Enhancement of vasculogenesis in mouse embryonic stem cells by alpha 2 macroglobulin activating the LRP1 signaling pathway

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Dedicated to my beloved father

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1. Introduction

1.1 α2m

Alpha 2 macroglobulins (α2m) are a family of abundant plasma proteins best known for their ability to inhibit broad spectrum of proteases. α2m is the major representative of the alpha macroglobulin family that also comprises pregnancy zone protein (PZP) and complement components C2 and C3 (Jensen 1989, Borth 1992). Its structural and functional properties make it a very unique protein because of its protease trapping mechanism. Besides proteinase inhibition it also functions as a carrier of cytokines and growth factors by covalently binding to them thereby linking its role in growth promoting activities by targeting the α2m-cytokine/growth factor complex to α2m receptor expressing cells (Birkenmeier et al., 2003, Esadega et al., 2003). α2m is known to bind to two distinct receptors namely LRP1 and GRP78 which are expressed on several tissues.

α2m is a typical acute phase protein in many mammalian species such as rabbit, rat, mouse and humans with its concentration increasing several-fold upon diseased states like pancreatitis, multiple sclerosis, sepsis etc. Several groups have described the anti-inflammatory effects of α2m, for example in induced hepatitis, in burn trauma, anaphylactic shock and in inflammation caused by histamine, bradykinin or prostaglandins (Hochepied et al., 2000). Oxidation of α2m increases its binding affinity to inflammatory mediators like TNFα thereby inhibiting progression of the inflammatory cascade (Wu et al., 1998). α2m was also reported to play a role in immunity where α2m isolated from blood of tumor bearing mice was bound with tumor antigens that were immunogenic (Pawaria et al., 2012). α2m also inhibits coagulation and fibrinolysis by binding to thrombin and plasmin respectively (Schaller et al., 2011). α2m was reported to possess chaperone activity by binding to unfolded proteins resulting from heat or oxidative stress and their subsequent disposal (Wyatt et al., 2013). α2m facilitates removal of various disease associated proteins like β2-microglobulin in amyloidosis, prion protein in Creutzfeldt-Jakob disease and Amyloid β in Alzheimer's disease (Wyatt et al., 2013).

α2m is predominantly synthesized by the liver and is mainly present in plasma, cerebrospinal fluid and other extravascular fluids. α2m is also locally produced by fibroblasts, monocytes, macrophages, hepatocytes and astrocytes (Jensen 1989).

1.1.1 Structure

The primary structure of α2m was elucidated by Sottrup-Jensen et al. in 1983. It has a molecular weight of 720 KDa and is the largest protease inhibitor known. It is a homo-tetramer consisting of four identical subunits each of 180 KDa where two subunits are joined by a disulphide bond and these two pairs are covalently linked to each other forming a cylindrical structure with an enclosed central cavity. Each α2m subunit contains four distinct regions; the bait region, growth factor binding domain, the β-cysteinyl-γ-glutanyl thiol ester containing domain and a receptor recognition site. The bait region contains a specific set of amino acids where serine, cysteine, aspartic and metallo-proteinases gets bound. The thiol ester rupturing imparts α2m its conformational structural change with α2m receptor exposure. Growth factors and cytokines are known to bind to growth factor binding site covalently and non-covalently to other sites (Wyatt et al., 2013).

