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DIPLOMARBEIT

Thrombin Receptor Activating Peptide-6 (TRAP-6) Promotes Human Umbilical Vein Endothelial Cells Proliferation

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I. Abstract

Objectives: Thrombin, a dominant coagulation protease has a vascularization-promoting function. Previous studies showed that the interaction between thrombin and human umbilical vein endothelial cell (HUVEC) results in upregulation in expression of vascular endothelial growth factor's (VEGF) receptor. Thrombin signaling in endothelial cells mediated by protease-activated receptors (PARs). Thrombin cleaves the PAR-1, the predominant thrombin receptor in endothelial cells, and results in the tethered ligand; SFLLRN interacts with the extracellular loop of receptor and causes receptor activation. TRAP-6, a synthetic peptide that contains motif SFLLRN, showed agonist activity for thrombin. In this study, we investigated the effect of TRAP-6 on the proliferation of human umbilical vein endothelial cells and adipose-derived stem cells (ASCs). Furthermore, vasculogenesis function of TRAP-6 was determined on human umbilical vein endothelial cells/ adipose-derived stem cells co-culture. Moreover, the ability of TRAP-6 in stimulation of human umbilical vein endothelial cells to express various surface markers was determined.

Approach and Results: Here, we used bromodeoxyuridine (BrdU) assay to measure the proliferation of HUVEC and ASC. The results demonstrated that the proliferation of both HUVEC and ASC is enhanced. The HUVEC/ASC co-culture treated with TRAP-6 resulted in denser microvascular network formation in vitro and vascular network parameters such as the number of junctions, the number of tubules and total tubule length are enhanced. Furthermore, immunohistochemically investigation of surface markers in stimulated HUVEC with TRAP-6 using flow cytometry showed slightly increase of E-selectin, CD 73, thrombomodulin and CD 34 expression, decrease in CD 31 expression and no change in CD 146, VE-cadherin, Tie2, and VEGFR2 expression pattern.

Conclusion: Our study provides detailed insight into angiogenetic behavior and influence of TRAP-6 on HUVECs. Our data demonstrate TRAP-6 improved either of HUVEC and ASC proliferation with no toxic effect. TRAP-induced HUVEC/ASC co-culture promotes microvascular network formation. However, the HUVEC surface markers do no indiacte significant change after stimulation with TRAP-6. Therefore, we concluded that TRAP-6 has potential in promoting angiogenesis and vascularization in tissue engineering.

Keywords: Vascularization, Endothelial cells, Adipose-derived stem cells, Thrombin, TRAP-6

II Zusammensfassun

Ziele: Thrombin, eine dominante Gerinnungsprotease, hat eine vaskularisationsfördernde Funktion. Frühere Studien zeigten, dass die Wechselwirkung zwischen Thrombin und der Endothelzelle der menschlichen Nabelschnurvene (HUVEC) zu einer Hochregulation der Expression des VEGF-Rezeptors (VEGF = Vascular Endothelial Growth Factor) führt. Thrombinsignalisierung in Endothelzellen vermittelt durch Protease-aktivierte Rezeptoren (PARs). Thrombin spaltet das PAR-1, den in Endothelzellen vorherrschenden Thrombinrezeptor, und führt zu dem gebundenen Liganden; SFLLRN interagiert mit der extrazellulären Schleife des Rezeptors und bewirkt eine Rezeptoraktivierung. TRAP-6, ein synthetisches Peptid, das das Motiv SFLLRN enthält, zeigte eine Agonistenaktivität für Thrombin. In dieser Studie untersuchten wir die Wirkung von TRAP-6 auf die Proliferation von Endothelzellen der menschlichen Nabelschnurvene und von Fettstammzellen abgeleiteten Stammzellen (ASCs). Darüber hinaus wurde die Vaskulogenese-Funktion von TRAP-6 an humanen Nabelvenen-Endothelzellen / Fettstammzellen-Co-Kultur bestimmt. Darüber hinaus wurde die Fähigkeit von TRAP-6 zur Stimulierung von Endothelzellen der menschlichen Nabelschnurvene zur Expression verschiedener Oberflächenmarker bestimmt.

Ansatz und Ergebnisse: Hier verwendeten wir einen Bromdeoxyuridin (BrdU) -Assay, um die Proliferation von HUVEC und ASC zu messen. Die Ergebnisse zeigten, dass die Proliferation von HUVEC und ASC verbessert ist. Die mit TRAP-6 behandelte HUVEC / ASC-Co-Kultur führte zu einer dichteren Bildung des mikrovaskulären Netzwerks in vitro, und vaskuläre Netzwerkparameter wie die Anzahl der Übergänge, die Anzahl der Röhrchen und die Gesamtlänge der Röhrchen wurden erhöht. Darüber hinaus zeigten immunhistochemische Untersuchungen von Oberflächenmarkern in stimuliertem HUVEC mit TRAP-6 mittels Durchflusszytometrie einen leichten Anstieg der Expression von E-Selectin, CD 73, Thrombomodulin und CD 34, eine Abnahme der CD 31-Expression und keine Veränderung von CD 146, VE-Cadherin. Tie2- und VEGFR2-Expressionsmuster.

Schlussfolgerung: Unsere Studie bietet detaillierte Einblicke in das angiogenetische Verhalten und den Einfluss von TRAP-6 auf HUVECs. Unsere Daten zeigen, dass TRAP-6 sowohl die HUVEC- als auch die ASC-Proliferation ohne toxische Wirkung verbessert. TRAP-induzierte HUVEC / ASC-Co-Kultur fördert die Bildung von mikrovaskulären Netzwerken. Die HUVEC-Oberflächenmarker zeigen jedoch nach Stimulation mit TRAP-6 keine signifikante Veränderung. Wir kamen daher zu dem Schluss, dass TRAP-6 das Potenzial hat, die Angiogenese und Vaskularisation beim Tissue Engineering zu fördern.

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Chapter 1:

Introduction

Homeostasis in the cardiovascular system controls blood loss in the time of vascular injury. Thrombin, a serine protease, is a natural product of inactive precursor, prothrombin that originally synthesized in the liver, produces in injury site and controls coagulation process and promotes clot formation. Several events such as cell signaling, fibrinolysis and inflammation are influenced by thrombin. The main criterion in thrombin function is a cleavage reaction that must be controlled. Otherwise results in either bleeding or thrombosis (Crawley et al, 2007). The early function of thrombin during vascular injury is the conversion of soluble fibrinolysis into insoluble fibrin clot, which traps red blood cells and inhibits blood loss (Church F. C et al, 1989; Coughlin, Shaun R et al, 2000).

1.1 Thrombin structure

Thrombin is a serine protease has a crystal structure which is highly homologous comparing to other serine proteases like chymotrypsin (Bode et al, 2005; Bodeet et al, 1992; Cera et al, 2007). In the crystallographic structure of these enzymes, the serine residue, S195 (chymotrypsin numbering) is placed in active site cleft. Specifically, in thrombin, S195 is in conjugation with H57 and D18 and built the essential portion of the active center. The center is necessary for the nucleophilic attack of the target peptide bond. Therefore, the chemical structure of thrombin makes its function highly specific. There are loops in the structure termed exosites that located on the surface and charge fragment and enclosed the active site. In the 60-and-γ loops, the catalytic triad is located. The 60-loop is hydrophobic and interacts with hydrophobic residues in the amino-terminal side of substrates cleavage part. It gives thrombin structure rigidity that induced by pro residues (P60b and P60c). On the other hand, the hydrophilic γ-loop interacts with carboxyl-terminal in substrate cleavage side to target scissile bond and gives more mobility to structure. (Fig. 1.1) The exosites I and II are two anion-binding loops made of separate residues. The exosite I is located around K36, H71, R73, R75, Y76, R77a, and K109/110, while exosite II surrounds residues R93, K236, K240, R101, and R233 (Huntington et al, 2005). Anionic exosites interaction with the negatively charged region in thrombin's substrate and

cofactors are specific. The cofactor promotes thrombin's specificity and the catalytic reaction of thrombin for different substrates. This explains paradox which thrombin as protease has multiple and opposing substrates reactions. The thrombin protease activity is controlled. The Na^+ - binding site in the loop modulates thrombin activity (Page et al, 2005). When it bound, thrombin function changes by enhancing access of protease coagulant substrates such as fibrinogen, factor V, factor VIII, and PAR1 to the active site. In particular, Na^+-binding supports pro coagulant substrates over anticoagulant ones like protein C (Pineda et al, 2004).

While Na^+ concentration in blood (140 mM) is slightly higher than the affinity of Na^+-binding site for this metal ion, it is considered under physiological condition, C. 60% of thrombin molecules' structure is occupied by Na^+. The procoagulant activity of mutants E217 K and W215A/E217A is less due to more "Na^+ free conformation" while keeping anticoagulant activity. Also, they are inactivated more slowly by antithrombin and may show more therapeutic anticoagulant activity (Gruber et al, 2002; Gruber et al, 2006). The complexity of thrombin is the product of a conformational change in structure.

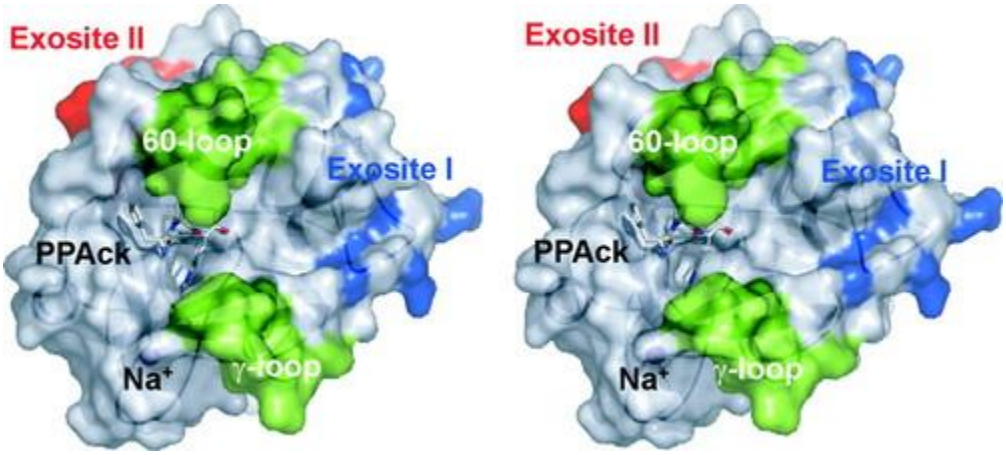

Figure 1. 1 Structure of thrombin. The figure (displayed with a transparent surface and underlying ribbon structure) shows positions of relevant specificity-determining sites: the 60- and γ-loops (green), residues that makeup exosite I (blue), and residues in exosite II (red). The position of coordinated Na^+ is indicated (Adams et al., 2006).

1.2 Thrombin generation

The maintaining of homeostasis is important and regulates by vascular endothelium. In physiological condition, several events such as vascular dilation, inhibiting platelet adhesion and thrombin generation are mediated by homeostasis. The vascular injury may start among acute or chronic inflammation such as atherosclerosis or during balloon angioplasty. The pro-inflammatory environment favors leukocyte transmigration toward the inflammatory site.(Popović et al, 2012). In detail, when tissue factor binds to proteinase factor, FVIIa, it activates FX. The FX activates prothrombin as an inactive precursor of thrombin that circulates in plasma at concentration C. 1.4 µM. The cleavage occurs at R320 to generate meizothrombin and then at R271 to generate thrombin (Orcutt et al, 2004). The prothrombin activation causes inefficient thrombin generation. Following thrombin generation, the coagulation cascade occurs. The activation of FV and VIII in cascade is thrombin dependent. The proteolytic reactions leave F1+2 containing prothrombin GI and kringle domains.

Thrombin is generated by activation of coagulation proteinase and attack to its target substrates. Therefore, to interact with many targets, it must be mobile. The activation of prothrombin exposes thrombin active site, charged binding sites such as exosites I and II are necessary for thrombin's specificity. (Fig. 1.1).

1.3 Homeostatic substrates of thrombin

1.3.1 Fibrinogen

The early function of thrombin is to generate fibrin clot from fibrinogen (Fig. 1.2) (Mosesson et al, 2005). Essentially, the two fibrin-peptides (FPA, 16 amino acids; FPB, 14 amino acids) release from fibrinogen Aα and Bβ chains respectively. At the first, the FPA cleavage generates fibrin monomer (termed fibrin I) then spontaneously polymerized to form protofibrils. The FPB cleavage is fibrin II protofibrils which undergo lateral aggregation and platelet traps into fibrin meshwork. This cause clot stabilization and prevents bleeding.

1.3.2 Factors V and VIII

Following thrombin generation by activation of prothrombin, the thrombin enters feedback activation loop and influences clotting cascade by activation FV and VIII factors. This enhances FXa and IXa and further sustained thrombin formation (Mann et al, 2003) (Fig.1. 2).

