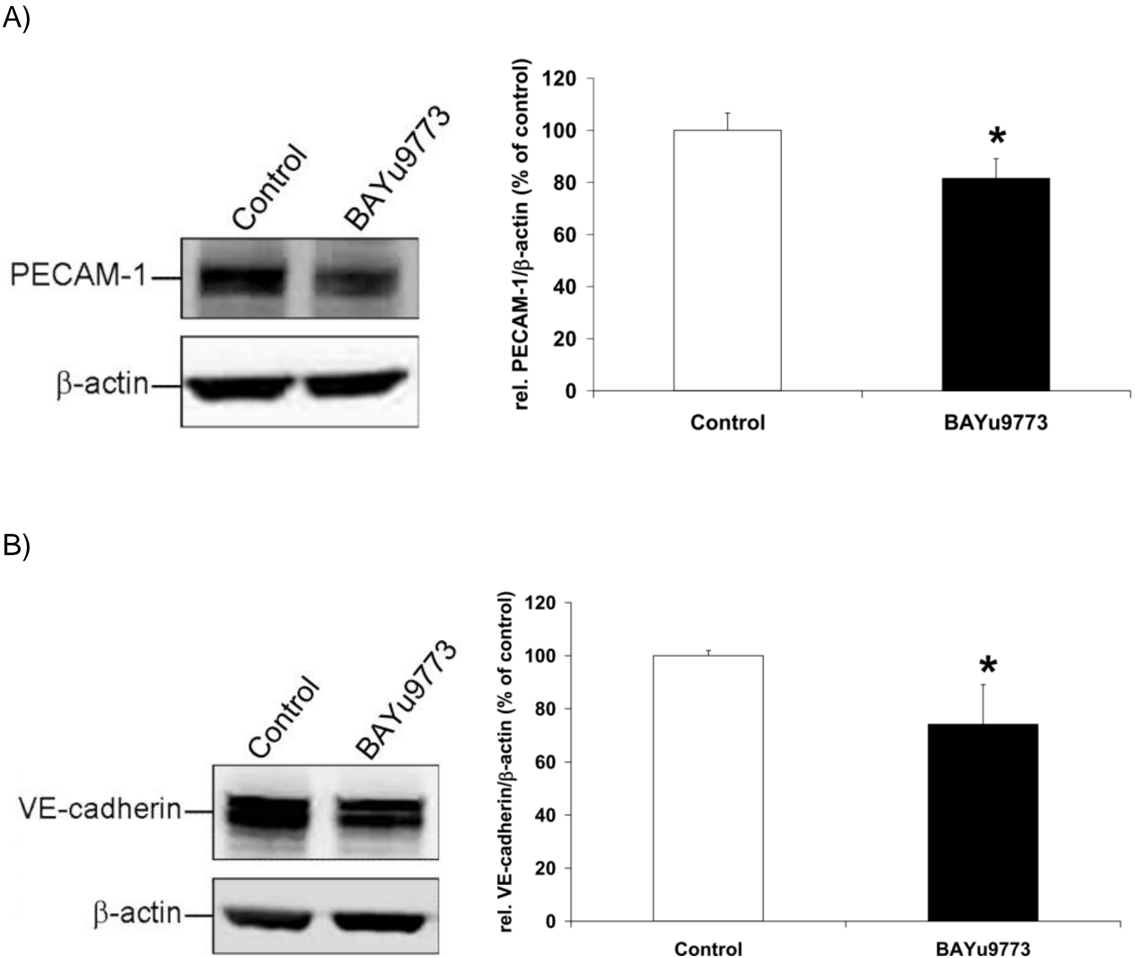
5.6 Impact of the CysLT inhibitor BAYu9773 on vasculogenesis of mouse ES cells

Based on the result shown in 5.4.3, LTD_4, which is the metabolite of AA and a part of the CysLT signaling pathway, is involved in the signaling pathway of AA-induced vasculogenesis. Furthermore, we found in result 5.5 that LTB_4, another metabolite of AA signaling pathway, involves in vasculogenesis. Therefore, we postulated that LTD_4 might regulate vasculogenesis via the CysLT receptor. To validate this hypothesis, the CysLT receptor inhibitor, BAYu9773, was used. EBs were treated with BAYu9773 (1 μM)

from day 3 to day 10 of differentiation and then collected on day 10. The expression of PECAM-1, VE-cadherin and Flk-1 and branching points of EBs were assessed.

5.6.1 Effect of BAYu9773 on the expression of PECAM-1, VE-cadherin and Flk-1

The expression of the endothelial markers PECAM-1, VE-cadherin and Flk-1 was examined after BAYu9773 treatment. EBs were treated from day 3 till day 10 of differentiation, with BAYu9773 (1 μM) and then collected on day 10 for western blot analysis. The expression of all three endothelial markers, PECAM-1, VE-cadherin and Flk-1 was down-regulated in EBs treated with BAYu9773 in comparison to the untreated controls (Figure 5.14).



C)

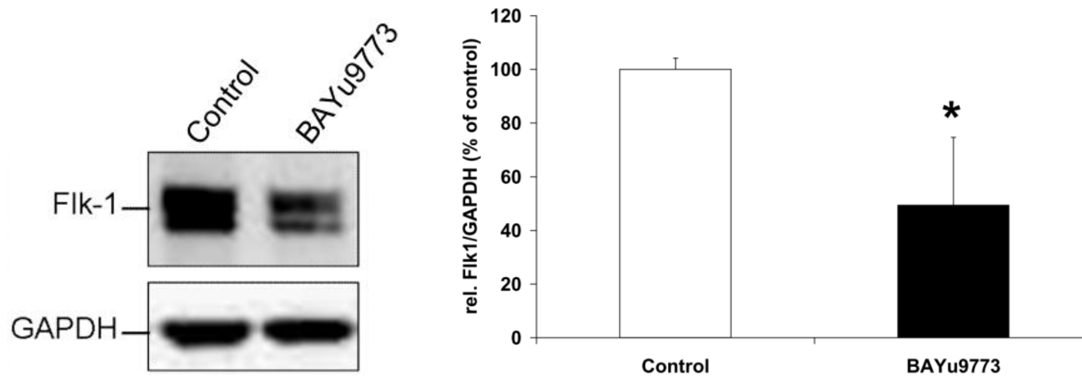
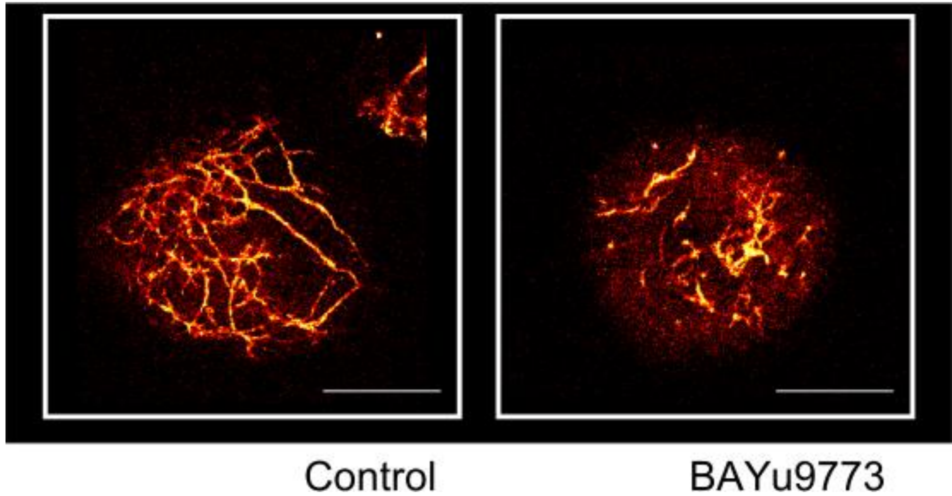


Figure 5.14 Effect of BAYu9773 on the expression of PECAM-1, VE-cadherin and Flk-1. EBs were collected after treatment with or without BAYu9773 (1 μM) from day 3 to day 10 of differentiation and then protein expression of endothelial markers was detected. As shown in blots and bar charts, (A) PECAM-1, (B) VE-cadherin, and (C) Flk-1 expression were all reduced after BAYu9773 treatment compared to the untreated control cells (n = 4 in the case of PECAM-1 and VE-cadherin, n = 3 in the case of Flk-1). $^*p < 0.05$, significantly different from the untreated control.

5.6.2 Effect of the CysLT receptor inhibitor BAYu9773 on the formation of vascular structures

To further confirm the effects of BAYu9773 treatment on vasculogenesis, PECAM-1 positive branching points were examined. The branching points in BAYu9773-treated EBs were significantly decreased compared to the untreated control (Figure 5.15). Taken together, the data from result 5.6 suggest that CysLTs take part in LT signaling pathway-regulated vasculogenesis through binding to the corresponding receptor.

A)



B)

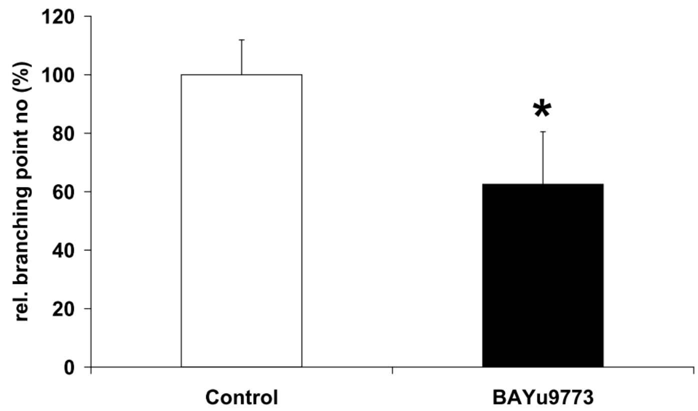


Figure 5.15 Effect of the CysLT receptor inhibitor BAYu9773 on the formation of vascular structures. From day 3 to day 10 of differentiation, EBs were treated with the CysLTs receptor inhibitor BAYu9773 (1 μM), and then collected on day 10 for whole EB staining to analyze PECAM-1 positive branching points. A) The EBs treated with BAYu9773 displayed reduced vascular differentiation in comparison to the untreated control EBs. B) Bar charts demonstrated that the branching point numbers of EBs treated with BAYu9773 were significantly decreased compared to the untreated control EBs. The scale bar represents 300 μm. The bar chart represents mean ± SD of 3 separate experiments in which at least 30 individual EBs were analyzed. *$p < 0.05$, significantly different from the untreated control.

5.7 Influence of combined LTB_4 and CysLT receptor inhibitors on

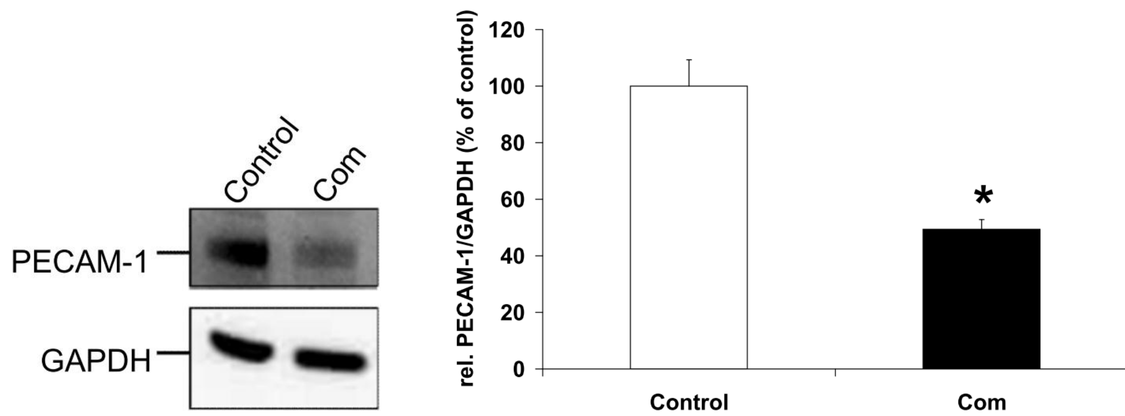
vasculogenesis of mouse ES cells

Based on result 5.5 and 5.6, LTB_4 and CysLTs involve in FLAP-dependent vasculogenesis; however, each of them seems to participate only partially in this signaling pathway. To delineate the effect of the entire LT signaling pathway on vasculogenesis more precisely, three different LT receptor inhibitors, U75302, LY255283, and BAYu9773, were used together to examine the influence of overall blocking of the LT signaling pathway on vasculogenesis. Differentiated EBs were treated with or without the combination of three inhibitors, U75302 (1 μM), LY255283 (1 μM) and BAYu9773 (1 μM) from day 3 to day 10 of differentiation. EBs were then collected to measure branching points of vascular structures and PECAM-1, VE-cadherin and Flk-1 expression.

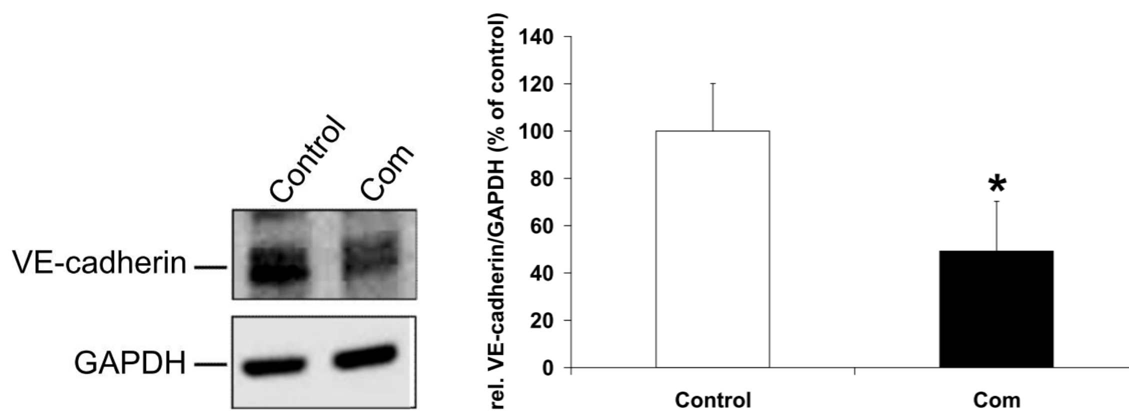
5.7.1 Effect of combined treatment of EBs with LTB_4 and CysLTs receptor inhibitors on PECAM-1, VE-cadherin and Flk-1 expression

To verify the occurrence of vasculogenesis, the expression of endothelial cell markers i.e. PECAM-1, VE-cadherin and Flk-1 were examined after combined treatment with the inhibitors U75302, LY255283 and BAYu9773. From day 3 to day 10 of differentiation, EBs were treated with or without combined inhibitors (U75302 (1 μM), LY255283 (1 μM), and BAYu9773 (1 μM)). A significant reduction in PECAM-1 expression was observed in EBs treated with combined inhibitors U75302, LY255283, and BAYu9773 (Figure 5.16A); similarly, the VE-cadherin expression was down-regulated in treated EBs (Figure 5.16B) compared to the untreated control cells. In parallel, Flk-1 expression was significantly decreased in EBs treated with combined inhibitors in comparison to the untreated EBs (Figure 5.16C).

A)



B)



C)

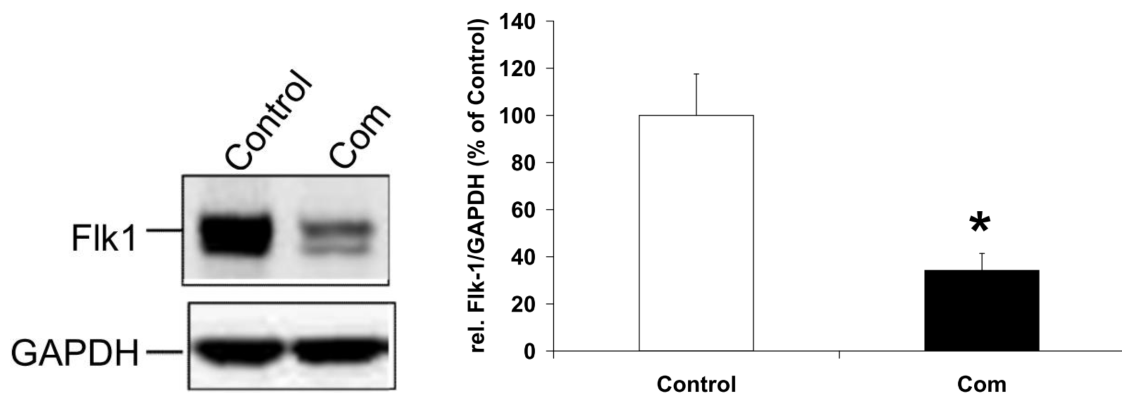


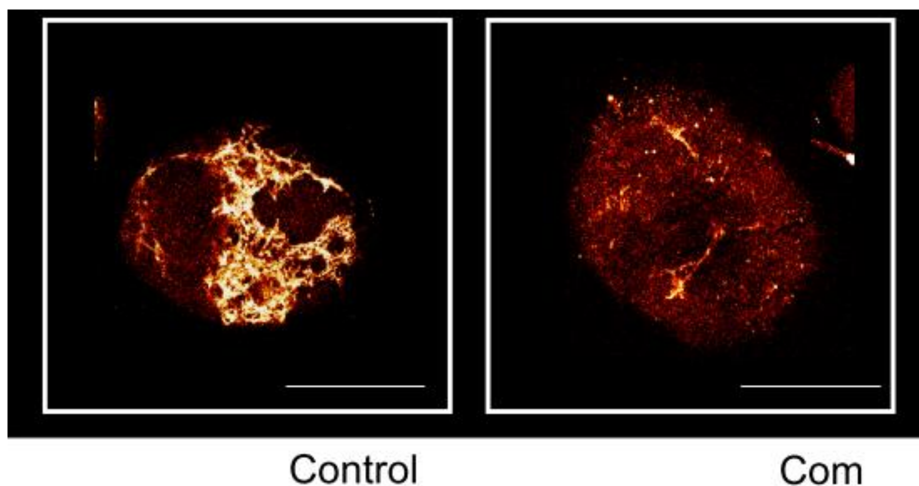
Figure 5.16 Effect of combined treatment of EBs with LTB_4 and CysLTs receptor inhibitors on PECAM-1, VE-cadherin and Flk-1 expression. To measure the expression of endothelial markers, EBs were collected on day 10 after treatment for 7 days with or without LTB_4 and CysLT receptor inhibitors (U75302 (1 μM), LY255283 (1 μM), and BAYu9773 (1 μM)). The representative images and bar charts showed that the

expression of (A) PECAM-1, (B) VE-cadherin, and (C) Flk-1 were decreased in treated EBs compared to the untreated control cells (Com = inhibitor combination of U75302, LY255283, and BAYu9773). Each value represents the mean ± SD of 3 separate experiments. *$p < 0.05$ compared to the untreated control.

5.7.2 Effect of combined treatment of EBs with LTB_4 and CysLTs receptor inhibitors on vasculogenesis

Vasculogenesis of EBs treated with LTB_4 and CysLT receptor inhibitors was significant decreased as indicated by the reduction of branching points compared to the untreated control cells (Figure 5.17). Taken together, the result of 5.7 suggested that combined inhibition of LTB_4 and CysLTs receptors exerted a stronger inhibition of vasculogenesis as compared to selective inhibition of either the LTB_4 or the CysLT signaling pathway.