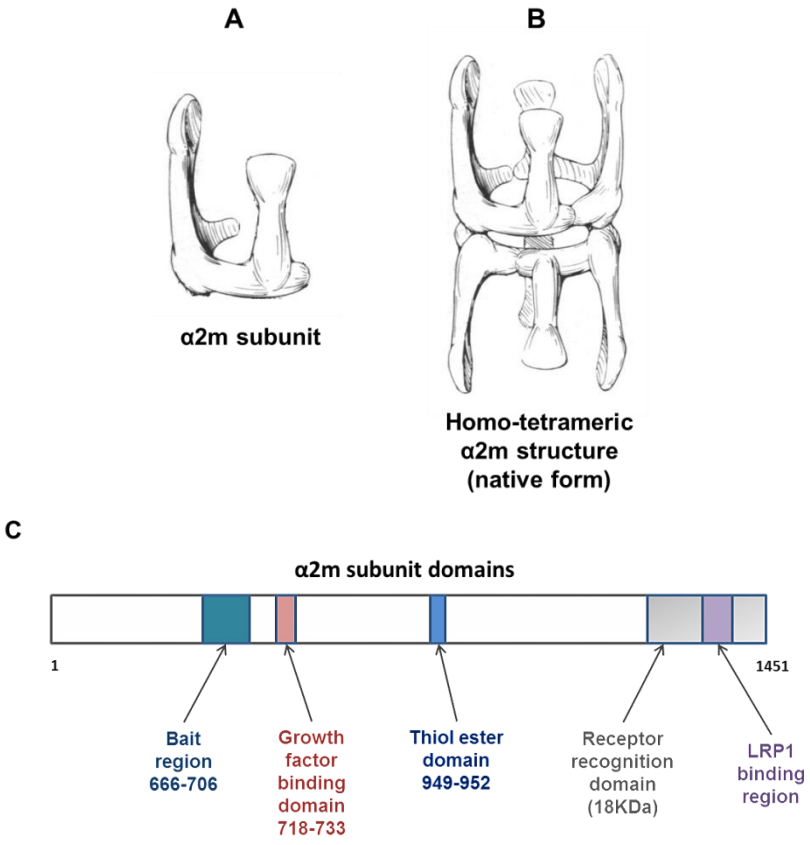


Figure 1.1.1: α2m structure and domains within α2m subunit. A: representation of a single 180 KDa α2m subunit, **B:** α2m homo-tetramer joined by disulphide bonds (Courtesy: Feldman et al., 1985) **C:** Representation of domains present in each α2m subunit. The numbers denote the amino acid sequence in which each domain is present. (Modified from Mettenburg et al., 2002)

1.1.2 Protease binding and clearance by α2m

Native α2m found in the plasma is not recognized by its receptors. The inhibition of proteinases by α2m is a unique mechanism. As illustrated in Figure 1.2 upon a protease binding to a native α2m, the bait region gets cleaved trapping the protease in its cage like structure. This protease then forms a covalent linkage with the thiol ester bond which subsequently ruptures and induces a conformational change. This structural change in the α2m molecule results in the exposure of a cryptic recognition site at the carboxyl terminus of each α2m subunit and results in the activated form of α2m (Misra et al., 2006). Other than proteinases small nucleophiles like methylamine or ammonia can also rupture the thiol esters resulting in the activated form. Proteinase binding to α2m is irreversible but the active site of proteinase remains 80 – 100% active against low molecular substrates (French et al., 2008). Once in active form, this protease-α2m complex gets recognized by α2m receptors LRP1 and GRP78. Upon binding to LRP1, the complex gets rapidly cleared via receptor mediated endocytosis. On the other hand when bound to GRP78 it can mediate several cellular signaling pathways. The active form of α2m migrates faster on gel electrophoresis under non denaturing conditions compared to the native form because of its compact structure (Bhattacharjee et al., 2000).

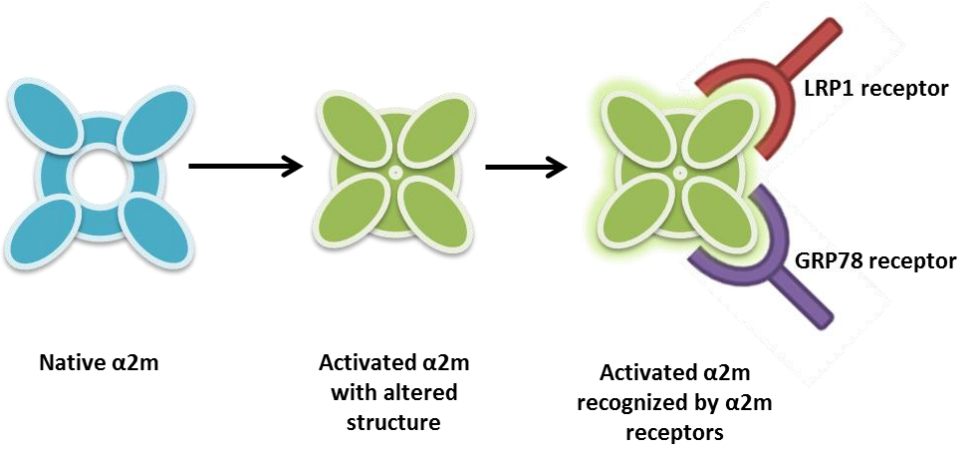


Figure 1.1.2: α2m activation and receptor recognition. Native α2m is having an open structure. Upon activation by a nucleophile or protease structural change is induced which is recognized by its receptors LRP1 and GRP78