1.3.3 Factor XIII

Thrombin enters different events such as anticoagulation, antifibrinolytic which promotes anti-inflammatory activity. It also has a function in a procoagulant reaction that activates FXIII proteolytically. This reaction is enhanced by fibrin. Thrombin cleaves FXIII A subunit after R37 and causes amino-terminal active peptide release. By exposure of active peptide site C314 residue, the active transglutaminase generates and catalyzes covalent crosslinking and results in fibrin fibrils stabilization. (Fig.1.2) (Mosesson et al, 2005).

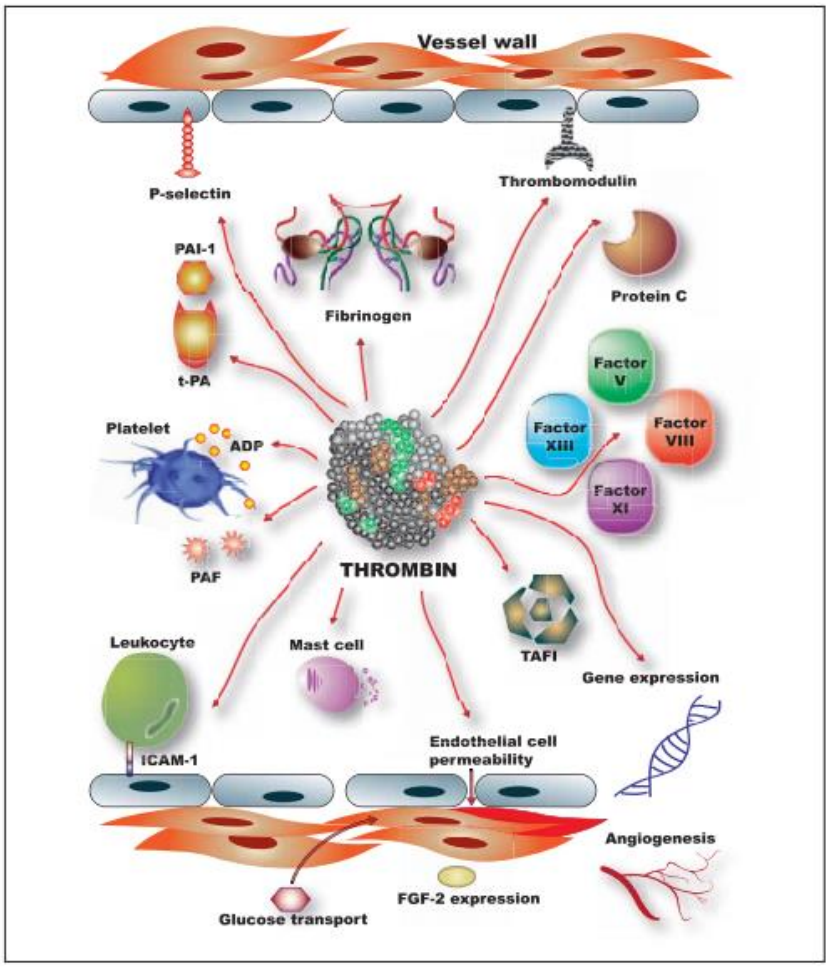

Figure 1. 2 Multifunctional roles of thrombin. When thrombin activated through the coagulation cascade, has a function in procoagulant, anticoagulant, antifibrinolytic, and pro/anti-inflammatory pathways.PAI-1, plasminogen activator inhibitor 1; t-PA, tissue plasminogen activator; PAF, platelet activation factor; TAFI, thrombin activable fibrinolysis inhibitor; ICAM-1, intercellular adhesion molecule 1; FGF-2, fibroblast growth factor-2. (Siller-Matula and Jolanta M., et al,.2011).

1.3.4 Protease activated receptors

Thrombin has multiple functions such as anticoagulant, anti-inflammatory, and antifibrinolytic. It has been reported that thrombin activates different cell types such as endothelial cells, platelets and dendritic cells (DCs). (Coughlin et al, 2000; Davey et al, 1969; Li et al 2008). These cellular reactions are mediated by cell surface receptors termed protease-activated receptors (PAR) 1, 3 and 4. (Crawley et al, 2007). The PARs consists of seven-transmembrane G-protein-coupled

receptors which are activated through cleavage of proteases such as thrombin at amino terminus extracellular domain. The new amino-terminal domain unmasked and acts like a tethered ligand that auto activates the receptor. (Coughlin et al, 1999;Coughlin et al, 2000).

1.3.4.1 G-protein- coupled receptors

The G-protein-coupled receptors superfamily is a group of molecules with different important roles in biological events. They have a significant role in human's disease like cancer. The most important agonist for GPCRs is thrombin. It has been reported that protease has involved in tumor progression, promoting tumor cell invasion and metastasis by extracellular matrix cleavage(Mook et al, 2004). However, studies reported that protease target divers substrates and promotes tumor evolution (López-Otín et al, 2007). The subunits if protease acts like signaling molecules and mediates cellular reactions through specific GPCRs called PARs. Four types of PARs have been cloned and all have a common mechanism of activation (Déry et al, 1998). PARs activated by even low concentration of protease at a specific site within extracellular N-terminal and new N-terminus unmasked and further activates the receptor. PAR 1, 3, and 4 are activated by thrombin while PAR 2 is activated by other proteases such as trypsin, tryptase, coagulation protease upstream of thrombin, tissue factor (TFs) VIIa and Xa (Macfarlane et al, 2001; Hollenberg et al, 2002; Ossovskaya et al, 2004; Vu et al 1991; Ishihara et al, 1997;Xu et al 1998).

1.3.5 Thrombin and PARs interaction in angiogenesis

1.3.5.1 Action of PAR-mediated thrombin on endothelial cells

Thrombin as a protease has a significant role in clot formation, platelet activation and inhibition of blood loss. Thrombin influences cancer by promoting angiogenesis. (Maragoudakis et al, 2002;Tsopanoglou et al, 1993; Haralabopoulos et al, 1997). The effect of thrombin on endothelial cells and activation of these cells may have a significant role in angiogenesis and further cancer development (Fujiwara et al, 2005; Vliagoftis et la, 2002; Kataoka et al, 2003;Dimitropoulou et al, 1998; Tsopanoglou et al, 1997; Maragoudakis et al, 2001; Marutsuka et al, 2002). Essentially

angiogenesis is described by activation and invasion of endothelial cells through the basement membrane and migration into the distal site. (Tsopanoglou et al, 1993; (Haralabopoulos et al, 1997; Dimitropoulouet al, 1998; Tsopanoglou et al, 1997; Maragoudakis et al, 2001). Thrombin influences endothelial cells adhesion and activates normally inactive cells at in the initiation of angiogenesis cascade. It starts with the local decomposition of basement membrane. This eases endothelial cells migration into the distal site. When endothelial cells exposed to thrombin, the cell adhesion to basement membrane components decreases with the time-and dose-dependent manner and increases in matrix metalloproteinase (MMP-2) activation (Dimitropoulou et al, 1998; Tsopanoglou et al, 1997; Maragoudakis et al, 2001). The release of endothelial cells is concomitant with the liberation of other angiogenic factors are hidden in ECM. Integrin αv β3 is known as a marker of the angiogenic phenotype of endothelial cells in vascular tissue (Brooks et al, 1994). The rabbit cornea model demonstrated that some of antibodies or peptides antagonist for this integrin, induced by fibroblast growth factor and inhibits tumor-induced angiogenesis by apoptosis in angiogenic blood vessels without affecting mature cells. (Brooks et al, 1996).Indeed ECs attach to thrombin by αv β3 and supply survival signal for cells during anchorage-independent migration. It is noteworthy to mention αv β3 and MMP-2 are coexist in the surface of angiogenic capillaries. (Haralabopoulos et al, 1997; Maragoudakis et al, 2001;Brooks et al, 1996). Thrombin enhanced vascular endothelial growth factor (VEGF) receptors expression (KDR and Flt1) in ECs which results in cells proliferation (Tsopanoglou et al, 1999). The study revealed that 8-12 h after exposure of ECs to thrombin, the VEGF receptors started to synthesize, higher than thrombin and VEGF alone. PARs 1-4 are expressed in arterial venous ECs (Fujiwara et al, 2005; Kataoka et al, 2003; Nelken et al, 1992; Mirza, et al, 1996;Schmidt et al, 1998). The receptors transmit signals after cleavage by a protease as thrombin. Activation of PAR 1 by thrombin generates cytoskeletal rearrangement and cell contraction and rounding in ECs. This effect destabilizes cell-cell contacts and increases vascular permeability. (Camerer et al, 2000; Riewald et al, 2001;Garcia et al, 1995; Vouret-Craviari et al, 2002). Following that, cells and molecules migrate to the subendothelial compartment and cell adhesion molecules expression such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, P-selectin and E-selectin increase (Minami et al, 2004).

1.3.5.2 Thrombin receptor activating peptide-6 (TRAP-6)

Human umbilical vein endothelial cells (HUVECs) express PARs 1, 2 and 3 except 4 (O'Brien et al, 2000; Schmidt et al, 1998). One study reported the existence of PAR 4 in the endothelium of human coronary artery segments. PAR 1 is a significant receptor in ECs (Hamilton et al, 2000). Thrombin activates the receptor and generates new N-terminus, a soluble peptide acts as a tethered ligand and auto-activates the receptor. To explain in detail, thrombin activates PAR 1 in two stages (Fig. 1.3) (Vu et al, 1991). First, thrombin specifically binds to cleavage site in PAR 1 and secondly, it cleaves the Arg 41 and Ser 42 to generate the new N-terminal domain SFLLRN (seryl-phenylalanyl--leucyl-leucyl-arginyl-asparagine). It interacts with domains in extracellular loop 2 and causes conformation of G-protein receptor to alter. Other PARs such as PAR 3 contains the active site while PAR 4 lacks it. Therefore, it only responds to a high concentration of thrombin. (Coughlin et al, 2000; Xu et al 1998).

The resulting peptide SFLLRN cause auto-activation of the receptor. A synthetic peptide containing this motif able to activate the receptor (Vu et al, 1991.). Several structures synthesized in laboratories. Sequences with five amino acids (SFLLR-NH2) showed good agonist potency while sequence with 3 amino acids length showed a weaker effect. The phenylalanine residue at position 2 is crucial for agonist activity while N-terminal free amino group, a basic or aromatic residue at position 5 and aliphatic residue at position 4 have a moderate effect. Amino acids at positions 1 and 3 (S, L, and N) replaced with alanine and do affect agonist properties. Positions 3 and 6 would diversely substitute and position 1 and 3 substitutes with proline. (Scarborough et al, 1991; Sabo et al; 1992; Feng et al, 1995) Trap-6 is synthetic peptide contains six amino acids sequence with formula C_{34} H_{56} N_{10} O_9. Its function as tethered ligand is to cause PAR 1 activation independent of receptor cleavage. It is probed, PAR 1 in various cells and tissues. It is activated PAR 2. Therefore it is used as an agonist for thrombin and able to activate the PAR 1 receptor on the surface of ECs. (Available at: https://pubchem.ncbi.nlm.nih.gov/compound/trap-6#section)

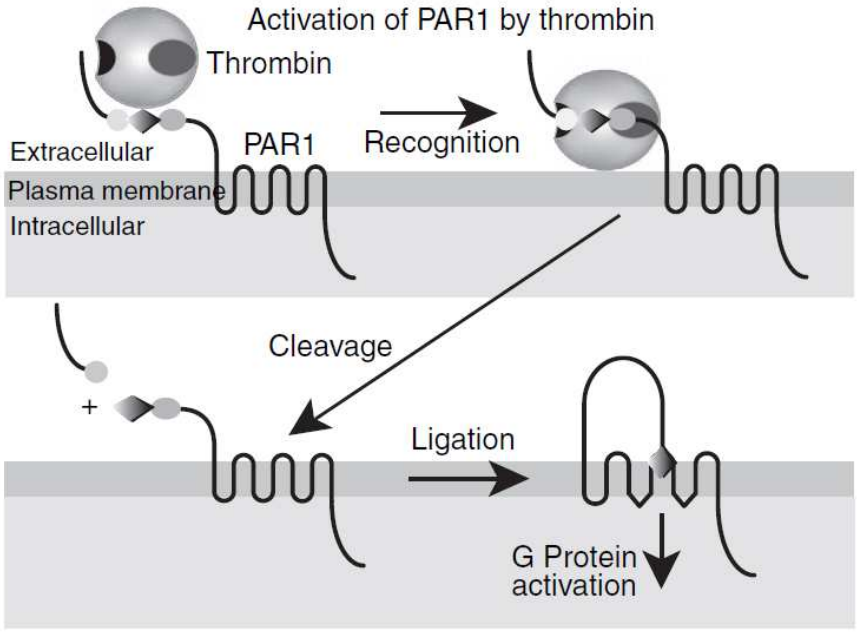

Figure 1. 3 Thrombin, serine protease acts like a hormone: Mechanism of the PAR1, G protein-coupled receptor for thrombin (large sphere) function. The N-terminal exodomain of the PAR1 is recognized by thrombin which interacts with both amino (P1-P4, small sphere) and carboxyl-terminal (P9'-P14'; small oval) and cleaves them. Thrombin cleavages peptide bond between receptor residues Arg41 and Ser42.The resulting sequence is new amino terminus beginning with sequence SFLLRN (Diamond) that functions as a tethered ligand interacts intermolecular with the body of the receptor to effect transmembrane signaling. The synthetic peptide of sequence SFLLRN mimics tethered ligand sequence and play a role as an agonist for PAR1 receptor cleavage independently. Therefore, PAR1 carries its ligand which after receptor cleavage being activated.(Vu et al, 1991; Vu et al, 1991; Liu et al; 1991; Mathews et al, 1994).