A)



B)

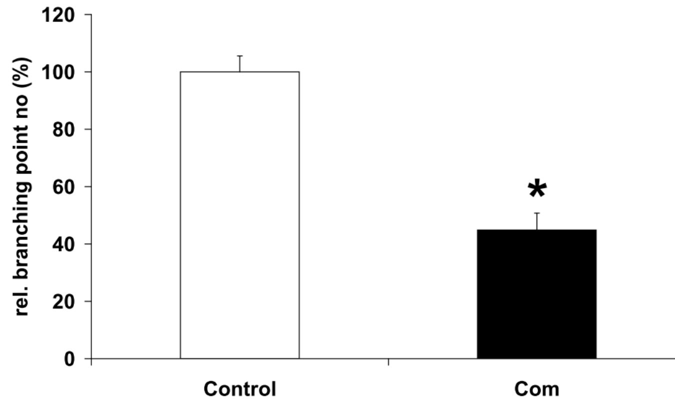


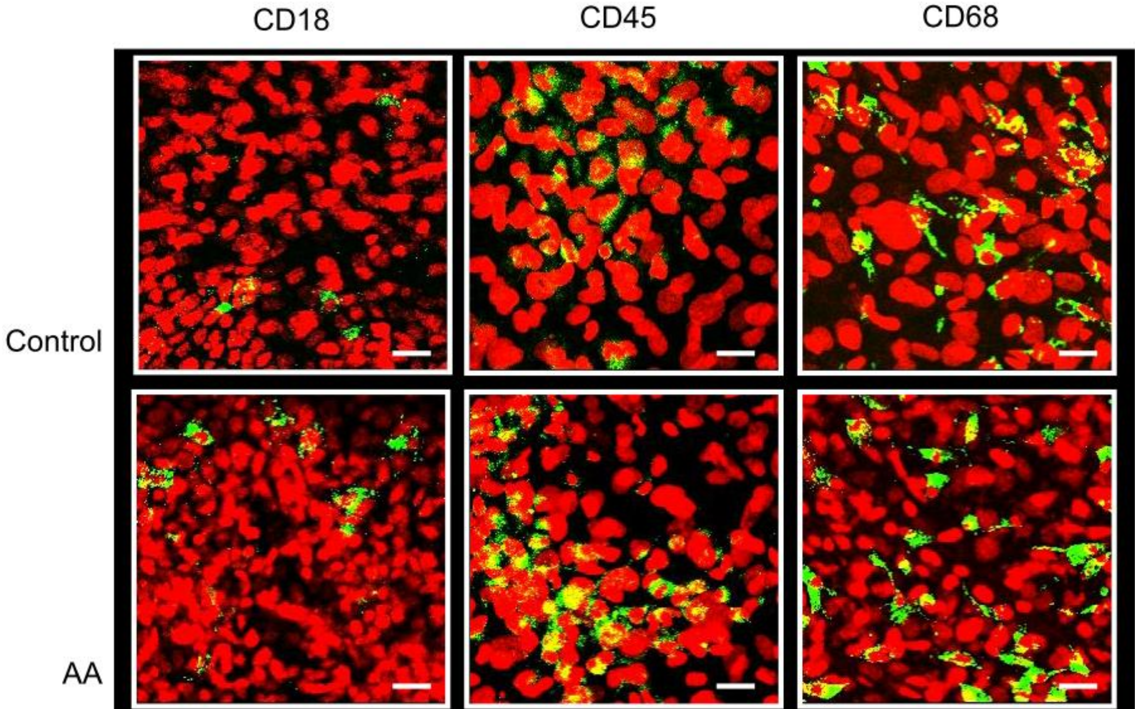
Figure 5.17 Effect of combined treatment of EBs with LTB_4 and CysLTs receptor inhibitors on vasculogenesis. From day 3 to day 10 of differentiation, differentiated EBs were treated with or without the LTB_4 and the CysLT receptor inhibitors U75302 (1 μM), LY255283 (1 μM), and BAYu9773 (1 μM), and were then collected on day 10 for experiments. A) Compared to the untreated control, branching points of EBs treated with combination of inhibitors was decreased. B) The bar charts showed the significance of vasculogenesis reduction in the LTB_4 and the CysLT receptor inhibitor-treated EBs in comparison to the untreated control (Com = inhibitor combination of U75302, LY255283, and BAYu9773). The upper panel shows representative images of EBs labeled with anti PECAM-1 antibody. The scale bar represents 300 μm. The bar chart represents mean ± SD of 3 separate experiments in which at least 30 individual EBs were analyzed. *$p < 0.05$ compared to the untreated control.

5.8 Effect of AA on leukocyte differentiation of mouse ES cells

Previous studies have shown that inflammatory responses, in which LTs are key mediators, not only contribute to vasculogenesis but also promote leukocyte accumulation (186). In result 5.1 we have proved that AA can enhance vasculogenesis in EBs. Interestingly, evidences have indicated that endothelial cells and leukocytes originally arise from the same progenitor cells (187; 188). Therefore we postulated that AA treatment might also influence leukocyte differentiation. For analyzing whether AA regulates leukocyte differentiation, EBs were plated on cover slips on day 4 after differentiation induction and collected on day 14. Treatment of EBs with AA (100 μM)

was performed from day 3 until day 14. Two common leukocyte markers, CD18 and CD45 and the macrophage marker CD68 were used to examine leukocyte differentiation (189-191). Leukocyte differentiation was significantly up-regulated after AA treatment in comparison to the untreated control cells as indicated by increased numbers of cells expressing leukocyte cell markers (Figure 5.18). Taken together, AA not only increased vasculogenesis but also enhanced leukocyte differentiation.

A)



B)

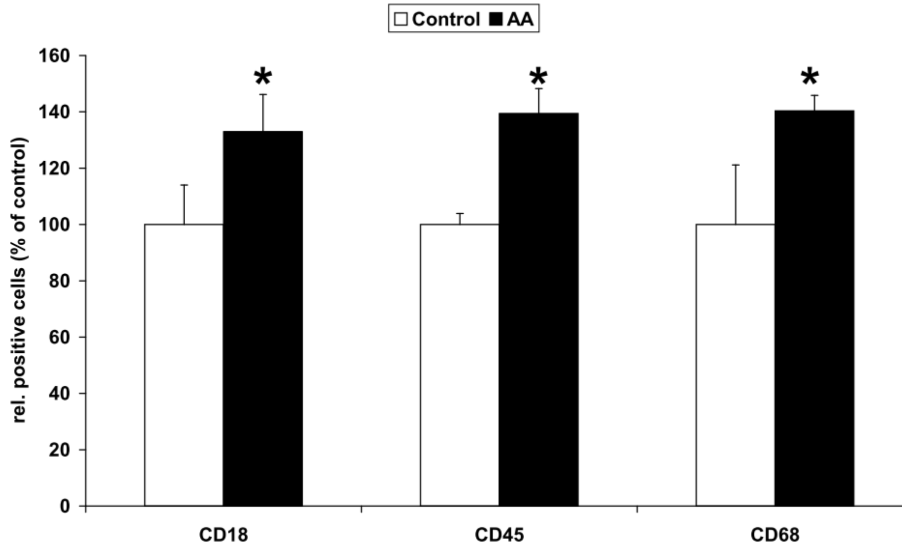


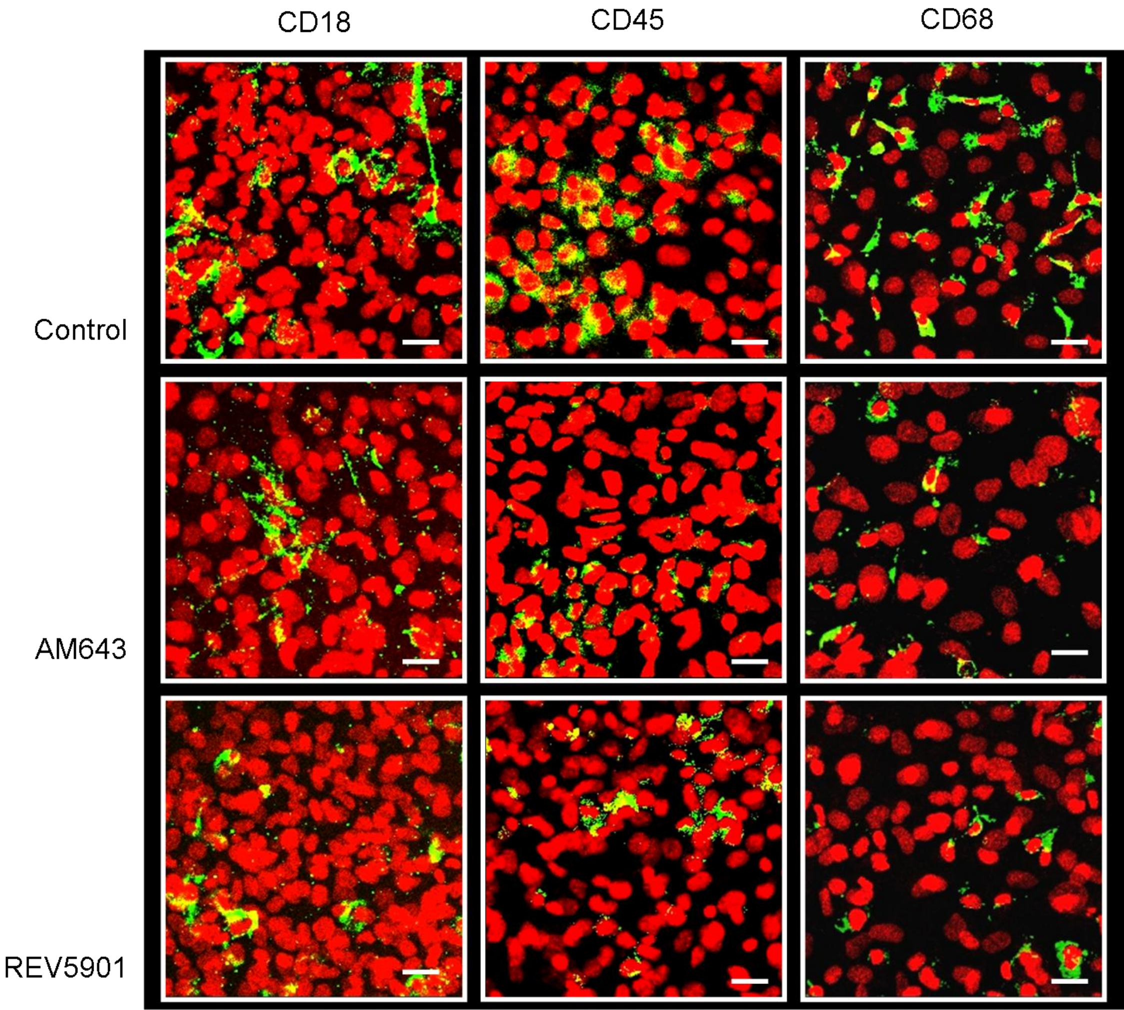
Figure 5.18 Effect of AA on leukocyte differentiation of mouse ES cells. After differentiation induction, 4-day-old EBs were plated on cover slips and treated with AA (100 μM) from day 3 to day 14 of differentiation. The plated-EBs were collected on day 14 for leukocyte differentiation detection. A) The representative images showed that the number of cells positive for the leukocyte cell markers CD18, CD45, and CD68 in AA-treated EBs was increased compared to the untreated control (n = 3 in the case of CD18 and CD45, n = 4 in the case of CD68). B) The bar charts indicated that leukocyte differentiation of EBs treated with AA was significantly enhanced compared to the untreated control. $^{*}p < 0.05$ compared to the untreated control. The scale bar represents 20 μm.

5.9 Impact of FLAP inhibitors on leukocyte differentiation of mouse ES cells

According to the result 5.2, 5.3 and 5.8, FLAP inhibitors down-regulated vasculogenesis while LT signaling pathways participated in leukocyte differentiation. Therefore, we aimed to address whether the FLAP inhibitors AM643 and REV5901 would down-regulate leukocyte differentiation in addition to attenuate vasculogenesis. We treated EBs with the FLAP inhibitors AM643 (5 μM) and REV5901 (2 μM) from day 3 to day 14 of differentiation. 4-day-old differentiated EBs were plated on cover slips and were then collected on day 14. The leukocyte and macrophage markers CD18, CD45

and CD68 were examined. Our results showed that FLAP inhibitors AM643 and REV5901 down-regulated leukocyte differentiation compared to the untreated control (Figure 5.19). Based on result 5.2 and 5.9, FLAP inhibitors interfered not only with leukocyte differentiation but also with vasculogenesis.

A)



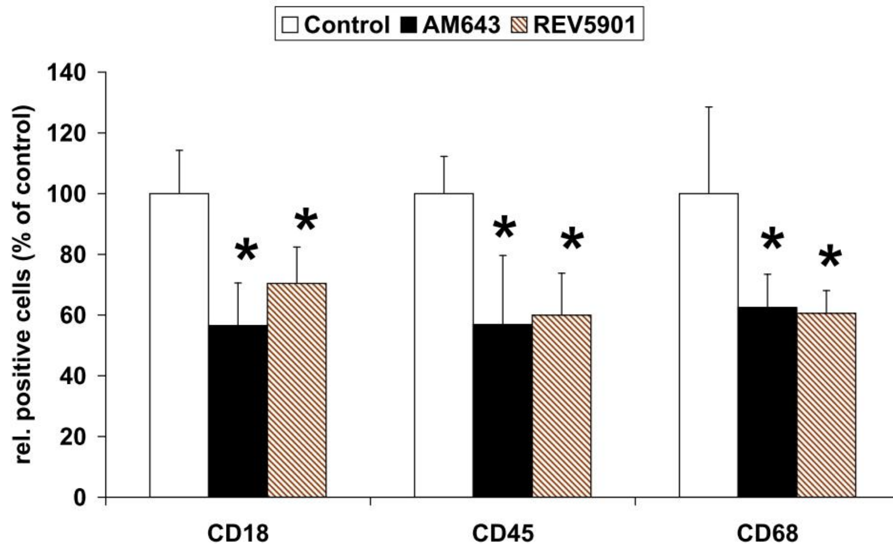


Figure 5.19 Impact of FLAP inhibitors on leukocyte differentiation of mouse ES cells. EBs were treated with the FLAP inhibitors AM643 (5 μM) or REV5901 (2 μM) from day 3 to day 14 and plated on cover slips from day 4 to day 14 of differentiation. EBs were then harvested to measure the expression of CD18, CD45, and CD68. A) The representative images showed that the number of cells positive for the cell markers CD18, CD45, and CD68 were decreased after treatment of EBs with AM643 and REV5901 compared to the untreated control. B) The bar charts and statistic results showed that EBs treated with AM643 or REV5901 contained significantly decreased numbers of CD18, CD45, and CD68-positive cells in comparison to the untreated control (n = 4). $^{*}p < 0.05$ compared to the untreated control. The scale bar represents 20 μm.

5.10 Influence of AA on intracellular calcium concentration and intracellular calcium-regulated vasculogenesis of mouse ES cells

Previous studies have suggested that changes of the intracellular calcium concentration are important for cellular communication and are initiated by several cellular interactions (192). Additionally, intracellular calcium has been proposed to enhance the secretion of LTs (132). Therefore, we evaluated whether the intracellular calcium concentration is regulated by AA, and whether changes of the intracellular calcium concentration are involved in AA-regulated vasculogenesis.

5.10.1 Impact of AA on intracellular calcium concentration

In order to investigate the effect of AA on the intracellular calcium concentration, we assessed intracellular calcium upon AA treatment at different times of cell culture. ES cells were induced for differentiation until one day before intracellular calcium measurement. On the previous day, EBs were dissociated into single cells and plated onto cover slips. On the day of calcium measurement, plated cells were incubated with 10 μM fluo-4, AM with or without BAPTA-AM (10 μM) in serum free-medium for 30 min and then switched back to normal fresh medium. The intracellular calcium concentration following treatment with AA (100 μM) was measured on day 2, day 4, day 6 and day 8 of differentiation.

The data showed that the percentage of cells displaying intracellular calcium transients upon treatment with AA increased from day 2 (approximately 10%) to day 4 (approximately 14%) and decreased at later stages of differentiation (Figure 5.20). To investigate whether calcium from intracellular stores is involved in the AA-mediated calcium response, cells were pre-incubated for 30 min with the intracellular calcium chelator BAPTA-AM. As shown in figure 5.21 this treatment completely abolished the calcium signal elicited by AA, indicating calcium release from intracellular stores.

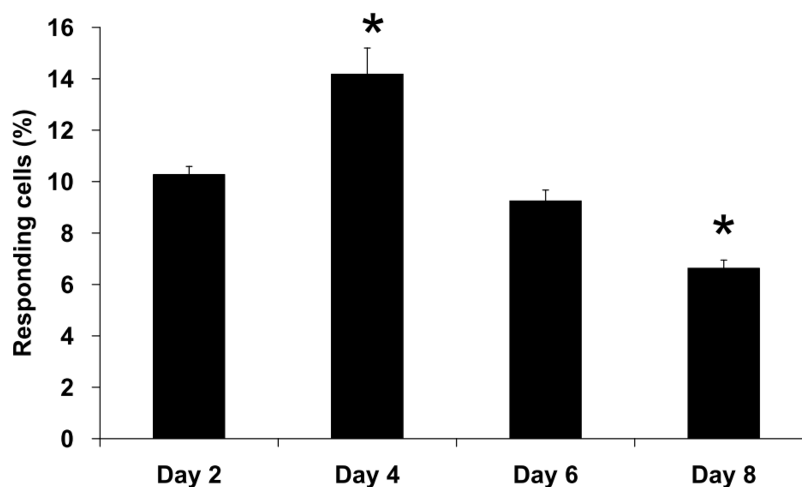
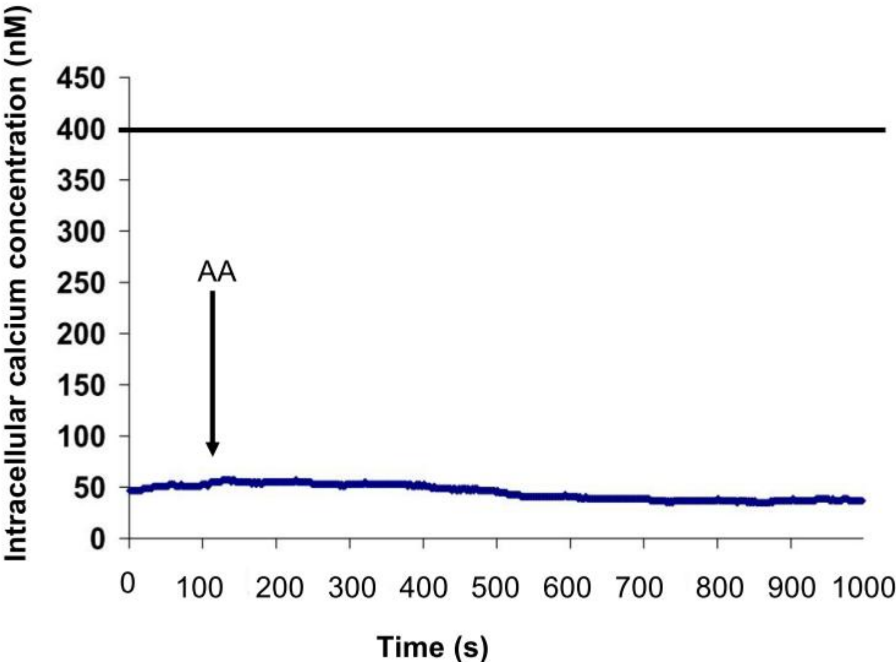


Figure 5.20 Number of cells showing calcium signals upon treatment with AA. Intracellular calcium changes upon treatment with AA were assessed on day 2, day4, day 6 and day 8 of differentiation. Single cells isolated from EBs were loaded for 30 min with fluo-4, AM (10 μM). AA treatment elicited calcium responses in approximately 10 % of positive cells on day 2 and 14 % on day 4. During subsequent days the percentage of responding cells decreased to approximately 7% on day 8 (n = 3). *$p < 0.05$ compared to intracellular calcium concentration on day 2 of differentiation.