Aim of the study

TRAP-6, a tethered ligand from thrombin receptor cleavage, showed agonist effect for thrombin. Studies demonstrated the agonist activity of TRAP-6 on human platelet aggregation and upregulation of vascular endothelial growth factor's (VEGF) mRNA expression. However, angiogenesis potential and influence of TRAP-6 independent of thrombin has remained unclear. Furthermore, data on TRAP-6 influence on endothelial cell activation and proliferation behavior are lacking. Besides, the expression pattern of various cell adhesion molecules on the surface of vascular endothelium exclusively by TRAP-6 have remained unsolved. In this study, we determine the effect of TRAP-6 on human umbilical vein endothelial cells (HUVEC) and adipose-derived stem cells (ASCs) proliferation using the bromodeoxyuridine (BrdU) assay. TRAP-6 vasculogenesis function in HUVECs/ASCs co-culture on fibrin matrix is determined quantitatively. In addition, the expression pattern of surface markers on HUVEC is determined using flow cytometry.

Chapter 2

Materials and methods

1.4 Antibodies

To measure HUVEC markers by flow cytometry, PE, Cy5 and FITC labeled mouse anti-human antibodies were used. All objective markers were cell surface antigen. Thrombomodulin (CD141, PE-labeled, catalog 559781 form BD Pharming™), VEGFR-2 (CD 309, PE-labeled, catalog 130-102-559 from Miltenyi Biotech), CD 146 (PE-labeled, catalog 561013 BD Pharmingen™), CD 73 (PE-labeled, catalog 560847 BD Pharmingen™), Tie2/Tek (CD202b, PE-labeled, catalog 334205 BioLegend), CD 34 (FITC labeled, catalog 11-0349-42 Invitrogen), E-selectin (CD 62E, Cy 5 labeled, catalog 555648 BD Pharmingen™), PECAM (CD31, FITC labeled, catalog 560984 BD Pharmingen™), VE-cadherin (CD 144, FITC labeled, catalog 560411 BD Pharmingen™), and PE-Cy™5 Mouse Anti-Human isotype control (IgG2a , catalog 555975 BD Pharmingen™) and FITC anti-human Ig (Mouse IgG2a , catalog 316606 BioLegend) were stored in cold room.

1.5 Reagents

The phosphate buffer saline (PBS, catalog 17-516F, Lonza) used with composition KCl 0.2g/l, KH_2PO_4 0.2 g/l, NaCl 8.0 g/l and Na_2HPO_4 anhydrous 1.15 g/l without Ca and Mg with pH between 7.0 and 7.5. Trypsin/EDTA 10 X consisted of trypsin 5 mg/ml, EDTA 2.2 mg/ml diluted in PBS 1:10 (catalog 17-161E, Lonza). Accutase® Cell Dissociation Reagent, fetal bovine serum (FCS, Sigma-Aldrich Co. LLC., St. Louis, MO, USA), dimethyl sulfoxide (DMSO), Endothelial basal medium-2 500 ml (EBMTM-2, catalog CC-3156, Lonza Walkersville, MD, USA), Thrombin 4 U/ml and 500 and fibrinogen (TISSEEL®, Baxter), aprotinin 3000 KIE/ml, TNF α 10 µg/ml, thrombin receptor activating peptide-6 10 mM (SFLLRN, CAS number 141136-83-6 purchased from Abcam), and CaCl 2 40 mM, BrdU assay kit (Cat. Number 11 647229001 purchased from Roche).

1.6 Full EGM-2 preparation

In this study, Cells consume full EGM-2 contain 5 % fetal Calf serum (FCS). The endothelial basal medium (EBM-2) purchased from Lonza and keep at -4^{0}C. The following reagents added as following: 5 % FCS (10 ml in supplement and 15 ml prepared and stored in -20^0 C), Fetal Bovine Serum, Hydrocortisone, hFGF-B, VEGF, R3-IGF-1, Ascorbic acid, hEGF, GA-1000 (antibiotics gentamicin and amphotericin-B) and heparin. The cocktail mixed well, was aliquot into 50 ml falcon tubes, and stored at -20^0 C. HUVECs medium changed each 2 days and whenever they thawed, medium should be change after 4 h due to they cannot survive DMSO. In addition, to make fibrin clot, for cell suspension step and fibrinogen mixture preparation, Full EGM-2 containing 5 % FCS without antibiotic was used.

1.6.1 Isolating cell

In this project, human umbilical vein endothelial cells (HUVECs) and adipose-derived stem cells (ASCs) were used. The isolation of EC as well as ASC were approved by the ethics committee of Upper Austria (ethics vote #200) and performed after patients gave written informed consent. Research was performed in accordance with relevant guidelines and regulations. HUVEC were isolated as described elsewhere (Petzelbauer et al., 1993). ASC were isolated from liposuction material as previously described (Wolbank et al., 2007; Priglinger et al., 2017) and cultured in EGM-2 supplemented with additional FCS to a final concentration of 5%. GFP-HUVEC were retrovirally infected using protocol described elsewhere (Mühleder et al, 2018).

1.6.2 Passaging cells

After cells cultured into T25 or T75 (Adipose-derived stem cells only cultured into T75 because of the high confluency after 24 h), they got confluent approximately after one week. To transfer cells to new flasks, we splitedd or passage them as following: First, medium was removed and the flask was washed twice with 5 ml or 10 ml for T25 and T75 respectively. After washing step, 1ml trypsin for T25 and 2 ml for T75 flasks were usedw. Flask was incubated for 5 minutes at 37^0 C and cells checked under the microscope. Cells detached during this time and if some aggregations were observed, flask was shaked a little and 4 ml full EGM-2 added to T25 or 8 ml to T75 flask to inhibit trypsinization. The flask was washed well, alle suspension was taken off,

and transferd into 50 ml falcon tube. Depending on the experiment, cell suspension was wcentrifuged (like fibrin clot experiment) at 1000 rpm for 5 minutes, to collect cells at the bottom of the tube in a pellet. The supernatant removed and the cells were suspended in a specific amount of medium depends on the type of flask and number of cells. Otherwise, the suspensions were transfered to new flask or plate. Besides, to identify exact cell numbers, the protocol was eused: 10µl of cell suspension is taken and carefully added to the Neubauer chamber and put under the microscope to count 4 squares and take the mean value of them, then the number multiplied into dilution factor (5 ml for T25 and 8 ml for T75). This gives a total number of cells in the whole flask.

1.6.3 Freezing cell

The cells were detached from the flask, first were centrifuged and 900 µl of FSC added to the cell pellet. 100µl of DMSO was added to cryotube and the cell suspensions were added afterward, mixed well and transferred to thawed isopropanol, which guarantees slow cell freezing, and helped to avoid cell breaking and degradation. The cell keeps overnight at -800 ^{0} C. After 24 h, cryotube will transfer to -196^{0} C.

1.6.4 Thawing cell

Based on how many cells is needed to be thawed, the appropriate flask was used and medium was added and put into incubator before bringing cells from -196^{0} C. The frozen cells put into 37^{0} C water bath and thawed until only small ice-clot left. Then immediately cells were transferred into medium and incubated it at 37^{0} C. In this study, the main cells are HUVEC, so due to this cell cannot tolerate DMSO condition; the medium should be changed after 4 h. In addition, the medium was changed every two days.

1.7 TRAP-6 preparation

Thrombin receptor activating peptide-6 (from here on termed TRAP-6) supplied with molecular weight 1207.4 g/mole and 240 mg was freeze at -20^{0} C. To preparing 10 mM TRAP-6 solution, 24 mg was weighted. 1.5 ml PBS added to Eppendorf tube. PH measured with tornasol paper and

adjusted with HCL and NaOH. After reaching pH 7, 0.488µl PBS was added to reach 1.988µl final solution. The stock solution then was freezed at -20 ^{0}C for further experiment.

1.8 Cell proliferation investigation, using BrdU assay

1.8.1 HUVEC proliferation assay

Confluent HUVEC #41 passage 2 first were washed twice with 5ml PBS and 1ml trypsin 1X used to detach them and incubated for 5 minutes at 37^{0}C. 4 ml full EGM-2 containing 5% FCS was taken to inhibit trypsinization. The cell suspension was transferred into a new 50 ml falcon. 10 µl of cell suspension was taken and counted by cell counter. The cell suspension was counted, which included 2×10^{5} cells. The volume was 0.278 µl, this amount multiplied in 3 because we wanted to have three different cell numbers in the designed plate. 0.836 µl of cell suspension were taken and medium added up to 3 ml total volume. 96 wells plate was chosen, it was divided into three different cell numbers including 10^{4}, 5×10^{3} and 2.5×10^{3} with four replications. 50 µl full EGM-2 containing 5% FCS was added to 84 wells. 100 µl of the cell suspension was taken, added to the first column that assigned to 10^{4} cells, another 100 µl of the cell suspension was taken and added to the first column that assigned to 5×10^{3}, mixed well and the whole 100 µl was taken and added to the first column of 2.5×10^{3} cells.100 µl from 2.5×10^{3} was taken and discarded. Therefore, the final 96 wells plate was prepared with three different cell numbers including 10^{4}, 5×10^{3} and 2.5×10^{3} with four replications. The plate was shaken a little and incubated overnight at 37^{0}C. Cells morphology was checked after 24 h with 5X and 10X magnification with a light microscope (Zeiss Observer A1, Zeiss).

1.8.2 Stimulating HUVEC with TRAP-6

To investigate cells proliferation, Roche protocol for BrdU assay was used. BrdU labeling reagent 1000X was diluted into 1X with 10 ml full EGM-2 containing 5% FCS. To check the different concentration of TRAP-6, seven different solutions were prepared as following:

500, 250, 100, 50, 10, 1 µM and zero (only EGM-2 with BrdU reagent, termed control). The serial dilution was used to prepare the concentrations. Seven tubes for each concentration were prepared. 1250 µl of full EGM-2 including 1X BrdU was added to each tube, except tube 500 µM which contained 250 µl of medium- BrdU reagent mixture. TRAP-6 10 mM was thawed, 125 µl was added to falcon 500 µM, mixed well and 1250 µl pipetted to next one and this was repeated until the sixth falcon. As a result, TRAP-6 was diluted in six different falcons with dilution factor 1:2. One falcon was assigned as negative control, which did not contain any TRAP-6. With 100-µl multi pipet channels solution, solution was pipetted to each well, in total for each concentration 12 wells. Plate was incubated for 24 h and cells morphology was checked under the light microscope.

1.8.3 Staining and detection step

First, the culture medium was aspirated from lower to higher concentrations, outside of laminar. According to Roche protocol, Fix Denat solution was wadded 200 µl/well from lower to higher concentrations. After 30 minutes FixDenat solution was removed by flicking off and tapping. Anti-BrdU-POD stock solution 1:100 in antibody dilution solution was diluted and added 100 µl/well for ideally 90 minutes at 25^0C. The solution was removed by flicking off and rinsed wells three times with 100 µl/well PBS. After the third washing step, substrate solution was added 100µl/well. After incubating for 5 minutes at 25^0C, until the color changed to blue and sufficient for photometric detection, the plate was inserted into ELISA reader. We measured the absorption of the sample in an ELISA reader at 370 nm (reference wavelength is approximately 492 nm). In this project, we did not use stopping solution and let the substrate reaction continue in 5, 10 and 15 minutes to reach optimal time for substrate reaction.

1.8.4 Adipose-derived stem cell proliferation assay

To investigate the influence of TRAP-6 on the proliferation of ASCs, BrdU assay was used the same as HUVEC. Briefly, ASC N314 passage 3 were thawed and were cultured into T75, a day before the experiment. 10 ml fresh full EGM-2, 5 % FCS added to T75 flask. ASC was thawed and added to the flask and incubate for 24 h. Confluent ASC first were washed twice with 10 ml PBS and used 2 ml trypsin 1X to detached cells and were incubated for 5 minutes at 37^0C. 8 ml of full EGM-2 contains 5% FCS to inhibit trypsinization was used. The cell suspension was

transferred into new 50 ml falcon. The total amount of cells were calculated and to seed 2×10^5 cells into 96 well plates, 0.547 µl of cell suspension was taken and because of three cell numbers (10^4 , 5×10^3 and 2.5×10^3), this number multiplied by 3. According to previously described procedure, cells were pipetted into columns (10^4, 5×10^3 and 2.5×10^3) with four replications. The plate was shacked well and incubated overnight at 37^0C. Cells morphology was checked after 24 h. The stimulating and staining steps were performed at the same as HUVEC and the absorption was detected after 5, 10 and 15 minutes with ELISA.