A)



B)

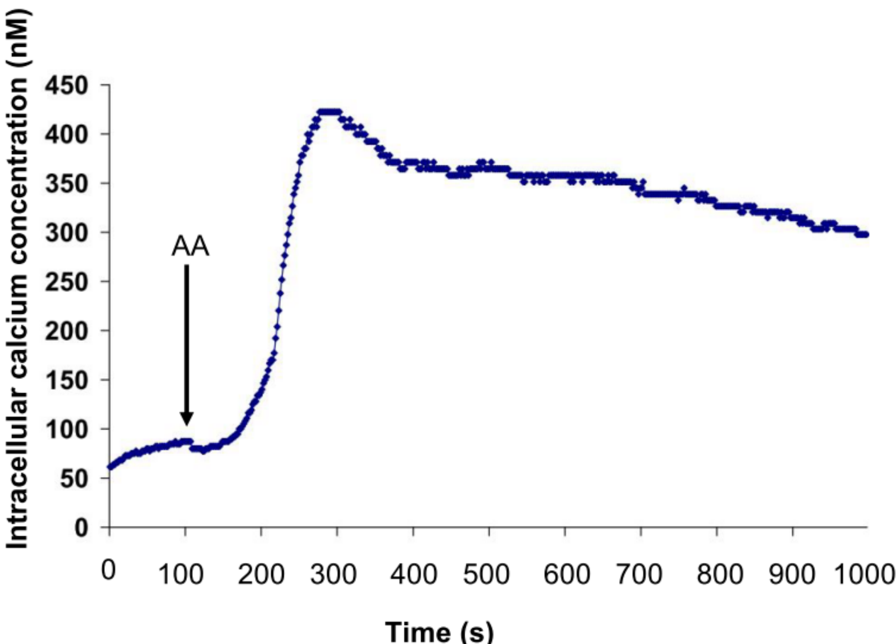
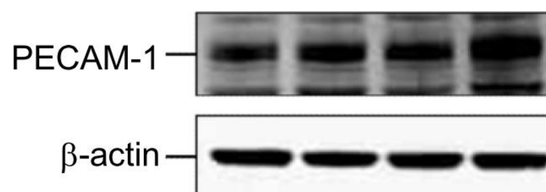


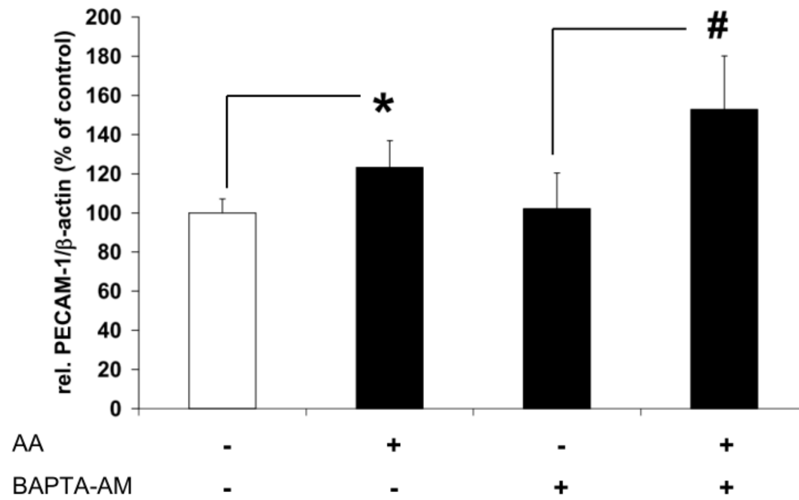
Figure 5.21 Representative tracings of intracellular calcium responses upon treatment with AA. Isolated single cells were plated on cover slips on day 3. On day 4, cells were pretreated for 30 min with fluo-4, AM (10 μM) either in the presence (A) or absence (B) of BAPTA-AM (10 μM). Subsequently, the cells were switched back to fresh medium and intracellular calcium was recorded. A) In the presence of BAPTA-AM, AA (100 μM) failed to increase intracellular calcium concentration. B) In the absence of BAPTA-AM, AA transiently increased intracellular calcium concentration in single cells isolated from EBs. (n = 3)

5.10.2 Effect of intracellular calcium on PECAM-1 and VE-cadherin expression

The data of the previous experiment (5.10.1) demonstrated that AA raised the intracellular calcium concentration in differentiating ES cells. To investigate whether these changes in intracellular calcium are necessary for the stimulation of vasculogenesis achieved by AA, we cultivated EBs from day 3 to day 10 of differentiation with AA (100 μM) either in the presence or absence of BAPTA-AM (10 μM). On day 10, EBs were collected and the expression of PECAM-1 and VE-cadherin was analyzed by western blot technique. It was evident that BAPTA-AM did not inhibit the increase of PECAM-1 and VE-cadherin expression achieved upon treatment with AA alone, suggesting that the intracellular calcium response was not required for the stimulation of vasculogenesis upon AA treatment (Figure 5.22).

A)





B)

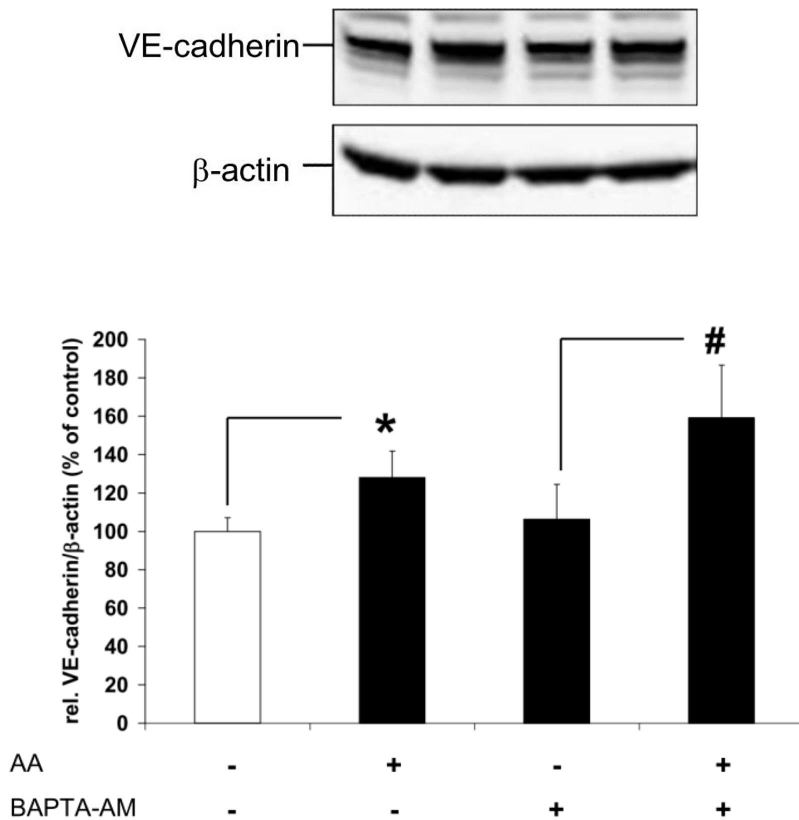


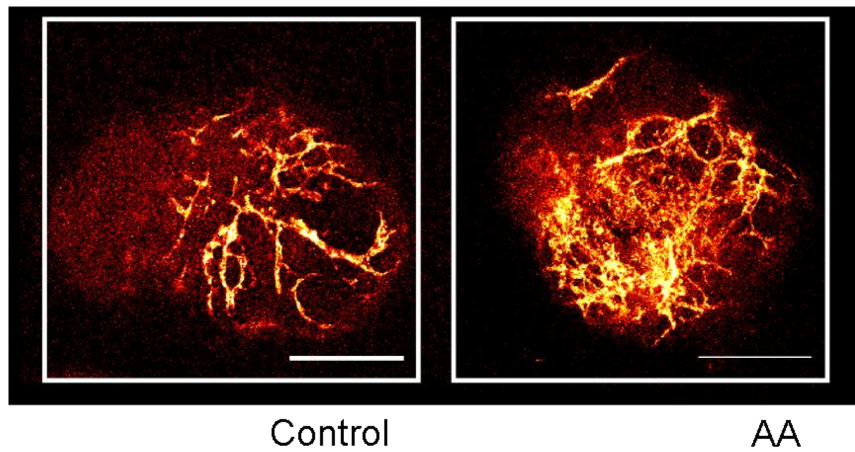
Figure 5.22 Effect of intracellular calcium on PECAM-1 and VE-cadherin expression. EBs were treated with BAPTA-AM (10 μM), AA (100 μM) or pre-treated with BAPTA-AM (10 μM) for 1 h and then AA (100 μM) from day 3 to day 10 of differentiation. A) The representative western blots showed that PECAM-1 expression in AA-treated EBs was increased in comparison to the untreated control. PECAM-1 expression in BAPTA-AM-treated EBs was not changed compared to the untreated

control. In the presence of BAPTA-AM AA still increased PECAM-1 expression. The bar charts demonstrated that the PECAM-1 expression of the AA-treated EBs were significant increased. In AA and BAPTA-AM co-treated EBs PECAM-1 expression remained elevated (n = 4). B) The representative western blots and bar charts showed VE-cadherin expression in EBs treated with AA was increased in comparison to the untreated control. BAPTA-AM treatment had no obvious effect on VE-cadherin expression in EBs in comparison to the untreated control. VE-cadherin expression of EBs pre-treated with BAPTA-AM and then AA was increased compared to BAPTA-AM-treated EBs but no significant change in comparison to the AA-treated EBs was observed. The bar charts suggested that the VE-cadherin expression of the AA-treated EBs was significantly increased. In AA and BAPTA-AM co-treated EBs still significant increased VE-cadherin expression was observed (n = 4). *$p < 0.05$ compared to the untreated control. #$p < 0.05$ compared to BAPTA-AM treated EBs.

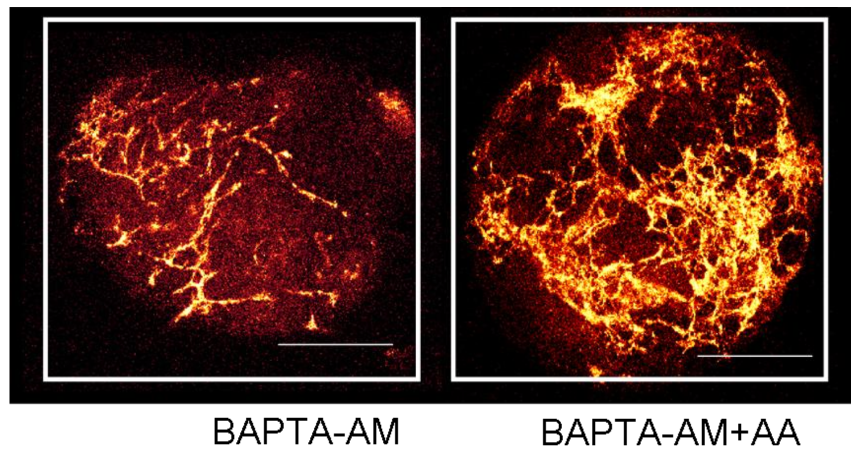
5.10.3 Impact of calcium for AA-mediated vasculogenesis

To further clarify whether the change of intracellular calcium concentration upon AA treatment affects ES cell vascular differentiation, branching points of EBs were examined. Consistent with result 5.10.2 branching points of BAPTA-AM-treated EBs remained unchanged compared to the untreated control. When EBs were pre-treated with BAPTA-AM and then incubated with AA an increased number of branching points compared to BAPTA-AM-treated EBs (Figure 5.23) was observed, suggesting that the calcium response elicited by AA was not necessary for the stimulation of vasculogenesis.

Aa)



Ab)



B)

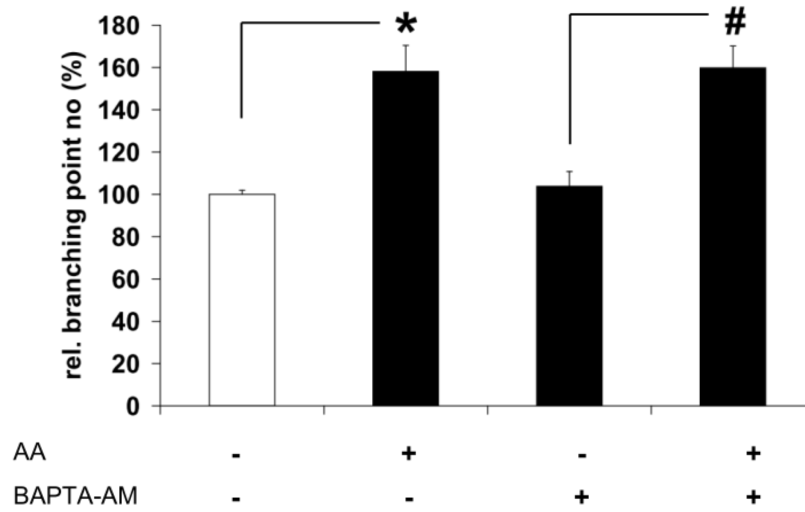


Figure 5.23 Impact of calcium for AA-mediated vasculogenesis. In order to investigate whether intracellular calcium changes elicited upon AA treatment are involved in vasculogenesis, EBs were treated with AA (100 μM), BAPTA-AM (10 μM) or pre-treated with BAPTA-AM (10 μM) for 1 h and then AA (100 μM) from day 3 to day 10 of differentiation. Whole EBs were collected for branching point analysis. Aa) The representative images showed that branching points of EBs were increased in comparison to the untreated control. Ab) The number of branching points of EBs treated with BAPTA-AM did not significantly change compared to the untreated control. Branching points of EBs pre-treated with BAPTA-AM and then AA were increased compared to BAPTA-AM-treated EBs. B) The bar charts showed the significantly up-regulated branching points in AA-treated EBs compared to the untreated control. In BAPTA-AM co-treated EBs the number of branching points was still significantly increased in comparison to EBs treated with BAPTA-AM alone. The upper panel shows representative images of EBs labeled with anti PECAM-1 antibody. The scale bar represents 300 μm. The bar chart represents mean ± SD of 3 separate experiments in which at least 30 individual EBs were analyzed. *$p < 0.05$ compared to the untreated control. #$p < 0.05$ compared to BAPTA-AM treated EBs.

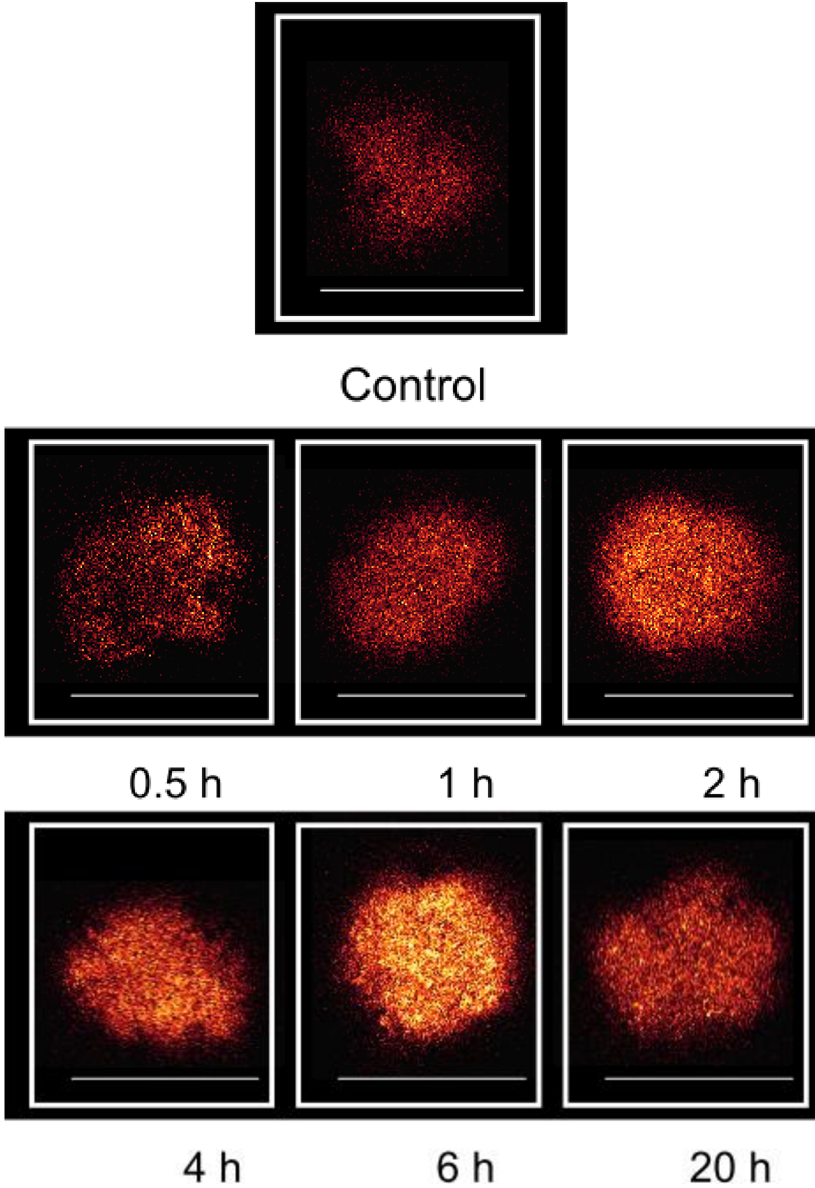
5.11 Role of ROS in AA-induced vasculogenesis of mouse ES cells

ROS arising in inflammatory processes can stimulate cell proliferation and differentiation (143). In the LT signaling pathway, AA and its metabolites have been shown to play a role as ROS inducers (152). Hence, we supposed that ROS may be involved in AA-induced vasculogenesis. To understand whether ROS participate in vasculogenesis stimulated by AA, the free-radical scavengers VAS2870, NMPG, and Trolox were used to inhibit ROS production during ES cell differentiation. First, the ability of AA to induce ROS production was examined. EBs were treated with AA (100 μM) on day 4 for 0.5 h, 1 h, 2 h, 4 h, 6 h or 20 h and ROS production was measured by confocal laser microscopy (Leica LCSM TCS-SP2). Second, the influence of AA-induced ROS production on vasculogenesis was investigated by applying free-radical scavengers to differentiated EBs before assessing the branching points.

5.11.1 AA induces ROS production in a time-dependent manner

The representative images showed that ROS production was induced by AA in a time-dependent manner (Figure 5.24). Compared to the untreated control, ROS production in EBs treated with AA was significant increased following 2 h, 4 h and 6 h post-AA treatment and remained on an elevated level until 20 h of incubation with AA.