1.9 HUVEC/adipose-derived stem cell co-culture, analysis of in-vitro microvascular network formation

To determine effect of TRAP-6 on microvascular network formation of GFP-HUVEC/ASC co-culture, culture medium was treated with TRAP-6 100 and 500 µM.

1.9.1 GFP-HUVEC and ASC culture

HUVEC were retrovirally infected with green fluorescent protein (GFP) to document network-formation over time as previously described (Knezevic et al., 2017). Both ASC and GFP-HUVEC were cultured until reached enough confluency.

1.9.2 Fibrin matrix co-culture

To prepare fibrin matrix, developed protocol by Rohringer et al.,2014 was used. Fibrinogen 100 mg/ml powder was mixed with 5 ml full EGM-2, 5% FCS to produce fibrinogen 100 mg/ml and put it into incubator 37^0C for 10-15 minutes. Fibrinogen solution was stirred on warm (37^0C) magnetic stirrer (with the small metal piece inside of fibrinogen bottle) for 3-4 minutes. Fibrinogen solution was aliquoted into Eppendorf tubes (50-100 µl per tube), freezed, and stored at -20^0C. Fibrinogen was thawed at room temperature for 15-20 minutes. Fibrinogen 100 mg/ml was diluted with factor 1:20 in full EGM-2 containing 5% FCS without antibiotic to keep clot integrity, resulted in final 5mg/ml fibrinogen. Six Eppendorf tubes were prepared. Three tubes for GFP-HUVEC monoculture and the rest for GFP-HUVEC/ASC co-culture. 10µl of fibrinogen was

pipetted to each Eppendorf tube, and 140 µl and 90 µl of full EGM-2 containing 5% FCS without antibiotic was added to monoculture and co-culture tubes respectively.

Thrombin 4 U/ml was diluted with factor 1:10 in $CaCl_2$. 140 µl of thrombin was diluted with 1260 µl of $CaCl_2$ and was kept into the fridge. Meanwhile, twelve coverslips 15 mm diameter, which were immersed in ethanol 70%, and after they got dry, were placed into 12 wells plate.

GFP-HUVEC were detached, the total number were counted which was 4737500. Because the number of cells in 50 µl should be 2 ×105, the total number of cells was divided into 5×106, the resulting number was the amount of medium that should be added to cell pallet. Cell suspension were centrifuged and the pallet was suspended into 1.184 ml EGM-2. 50µl of cell suspension was pipetted in each Eppendorf tube and mixed 7-8 times with previously added fibrinogen. The final number of cells was 105 in each clot. To achieve a microvascular network, ASC should be co-cultured with GFP-HUVEC. To do this, cultured ASC was detached and counted to calculate the total number of cells, which was 39 ×105. Like GFP-HUVEC, cell suspension was centrifuged and suspended with 975 µl of EGM-2. To prepare GFP-HUVEC /ASC with a 1:1 ratio, 50 µl of ASC suspension was added to co-culture tubes and mixed 7-8 times up and down. For 1: 10 ratios, 5 µl of ASC suspension and 50 µl of GFP-HUVEC were added to Eppendorf tube. 200 µl of diluted thrombin was pipetted into one Eppendorf tube (both mono and co-cultures), suspended 5-6 times, and 200 µl of mixture was pipetted on each coverslip to form a clot. Therefore, each Eppendorf tube containing 400 µl thrombin/Fibrinogen-cells used to form 2 same clots. Clots were incubated at 370C for 30 minutes. The clots looked opaque after incubation, 1.5 ml of full EGM-2 containing 5% FCS added to each clot. To prevent clot degradation, only to GFP-HUVEC/ ASC co-culture 1:1 and GFP-HUVEC monoculture, 50 µl of aprotinin was added to one of each same clots.TRAP-6 10 mM was thawed. To check the effect of TRAP-6 on the maturation of microvascular tube formation, three conditions was designed. One raw including four clots, without TRAP-6 (termed control), the other raw was treated with 100 µM, and the other four were clots treated with TRAP-6 500 µM. Clots were incubated at 37^0C. Medium was changed every two days, aprotinin, and TRAP-6 with the same instruction were added as well. The images were taken from the same area of each matrix after day1, day 3 and day 7 under the epifluorescence microscope (Zeiss Observer A1, Zeiss) for further analysis, leading to 16 different values for each data set. Images were taken from co-cultures on day 7 and the network parameters, number of junctions, number of tubules, mean tubule length, and total tubule length were quantified. To do this, all images were randomized

using Adobe Photoshop CS5 (Adobe Systems, San José, CA, USA) and were analyzed in a blinded manner, and processed subsequently with Angiosys software (TCS Cell works) (Charwat et al., 2015; Knezevic et al., 2017).

1.10 Characterization of HUVEC surface markers by flow cytometry

1.10.1 Flow cytometry principles

Flow cytometry analyzes the optical properties of single cells and particles both qualitatively and quantitatively. Physical characteristics of certain cells population such as size (determine by forwarding angle light scatter) and internal complexity (determined by right-angle scatter) can be measured by flow cytometry. In principle, cells stained with antibody conjugated with fluorescent dyes and able to bind to specific proteins on the cell surface or inside cells. By passing the cells into the light source, the fluorescent molecules excited and jump to a higher state of energy and by returning to a relaxing state emit light at a higher wavelength (Keren et al, 1994; Shapiro et al, 1995; Orfao et al, 1995).

Emitted light scatter in all direction, collect via optics, and directed to series of filters and dichroic mirror in order to isolate specific wavelength. (Fig. 2.1.1) The photomultiplier tubes detect signals and digitize them for computer analysis for displaying in histogram or 2-D dot-plot formats.

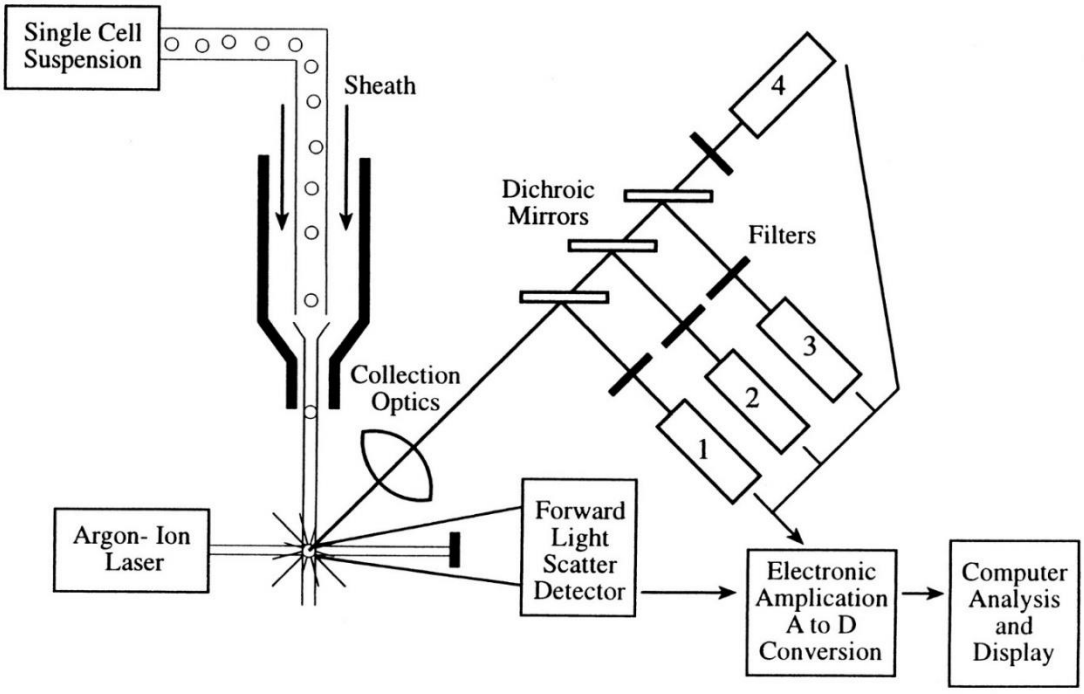

Figure 2.1. 1 Schematic of a flow cytometer. A single cell suspension hydrodynamically focused with sheath fluid to intersect an argon-ion laser. Signals collected by a forward angle light scatter detector, a side scatter detector (1), and multiple fluorescence emission detectors (2–4). The signals amplified and converted to digital form for analysis and display on a computer screen (Brown et al., 2000).

1.10.2 Characterization of E-selectin expression after 4 and 24 hours

HUVEC were washed with 2 ml PBS in nonsterile condition. 500 µl of accutase was used for cells detaching. Cells were incubated at 37^0C for 5 minutes. Meanwhile, four flow cytometry tubes werew labeled and kept into ice. The detached were cells suspended into 500 µl PBS 1% Bovine serum albumin (BSA), transferred into prepared tubes, and were centrifuged at 1300 rpm, at 4^0C for 5minutes. CD 62-E wconjugated with Cy 5 (ELAM), was transferred into ice. The supernatant was discarded and cell pallet was suspended into 200 µl of PBS 1% BSA. Each tube containedw 200-µl cells suspension and was splited into two tubes contain 100 µl of cell suspension. 2µl of E-selectin was wadded to each of 4- and 24-h prepared tube. Then, 2 µl of Immunoglobulin isotype control PE-CY 5 was added to each 4- and 24-h tube and tubes were kept into ice, in dark for 45 minutes.

1ml PBS 1% BSA was added to each tube and was centrifuged at 1300 rpm for 5 minutes at 4^0C. The washing step with the same volume of PBS 1%w BSA was repeated and the 300 µl of PBS 1% BSA was added to cells pallet and put on ice for flow cytometry analysis. First, the isotype measured and the CD 62E treated samples. Data were analyzed using Flow Jo VX software.

1.10.3 Characterization of HUVEC inflammatory surface markers after 24 hours

HUVEC # 41 p 6 were cultured into three T75. After a week, confluent cells were detached, and Trap-6 500 µM which was diluted into 9.5 ml full EM-2, thrombin 1 U/ml and $CaCl_2$ 40 mM as negative control were added to each of three T75 flasks. After 24 h, images were taken with 5X and 10X objectives under the light microscope.

For flow cytometry analysis, the same steps of staining were performed. Briefly, the medium was discarded and flasks were washed with 10 ml PBS. 2 ml of was accutase added to each flask and flasks were incubated for 5 minutes at 37^0C. 2 ml of PBS 1% BSA was added to each flask and cell suspension was transferred into 15 ml tubes, and centrifuged for 5 minutes at 4^0C at 1300 rpm. After discarding the supernatant, 800 µl of PBS 1% BSA was added to thrombin and TRAP-6 and 1 ml into negative control ($CaCl_2$) flasks. 100 µl of cell suspension was pipetted into each tube. There should be two more tubes for PE and FITC immunoglobulin isotype controls. 2 µl of each thrombomodulin, CD 31, CD 146, CD 73, VE-cadherin, VEGFR- 2, CD w34 and Tie-2 antibodies were added to specific tube. 2 µl of PE and FITC isotype controls was added into isotype tubes. Tubes were vortexed and incubated into ice in dark for an hour. 1 ml PBS 1% BSA was added to each tube and all were centrifuged for 5 minutes at 1300 rpm at 4^0C. After two times washing with 1 ml of PBS 1% BSA, and finally adding 300 µl of PBS 1% BSA, samples were analyzed by flow cytometry. Data analyzed using flow Jo VX software.

Statistics

Statistical analysis was performed using GraphPad Prism Version 5 (GraphPad). For comparing vascular parameters were obtained through network quantification on day 7 of co-culture treated with TRAP-6 100 and 500 µM compared to control, two tailed Mann-Withney tests was performed. To compare surface markers expression of TRAP-6 and thrombin

stimulated HUVEC with control, two tailed Mann-Withney tests were performed. Proliferation of HUVEC treated with TRAP-6 compared to the non-treated HUVEC, using one-way ANOVA test, and non-parametric test.

Chapter 3

Results and Discussions

1.11 TRAP-6 enhanced HUVEC and ASC proliferation

To investigate HUVEC proliferation in response to TRAP-6, BrdU assay was incorporated. Incubation of 10^4, 5×10^3 and 2.5×10^3 HUVEC with TRAP-6 resulted in increasing HUVEC proliferation compared to control. HUVEC morphology before treatment with TRAP-6 (Fig. 3.1a) and 24 hours after treatment with TRAP-6 (Fig. 3.1b) Images clearly demonstrate the proliferation of HUVEC is increased in response to TRAP-6. The proliferation is significantly enhanced by TRAP-6 500 and 250 µM compared to control (Fig. 3.1c) (Data shown only 10 minutes after using substrate solution). No toxic effect was observed due to TRAP-6 incorporation. This ensures TRAP is not toxic and using high concentration as 500 µM in culture medium did not show cytotoxic effect.