A)



B)

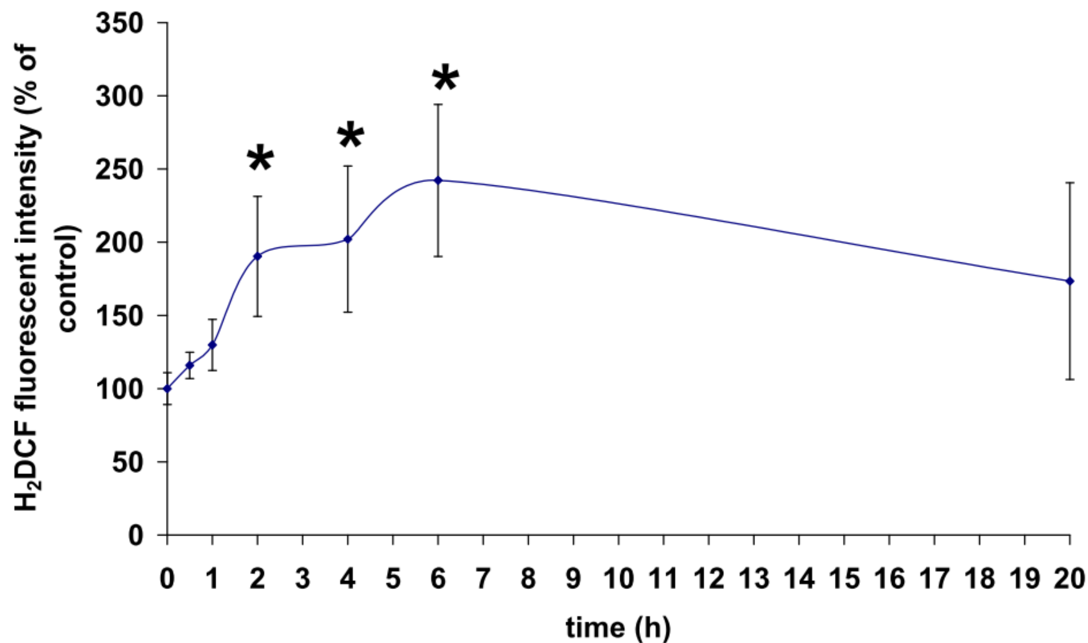


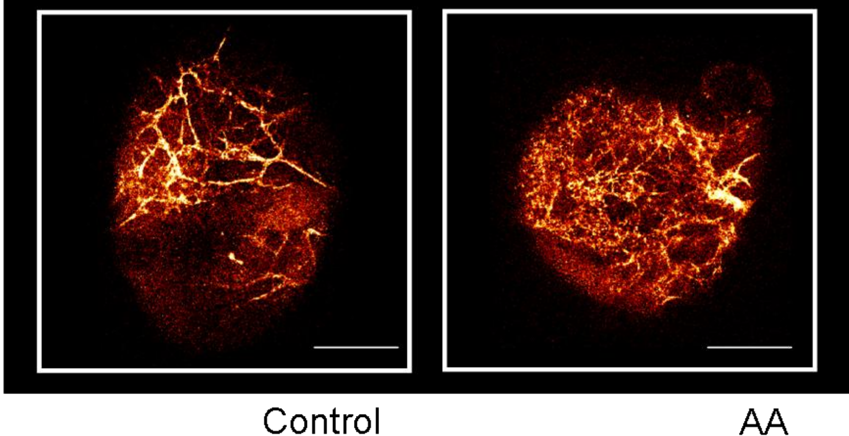
Figure 5.24 AA induces ROS production in a time-dependent manner. On day 4 of differentiation, EBs were treated with AA (100 μM) for 0.5 h, 1 h, 2 h, 4 h, 6 h and 20 h, and then the ROS fluorescence indicator H_2DCF-DA was applied for 30 min. A) Representative images showed that ROS production was increased upon treatment with AA in a time-dependent manner. B) The statistic analysis indicated that ROS production increased significantly after AA treatment for 2, 4, and 6 h and remained on an elevated plateau after 20 h of AA treatment in comparison to the untreated control. The scale bar represents 300 μm (n = 3). $^*p < 0.05$ compared to the untreated control.

5.11.2 Effect of AA-induced ROS production on vasculogenesis

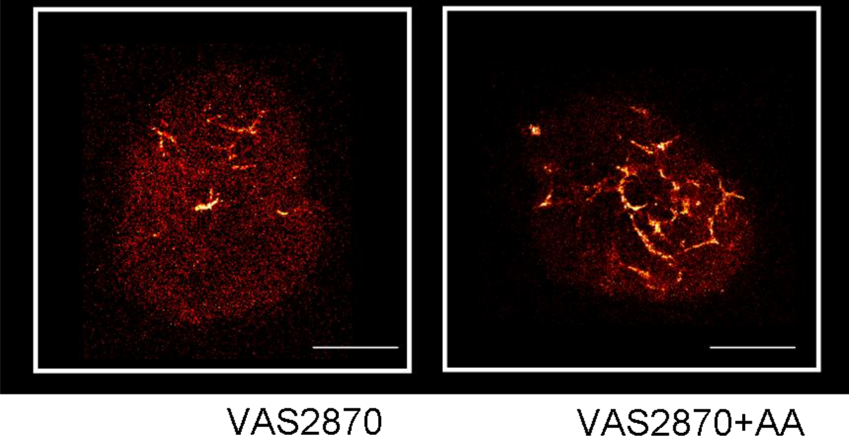
Based on the result above, we provided evidence that AA is able to induce ROS production in a time-dependent manner. To further understand whether AA-induced ROS production also participates in vasculogenesis, whole EBs were collected on day 10 of differentiation and branching points were examined. On day 3, EBs were treated with either AA (100 μM) or two different ROS scavengers (NMPG (100 μM) and Trolox (100 μM)) or the NADPH oxidase inhibitor VAS2870 (50 μM), respectively, or a combination of ROS scavengers, NADPH oxidase inhibitor and AA until day 10 of differentiation. As shown in figure 5.25, the branching points of EBs treated with ROS

scavengers together with AA were significantly decreased in comparison to that of AA-treated EBs (Figure 5.25). Comparable results were obtained upon treatment with VAS2870. Notably, ROS scavengers as well as VAS2870 significantly decreased branching points already in the absence of AA. Taken together, ROS can be produced in response to AA treatment and AA-induced ROS generation is involved in vasculogenesis.

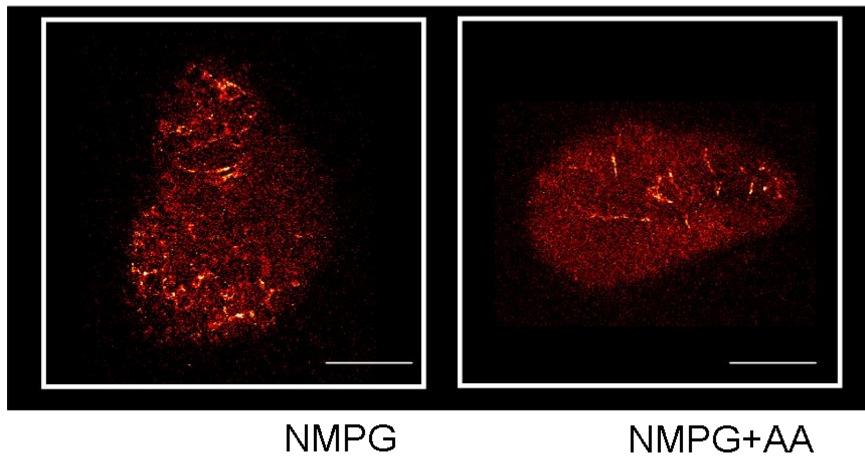
Aa)



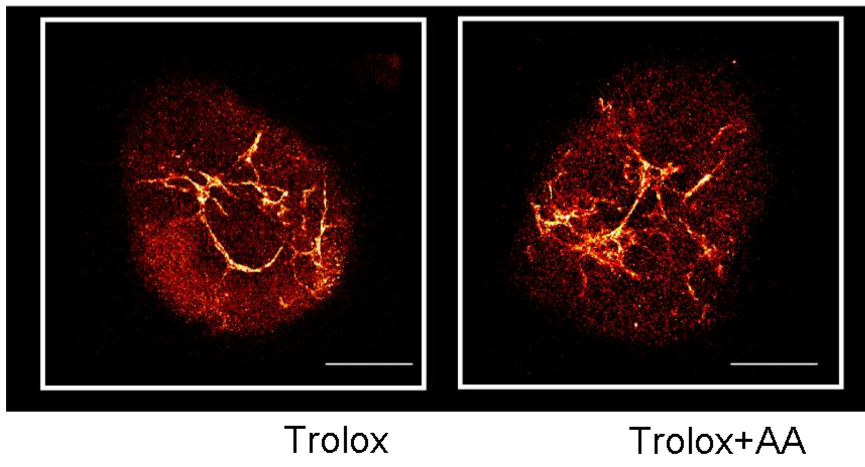
Ab)



Ac)



Ad)



B)

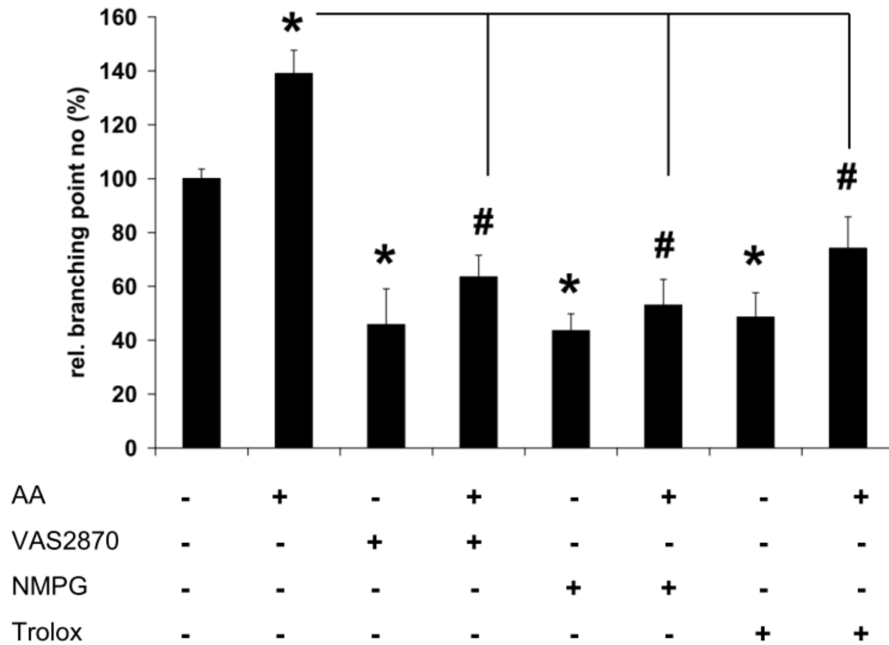


Figure 5.25 Effect of AA-induced ROS production on vasculogenesis. EBs were treated from day 3 to day 10 of differentiation with AA (100 μM) either in presence or absence of ROS scavengers (NMPG (100 μM) and Trolox (100 μM)) or the NADPH oxidase inhibitor VAS2870 (50 μM). The branching points of vascular structures were examined on day 10. A) The representative images showed that branching points of AA-treated EBs were significantly increased, while branching points of ROS scavenger and NADPH oxidase inhibitor treated EBs were decreased compared to the untreated control. B) The bar charts showed the significantly decreased branching points in NMPG, Trolox or NADPH-treated EBs compared to the untreated control. The branching points in NMPG, Trolox or the NADPH-co-treated EBs were significant reduced in comparison to the AA alone. The upper panel shows representative images of EBs labeled with anti PECAM-1 antibody. The scale bar represents 300 μm. The bar chart represents mean ± SD of 3 separate experiments in which at least 30 individual EBs were analyzed (n = 3). *$p < 0.05$ compared to the untreated control. #$p < 0.05$ compared to AA treated cells.

6. Discussion

Blood vessel formation including vasculogenesis and angiogenesis is the most necessary event during embryo development for maintaining homeostasis and survival. In addition, blood vessel formation is associated with inflammation in wound healing and tumor growth (154). Inflammation, which can be divided into acute inflammation and chronic inflammation, is not only a physiological but also a pathological response to injury and pathogen infections to cells and tissues inside the body. During inflammation immune cells such as leukocytes are recruited to the inflamed tissues for pathogen elimination (109). Short period acute inflammation is characterized by leukocyte accumulation in the injury region, removal of the inflammatory stimulus and tissue repair, while chronic inflammation coincides with continuing and long-lasting inflammation and tissue destruction before tissue repair can occur (193). LTs, one class of inflammatory mediators, have been previously reported to regulate inflammation by enhancing protein production and leukocyte accumulation, promoting permeability of vessels and smooth muscle constriction (194; 195). Moreover, novel studies have pointed out that LTs also play key roles in vasculogenesis. Previously it has been indicated that the receptors of LTB_4, BLT1 and BLT2, are involved in regulating endothelial cell proliferation, differentiation and migration (56; 57). However, more evidence is required to confirm the effect of LTs to modulate vasculogenesis and leukocyte differentiation. Therefore, the specific aim of this thesis was to investigate whether the AA/LT signaling pathway could contribute to vasculogenesis and leukocyte differentiation.

Based on our result (5.1, 5.2, 5.8 and 5.9) the AA/LT signaling pathway indeed plays a role in both vasculogenesis and leukocyte differentiation. Result 5.3 indicated that AA-induced vasculogenesis is FLAP-dependent. Result 5.4, 5.5 and 5.6 provided

evidence that LTB_4, 12(S)-HETE, LTD_4, and their corresponding receptors participate in vasculogenesis. Last but not the least, as common intracellular signaling factors, ROS but not intracellular calcium involve in the stimulation of vasculogenesis achieved by AA. Taken together, our findings suggest that the AA/LT signaling pathway contributes to vasculogenesis and leukocyte differentiation from ES cells.

Regulation of vasculogenesis by AA

AA can be synthesized by human cells or taken-up from diet. Imbalanced AA levels are found to be related with the occurrence of Alzheimer's disease, coronary artery disease and chronic inflammation (196; 197). According to previous studies, there are three main pathways of AA metabolism: the LO pathway, the COX pathway and the cytochrome P450 pathway. In the LO pathway, 5, 12 and 15 HPETE and their metabolites are produced, while in the COX pathway, PGH_2 and its metabolites are synthesized. In the cytochrome P450 pathway, four epoxyeicosatrienoic acids (EETs), including 5,6-, 8,9-, 11,12-, and 14,15-EETs, are generated (198). Due to our specific interest on the regulation of vasculogenesis and leukocyte differentiation by LTs, we mainly focused on 5-LO signaling pathway in this project.

Previous studies have demonstrated that AA facilitates cell differentiation of adipose tissue, decidual cells and neuronal cells (199-201). However, some of the AA-dependent cell commitments are mediated through other AA metabolites such as 15-LO (202). To date, the impact of LTs on regulating vascular formation is still poorly understood. Hence in this study we analyzed how LTs modulate vasculogenesis by examining the influence of LT receptor inhibition on LT signaling pathways in order to clarify which signaling cascade plays a role in vasculogenesis. Before further investigation with specific AA metabolites we firstly confirmed with exogenous AA

treatment that AA successfully stimulated vasculogenesis and the expression of PECAM-1 and VE-cadherin (results 5.1).

Selection of FLAP inhibitors

5-LO is the enzyme initiating the AA/LT signaling pathway in bone marrow (203). Additionally, FLAP is the key player in AA to 5-LO conversion and enhances the 5-LO signaling pathway. There are several kinds of FLAP inhibitors each of which has its own distinguishing feature. The most common inhibitor of FLAP is MK886. However, MK886 is also an antagonist of PPARα, which is one of the receptors of LTB_4 (204). To prevent cross-interference and to obtain better specificity, we used AM643 and REV5901 as FLAP inhibitors. AM643 directly and specifically inhibits FLAP expression. REV5901 is a FLAP inhibitor as well as inhibitor of CysLTs receptors, 5-LO and LTs (205). In result 5.2, we found that FLAP inhibitors did not fully suppress vasculogenesis. We speculated that this might be due to the complex and complementary regulatory networks of vasculogenesis, among which the FLAP-regulated AA/LT signaling pathway plays a partial but still important role.

Regulation of vasculogenesis by FLAP

LTs in the cells are converted from membrane-released AA, mainly from pulmonary and vascular tissues (206). LT formation requires FLAP for enhancing AA binding to 5-LO. Due to the significance of AA under physiological and pathological conditions, dysfunction of FLAP is related to Alzheimer’s disease, allergy, metabolic syndromes, respiratory and cardiovascular disease (207-210).

In this project, we used FLAP inhibitors to examine the effect of FLAP on the LT signaling pathway and on the pathway mediating vasculogenesis. Result 5.2 showed

that blocking FLAP expression not only suppressed vasculogenesis, but also down-regulated the expression of the endothelial markers PECAM-1 and VE-cadherin. Additionally, result 5.3 proved that the stimulation of vasculogenesis by AA is dependent on the FLAP-enhanced AA/LT signaling pathway. However, FLAP inhibition could not fully abolish AA-regulated vasculogenesis. This may be due to the fact that FLAP is required for LT synthesis from endogenous AA but it is not essential for LT synthesis from exogenous AA (211). When cells encounter FLAP deficiency, the endogenous AA conversion is stopped, yet the conversion of exogenous AA from other cells is remained and capable of enhancing vasculogenesis. Exogenous AA, derived from plasma or neighboring cells and endogenous AA are metabolized through two distinct signaling pathways, in which exogenous AA has the preference to produce LTA_4 and then convert to LTC_4 instead of LTB_4 in the case of endogenous AA (212).

LTB_4 and vasculogenesis

Previous studies have shown that LTB_4, one of the LT-derived inflammatory mediators derived from AA, involves in the inflammatory response through promoting trans-endothelial migration of neutrophils *in vitro* (213). *In vivo*, LTB_4 enhances the permeability of blood vessels and triggers neutrophil adhesion and consecutive migration across microvessel walls (214; 215). In addition, LTB_4 has been proven to promote differentiation and proliferation of osteoclasts and neuronal stem cells (216; 217). In our study, we deciphered whether LTB_4 takes part in vasculogenesis of ES cells. Thus inhibitors of LTB_4 receptors, U75302 and LY255283, were separately applied. We observed that LTB_4 restored the level of vasculogenesis after FLAP inhibition (result 5.4.1 and 5.4.4). Regarding endothelial markers, LTB_4 also restored the expression of both PECAM-1 and VE-cadherin, which were decreased by FLAP inhibitors. Additionally, in result 5.5 both U75302 and LY255283 inhibitors down-regulated vascular structure

formation, which suggests that LTB_4 is pivotal for this process. Since another ligand of BLT2 receptor, 12(S)-HETE, is from another signaling pathway of AA and is as inducible as LTB_4 by VEGF, we postulated that 12(S)-HETE would exert similar effect as LTB_4 in mediating vasculogenesis (218). We found that 12(S)-HETE partially restored vasculogenesis after FLAP inhibition, but without reaching statically significance (result 5.4.4). This might result from that the low stability of 12(S)-HETE in the cell culture medium. In addition, Klampfl T, *et al.* previous demonstrated that 12(S)-HETE inhibition can promote clonogenicity, which implied that 12(S)-HETE might have negative effect on cell proliferation (219).

CysLTs and vasculogenesis

CysLTs include LTC_4, LTD_4 and LTE_4, which contain cysteine and are able to induce inflammatory reactions. LTD_4 and LTE_4 are metabolites of LTC_4, which is highly expressed in differentiated human U937 monoblast leukemia cells incubated with dimethyl sulfoxide (220). While LTD_4 has been known to be required for granulocytic differentiation, the influence of LTE_4 on cell differentiation is still unknown (221). CysLTs can bind to at least three different types of receptors including CysLT1 and CysLT2, both of them have high affinity for LTD_4. To investigate whether CysLTs play roles in vasculogenesis we treated differentiating ES cells with exogenous LTD_4. We observed that the inhibitory effect of AM643 on PECAM-1 and VE-cadherin expression could be restored by exogenous LTD_4 in the EBs. This was not the case when cells were treated with REV5901 since REV5901 is also an antagonist of CysLTs receptors.

BAYu9773, a specific antagonist of CysLT2 was used to further analyze the impact of LTD_4 on vasculogenesis. BAYu9773 decreased expression of endothelial markers in

comparison to untreated control cells. This may indicate that CysLTs especially LTD_4 take part in AA-induced vasculogenesis.