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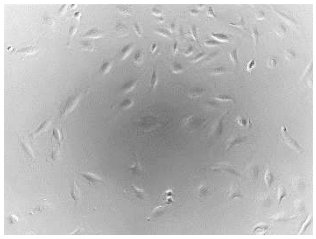

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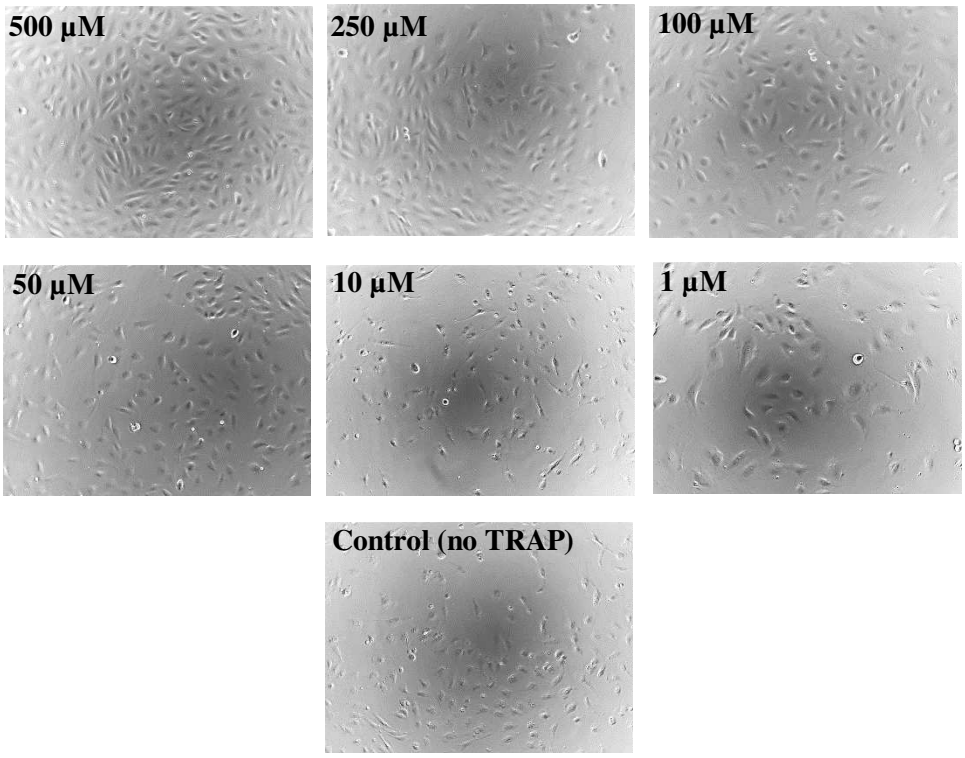

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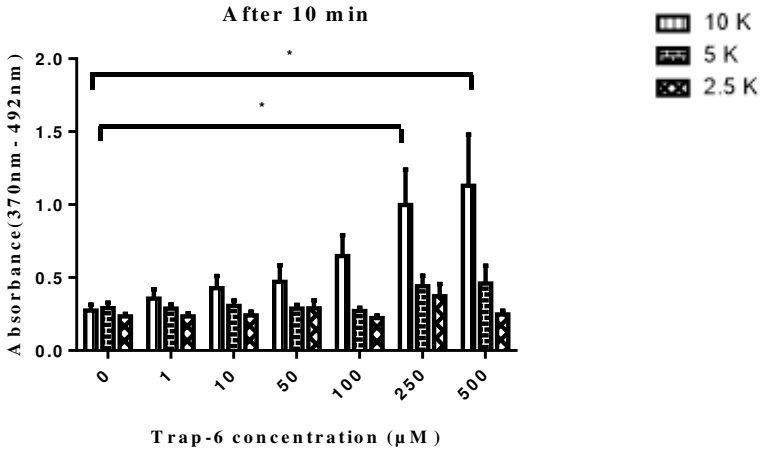

Figure 3.1 1 TRAP-6 induces human umbilical vein endothelial cells' (HUVEC) proliferation. a) Images of HUVEC passage 2 before treatment with TRAP-6. b) Images of HUVEC treated with 1-500 µM TRAP-6 after 24 hours incubation. The control sample includes cells with BrdU without TRAP-6, to compare proliferation with TRAP treated samples. The number of HUVEC treated with TRAP- 6 500 and 250 µM are higher compared to other concentrations. c) Samples were treated with BrdU antibody solution and stained with substrate solution, and the absorption was determined using ELISA. TRAP-6 500 and 250 µM enhanced proliferation, both significantly compared to control. This is clearly shown in images after treatment. $*p < 0.05$. Scale bar 100 µm.

Adipose-derived stem cell proliferation was measured as a function of TRAP-6. Likewise HUVEC, 10^4, 5×10^3 and 2.5×10^3 , ASC were incubated with TRAP for 24 h and results are showing that, the number of cells are increased compared to samples before treatment (Fig. 3.2a) especially samples treated with TRAP-6 with concentration 50 µM compared to control (Fig. 3.2b) showe higher number of cells as a result of treating with TRAP-6 (data only represents the morphology before and after treatment for 10^4). The incubation of ASC with TRAP-6 results in higher proliferative response which TRAP 50 µM enhanced proliferation significantly compared to control (Fig. 3.2c) (Data shown only 15 minutes after using substrate solution due to cause significantly increase in proliferation compared to 5 and 10 minutes). The data are in agreement with the change in morphology of TRAP-treated ASC. Moreover, similar to HUVEC, TRAP is not cytotoxic for ASC and cells proliferate when were treated with high amount of TRAP.

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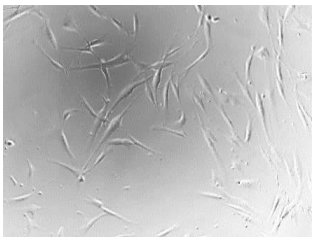

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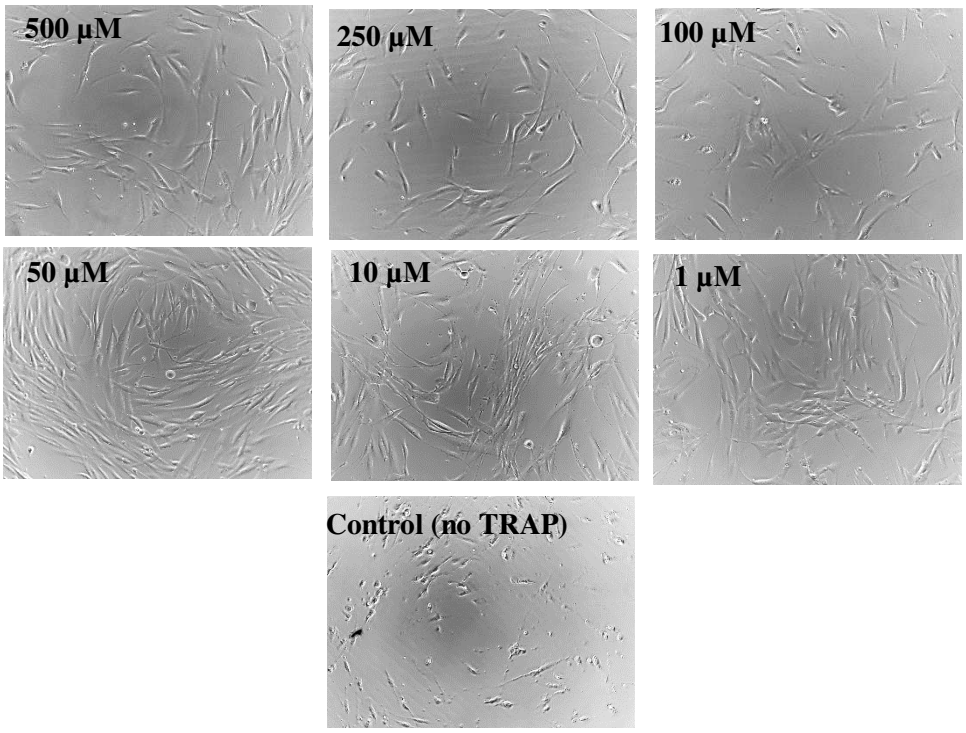

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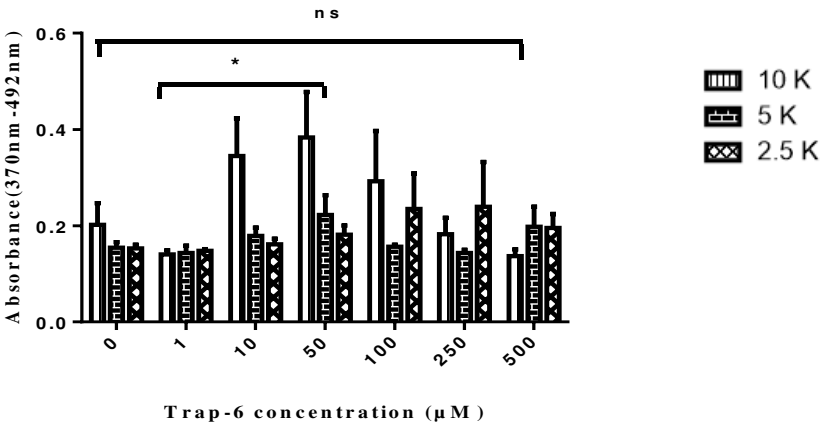
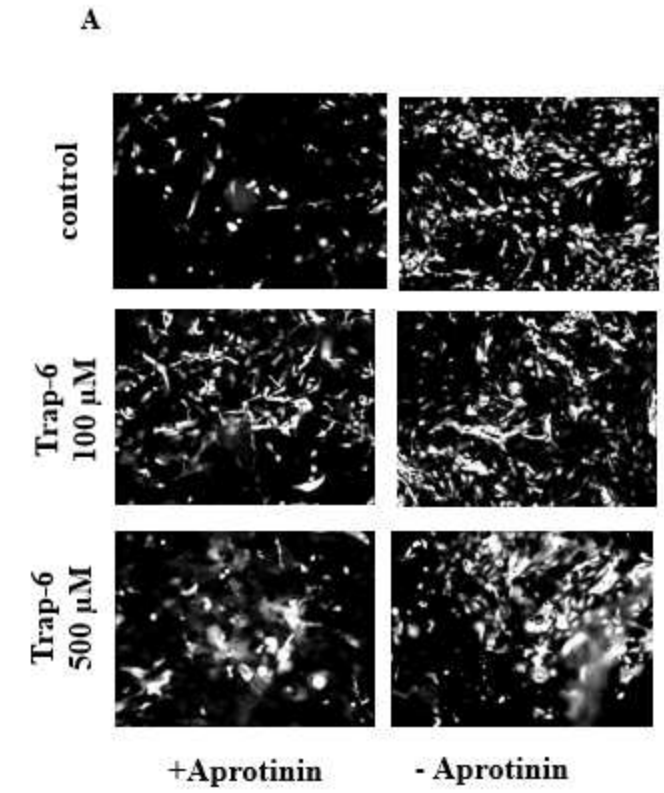
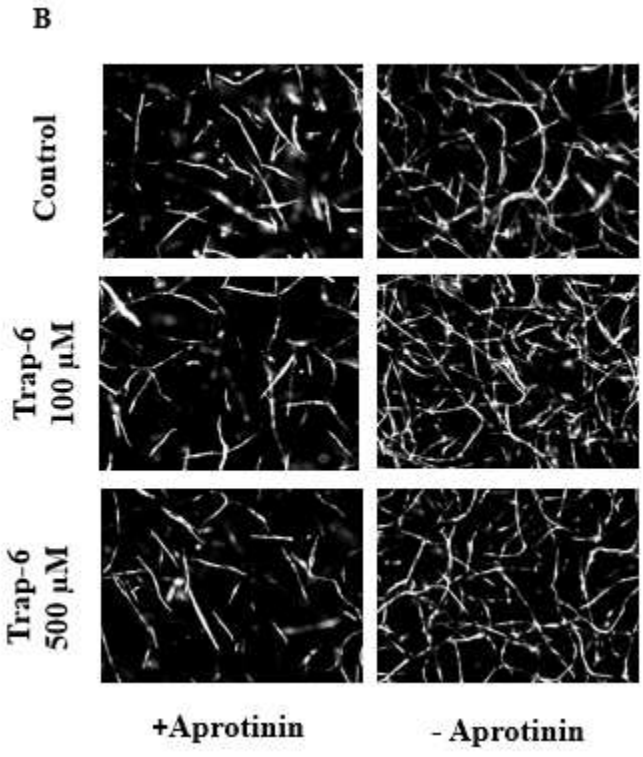


Figure 3.1. 2 TRAP-6 induces adipose-derived stem cells (ASCs) proliferation. Images of ASC passage 4 before treatment with TRAP-6. b) Images of ASC treated with 1-500 µM TRAP-6 after 24 hours incubation. The control sample includes cells with BrdU without TRAP-6, to compare proliferation with TRAP treated samples. The number of ASC that were treated with TRAP- 6 50 µM are higher compared to control. c).Samples were treated with BrdU antibody solution and stained with substrate solution, and the absorption was determined using ELISA. TRAP-6 50 µM enhanced proliferation significantly compared to control. This is clearly shown in images after treatment. *p < 0.05, Ns= not significant, Scale bar 100 µm.

1.12 TRAP-6 improved microvascular network formation in fibrin matrix

GFP-HUVEC/ASC with ratio 1:1 embedded in fibrin matrix, were treated with TRAP-6 100 and 500 µM with and without aprotinin. The microvascular networks on day 7 on a fluorescence microscope demonstrate, ASC started to differentiate into endothelial cells and formed microvascular tubules, which is not visible in GFP-HUVEC mono-culture (Fig. 3.3a). The influence of TRAP-6 on vascular tubule formation in co-culture is hardly recognizable from control (no TRAP) (Fig. 3.3b). Quantification of vascular network demonstrates an increase in number of junctions, tubules and total tubule length of GFP-HUVEC/ASC, treated with TRAP-6 100 µM and 500 µM with no aprotinin compared to samples with aprotinin (Fig 3.3c). Indeed, TRAP-6 500 µM improved number of junctions and total tubule length significantly compared to control, and 100 µM improved number of junctions, number of tubules and total tubule length significantly compared to control**.**





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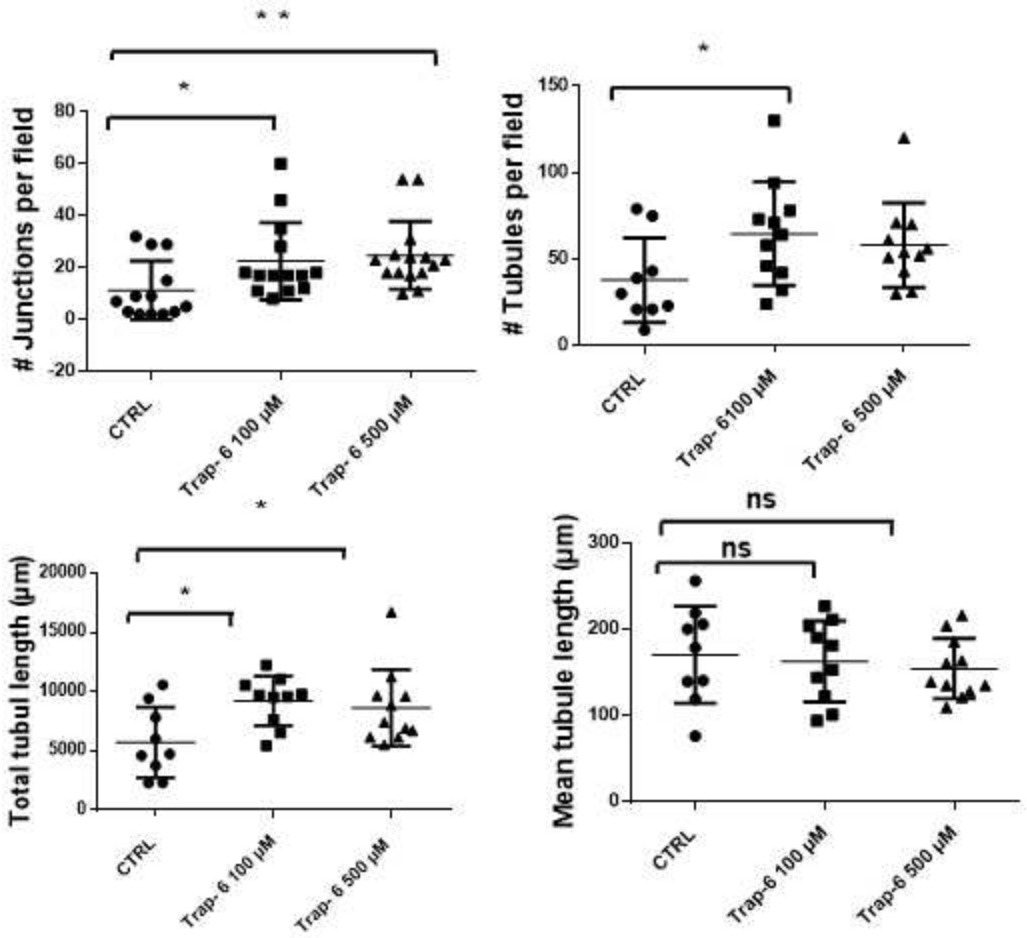

Figure 3.1 .3 TRAP-6 improves microvascular network formation in fibrin matrix. a) GFP-HUVEC monoculture in fibrin matrix treated with TRAP-6 100 and 500 µM on day 7. Because of the absence of ASC, vascular tubule did not form. b) GFP-HUVEC/ASC co-culture were treated with TRAP-6 100 and 500 µM forms microvascular network. The aprotinin impaired network formation while inhibited fibrinolysis. c) Quantified network showing parameters such as number of junctions, number of tubules and total tubule length were improved significantly with TRAP-6 500 and 100 µM. Results from triple separate experiments with different donors to eliminate effect only in one special donor. *$p < 0.05$; **$p < 0.01$. Ns= not significant, scale bar 100 µm.

GFP-HUVEC/ASC co-culture ratio 10:1 in fibrin matrix without using aprotinin, were treated with TRAP-6 100 and 500 µM. On day 7, images were taken on a fluorescence microscope. Lower number of ASC results in immature microvascular network formation (Fig. 3.4a). TRAP-6 influences maturation of network and the vascular network is hardly visible. While quantified networks show, TRAP-6 100 µM significantly increases number of junctions, tubules and total tubule length compared to control (Fig. 3.4b).

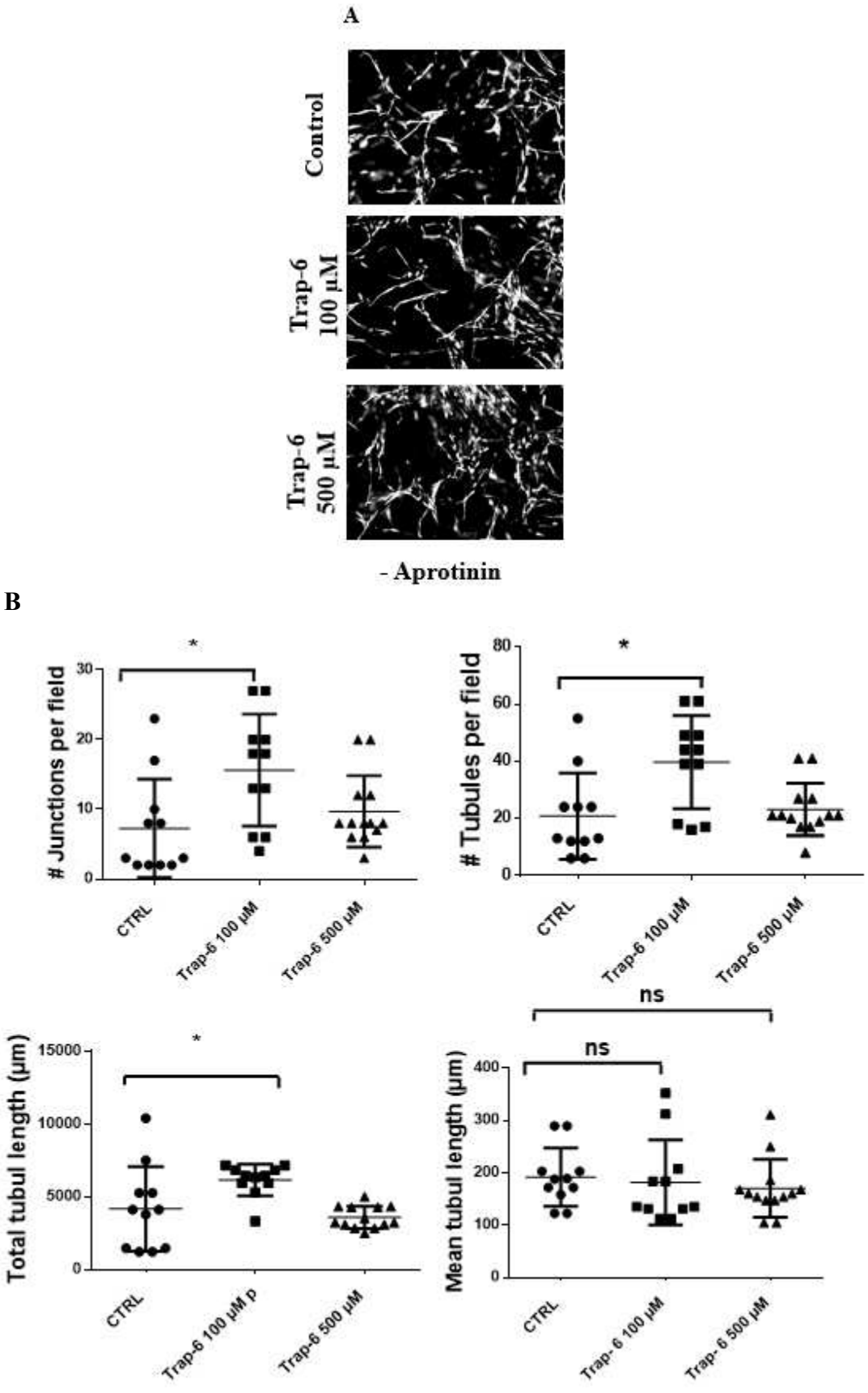

Figure 3.1. 4 TRAP-6 improved microvascular network formation in GFP-HUVEC/ASC co-culture with ratio 10:1. a) GFP-HUVEC/ASC co-culture were treated with TRAP-6 100 and 500 µM form microvascular network. Due to lower number of ASC in co-culture than HUVEC, resulting network does not show mature vascular tubules compared to co-culture with ratio 1:1 for HUVEC/ASC. b) Quantified network demonstrates significantly higher number of junctions, tubules and total tubule length in co-culture treated with TRAP-6 100 µM compared to control. $*p < 0.05$. Ns= not significant, scale bar 100 µm.

1.13 HUVEC-stimulated TRAP-6 expresses various surface markers

To investigate whether TRAP-6 influences expression of endothelial cell adhesion molecule, E-selectin (CD62E), HUVEC were cultured and stimulated with TRAP-6. Images were taken from stimulated-HUVEC with TRAP-6 100 µM and thrombin 1U/ml morphology compared to TNF-α 10 ng/ml as positive control and $CaCl_2$ as negative control (Fig. 3.5a and b). Although cells morphology does not show significant change like TNF-α, TRAP-induced HUVEC expressed more E-selectin after 4 h compared to thrombin and control (Fig. 3.5c). While expression was started to downregulate until 24 h. The fluorescence intensity determined by flow cytometry shows increasing in E-selectin expression during first 4 h and downregulating up to 24 h (Fig. 3.5d).