Combination of three LT receptor inhibitors

To underscore that LTs regulate vasculogenesis, we combined the use of three inhibitors, U75302, LY255283 and BAYu9773. Combined treatment of all three inhibitors of LT receptors inhibited AA-induced vasculogenesis and the expression of PECAM-1, VE-cadherin and Flk-1 stronger than each inhibitor alone. This corroborated our finding that LTs play crucial roles in AA-induced vasculogenesis.

Involvement of LTs in leukocyte differentiation

Inflammation induces leukocytes to proliferate and accumulate for defending the pathogen invasion. Leukocytes belong to the cellular immune system which protects the body from foreign pathogens and microbes. The functions of neutrophils, lymphocytes and monocytes all require the initiation of leukocyte adhesion and migration through the blood vessel wall (222; 223). Leukocyte adhesion is also one of the typical inflammatory features. LTs are converted from acute-inflammation-triggered AA release to regulate subsequent inflammatory responses by enhancing leukocyte accumulation. For characterizing the effect of LTs on leukocyte differentiation, we examined the expressions of CD18, CD45 and CD68 as indicators of leukocyte differentiation under conditions of exogenous AA treatment and FLAP inhibition. By detecting the levels of CD18, CD45, and CD68, we found that AA treatment stimulated leukocyte differentiation (result 5.8). On the contrary, FLAP inhibition decreased leukocyte differentiation (result 5.9). These results indicate that AA/LT signaling pathway involves in leukocyte commitment from ES cells.

Intracellular calcium changes are not required for AA induced vasculogenesis

Calcium is essential in human body and acts as an intracellular signaling messenger to promote muscle contraction, bone formation and hormone release. Extracellular calcium binds to calcium receptors to initiate the differentiation of human keratinocytes and HL-60 cells (224-227). Moreover, intracellular calcium plays a central role in enhancing the process of AA-induced breast tumour cell proliferation and tubulogenesis (228). Since we observed a transient increase of intracellular calcium in a subpopulation of differentiating ES cells we investigated whether intracellular calcium changes would underlie the stimulation of vasculogenesis observed upon AA treatment. We therefore treated differentiating ES cells in presence of the calcium chelator BAPTA-AM and assessed endothelial cell marker expression and capillary branching structures. However, we observed that under these conditions AA still stimulated vasculogenesis which precludes that calcium plays a role in this process. On the other hand, calcium has been shown to be involved in LT synthesis. Extracellular calcium can promote AA conversion and LT release in macrophages and neutrophils (132). Intracellular calcium also promotes LTB_4 synthesis, but the optimal intracellular calcium concentrations to enhance LT synthesis are between 150 nM and 350 nM (229). In our experiments intracellular calcium concentrations upon treatment with BAPTA-AM amounted to about 50 nM, while exogenous AA increased the intracellular calcium concentration to more than 400 nM, which is not within the optimal range for promoting LTB_4 synthesis. Furthermore, LTB_4 synthesis from high concentrations of exogenous AA is independent of intracellular calcium levels since AA can be induced even in the absence of intracellular calcium (230; 231).

Involvement of ROS in vasculogenesis stimulated by AA

ROS arise from daily oxygen metabolism within the mitochondrial respiratory chain,

especially complex I and III, which generate ROS during electron transfer (232). Moreover, there are other enzymes within mitochondria including monoamine oxidase (MAO) and glycerol-3-phosphate dehydrogenase (GPDH) that can produce ROS (233). Several antioxidants such as vitamin C and E act as ROS scavengers to prevent cell damage from ROS (234). In the stem cell niche of the bone marrow, a low-oxygen environment causes stem cells to escape from ROS effects and maintain self-renewal ability (235). However, AA may destroy the function of mitochondria and increase ROS production (236). By interfering with the formation of cytochrome c in mitochondria, AA stimulates superoxide anion generation and more ROS release (237). Consistent with previous findings, we also demonstrated that AA treatment in differentiating ES cells raised intracellular ROS levels. Previous studies have shown that up-regulated NADPH oxidases and ROS promote cardiomyogenesis of mouse ES cells (238; 239). ROS were also reported to participate in Wnt/β-catenin and metabotropic glutamate receptor 5-mediated cardiomyogenesis (240; 241). Physiologically, low concentration of ROS stimulated proliferation, migration and differentiation as well as vasculogenesis of ES cells (242; 243). Pathologically, excess ROS generation reduces vasculogenesis by triggering apoptosis and aging of ES cells. Moreover oxidative stress is known to promote cardiovascular disorder development especially in the heart (242; 244; 245). To unravel the potential impact of ROS for vasculogenesis the capability of AA-induced ROS to regulate vasculogenesis was examined. Differentiating ES cells were co-treated with AA and either free radical scavengers (trolox or NMPG) or the NADPH oxidase inhibitor VAS2870. All these treatments successfully prevented AA-induced vasculogenesis in our study. This result implies that ROS participate in AA-mediated vasculogenesis.

In conclusion, our study uncovers mechanisms underlying the effects of

AA-derived LT synthesis on vasculogenesis and leukocyte differentiation of ES cells as well as AA-increased ROS production on vasculogenesis. In our study, AA up-regulates vasculogenesis and leukocyte differentiation. Inhibition of FLAP, the pivotal enhancer of AA signaling pathway, down-regulates vasculogenesis and leukocyte differentiation. Furthermore, AA-derived LTs increase vasculogenesis. ROS production can be induced by the treatment of AA and then up-regulates vasculogenesis. However, intracellular calcium does not participate in the AA-induced vasculogenesis. Our study provides a novel view of AA and LT-regulated signaling on vasculogenesis of ES cells. Furthermore our data shed light on the mechanism of blood vessel formation and leukocytogenesis under pathological conditions of inflammation and wound healing.

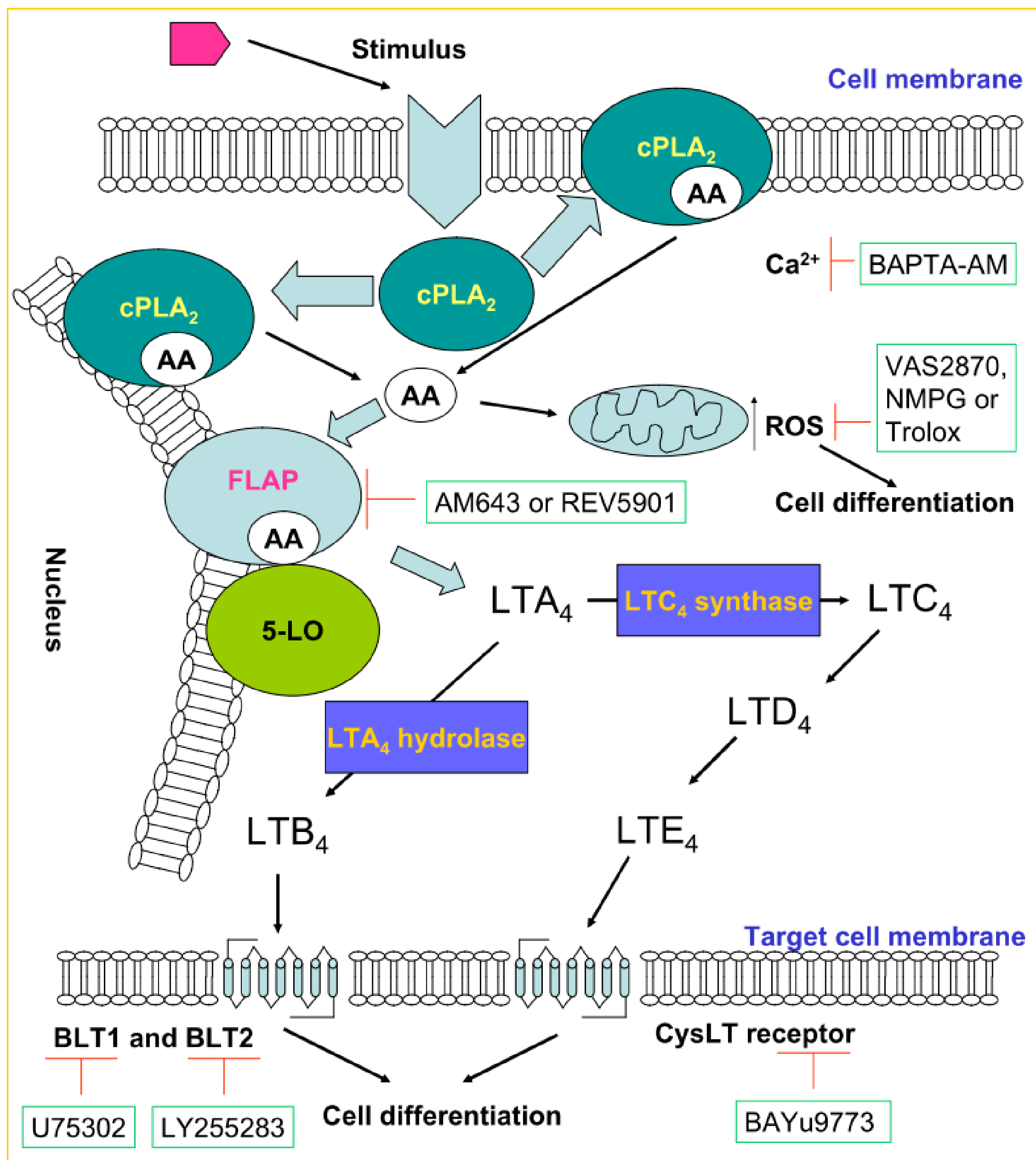


Figure 6.1 Scheme of AA-mediated LT generation: After stimulus, AA is released from nuclear membranes and combined with FLAP and 5-LO to convert into LTA_4. LTA_4 is then converted to either LTB_4 by LTA_4 hydrolase or LTC_4 by LTC_4 synthase. After binding to their correspondent receptors, LTB_4 and LTC_4-derived metabolites regulate vasculogenesis or leukocyte differentiation. Moreover, ROS, the by-product of the AA/LT signaling pathway also takes part in promoting AA-induced vasculogenesis.

7. Summary

Previous studies have shown the close relation between inflammation and vascular structure formation (155). LTs are important mediators of inflammation and are derived from cell membrane released-AA after the cells are stimulated by inflammatory stress or other factors. To shed light on whether LTs involve in vasculogenesis and leukocyte differentiation, pluripotent mouse ES cells were used as in vitro model. ES cells can be induced to differentiate to several types of cells including endothelial cells and leukocytes.

The following experimental results were achieved in the present study:

1) Exogenous addition of AA to EBs from differentiating ES cells dose-dependent stimulated vascular structure formation and increased the expression of the vascular markers PECAM-1 and VE-cadherin. Furthermore a stimulation of leukopoiesis was observed.

2) Inhibition of FLAP which is a key enzyme in LT synthesis with either AM643 or REV5901 significantly downregulated vascular structure formation as well as expression of endothelial cell markers. Furthermore FLAP inhibition abolished leukopoiesis achieved upon treatment with AA.

3) Exogenous addition of AA metabolites, i.e. LTB_4 and LTD_4 and 12(S)-HETE partially restored vasculogenesis inhibited by the FLAP inhibitors AM643 and REV5901.The LTB_4 receptor antagonist U75302 and the BLT2 receptor antagonist LY255283 inhibited vasculogenesis and the expression of PECAM-1, VE-cadherin and Flk-1.

Comparable effects were achieved using the CysLTs receptor inhibitor BAYu9773. Combined treatment of U75302, LY255283 and BAYu9773 exerted additive effects on the inhibition of vasculogenesis and the expression of PECAM-1, VE-cadherin and Flk-1.

4) AA treatment of differentiating ES cells elicited a transient calcium response in a subpopulation of cells. The intracellular calcium response was not related to the AA-induced vasculogenic process since chelation of intracellular calcium by BAPTA-AM did not abolish vascular structure formation.

5) AA treatment of differentiating ES cells within EB increased intracellular ROS. Co-incubation with either free radical scavengers or the NADPH oxidase inhibitor VAS2870 abolished ROS generation as well as AA-stimulated vasculogenesis.

Conclusion:

AA stimulates vasculogenesis and leukopoiesis of ES cells via activation of LT signalling pathways and elevation of intracellular ROS.

Zusammenfassung

Frühere Studien haben eine enge Beziehung zwischen entzündlichen Prozessen und dem Wachstum vaskulärer Strukturen aufgezeigt. Leukotriene (LT) sind wichtige Entzündungsmediatoren und werden aus Arachidonsäure (AA) nach deren Freisetzung aus der Membran durch inflammatorischen Stress und andere Faktoren gebildet. Um zu erhellen, ob LTs Prozesse der Vaskulogenese und Leukozyten-Differenzierung regulieren, wurden pluripotente embryonale Stammzellen (ES) der Maus als in vitro Modell verwendet. ES Zellen können zur Bildung verschiedener Zelltypen einschließlich endothelialer Zellen und Leukozyten stimuliert werden.

In der vorliegenden Studie wurden die folgenden Ergebnisse erzielt:

1) Exogene Behandlung von Embryonalkörperchen (EBs) aus differenzierenden ES Zellen mit AA stimulierte Dosis-abhängig die Bildung vaskulärer Strukturen und die Expression der endothelialen Marker PECAM-1 und VE-cadherin. Weiterhin wurde eine Stimulation der Leukopoiese beobachtet.

2) Inhibition von 5-Lipoxygenase-aktivierendem Protein (FLAP) eines Schlüsselenzyms der LT Synthese durch entweder AM643 oder REV5901 verhinderte die Bildung vaskulärer Strukturen und die Expression endothelialer Marker. Weiterhin wurde eine Inhibierung der Leukopoiese erzielt.

3) Exogene Zugabe der AA Metabolite LTB_4, LTD_4 und 12(S)-HETE revertierte teilweise den inhibierenden Effekt der FLAP Antagonisten AM643 und REV5901. Der LTB_4 Rezeptor Antagonist U75302 und der BLT2 Rezeptor Antagonist

LY255283 inhibierten sowohl die Bildung vaskulärer Strukturen als auch die Expression der endothelialen Marker PECAM-1, VE-Cadherin und Flk-1. Vergleichbare Effekte wurden für den CysLT Rezeptor Inhibitor BAYu9773 beobachtet. Die kombinierte Behandlung der Zellen mit U75302, LY255283 und BAYu9773 führte zu additiven Effekten bei der Inhibition der Vaskulogenese und der Expressionsverminderung von PECAM-1, VE-Cadherin und Flk-1.

4) Behandlung differenzierender ES Zellen mit AA induzierte in einer Subpopulation der Zellen eine transiente Kalzium-Antwort. Die intrazelluläre Kalzium-Antwort war jedoch nicht mit dem vaskulogenen Effekt von AA korreliert, da Kalzium-Chelierung durch BAPTA-AM die AA-vermittelte Vaskulogenese nicht beeinflusste.

5) Behandlung differenzierender ES Zellen in EBs mit AA führte zu einem Anstieg intrazellulärer Sauerstoff-Intermediate (ROS). Ko-Inkubation mit entweder freien Radikalfängern oder dem NADPH Oxidase Inhibitor VAS2870 inhibierte die ROS Bildung und die AA-vermittelte Vaskulogenese.

Schlussfolgerung:

AA stimuliert die Vaskulogenese und Leukopoiese von ES Zellen. Der unterliegende Signalweg wird durch LTs und einen Anstieg intrazellulärer ROS vermittelt.

8. Abbreviations

5-HPETE	5-hydroperoxyeicosatetraenoic acid
5-LO	5-lipoxygenase
12(S)-HETE	12S-hydroxy-5Z, 8Z, 10E, 14Z- eicosatetraenoic acid
AA	Arachidonic acid
ABCG2	ATP-binding cassette sub-family G member 2
AFP	α-fetoprotein
AM643	3-{3-tert-Butylsulfanyl-1-[4-(5-methoxy-pyrimidin-2-yl)-benzyl]-5-(5-methyl-pyridin-2-ylmethoxy)-1H-indol-2-yl]-2,2-dimethyl-propionic acid
AP	Alkaline phosphatase
AP1	Activator protein 1
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis
BAYu9773	4-[[(1R,2E,4E,6Z,9Z)-1-[(1S)-4-carboxy-1-hydroxybutyl]-2,4,6,9-pentadecatetraen-1-yl]thio]-Benzoic acid
B cells	B lymphocytes
BLT1, 2	LTB_4 receptor-1 and 2
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CO_2	Carbon dioxide
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
Cy5	Indodicarbocyanine

CysLTs	Cysteinyl leukotrienes
CysLT1-R	CysLT receptor 1
CysLT2-R	CysLT receptor 2
D-LIF	Diffusible form LIF
DCF	Dichlorofluorescein
Dll4	Delta like ligand 4
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulfoxide
EBs	Embryoid bodies
EC cells	Embryonic carcinoma stem cell
ECL	Enhanced chemi-Luminescence
EDTA	Ethylendiamintetraacetat
EETs	Epoxyeicosatrienoic acids
EG cells	Embryonic germ cells
ERK1, 2	Extracellular signal-regulated kinase 1and 2
ES cells	Embryonic stem cells
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FLAP	5-lipoxygenase activating protein
Flk-1	Fetal liver kinase -1
Fluo,4-AM	1-[2-Amino-5-(2,7-difluoro-6-hydroxy-3-oxo-9-xanthenyl) phenoxy]-2-(2-amino-5-methyl-phenoxy) ethane-N,N,N',N'-Tetraessigsäure, pentaacetoxymethyl ester
Foxa2	Forkhead box protein A2

g	Gravity 1*g* = 9,81m/s²
g	Gram
gp130	Glycoprotein 130
GPDH	Glycerol-3-phosphate dehydrogenase
h	Hour
H_2DCF	2',7'-dichlorodihydrofluorescein
H_2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
He	Helium
HETEs	Eicosatetraenoic acids
HSC	Hematopoietic stem cell
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
IMDM	Basal Iscoves modified Dulbecco's medium
Lhx1	LIM homeobox 1
LIF	Leukemia inhibitory factor
LIFRβ	Low-affinity LIF receptor
JAK	Janus kinase
KCl	Potassium chloride
kDa	kilo Dalton
KH_2PO_4	Potassium phosphate monobasic
Klf4	Kruppel-like factor 4
LIF	Leukemia inhibitory factor

LO	Lipoxygenase
LT	Leukotriene
LTA_4	Leukotriene A_4
LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
LTD_4	Leukotriene D_4
LTE_4	Leukotriene E_4
LY255283	1-[5-ethyl-2-hydroxy-4-[[6-methyl-6-(1H-tetrazol-5-yl)heptyl]oxy]phenyl]-ethanone
M	Molar
M-LIF	Matrix–bound form LIF
MAO	Monoamine oxidase
MAP	Mitogen-activated protein
MEM	Minimal essential aminoacids
Mg	Magnesium
mg	Milligramm
min	Minute
ml	Milliliter
µM	Micromolar
µm	Micrometer
mM	Millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
Na_2HPO_4	Sodium hydrogen phosphate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate

Ne	Neon
NEA	Non-essential aminoacids
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nM	Nanomolar
NMPG	N-(2-Mercaptopropionyl)glycin
Oct 4	Octamer-binding transcription factor 4
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline and Triton
PECAM-1	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PGH_2	Prostaglandin H_2
PMNs	Polymorphonuclear neutrophils
PPARα	Peroxisome proliferator-activated receptor alpha
REV5901	6-(6-(3R-hydroxy-1E,5Z-undecadien-1-zl)-2-pyridinyl)-1,5S-hexanediol
ROS	Reactive oxygen species
RT	Room temperatur
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Ser	Serine
SHH	Sonic hedgehog
Sox2	Sex determining region Y-box 2
SRS	Slow reacting substance
SRS-A	Slow reacting substance of anaphylaxis
STAT-3	Signal transducers and activators of transcription 3

SSEA-1	Stage-specific embryonic antigen
TBS	Tris-buffered saline
TBST	Tris-buffered saline and Tween 20
T cells	T lymphocytes
TNFα	Tumor necrosis factor α
Trolox	α-tocopherol (vitamin E) derivative
U75302	6-(6-3R-hydroxy-1E,5Z-undecadien-1-yl)-2-pyridinyl)-1,5S-hexanediol
VAS2870	VAS2870, 3-benzyl-7-(benzoxazolyl)thio- -1,2,3-triazolo[4,5]pyrimidin
VE-Cadherin	Vascular endotheliam cadherin (Syn. CD144)
VEGF	Vascular endothelial growth factor
VEGFR1, 2	Vascular endothelial growth factor receptor-1 and 2

9. List of figures and tables

Figures

Figure 1.1	Development of ES cell.	P2
Figure 1.2	Development of pluripotent stem cells.	P3
Figure 1.3	Cellular metabolism of AA.	P6
Figure 1.4	Notch signaling pathway regulates the differentiation of tip cells and stalk cells.	P14
Figure 2.1	Aim of the study	P19
Figure 4.1	ES cells co-cultured with mitotically inactivated feeder layers.	P34
Figure 4.2	Silicon-coated spinner flask for ES cell differentiation.	P35
Figure 4.3	EBs in suspension at day 3 of cell culture.	P36
Figure 5.1	AA regulates the expression of PECAM-1 in a dose-dependent manner.	P43
Figure 5.2	Influence of AA on the expression of FLAP and VE-cadherin.	P45
Figure 5.3	Evaluation of vasculogenesis upon AA treatment.	P47
Figure 5.4	Evaluation of FLAP expression upon FLAP inhibitor treatment.	P49
Figure 5.5	Effect of FLAP inhibitors on the expression of PECAM-1 and VE-cadherin.	P51
Figure 5.6	Impact of FLAP inhibitors on vasculogenesis.	P53
Figure 5.7	AA regulated vasculogenesis of mouse ES cells is FLAP-dependent.	P55
Figure 5.8	Exogenous LTB_4 restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors.	P58
Figure 5.9	Exogenous 12(S)-HETE restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors.	P60
Figure 5.10	Exogenous LTD_4 restores the decrease of PECAM-1 and VE-cadherin expression upon treatment with FLAP inhibitors.	P62
Figure 5.11	Effects of LTs on vasculogenesis upon treatment with FLAP inhibitors.	P66
Figure 5.12	Impact of LTB_4 receptors for the expression of PECAM-1, VE-cadherin and Flk-1.	P69
Figure 5.13	Effect of LTB_4 receptor inhibition on vascular structures.	P71
Figure 5.14	Effect of BAYu9773 on the expression of PECAM-1, VE-cadherin and Flk-1.	P73
Figure 5.15	Effect of the CysLT receptor inhibitor BAYu9773 on the formation of vascular structures.	P74
Figure 5.16	Effect of combined treatment of EBs with LTB_4 and CysLTs	P76

receptor inhibitors on PECAM-1, VE-cadherin and Flk-1 expression.