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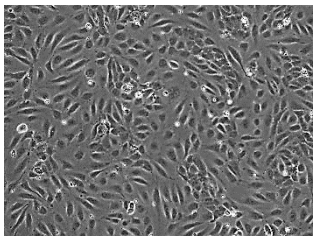

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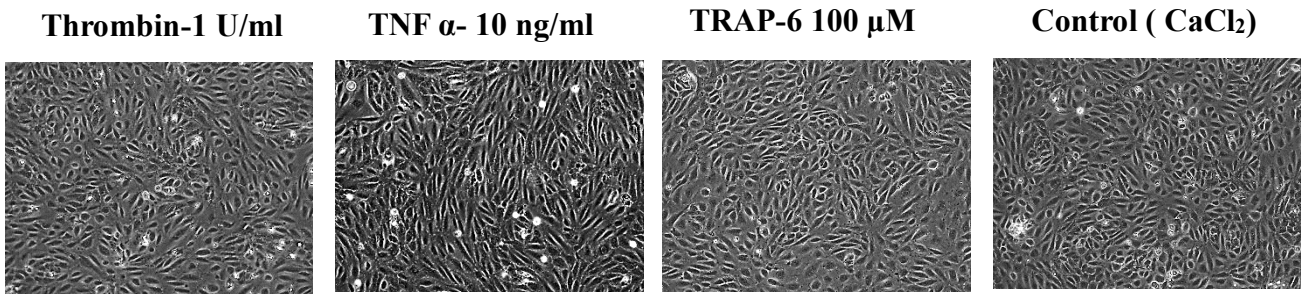

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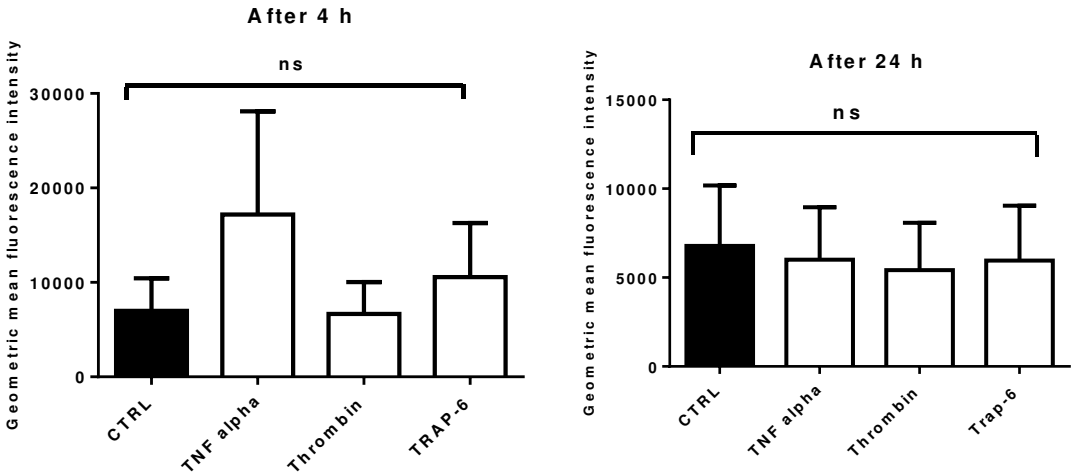

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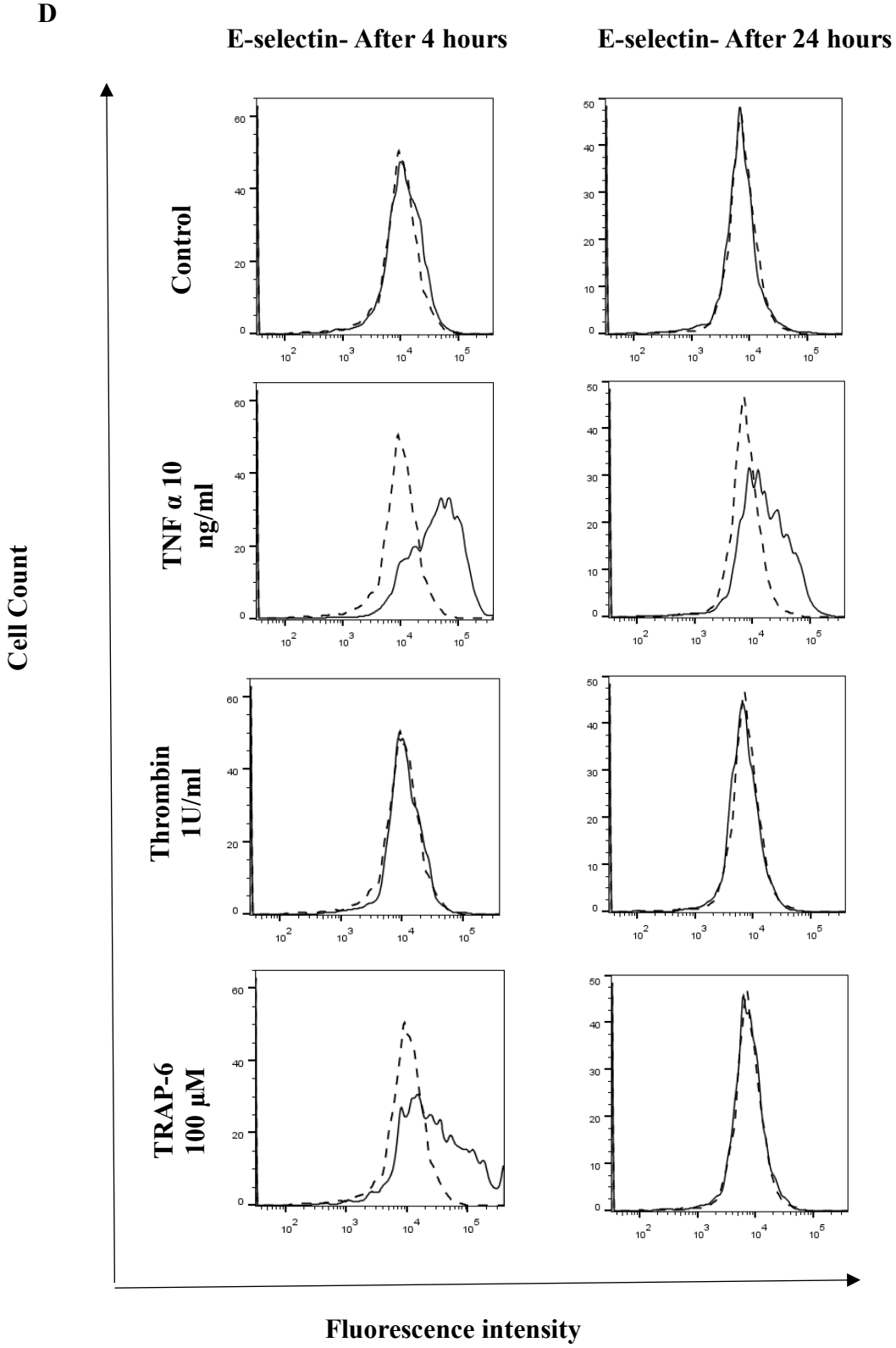
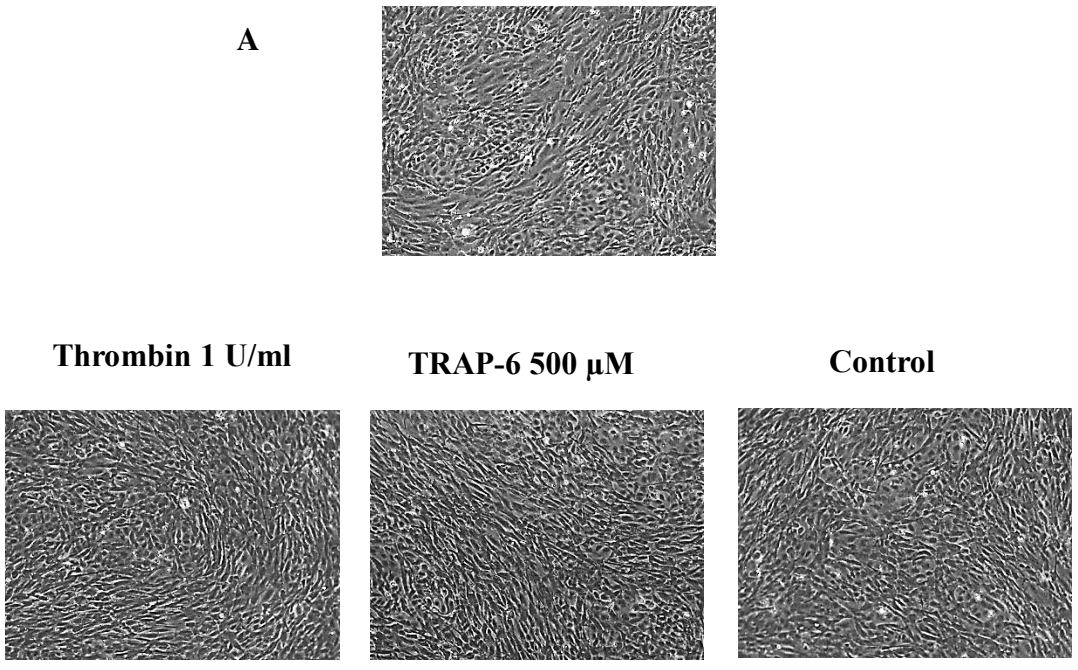


Figure 3.1 .5 E-selectin expression is enhanced in HUVEC-stimulated TRAP-6 100 µM. a) HUVEC morphology before stimulation, b) HUVEC morphology after stimulation with TRAP-6 100 µM, TNF-α 10 ng/ml, and 1U/ml thrombin were added to cell medium and $CaCl_2$ serves as a negative control. Stimulated cells with TNF-α as positive control show narrowed shape, but the change is not observable in HUVEC-stimulated with TRAP-6 and thrombin. c) Quantification of flow cytometric data reveals, E-selectin expression in TRAP-6 stimulated cells was increased after 4 hours, but no significant difference in thrombin can be determined compared to control, and expression was downregulated until 24 hours. d) Representative flow cytometric dot plots of TRAP-6$^+$/TNF$^+$/thrombin$^+$ HUVEC are shown. In TRAP-6 stimulated HUVEC, E-selectin expression is visible, but not significantly, while this effect cannot be observed in HUVEC-stimulated with thrombin. Ns= not significant. Scale bar 100 µm.

To evaluate different HUVEC surface markers such as thrombomodulin, CD31, CD34, CD146, VE-cadherin, VEGFR-2 and Tie-2 that all have whether anti or pro-inflammatory functions, HUVEC-stimulated TRAP-6 500 µM, thrombin 1 U/ml and $CaCl_2$ (as negative control) were characterized with flow cytometry. (Fig. 3.6a and b) HUVEC's morphology before and after stimulation are shown and no significant change in shape of cells is visible. We detected no significant difference in expression of all mentioned surface markers in TRAP-6 and thrombin-stimulated cells compared to control (Fig. 3.6c). Quantification of surface markers expression reveals slightly increase in CD73 expression of cells stimulated TRAP-6, decrease in CD31 expression and no change in VEGFR-2, VE-cadherinTie-2 and CD146 expression, no significant increase in CD34 and decrease in thrombomodulin expression in cells-stimulated with thrombin (Fig. 3.7d).



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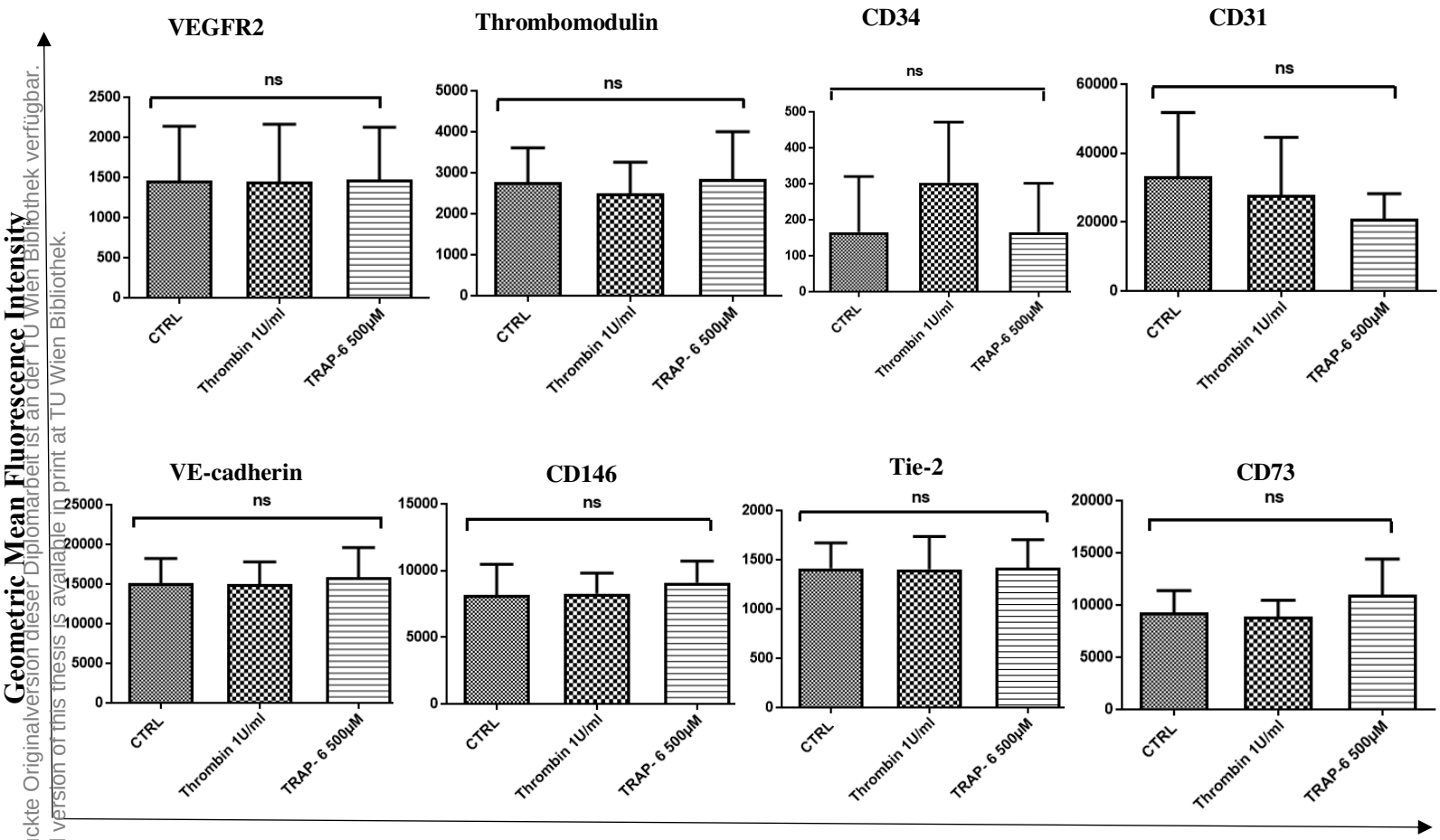