Figure 5.17 Effect of combined treatment of EBs with LTB_4 and CysLTs receptor inhibitors on vasculogenesis. P78

Figure 5.18 Effect of AA on leukocyte differentiation of mouse ES cells. P80

Figure 5.19 Impact of FLAP inhibitors on leukocyte differentiation of mouse ES cells. P82

Figure 5.20 Number of cells showing calcium signals upon treatment with AA. P84

Figure 5.21 Representative tracings of intracellular calcium responses upon treatment with AA. P85

Figure 5.22 Effect of intracellular calcium on PECAM-1 and VE-cadherin expression. P86

Figure 5.23 Impact of calcium for AA-mediated vasculogenesis. P89

Figure 5.24 AA induces ROS production in a time-dependent manner. P91

Figure 5.25 Effect of AA-induced ROS production on vasculogenesis. P94

Figure 6.1 Scheme of AA-mediated LT generation: P104

Tables

Table 4.1 Final concentration and source of substrates used in the experiments. P36

10. References

1. Pardanaud L, Altmann C, Kitos P, Dieterlen-Lievre F, Buck C: Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. Development. 100:339-349, 1987

2. Masuda H, Asahara T: Post-natal endothelial progenitor cells for neovascularization in tissue regeneration. Cardiovasc Res. 58:390-398, 2003

3. Adams RH, Alitalo K: Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol. 8:464-478, 2007

4. Risau W: Mechanisms of angiogenesis. Nature. 386:671-674, 1997

5. Balaji S, King A, Crombleholme TM, Keswani SG: The Role of Endothelial Progenitor Cells in Postnatal Vasculogenesis: Implications for Therapeutic Neovascularization and Wound Healing. Adv Wound Care (New Rochelle) 2:283-295, 2013

6. Wong VW, Crawford JD: Vasculogenic cytokines in wound healing. Biomed Res Int, 2013

7. Carmeliet P: Angiogenesis in health and disease. Nat Med. 9:653-660, 2003

8. Carmeliet P, Jain RK: Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nat Rev Drug Discov. 10:417-427, 2011

9. Tuch BE: Stem cells--a clinical update. Aust Fam Physician. 35:719-721, 2006

10. Potten CS, Loeffler M: Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the Crypt. Development 110:1001-1020, 1990

11. Hasegawa K, Namekawa SH, Saga Y: MEK/ERK signaling directly and indirectly contributes to the cyclical self-renewal of spermatogonial stem cells. Stem Cells. 10, 2013

12. Luca GD, Ferretti R, Bruschi M, Mezzaroma E, Caruso M: Cyclin D3 critically regulates the balance between self-renewal and differentiation in skeletal muscle stem

cells. Stem Cells., 2013

13. McDonald J, Liu X, Qu Y, Liu S, Mickey S, Turetsky D, Gottlieb D, Choi D: Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat Med. 5:1410-1412, 1999

14. Johnson M, Ziomek C: Induction of polarity in mouse 8-cell blastomeres: specificity, geometry, and stability. J Cell Biol. 91:303-308, 1981

15. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM: Embryonic stem cell lines derived from human blastocysts. Science 282:1145-1147, 1998

16. Usui J, Kobayashi T, Yamaguchi T, Knisely A, Nishinakamura R, Nakauchi H: Generation of kidney from pluripotent stem cells via blastocyst complementation. Am J Pathol. 180:2417-2426, 2012

17. Boheler KR, Czyz J, Tweedie D, Yang H-T, Anisimov SV, Wobus AM: Differentiation of pluripotent embryonic stem cells Into cardiomyocytes. Circ Res. 91, 2002

18. Weissman IL: Stem cells: units of development, units of regeneration, and units in evolution. Cell 100:157-168, 2000

19. Beltrami A, Cesselli D, Bergamin N, Marcon P, Rigo S, Puppato E, D'Aurizio F, Verardo R, Piazza S, Pignatelli A, Poz A, Baccarani U, Damiani D, R RF, Mariuzzi L, Finato N, Masolini P, Burelli S, Belluzzi O, Schneider C, Beltrami C: Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow). Blood. 110:3438-3446, 2007

20. Stemple D, Anderson D: Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell. 71:973-985, 1992

21. He Z, Hua J, Song Z: Concise Review: Mesenchymal Stem Cells Ameliorate Tissue Injury via Secretion of Tumor Necrosis Factor-α Stimulated Protein/Gene 6. Stem Cells Int. , 2014

22. Saito S, Liu B, Yokoyama K: Animal embryonic stem (ES) cells: self-renewal, pluripotency, transgenesis and nuclear transfer. Hum Cell. 17:107-115, 2004

23. Kanno M, Yazawa T, Kawabe S, Imamichi Y, Usami Y, Ju Y, Matsumura T, Mizutani T, Fujieda S, Miyamoto K: Sex-determining region Y-box 2 and GA-binding proteins regulate the transcription of liver receptor homolog-1 in early embryonic cells. Biochim Biophys Acta. 1839:406-414, 2014

24. Pan G, Thomson J: Nanog and transcriptional networks in embryonic stem cell pluripotency. Cell Res. 17:42-49, 2007

25. Zhang P, Andrianakos R, Yang Y, Liu C, Lu W: Kruppel-like factor 4 (Klf4) prevents embryonic stem (ES) cell differentiation by regulating Nanog gene expression. J Biol Chem. 285:9180-9189, 2010

26. Galic Z, Kitchen S, Kacena A, Subramanian A, Burke B, Cortado R, Zack J: T lineage differentiation from human embryonic stem cells. Proc Natl Acad Sci U S A. 103:11742-11747, 2006

27. Kayama M, Kurokawa M, Ueno H, Suzuki N: Recent advances in corneal regeneration and possible application of embryonic stem cell-derived corneal epithelial cells. Clin Ophthalmol. 1:373-382, 2007

28. Evans M, Kaufman M: Establishment in culture of pluripotential cells from mouse embryos. Nature. 292:154-156, 1981

29. Martin GR: Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 78:7634-7638, 1981

30. Ying Q-L, Nichols J, Chambers I, Smith A: BMP induction of id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115:281-292, 2003

31. Smith AG, Hooper ML: Buffalo rat liver cells produce a diffusible activity which

inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev Biol. 121:1-9, 1987

32. Youngblood B, Alfano R, Pettit S, Zhang D, Dallmann H, Huang N, Macdonald C: Application of recombinant human leukemia inhibitory factor (LIF) produced in rice (Oryza sativa L.) for maintenance of mouse embryonic stem cells. J Biotechnol. , 2014

33. Rajasingh J, Bord E, Hamada H, Lambers E, Qin G, Losordo D, Kishore R: STAT3-dependent mouse embryonic stem cell differentiation into cardiomyocytes: analysis of molecular signaling and therapeutic efficacy of cardiomyocyte precommitted mES transplantation in a mouse model of myocardial infarction. Circ Res. 101:910-918, 2007

34. Matsuda T, Nakamura T, Nakao K, Arai T, Katsuki M, Heike T, Yokota T: STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. EMBO J. 18:4261-4269, 1999

35. Raz R, Lee C, Cannizzaro L, d'Eustachio P, Levy D: Essential role of STAT3 for embryonic stem cell pluripotency. Proc Natl Acad Sci U S A. 96:2846-2851, 1999

36. Bader A, Al-Dubai H, Weitzer G: Leukemia inhibitory factor modulates cardiogenesis in embryoid bodies in opposite fashions. Circ Res. 86:787-794, 2000

37. Keller G, Kennedy M, Papayannopoulou T, Wiles MV: Hematopoietic commitment during embryonic stem cell differentiation in culture. Mol Cell Biol. 13:473-486, 1993

38. Boyer L, Lee T, Cole M, Johnstone S, Levine S, Zucker J, Guenther M, Kumar R, Murray H, Jenner R, Gifford D, Melton D, Jaenisch R, Young R: Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122:947-956, 2005

39. Davidson KC, Adams AM, Goodson JM, McDonald CE, Potter JC, Berndt JD, Biechele TL, Taylor RJ, Moon RT: Wnt/β-catenin signaling promotes differentiation, not self-renewal, of human embryonic stem cells and is repressed by Oct4. Proc Natl Acad Sci U S A. 109:4485-4490, 2012

40. Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeannet J, He X: LDL-receptor-related proteins in Wnt signal transduction. Nature. 407:530-535, 2000

41. Miki T, Yasuda S-y, Kahn M: Wnt/β-catenin Signaling in Embryonic Stem Cell Self-renewal and Somatic Cell Reprogramming. Stem Cell Rev and Rep 7:836-846, 2011

42. Lien W-H, Fuchs E: Wnt some lose some: transcriptional governance of stem cells by Wnt/β-catenin signaling. Genes Dev. 28:1517-1532, 2014

43. Hammarström S: Leukotrienes. Annu Rev Biochem. 52:355-377, 1983

44. Feuerstein G, Hallenbeck J: Leukotrienes in health and disease. FASEB J. 1:186-192, 1987

45. Chwieśko-Minarowska S, Kowal K, Bielecki M, Kowal-Bielecka O: The role of leukotrienes in the pathogenesis of systemic sclerosis. Folia Histochem Cytobiol. 50:180-185, 2012

46. Riccioni G, Back M, Capra V: Leukotrienes and atherosclerosis. Curr Drug Targets. 11:882-887, 2012

47. Magnusson C, Mezhybovska M, Lörinc E, Fernebro E, Nilbert M, Sjölander A: Low expression of CysLT1R and high expression of CysLT2R mediate good prognosis in colorectal cancer. Eur J Cancer. 46:826-835, 2010

48. Beller TC, Friend DS, Maekawa A, Lam BK, Austen KF, Kanaoka Y: Cysteinyl leukotriene 1 receptor controls the severity of chronic pulmonary inflammation and fibrosis. Proc Natl Acad Sci U S A. 101:3047-3052, 2004

49. Okunishi K, Peters-Golden M: Leukotrienes and airway inflammation. Biochim Biophys Acta. 1810:1096-1102, 2011

50. Rådmark O, Werz O, Steinhilber D, Samuelsson B: 5-Lipoxygenase: regulation of expression and enzyme activity. Trends Biochem Sci. 32:332-341, 2007

51. Fukaya T, Gondaira T, Kashiyae Y, Kotani S, Ishikura Y, Fujikawa S, Kiso Y, Sakakibara M: Arachidonic acid preserves hippocampal neuron membrane fluidity in senescent rats. Neurobiol Aging. 28:1179-1186, 2007

52. Camara-Lemarroy CR, Gonzalez-Moreno EI, FJ G-dlG, Fernandez-Garza NE: Arachidonic acid derivatives and their role in peripheral nerve degeneration and regeneration. ScientificWorldJournal. 2012, 2012

53. Brash AR: Arachidonic acid as a bioactive molecule. J Clin Invest 107:1339-1345, 2001

54. Sakai M, Kakutani S, Horikawa C, Tokuda H, Kawashima H, Shibata H, Okubo H, Sasaki S: Arachidonic acid and cancer risk: a systematic review of observational studies. BMC Cancer 12, 2012

55. Chu J, Praticò D: The 5-lipoxygenase as a common pathway for pathological brain and vascular aging. Cardiovasc Psychiatry Neurol. , 2009

56. Finkensieper A, Kieser S, Bekhite MM, Richter M, Mueller JP, Graebner R, Figulla HR, Sauer H, Wartenberg M: The 5-lipoxygenase pathway regulates vasculogenesis in differentiating mouse embryonic stem cells. Cardiovasc Res. 86:37-44, 2010

57. Molin DG, Akker NMvd, Post MJ: 'Lox on neovascularization': leukotrienes as mediators in endothelial biology. Cardiovasc Res. 86:6-8, 2010

58. Tian W, Jiang X, Sung Y, Qian J, Yuan K, Nicolls M: Leukotrienes in pulmonary arterial hypertension. Immunol Res. 58:387-393, 2014

59. Abramovitz M, Wong E, Cox ME, Richardson CD, Li C, Vickers PJ: 5-lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase. Eur J Biochem. 215:105-111, 1993

60. Luo M, Jones SM, Peters-Golden M, Brock TG: Nuclear localization of 5-lipoxygenase as a determinant of leukotriene B4 synthetic capacity. PNAS 100:12165-12170, 2003

61. Gijón MA, Zarini S, Murphy RC: Biosynthesis of eicosanoids and transcellular metabolism of leukotrienes in murine bone marrow cells. J Lipid Res. 48:716-725, 2007

62. Ford-Hutchinson AW: Leukotriene B4 and neutrophil function: a review. Journal of the Royal Society of Medicine 74:831-833, 1981

63. Rådmark O, Samuelsson B: Regulation of the activity of 5-lipoxygenase, a key enzyme in leukotriene biosynthesis. Biochem Biophys Res Commun. 396:105-110, 2010

64. Peters-Golden M, Brock T: 5-lipoxygenase and FLAP. Prostaglandins Leukot Essent Fatty Acids. 69:99-109, 2003

65. Basavarajappaa D, Wana M, Lukica A, Steinhilberb D, Samuelssona B, Rådmarka O: Roles of coactosin-like protein (CLP) and 5-lipoxygenase-activating protein (FLAP) in cellular leukotriene biosynthesis. PNAS 111:11371–11376, 2014

66. Crooks S, Stockley R: Leukotriene B4. Int J Biochem Cell Biol. 30:173-178, 1998

67. Brain SD, Williams TJ: Leukotrienes and inflammation. Pharmac. Ther 46:57-66, 1990

68. Yared A, Albrightson-Winsbow C, Griswold D, Takahashi K, Fogo A, Badr KF: Functional signifcance of leukotriene B4 in normal and glomerulonephritc kidneys. Journal of the American Society of Nephrology 2:45-56, 1991

69. Rola-Pleszczynski M, Stanková J: Leukotriene B4 enhances interleukin-6 (IL-6) production and IL-6 messenger RNA accumulation in human monocytes in vitro: transcriptional and posttranscriptional mechanisms. Blood. 80:1004-1011, 1992

70. Aoki Y, Qiu D, Zhao G, Kao P: Leukotriene B4 mediates histamine induction of NF-kappaB and IL-8 in human bronchial epithelial cells. Am J Physiol. 274:L1030-1039, 1998

71. Goldman G, Welbourn R, Kobzik L, Valeri C, Shepro D, Hechtman H: Lavage with leukotriene B4 induces lung generation of tumor necrosis factor-alpha that in turn

mediates neutrophil diapedesis. Surgery. 113:297-303, 1993

72. Wada K, Arita M, Nakajima A, Katayama K, Kudo C, Kamisaki Y, Serhan C: Leukotriene B4 and lipoxin A4 are regulatory signals for neural stem cell proliferation and differentiation. FASEB J. 20:1785-1792, 2006

73. Wang Z, Filgueiras L, Wang S, Serezani A, Peters-Golden M, Jancar S, Serezani C: Leukotriene B4 enhances the generation of proinflammatory microRNAs to promote MyD88-dependent macrophage activation. J Immunol. 192:2349-2356, 2014

74. Ohnishi H, Miyahara N, Gelfand EW: The role of leukotriene B4 in allergic diseases. Allergol Int. 57:291-298, 2008

75. Fiedler J, Simon F, Iwahashi M, Murphy R: Effect of peroxisome proliferator-activated receptor alpha activation on leukotriene B4 metabolism in isolated rat hepatocytes. J Pharmacol Exp Ther. 299:691-697, 2001

76. Narala V, Adapala R, Suresh M, Brock T, Peters-Golden M, Reddy R: Leukotriene B4 is a physiologically relevant endogenous peroxisome proliferator-activated receptor-alpha agonist. J Biol Chem. 285:22067-22074, 2010

77. Iizuka Y, Yokomizo T, Terawaki K, Komine M, Tamaki K, Shimizu T: Characterization of a mouse second leukotriene B4 receptor, mBLT2: BLT2-dependent ERK activation and cell migration of primary mouse keratinocytes. J Biol Chem. 280:24816-24823, 2005

78. Lundeen K, Sun B, Karlsson L, Fourie A: Leukotriene B4 receptors BLT1 and BLT2: expression and function in human and murine mast cells. J Immunol. 177:3439-3447, 2006

79. Sun R, Ba X, Cui L, Xue Y, Zeng X: Leukotriene B4 regulates proliferation and differentiation of cultured rat myoblasts via the BLT1 pathway. Mol Cells. 27:403-408, 2009

80. Laidlaw TM, Boyce JA: Cysteinyl leukotriene receptors, old and new; implications for

asthma. Clin Exp Allergy. 42:1313-1320, 2012

81. Lee TH, Woszczek G, Farooque SP: Leukotriene E4: perspective on the forgotten mediator. J Allergy Clin Immunol. 124:417-421, 2009

82. Ciana P, Fumagalli M, Trincavelli ML, Verderio C, Rosa P, Lecca D, Ferrario S, Parravicini C, Capra V, Gelosa P, Guerrini U, Belcredito S, Cimino M, Sironi L, Tremoli E, Rovati GE, Martini C, Abbracchio MP: The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. EMBO J. 25:4615-4627, 2006

83. Singh RK, Gupta S, Dastidar S, Ray A: Cysteinyl leukotrienes and their receptors: molecular and functional characteristics. Pharmacology. 85:336-349, 2010

84. Stamper IJ, Byrne HM, Owen MR, Maini PK: Modelling the role of angiogenesis and vasculogenesis in solid tumour growth. Bull Math Biol. 69:2737-2772, 2007

85. Patan S: Vasculogenesis and angiogenesis. Cancer Treat Res. 117:3-32, 2004

86. Anderson L, Gibbons G: Notch: a mastermind of vascular morphogenesis. J Clin Invest. 117:299-302, 2007

87. Patel-Hett S, D'Amore PA: Signal transduction in vasculogenesis and developmental angiogenesis. Int J Dev Biol. 55:353-363, 2011

88. Marcelo KL, Goldie LC, Hirschi KK: Regulation of endothelial cell differentiation and specification. Circ Res. 112:1272-1278, 2013

89. Geudens I, Gerhardt H: Coordinating cell behaviour during blood vessel formation. Development. 138:4569-4583, 2011

90. Vokes SA, Krieg PA: Endoderm is required for vascular endothelial tube formation, but not for angioblast specification. Development. 129:775-785, 2002

91. Moser M, Patterson C: Bone morphogenetic proteins and vascular differentiation: BMPing up vasculogenesis. Thromb Haemost. 94:713-718, 2005

92. Cleaver O, Krieg PA: VEGF mediates angioblast migration during development of the dorsal aorta in Xenopus. Development 125:3905-3914, 1998

93. Cox CM, Poole TJ: Angioblast differentiation is influenced by the local environment: FGF-2 induces angioblasts and patterns vessel formation in the quail embryo. Dev Dyn. 218:371-382, 2000

94. Liu Z-J, Shirakawa T, Li Y, Soma A, Oka M, Dotto GP, Fairman RM, Velazquez OC, Herlyn M: Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. Mol Cell Biol. 23:14-25, 2003

95. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z: Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. 13:19-22, 1999

96. Atkins GB, Jain MK, Hamik A: Endothelial differentiation: molecular mechanisms of specification and heterogeneity. Arterioscler Thromb Vasc Biol. 31:1476-1484, 2011

97. Ferguson JEI, Kelley RW, Patterson C: Mechanisms of endothelial differentiation in embryonic vasculogenesis. Arterioscler Thromb Vasc Biol. 25:2246-2254, 2005

98. Vokes SA, Krieg PA: Endoderm is required for vascular endothelial tube formation, but not for angioblast specification. Development 129:775-785, 2002

99. Caprioli A, Minko K, Drevon C, Eichmann A, Dieterlen-Lie`vre F, Jaffredo T: Hemangioblast commitment in the avian allantois: cellular and molecular aspects. Dev Biol. 238:64-78, 2001

100. Ribatti D, Crivellato E: "Sprouting angiogenesis", a reappraisal. Dev Biol. 372:157-165, 2012

101. Hellström M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, Alva J, Nilsson AK, Karlsson L, Gaiano N, Yoon K, Rossant J, Iruela-Arispe ML, Kalén M, Gerhardt H, Betsholtz C: Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature. 445:776-780, 2007

102. Herbert SP, Cheung JYM, Stainier DYR: Determination of endothelial stalk versus tip cell potential during angiogenesis by H2.0-like Homeobox-1. Current Biology

22:1789-1794, 2012

103. Herbert S, Cheung J, Stainier D: Determination of endothelial stalk versus tip cell potential during angiogenesis by H2.0-like homeobox-1. Curr Biol. 22:1789-1794, 2012

104. Stockmann C, Schadendorf D, Klose R, Helfrich I: The impact of the immune system on tumor: angiogenesis and vascular remodeling. Front Oncol. 4, 2014

105. Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T: Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev. 18:2469-2473, 2004

106. Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP, Gallahan D, Closson V, Kitajewski J, Callahan R, Smith GH, Stark KL, Gridley T: Notch signaling is essential for vascular morphogenesis in mice. Genes Dev. 14:1343-1352, 2000

107. Hainaud P, Contrerès J, Villemain A, Liu L, Plouët J, Tobelem G, Dupuy E: The role of the vascular endothelial growth factor-Delta-like 4 ligand/Notch4-ephrin B2 cascade in tumor vessel remodeling and endothelial cell functions. Cancer Res. 66:8501-8510, 2006

108. Imhof BA, Aurrand-Lions M: Angiogenesis and inflammation face off. Nat Med. 12:171-172, 2006

109. Medzhitov R: Origin and physiological roles of inflammation. Nature:428-435, 2008

110. Ren L, Hu H, Sun X, Li F, Zhou JJ, Wang YM: The roles of inflammatory cytokines in the pathogenesis of ossification of ligamentum flavum. Am J Transl Res. 5:582-585, 2013

111. Galea J, Brough D: The role of inflammation and interleukin-1 in acute cerebrovascular disease. J Inflamm Res. 6:121-128, 2013

112. Eun J, Moore E, Banerjee A, Kelher M, Khan S, Elzi D, McLaughlin N, Silliman C: Leukotriene b4 and its metabolites prime the neutrophil oxidase and induce proinflammatory activation of human pulmonary microvascular endothelial cells. Shock.

35:240-244, 2011

113. Kazani S, Arm J, Boyce J, Chhay H, Wechsler SDM, Govindarajulu U, Ivester P, Ainsworth H, Sergeant S, Chilton F, Israel E: LTC4 synthase polymorphism modifies efficacy of botanical seed oil combination in asthma. Springerplus. 3, 2014

114. Sheshachalam A, Srivastava N, Mitchell T, Lacy P, Eitzen G: Granule protein processing and regulated secretion in neutrophils. Front Immunol. 5, 2014

115. Gonzalez-Mejia M, Doseff A: Regulation of monocytes and macrophages cell fate. Front Biosci (Landmark Ed). 14:2413-2431, 2009

116. Guha D, Ayyavoo V: Innate Immune Evasion Strategies by Human Immunodeficiency Virus Type 1. ISRN AIDS. , 2013

117. Medzhitov R, Jr. CJ: Innate immunity: the virtues of a nonclonal system of recognition. Cell. 91:295-298, 1997

118. Kobayashi K, Inohara N, Hernandez L, Galán J, Núñez G, Janeway C, Medzhitov R, Flavell R: RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature. 416:194-199, 2002

119. Krause DS, Scadden DT, Preffer FI: The hematopoietic stem cell niche--home for friend and foe? Cytometry B Clin Cytom. 84:7-20, 2013

120. Wilson A, Trumpp A: Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol. 6:93-106, 2006

121. Müller-Sieburg CE, Cho RH, Thoman M, Adkins B, Sieburg HB: Deterministic regulation of hematopoietic stem cell self-renewal and differentiation. Blood 100, 2013

122. Vadillo E, Pelayo R: Toll-like receptors in development and function of the hematopoietic system. Rev Invest Clin. 64:461-476, 2012

123. Bonny O, Bochud M: Genetics of calcium homeostasis in humans: continuum between monogenic diseases and continuous phenotypes. Nephrol Dial Transplant. , 2014

124. Burgoyne R, Haynes L: Understanding the physiological roles of the neuronal calcium sensor proteins. Mol Brain. 5, 2012

125. Jaskova K, Pavlovicova M, Jurkovicova D: Calcium transporters and their role in the development of neuronal disease and neuronal damage. Gen Physiol Biophys. 31:375-382, 2012

126. Moccia F, Dragoni S, Lodola F, Bonetti E, Bottino C, Guerra G, Laforenza U, Rosti V, Tanzi F: Store-dependent Ca(2+) entry in endothelial progenitor cells as a perspective tool to enhance cell-based therapy and adverse tumour vascularization. Curr Med Chem. 19:5802-5818, 2012

127. Almadén Y, Canalejo A, Ballesteros E, Añón G, Cañadillas S, Rodríguez M: Regulation of arachidonic acid production by intracellular calcium in parathyroid cells: effect of extracellular phosphate. J Am Soc Nephrol. 13:693-698, 2002

128. Dragoni S, Laforenza U, Bonetti E, Lodola F, Bottino C, Berra-Romani R, Bongio GC, Cinelli M, Guerra G, Pedrazzoli P, Rosti V, Tanzi F, Moccia F: Vascular endothelial growth factor stimulates endothelial colony forming cells proliferation and tubulogenesis by inducing oscillations in intracellular Ca2+ concentration. Stem Cells. 29:1898-1907, 2011

129. Tran Q-K, Ohashi K, Watanabe H: Calcium signalling in endothelial cells. Cardiovasc Res. 48:13-22, 2000

130. Hammarberg T, Rådmark O: 5-lipoxygenase binds calcium. Biochemistry. 38:4441-4447, 1999

131. Hammarberg T, Provost P, Persson B, Rådmark O: The N-terminal domain of 5-Lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity. J. Biol. Chem., 2000

132. Alric L, Pinelli E, Carrera G, Vinel JP, Beraud M, Duffaut M, Pascal JP, Pipy B: Involvement of calcium in macrophage leukotriene release during experimental cirrhosis.

Hepatology. 23:614-622, 1996

133. Mansour MA, Al-Shabanah OA: Enhanced generation of leukotriene B4 from calcium ionophore-stimulated rat peritoneal inflammatory cells: a possible clinical relevance. Curr Drug Targets Inflamm Allergy. 2:47-52, 2003

134. Goldman DW, Gifford LA, Olson DM, Goetzl EJ: Transduction by leukotriene B4 receptors of increases in cytosolic calcium in human polymorphonuclear leukocytes. J Immunol. 135:525-530, 1985

135. Eaton A, Nagy E, Pacault M, Fauconnier J, Bäck M: Cysteinyl leukotriene signaling through perinuclear CysLT(1) receptors on vascular smooth muscle cells transduces nuclear calcium signaling and alterations of gene expression. J Mol Med (Berl). 90:1223-1231, 2012

136. Mittal M, Siddiqui M, Tran K, Reddy S, Malik A: Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal. 20:1126-1167, 2014

137. Richardson C, Yan S, Vestal C: Oxidative Stress, Bone Marrow Failure, and Genome Instability in Hematopoietic Stem Cells. Int J Mol Sci. 16:2366-2385, 2015

138. Dahle J, Arai Y: Environmental Geochemistry of Cerium: Applications and Toxicology of Cerium Oxide Nanoparticles. Int J Environ Res Public Health. 12:1253-1278, 2015

139. Kim C, Kim JY, Kim JH: Cytosolic phospholipase A(2), lipoxygenase metabolites, and reactive oxygen species. BMB Rep. 41:555-559, 2008

140. Simon HU, Haj-Yehia A, Levi-Schaffer F: Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis. 5:415-418, 2000

141. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD: Cell transformation by the superoxide-generating oxidase Mox1. Nature. 401:79-82, 1999

142. Cho KJ, Seo JM, Kim JH: Bioactive lipoxygenase metabolites stimulation of

NADPH oxidases and reactive oxygen species. Mol Cells. 32:1-5, 2011

143. Sauer H, Wartenberg M, Hescheler J: Reactive oxygen species as intracellular messengers during cell growth and differentiation. Cell Physiol Biochem. 11:173-186, 2001

144. Cocco T, Di M, Papa P, Lorusso M: Arachidonic acid interaction with the mitochondrial electron transport chain promotes reactive oxygen species generation. Free Radic Biol Med. 27:51-59, 1999

145. Colston J, Rosa Sdl, Strader J, Anderson M, Freeman G: H2O2 activates Nox4 through PLA2-dependent arachidonic acid production in adult cardiac fibroblasts. FEBS Lett. 579:2533-2540, 2005

146. Yun M, Park H, Seo K, Lee S, Im D, Kim C: 5-Lipoxygenase plays an essential role in 4-HNE-enhanced ROS production in murine macrophages via activation of NADPH oxidase. Free Radic Res. 44:742-750, 2010

147. Zeng Q, Han Y, Bao Y, Li W, Li X, Shen X, Wang X, Yao F, O'Rourke S, Sun C: 20-HETE increases NADPH oxidase-derived ROS production and stimulates the L-type Ca2+ channel via a PKC-dependent mechanism in cardiomyocytes. Am J Physiol Heart Circ Physiol. 299:H1109-H1117, 2010

148. Woo CH, You HJ, Cho SH, Eom YW, Chun JS, Yoo YJ, Kim JH: Leukotriene B(4) stimulates Rac-ERK cascade to generate reactive oxygen species that mediates chemotaxis. J Biol Chem. 277:8572-8578, 2002

149. Woo CH, Yoo MH, You HJ, Cho SH, Mun YC, Seong CM, Kim JH: Transepithelial migration of neutrophils in response to leukotriene B4 is mediated by a reactive oxygen species-extracellular signal-regulated kinase-linked cascade. J Immunol. 170:6273-6279, 2003

150. Ravasi S, Citro S, Viviani B, Capra V, Rovati G: CysLT1 receptor-induced human airway smooth muscle cells proliferation requires ROS generation, EGF receptor

transactivation and ERK1/2 phosphorylation. Respir Res. 7, 2006

151. Becher U, Ghanem A, Tiyerili V, Fürst D, Nickenig G, Mueller C: Inhibition of leukotriene C4 action reduces oxidative stress and apoptosis in cardiomyocytes and impedes remodeling after myocardial injury. J Mol Cell Cardiol. 50:570-577, 2011

152. Luchtefeld M, Drexler H, Schieffer B: 5-Lipoxygenase is involved in the angiotensin II-induced NAD(P)H-oxidase activation. Biochem Biophys Res Commun. 308:668-672, 2003

153. Griendling K, Sorescu D, Lassègue B, Ushio-Fukai M: Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol. 20:2175-2183, 2000

154. Mack JA, Maytin EV: Persistent inflammation and angiogenesis during wound healing in K14-directed Hoxb13 transgenic mice. J Invest Dermatol. 130:856-865, 2010

155. Fiedler U, Augustin HG: Angiopoietins: a link between angiogenesis and inflammation. Trends Immunol. 27:552-558, 2006

156. Côte C, Tremblay M, Duchesne E, Lapoite B: Inflammation-induced leukocyte accumulation in injured skeletal muscle: role of mast cells. Muscle Nerve. 37:754-763, 2008

157. Robertson E, Bradley A, Kuehn M, Evans M: Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323:445-448, 1986

158. Stock N, Baccei C, Bain G, Chapman C, Correa L, Darlington J, King C, Lee C, Lorrain DS, Prodanovich P, Santini A, Schaab K, Evans JF, Hutchinson JH, Prasit P: 5-Lipoxygenase-activating protein inhibitors. Part 3: 3-{3-tert-Butylsulfanyl-1-[4-(5-methoxy-pyrimidin-2-yl)-benzyl]-5-(5-methyl-pyridin-2-ylmethoxy)-1H-indol-2-yl]-2,2-dimethyl-propionic acid (AM643)-A potent FLAP inhibitor suitable for topical administration. Bioorg Med Chem Lett. 20:4598-4601, 2010

159. Liu S, Hu H, Gao C, Gao N, Wang G, Wang X, Gao X, Xia Y, Wood J: Actions of cysteinyl leukotrienes in the enteric nervous system of guinea-pig stomach and small intestine. Eur J Pharmacol. 459:27-39, 2003

160. Filliatre GL, Sayah S, Latournerie V, Renaud J, Finet M, Hanf R: Cyclo-oxygenase and lipoxygenase pathways in mast cell dependent-neurogenic inflammation induced by electrical stimulation of the rat saphenous nerve. Br J Pharmacol. 132:1581-1589, 2001

161. Inwegen RV, Khandwala A, Gordon R, Sonnino P, Coutts S, Jolly S: REV 5901: an orally effective peptidoleukotriene antagonist, detailed biochemical/pharmacological profile. J Pharmacol Exp Ther. 241:117-124, 1987

162. Bäck M, Bu D, Bränström R, Sheikine Y, Yan Z, Hansson G: Leukotriene B4 signaling through NF-kappaB-dependent BLT1 receptors on vascular smooth muscle cells in atherosclerosis and intimal hyperplasia. Proc Natl Acad Sci U S A. 102:17501-17506, 2005

163. Uğur M, Melli M: Inhibitory effect of LY 255283 on the synthesis of leukotriene B(4) and thromboxane A(2) in human peripheral blood polymorphonuclear leukocytes and monocytes. Mediators Inflamm. 2:407-409, 1993

164. Kim H, Kim D, Kim H, Koh J, Kim K, Noh M, Lee S, Kim S, Park S, Kim J, Kim S, Lee C: Involvement of the BLT2 receptor in the itch-associated scratching induced by 12-(S)-lipoxygenase products in ICR mice. Br J Pharmacol. 154:1073-1078, 2008

165. Tudhope SR, Cuthbert NJ, Abram TS, Jennings MA, Maxey RJ, Thompson AM, Norman P, Gardiner PJ: BAY u9773, a novel antagonist of cysteinyl-leukotrienes with activity against two receptor subtypes. Eur J Pharmacol. 264:317-323, 1994

166. Bäck M, Jonsson E, SE SD: The cysteinyl-leukotriene receptor antagonist BAY u9773 is a competitive antagonist of leukotriene C4 in the guinea-pig ileum. Eur J Pharmacol. 317:107-113, 1996

167. Tymianski M, Spigelman I, Zhang L, Carlen PL, Tator CH, Charlton MP, Wallace

MC: Mechanism of action and persistence of neuroprotection by cell-permeant Ca2+ chelators. J Cereb Blood Flow Metab. 14:911-923, 1994

168. Sancho P, Fabregat I: The NADPH oxidase inhibitor VAS2870 impairs cell growth and enhances TGF-β-induced apoptosis of liver tumor cells. Biochem Pharmacol. 81:917-924, 2011

169. Yum S, Park H, Hong S, Jeong S, Kim W, Jung Y: N-(2-Mercaptopropionyl)-glycine, a diffusible antioxidant, activates HIF-1 by inhibiting HIF prolyl hydroxylase-2: implication in amelioration of rat colitis by the antioxidant. Biochem Biophys Res Commun. 443:1008-1013, 2014

170. Simpson P, Mickelson J, Lucchesi B: Free radical scavengers in myocardial ischemia. Fed Proc. 46:2413-2421, 1987

171. Hamad I, Arda N, Pekmez M, Karaer S, Temizkan G: Intracellular scavenging activity of Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) in the fission yeast,Schizosaccharomyces pombe. J Nat Sci Biol Med. 1:16-21, 2010

172. Giordo R, Cossu A, Pasciu V, Hoa P, Posadino A, Pintus G: Different redox response elicited by naturally occurring antioxidants in human endothelial cells. Open Biochem J. 7:44-53, 2013

173. Paredes RM, Etzler JC, Watts LT, Lechleiter JD: Chemical Calcium Indicators. Methods. 46:143-151, 2008

174. Gee KR, Brown KA, Chen WN, Bishop-Stewart J, Gray D, Johnson I: Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. Cell Calcium. 27:97-106, 2000

175. Paddock SW: Principles and Practices of Laser Scanning Confocal Microscopy. Molecular Biotechnology 16:127-149, 2000

176. Allbritton N, Oancea E, Kuhn M, Meyer T: Source of nuclear calcium signals. Proc Natl Acad Sci U S A. 91:12458-12462, 1994