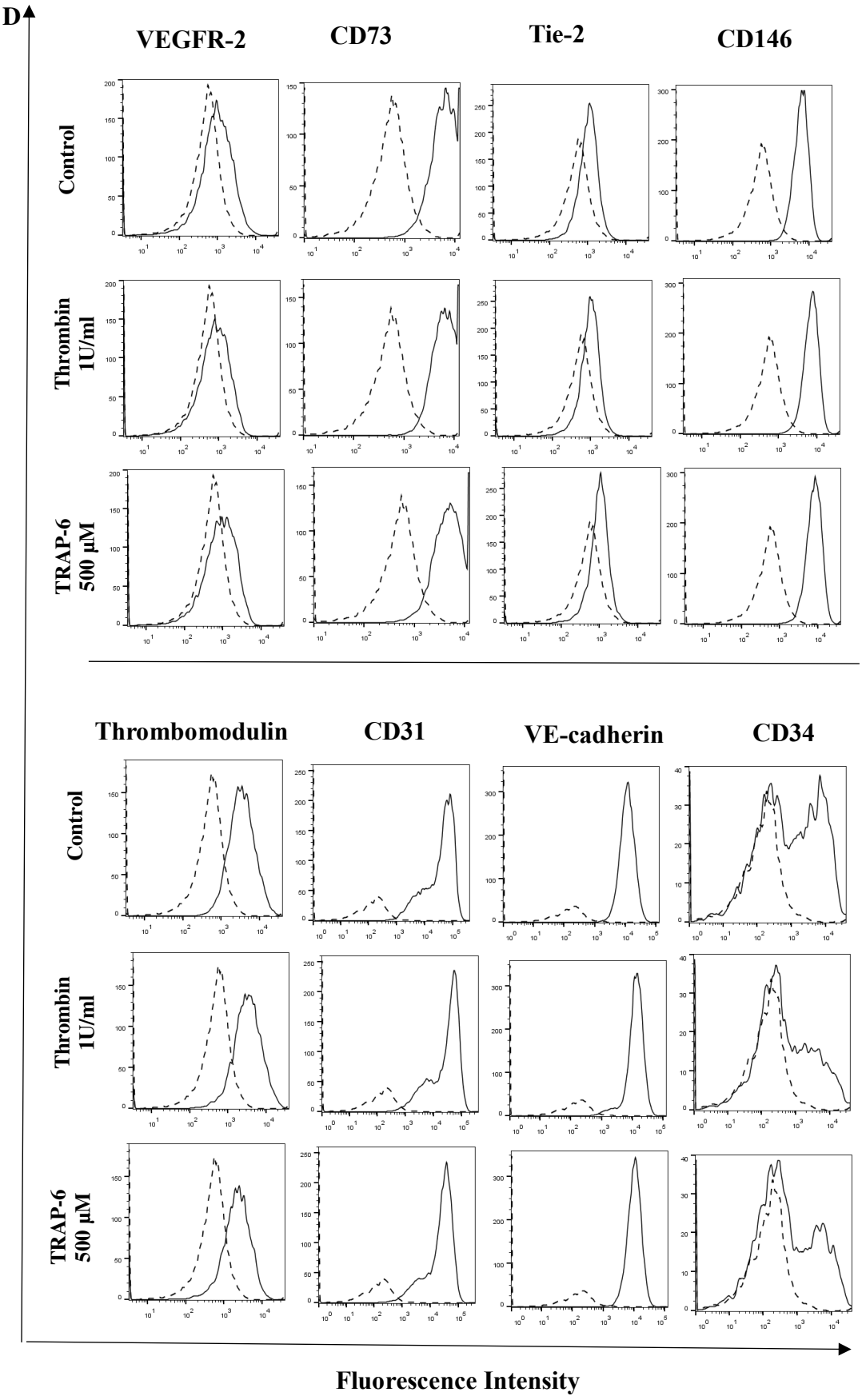

Figure 3.1.6 Characterization of HUVEC different surface markers. a and b) HUVECs' morphology before and after stimulation with TRAP-6 500 µM and thrombin 1 U/ml respectively compared to $CaCl_2$ as a negative control. Cells' shape did not change after stimulation. c) Quantification of flow cytometric data reveals, in HUVEC stimulated with TRAP-6, expression of CD73 was slightly increase. No change in pattern of expression of VEGFR-2, VE-cadherin, Tie-2 and CD146 in both TRAP-6 and thrombin stimulated cells were observed. Decrease in CD31 TRAP and thrombin stimulated cells but not significantly, an increase in CD43 and slightly decrease in thrombomodulin expression in thrombin-stimulated cells were resulted. d) Representative flow cytometric dot plots of TRAP-6$^+$/thrombin$^+$ HUVEC are shown. TRAP-6 could change HUVEC surface markers expression as well as CD31, CD73 although it is not significant. Ns=not significant. Scale bar 100µm.

1.14 Discussion

This study examined the potential effect of TRAP-6 as a thrombin alternative in angiogenesis promoting function. We determined that the influence of thrombin receptor activating peptide-6 (TRAP-6) on endothelial cells' proliferation, development of microvascular network formation and various surface markers expression.

TRAP is synthetic hexapeptide that activates the thrombin receptor independent of receptor cleavage. It corresponds to amino acid 42-47 of thrombin receptor and mimics many functions of thrombin such as platelet aggregation and angiogenesis promoting function. The efficacy of TRAP has demonstrated in platelet aggregation and promoting clot formation. (Landesberg et al., 2005). Furthermore, thrombin upregulates the VEGF receptors (KDR and flt-1) m-RNA expression that is mimicked by TRAP as well (Tsopanoglou et al, 1999).

Exposure of HUVEC to TRAP-6 increased the proliferative cells' response. At low concentrations, TRAP-6 seemed to be a weaker inducer of cells proliferation, but by increasing concentration of TRAP-6 up to 500 µM, cell's proliferation was enhanced significantly. Surprisingly, the increase in proliferation response because of high concentration of TRAP-6, did not observe in adipose-derived stem cells (ASCs) culture. Indeed, TRAP-6 affects ASC due to PARs are expressed on ASC surface like HUVEC. (Freyberg et al., 2009). Therefore, TRAP influences ASCs' proliferation via a pathway, which has not been revealed yet. TRAP-6 potential for microvascular network formation was assessed from HUVEC/ASC co-cultures. Here, HUVEC were used

exclusively. As “gold standard”, the integration of HUVEC with supporting cell types in a fibrin matrix has been the focus of many studies mainly by the Tranquillo, Putnam and Levenberg groups (Morin et al., 2013; Carrion et al., 2013; Blinder et al., 2015). Albeit, HUVEC close contact with ASC for microvascular formation is necessary (Rohringer et al., 2014). It has been shown that thrombin induces expression and secretion of VEGF from human FS4 fibroblasts, DU145 prostate cells, and CHRF megakaryocytes via the PI3K pathway (Huang et al., 2001). However, about the endothelial cells, the stimulation by thrombin as well as thrombin peptide led to DNA synthesis through activation of the ERK pathway (Olivot et al., 2001) Kataoka et al. showed that PAR1 is required for ERK activation in response to low concentrations of thrombin (Kataoka et al., 2003) Samdja et al. indicated that PAR1-induced proliferation of endothelial progenitor cells involves angiopoietin 2, which in turn activates Akt pathway (Smadja et al.,2006; Hildbrand st al., 2004). In in-vitro model, the influence of TRAP-6 on microvascular networks derived from co-cultures of HUVEC/ASC 1:1 and 10:1 ratio, with and without aprotinin in a fibrin matrix determined. In HUVEC/ASC 1:1 and 10:1 co-culture, TRAP-6 100 µM showed significantly higher number of junctions, tubules and total tubule length vascular network. According to proliferation assay, TRAP-6 500 µM induced HUVEC proliferation stronger, but in the co-culture model, TRAP 100 µM-treated samples exhibit mature network. Due to the existence of thrombin in fibrin clot mixture, the effect of TRAP-6 only considered on vascular tubule formation. The endothelial differentiation ability of ASC was first discovered in 2004. Miranville, A., et al demonstrated that the subset of ASC was capable of differentiating into ECs when cultured with endothelial growth medium supplemented with IGF and VEGF.ASC could secret range of different growth factors and miRNAs in therapeutic neovascularization. Under the hypoxic condition, ASC secrets high level of VEGF. The transplantation of preconditioned ASC increased the capillary density and restored cardiac function (Banai et al.,1994). The compensation potential of TRAP-6 for ASC tested by using HUVEC/ASC with ratio 10:1. When number of ASC diminished in co-culture in proportion to HUVECs, the number of junctions, tubules and total tubule length increase with TRAP-6 100 µM incorporation. Aprotinin was used in HUVEC/ASC 1: 1 co-culture to inhibit fibrin degradation, (Wolbank et al., 2015) but inhibition of fibrinolysis impairs vascular network formation. In our results, HUVEC/ASC 1:1 co-cultures treated with TRAP-6 100 µM, has higher number of junctions, tubules and total tubule length with and without aprotinin. As a result, TRAP

by inducing endothelial cell proliferation and upregulation of VEGF receptor is a potent component as thrombin in angiogenesis and microvascular network development.

Besides thrombin's function in angiogenesis, this molecule is involved in inflammation as well. Notably, thrombin induces endothelial neutrophil adhesion, P- and E-selectin expression, and chemokine production. Here, we investigated thrombin and TRAP-6 stimulated HUVEC surface marker which mediated by activating receptor on HUVEC. E-selectin (CD62E) on ECs mediate weak reversible bonds between PMN and ECs under physiological flow conditions and are responsible for rolling, the first step in the adhesion cascade (Lawrence et al., 1991). Thrombin induces E-selectin (dCD62E) expression as well as chemokine production in an IL-1a/b– and TNF α-independent way. The E-selectin expression in HUVEC stimulated with TRAP-6 and TNFα was slightly upregulated within the first 4 h. The E-selectin expression was not detectable After 24 h, which demonstrated before by Mühleder, et al (Mühleder et al, 2018). TRAP-6 showed similar effect in E-selectin expression as TNF α which means it has more potential than thrombin in activation of receptor mediated E-selectin expression.

Thrombomodulin expression on stimulated HUVEC with thrombin showed slightly decreased but it was not significant. This demonstrated that thrombomodulin's affinity for binding to TRAP and inhibiting coagulation by activating protein C is slightly lower than thrombin (Lu et al., 2005). Thrombin and TRAP-6 stimulated HUVEC showed the same pattern of expression for VE-cadherin and CD146, which demonstrate similar affinity of both adhesion molecules for TRAP and thrombin. Besides, VEGFR-2 and Tie-2 expression showed no change after TRAP-6 and thrombin stimulation. In contrast, CD34, which regulates early events in blood cells' migration and differentiation expression in thrombin, increased in stimulated samples. CD73 expression in TRAP-6 stimulated HUVEC is increased which confirmes that TRAP can activate G-protein coupled receptor independent of thrombin.CD 31 expression was decreased in TRAP-6 stimulated HUVEC compared to thrombin (but not significantly) which is significant according to CD31 thrombus formation inhibitory function, and demonstrates that TRAP is a better inhibitor of platelet function.

1.15 Limitations

In this study, we analyzed the angiogenesis promoting function of TRAP-6 exclusively. However, we did not study the mechanisms behind HUVEC and ASC proliferation response as a function of TRAP. HUVEC still is used as the "golden standard" in vascular biology and tissue engineering, it is not presented in the adult human body and therefore does not represent tissue-specific vasculature (Baiguera & Ribatti, 2013; Attalla et al., 2016). Therefore, it would worth to study the influence of TRAP on other endothelial cell types such as endothelial colony-forming cells from iPSC. The network formation of ECFC from peripheral blood and ASC in a fibrin matrix that both cell types can be used in an autologous setting that has been shown by Holnthoner et al., 2015. Moreover, the level of mRNA expression for VEGF receptors did not analyze to identify the clear reason behind microvascular tubes formation in TRAP-stimulated co-cultures. However, it is challenging to measure all-tube formed cells in co-culture. Also, because of the high proliferation of HUVEC in response to TRAP-6, using another medium instead of the full endothelial growth medium, as basal medium could be considered as a control to measure the TRAP-6 potential for cells proliferation in absence of growth factors.

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