177. Jackson JR, Seed MP, Kircher CH, Willoughby DA, Winkler JD: The codependence of angiogenesis and chronic inflammation. FASEB J. 11:457-465, 1997
178. Müller AM, Hermanns MI, Skrzynski C, Nesslinger M, Müller KM, Kirkpatrick CJ: Expression of the endothelial markers PECAM-1, vWf, and CD34 in vivo and in vitro. Exp Mol Pathol. 72:221-229, 2002
179. Iida M, Heike T, Yoshimoto M, Baba S, Doi H, Nakahata T: Identification of cardiac stem cells with FLK1, CD31, and VE-cadherin expression during embryonic stem cell differentiation. FASEB J. 19:371-378, 2005
180. Pusztaszeri M, Seelentag W, Bosman F: Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. J Histochem Cytochem. 54:385-395, 2006
181. Albelda S, Muller W, Buck C, Newman P: Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. J Cell Biol. 114:1059-1068, 1991
182. Fleming WH: Endothelial cell–specific markers: going . . . going . . . gone. Blood, 2005
183. Rudini N, Felici A, Giampietro C, Lampugnani M, Corada M, Swirsding K, Garrè M, Liebner S, Letarte M, Dijke Pt, Dejana E: VE-cadherin is a critical endothelial regulator of TGF-beta signalling. EMBO J. 27:993-1004, 2008
184. Hong SH, Avis I, Vos MD, Martı´nez A, Treston AM, Mulshine JL: Relationship of arachidonic acid metabolizing enzyme expression in epithelial cancer cell lines to the growth effect of selective biochemical inhibitors. Cancer research 59:2223-2228, 1999
185. Ells R, Kock JL, Albertyn J, Pohl CH: Arachidonic acid metabolites in pathogenic yeasts. Lipids Health Dis. 11, 2012
186. Wang D, DuBois RN, Richmond A: The role of chemokines in intestinal inflammation and cancer. Curr Opin Pharmacol. 2 9:688-696, 2009

187. Urbich C, Dimmeler S: Endothelial progenitor cells: characterization and role in vascular biology. Circ Res. 95:343-353, 2004

188. Harraz M, Jiao C, Hanlon H, Hartley R, Schatteman G: CD34- blood-derived human endothelial cell progenitors. Stem Cells. 19:304-312, 2001

189. Sindrilaru A, Peters T, Wieschalka S, Baican C, Baican A, Peter H, Hainzl A, Schatz S, Qi Y, Schlecht A, Weiss J, Wlaschek M, Sunderkötter C, Scharffetter-Kochanek K: An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. J Clin Invest. 121:985-997, 2011

190. Altin J, Sloan E: The role of CD45 and CD45-associated molecules in T cell activation. Immunol Cell Biol. 75:430-445, 1997

191. Gregorio GD, Yao-Borengasser A, Rasouli N, Varma V, Lu T, Miles L, Ranganathan G, Peterson C, McGehee R, Kern P: Expression of CD68 and macrophage chemoattractant protein-1 genes in human adipose and muscle tissues: association with cytokine expression, insulin resistance, and reduction by pioglitazone. Diabetes. 54:2305-2313, 2005

192. Sheng L, Leshchyns'ka I, Sytnyk V: Cell adhesion and intracellular calcium signaling in neurons. Cell Commun Signal. 13, 2013

193. Weiss U: Inflammation. Nature:427, 2008

194. Yoshikai Y: Roles of prostaglandins and leukotrienes in acute inflammation caused by bacterial infection. Curr Opin Infect Dis. 14:257-263, 2001

195. Moos M, Mewburn J, Kan F, Ishii S, Abe M, Sakimura K, Noguchi K, Shimizu T, Funk C: Cysteinyl leukotriene 2 receptor-mediated vascular permeability via transendothelial vesicle transport. FASEB J. 22:4352-4362, 2008

196. Booyens J, Merwe CFvd, Katzeff IE: Chronic arachidonic acid eicosanoid imbalance: a common feature in coronary artery disease, hypercholesterolemia, cancer

and other important diseases. Significance of desaturase enzyme inhibition and of the arachidonic acid desaturase-independent pathway. Med Hypotheses. 18:53-60, 1985

197. Sanchez-Mejia R, Mucke L: Phospholipase A2 and arachidonic acid in Alzheimer's disease. Biochim Biophys Acta. 1801:784-790, 2010

198. Chen C, Wei X, Rao X, Wu J, Yang S, Chen F, Ma D, Zhou J, Dackor RT, Zeldin DC, Wang DW: Cytochrome P450 2J2 is highly expressed in hematologic malignant diseases and promotes tumor cell growth. J Pharmacol Exp Ther. 336:344-355, 2011

199. Massiera F, Saint-Marc P, Seydoux J, Murata T, Kobayashi T, Narumiya S, Guesnet P, Amri EZ, Negrel R, Ailhaud G: Arachidonic acid and prostacyclin signaling promote adipose tissue development: a human health concern? J Lipid Res. 44:271-279, 2003

200. Tessier-Prigent A, Willems R, Lagarde M, Garrone R, Cohen H: Arachidonic acid induces differentiation of uterine stromal to decidual cells. Eur J Cell Biol. 78:398-406, 1999

201. Kan I, Melamed E, Offen D, Green P: Docosahexaenoic acid and arachidonic acid are fundamental supplements for the induction of neuronal differentiation. J Lipid Res. 48:513-517, 2007

202. Lundberg U, Serhan CN, Samuelsson B: Appearance of an arachidonic acid 15-lipoxygenase pathway upon differentiation of the human promyelocytic cell-line HL-60. FEBS letters 185:14-18, 1985

203. Talahalli R, Zarini S, Sheibani N, Murphy RC, Gubitosi-Klug RA: Increased synthesis of leukotrienes in the mouse model of diabetic retinopathy. Invest Ophthalmol Vis Sci. 51:1699-1708, 2010

204. Sharifpanah F, Wartenberg M, Hannig M, Piper HM, Sauer H: Peroxisome proliferator-activated receptor alpha agonists enhance cardiomyogenesis of mouse ES cells by utilization of a reactive oxygen species-dependent mechanism. Stem Cells.

26:64-71, 2008

205. Anderson G, Fennessy M: Effects of REV 5901, a 5-lipoxygenase inhibitor and leukotriene antagonist, on pulmonary responses to platelet activating factor in the guinea-pig. Br J Pharmacol. 94:1115-1122, 1988

206. Lefer AM, Roth DM, Lefer DJ, Smith JB: Potentiation of leukotriene formation in pulmonary and vascular tissue. Naunyn Schmiedebergs Arch Pharmacol. 326:186-189, 1984

207. Antoniu S: Targeting 5-lipoxygenase-activating protein in asthma and chronic obstructive pulmonary disease. Expert Opin Ther Targets. 18:1285-1292, 2014

208. Horrillo R, González-Périz A, Martínez-Clemente M, López-Parra M, Ferré N, Titos E, Morán-Salvador E, Deulofeu R, Arroyo V, Clària J: 5-lipoxygenase activating protein signals adipose tissue inflammation and lipid dysfunction in experimental obesity. J Immunol. 184:3978-3987, 2010

209. Giannopoulos P, Chu J, Joshi Y, Sperow M, Li J, Kirby L, Praticò D: 5-lipoxygenase activating protein reduction ameliorates cognitive deficit, synaptic dysfunction, and neuropathology in a mouse model of Alzheimer's disease. Biol Psychiatry. 74:348-356, 2013

210. Huang H, Zeng Z, Li J, Zhang L, Chen Y: Variants of arachidonate 5-lipoxygenase-activating protein (ALOX5AP) gene and risk of coronary heart disease: A meta-analysis. Arch Med Res. 41:634-641, 2010

211. Coffey MJ, Wilcoxen SE, Peters-Golden M: Increases in 5-lipoxygenase activating protein expression account for enhanced capacity for 5-lipoxygenase metabolism that accompanies differentiation of peripheral blood monocytes into alveolar macrophages. Am J Respir Cell Mol Biol. 11:153-158, 1994

212. Sala A, Zarini S, Folco G, Murphy RC, Henson PM: Differential metabolism of exogenous and endogenous arachidonic acid in human neutrophils. The Journal of

Biological Chemistry 274:28264-28269, 1999

213. Nohgawa M, Sasada M, Maeda A, Asagoe K, Harakawa N, Takano K, Yamamoto K, Okuma M: Leukotriene B4-activated human endothelial cells promote transendothelial neutrophil migration. J Leukoc Biol. 62:203-209, 1997

214. Pettipher ER, Salter ED, Breslow R, Raycroft L, Showell HJ: Specific inhibition of leukotriene B4 (LTB4)-induced neutrophil emigration by 20-hydroxy LTB4: implications for the regulation of inflammatory responses. Br. J. Pharmacol. 110:423-427, 1993

215. He P: Leucocyte/endothelium interactions and microvessel permeability: coupled or uncoupled? Cardiovasc Res. 87:281-290, 2010

216. Chen ZK, Lv HS, Jiang J: LTB4 can stimulate human osteoclast differentiation dependent of RANKL. Artif Cells Blood Substit Immobil Biotechnol. 38:52-56, 2010

217. Wada K, Arita M, Nakajima A, Katayama K, Kudo C, Kamisaki Y, Serhan CN: Leukotriene B4 and lipoxin A4 are regulatory signals for neural stem cell proliferation and differentiation. The FASEB Journal 20:1785-1792, 2006

218. Kim G-Y, Lee J-W, Cho S-H, Seo J-M, Kim J-H: Role of the low-affinity leukotriene B4 receptor BLT2 in VEGF-induced angiogenesis. Arterioscler Thromb Vasc Biol. 29:915-920, 2009

219. Klampfl T, Bogner E, Bednar W, Mager L, Massudom D, Kalny I, Heinzle C, Berger W, Stättner S, Karner J, Klimpfinger M, Fürstenberger G, Krieg P, Marian B: Up-regulation of 12(S)-lipoxygenase induces a migratory phenotype in colorectal cancer cells. Exp Cell Res. 318:768-778, 2012

220. Nicholson DW, Ali A, Klemba MW, Munday NA, Zamboni RJ, Ford-Hutchinson AW: Human leukotriene C4 synthase expression in dimethyl sulfoxide-differentiated U937 cells. J Biol Chem. 267:17849-17857, 1992

221. Hazel BA, O'Connor A, Niculescu R, Kalf GF: Benzene and its metabolite, hydroquinone, induce granulocytic differentiation in myeloblasts by interacting with

cellular signaling pathways activated by granulocyte colony-stimulating factor. Stem Cells. 13:295-310, 1995

222. Ley K, Laudanna C, Cybulsky MI, Nourshargh S: Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol. 7:678-689, 2007

223. Ley K: Molecular mechanisms of leukocyte recruitment in the inflammatory process. Cardiovasc Res. 32:733-742, 1996

224. Bikle DD, Xie Z, Tu CL: Calcium regulation of keratinocyte differentiation. Expert Rev Endocrinol Metab. 7:461-472, 2012

225. Xie Z, Singleton PA, Bourguignon LY, Bikle DD: Calcium-induced human keratinocyte differentiation requires src- and fyn-mediated phosphatidylinositol 3-kinase-dependent activation of phospholipase C-gamma1. Mol Biol Cell. 16:3236-3246, 2005

226. Pillai S, Bikle DD, Mancianti ML, Cline P, Hincenbergs M: Calcium regulation of growth and differentiation of normal human keratinocytes: modulation of differentiation competence by stages of growth and extracellular calcium. J Cell Physiol. 143:294-302, 1990

227. Yamaguchi T, Ye C, Chattopadhyay N, Sanders JL, Vassilev PM, Brown EM: Enhanced expression of extracellular calcium sensing receptor in monocyte-differentiated versus undifferentiated HL-60 cells: potential role in regulation of a nonselective cation channel. Calcif Tissue Int. 66:375-382, 2000

228. Antoniotti S, Fattori P, Tomatis C, Pessione E, Munaron L: Arachidonic acid and calcium signals in human breast tumor-derived endothelial cells: a proteomic study. J Recept Signal Transduct Res. 29:257-265, 2009

229. Krump E, Pouliot M, Naccache P, Borgeat P: Leukotriene synthesis in calcium-depleted human neutrophils: arachidonic acid release correlates with calcium influx. Biochem J. 310:681-688, 1995

230. Reynolds CH: 5-Lipoxygenase and leukotriene synthesis: effects of calcium ions and of inhibitors. Prostaglandins. 36:59-68, 1988

231. Lloret S, Martínez J, Moreno JJ: Influence of calcium on arachidonic acid mobilization by murine resident peritoneal macrophages. Arch Biochem Biophys. 323:251-257, 1995

232. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ: Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem. 278:36027-36031, 2003

233. Finkel T: Signal transduction by reactive oxygen species. J Cell Biol. 194:7-15, 2011

234. Lee S, Pagoria D, Raigrodski A, Geurtsen W: Effects of combinations of ROS scavengers on oxidative DNA damage caused by visible-light-activated camphorquinone/N,N-dimethyl-p-toluidine. J Biomed Mater Res B Appl Biomater. 83:391-399, 2007

235. Jang YY, Sharkis SJ: A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood. 110:3056-3063, 2007

236. Shin S, Kim S: Inhibition of arachidonic acid and iron-induced mitochondrial dysfunction and apoptosis by oltipraz and novel 1,2-dithiole-3-thione congeners. Mol Pharmacol. 75:242-253, 2009

237. Schönfeld P, Wojtczak L: Fatty acids as modulators of the cellular production of reactive oxygen species. Free Radic Biol Med. 45:231-241, 2008

238. Bartsch C, Bekhite M, Wolheim A, Richter M, Ruhe C, Wissuwa B, Marciniak A, Müller J, Heller R, Figulla H, Sauer H, Wartenberg M: NADPH oxidase and eNOS control cardiomyogenesis in mouse embryonic stem cells on ascorbic acid treatment. Free Radic Biol Med. 51:432-443, 2011

239. Sauer H, Ruhe C, Müller J, Schmelter M, D'Souza R, Wartenberg M: Reactive oxygen species and upregulation of NADPH oxidases in mechanotransduction of embryonic stem cells. Methods Mol Biol. 477:397-418, 2008

240. Zhou L, Huang Y, Zhang Y, Zhao Q, Zheng B, Lou Y, Zhu D: mGluR5 stimulating Homer-PIKE formation initiates icariin induced cardiomyogenesis of mouse embryonic stem cells by activating reactive oxygen species. Exp Cell Res. 319:1505-1514, 2013

241. Heo J, Lee J: β-Catenin mediates cyclic strain-stimulated cardiomyogenesis in mouse embryonic stem cells through ROS-dependent and integrin-mediated PI3K/Akt pathways. J Cell Biochem. 112:1880-1889, 2011

242. Ushio-Fukai M, Urao N: Novel role of NADPH oxidase in angiogenesis and stem/progenitor cell function. Antioxid Redox Signal. 11:2517-2533, 2009

243. Łuczak K, Balcerczyk A, Soszyński M, Bartosz G: Low concentration of oxidant and nitric oxide donors stimulate proliferation of human endothelial cells in vitro. Cell Biol Int. 28:483-486, 2004

244. Yao E, Yu Y, Fukuda N: Oxidative stress on progenitor and stem cells in cardiovascular diseases. Curr Pharm Biotechnol. 7:101-108, 2006

245. Maulik N: Redox signaling of angiogenesis. Antioxid Redox Signal. 4:805-815, 2002

Publications

SCI Journal

2012 Guo LY, Wang YL, **Huang YH**, Yang CH, Hou YY, Harn HI, You YL. Comparison of the electromyographic activation level and unilateral selectivity of erector spinae during different selected movements. Int J Rehabil Res 35: 345-51.

2008 Juan CC, Lien CC, Chang CL, **Huang YH**, Ho LT. Involvement of iNOS and NO in TNF-alpha-downregulated resistin gene expression in 3T3-L1 adipocytes. Obesity (Silver Spring) 16:1219-1225.

Oral presentations

2013 **Yu-Han Huang**. Impact of the arachidonic acid/leukotriene signaling pathway for vasculogenesis and leukocyte differentiation of mouse embryonic stem cells. 11th Annual Retreat of the International Graduate Programme Molecular Biology and Medicine of the Lung, Giessen, Germany

2012 **Yu-Han Huang**. Leukotrienes in vasculogenesis of embryonic stem cells. 10th Annual Retreat of the International Graduate Programme Molecular Biology and Medicine of the Lung, Giessen, Germany

Poster presentations

2015 **Yu-Han Huang**, Fatemeh Sharifpanah, Maria Wartenberg, Heinrich Sauer. Impact of arachidonic acid and the leukotriene synthesis pathway on vasculogenesis of mouse embryonic stem cells. International meeting of the German Society of Cell Biology, Cologne, Germany

2015 **Yu-Han Huang**, Fatemeh Sharifpanah, Maria Wartenberg, Heinrich Sauer. Impact of arachidonic acid and the leukotriene signaling pathway for vasculogenesis of embryonic stem cells. 94th Annual Meeting of the German Physiological Society, Magdeburg, Germany

2013 **Yu-Han Huang**, Fatemeh Sharifpanah, Maria Wartenberg, Heinrich Sauer. Leukotriens in vasculogenesis of embryonic stem cells. 92nd Annual Meeting of the German Physiological Society, Heidelberg, Germany

2012 **Yu-Han Huang**, Fatemeh Sharifpanah, Maria Wartenberg, Heinrich Sauer. Leukotrienes in vasculogenesis of embryonic stem cells. 7th Fraunhofer Life Science Symposium, Leipzig, Germany

2011 **Yu-Han Huang**, Heinrich Sauer. Impact of inflammation for vasculogenesis and differentiation of hematopoietic cells from embryonic stem cells. 9th Annual Retreat of the International Graduate Programme Molecular Biology and Medicine of the Lung, Giessen, Germany

2009 **Yu-Han Huang**, Chi-Chang Juan Low-Tone Ho. Effect of hypoxia on insulin sensitivity in 3T3-L1 adipocytes. 36th International Congress of Physiological Science, Kyoto, Japan

2007 **Y-H Huang**, I-C Harn, C-H Yang, L-Y Guo. The EMG Activation Selectivity of Unilateral Spinal Extensor for Different Lumbar Stabilization Exercises. XXIth International Society Of Biomechanics, Taipei, Taiwan

Erklärung zur Dissertation

„Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nichtveröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten sowie ethische, datenschutzrechtliche und tierschutzrechtliche Grundsätze befolgt. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, oder habe diese nachstehend spezifiziert. Die vorgelegte Arbeit wurde weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde Als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt und indirekt an der Entstehung der vorliegenden Arbeit beteiligt waren. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.“

Giessen, 15 April 2015 Yu-Han Huang

Acknowledgements

First, I would like to express my thanks to my supervisor Prof. Sauer for giving me the chance to study and supervise my PhD thesis. I thank Prof. Sauer for supporting all the necessary materials, the place and the assistance to accomplish my work.

Next, I would like to thank the MBML program team especially Prof, Werner Seeger and Dr Rory E. Morty. Thanks the program gave me the lectures to learn different skills, scientific knowledge.

I also want to thank all my colleagues. Dr. Sharifpanah strengthened my experimental ability and did the trouble shooting for me. Barbara Arnold prepared the ES cells and the medium. Sascha Behr, Lena Mascheck, Febina Ravindran, all of you helped me in my experiments and encouraged me for these past years. Ling Lee always helped me in my life no matter searching new house and shopping daily goods.

The most important, I want to thank my dad, my mom and my sister. Their love and big support made me stay here and finish my thesis. They always cheered me up, encouraged me and send the things that I missed from Taiwan.

Last but no least, I want to thank my dearest friend, she always accompanied with me no matter happy or sad, teached me, helped me, especially corrected the mistakes in my thesis. Hui-Ling and Ru-lin gave me so much useful information for experiments and thesis. Finally, I would like to thank all my friends from Taiwan, Meng-miao, Lucy, and the others a lot I haven't mentioned.