1.1.3 Growth factor binding to α2m

Numerous cytokines and growth factors are known to bind to α2m to regulate their physiological activity. Among them are transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), nerve growth factor (NGF), β-nerve growth factor (β-NGF), tumor necrosis factor a (TNFα), basic fibroblast growth factor (bFGF), interleukin (IL-6), interleukin 2 (IL-2) and vascular endothelial growth factor (VEGF). The cytokine and growth factor can bind to both native and activated forms of α2m. α2m acts both as a carrier protein in case of growth regulated processes and in clearance of cytokines in case of inflammatory conditions. VEGF has been shown to bind to α2m to a site other than the growth factor binding domain and that it binds to methylamine activated α2m to the interior and to the exterior of native and proteinase activated α2m (Bhattacharjee et al., 2000). Other studies have also demonstrated that inflammatory cytokines like TNFα, IL-2 and IL-6 exhibited increased affinity to bind to oxidized forms of native and activated α2m while growth factors like β-NGF, PDGF-BB, TGF-β1 and TGF-β2 showed decreased binding suggesting oxidation of α2m to be involved in down regulating progression of inflammation and up regulating tissue repair processes by releasing growth factors (Wu et al., 1998).

1.1.4 Implications of α2m

In rats, α2m is an acute phase protein with its concentration increasing 50 fold within 24 h under inflammatory conditions (Sultan et al., 2012). Treatment of rats with α2m enriched autologous plasma has been reported to diminish the progression of pancreatic damage in experimental pancreatitis (Di Loreto et al., 2012). α2m was reported to play a central role in survival upon irradiation with x-rays by activation of the IL6/JAK/STAT3 hepatoprotective signaling pathway (Mirjana et al., 2010). α2m treatment in rat ventricular cardiomyocytes was reported to induce hypertrophy by activating ERK and PI3K/AKT pathways (Padmasekar et al., 2007).

α2m like protein in zebra fish was shown to be essential for liver organogenesis, where its knock down resulted in disruption of growth and development of the liver with reduced cell proliferation without observable defects in pancreas and intestine (Hong et al., 2008).

α2m double knockout mice were viable and fertile, proving that α2m is not essential during embryonic development or adult life, but necessary during implantation and trophoblast invasion exerting its role in tissue remodeling both as a proteinase inhibitor

and TGFβ binding molecule (Esadeg et al., 2003). Upon induction of acute pancreatitis in double knockout mice mortality was 70% suggesting α2m to play an important role both as a protease inhibitor and as a carrier protein since mRNA levels of Lt-β, TNFα, IFN-ɣ, and TGF-β1 were increased (Umans et al., 1999). Mouse α2m was also reported to be a functional homologue of human α2m (Hudson et al., 1987).

Human α2m is the most widely studied one. In humans, α2m is a single-copy gene located on chromosome 12p12-13 also comprising the PZP gene. It is found in the plasma at a concentration of 4-5mg/ml and declines to 2-4mg/ml by adulthood (Tayade et al., 2007). Removal of α2m from blood significantly increases plasma protein. A cardiac isoform of α2m has been reported as an early marker for cardiac hypertrophy and its elevated upon myocardial infarction in diabetic patients (Annapoorani et al., 2006). The endothelial cells lining the endometrial blood vessels were reported to produce α2m with its concentration increasing 2-3 folds during the proliferative phase of menstrual cycle. This suggests a role in endometrial physiology and in the physiology of embryo development and implantation (Sayegh et al., 1997).

1.2 LRP1

Low density lipoprotein receptor related protein 1 (LRP1) is a member of the LDL receptor family mainly implicated in endocytosis of various ligands and in mediation of various cell signaling pathways. It was identified to be the receptor responsible for clearing α2m-proteinase complexes found in plasma. Other than α2m-proteinase complexes, LRP1 recognizes and binds to 40 other various ligands known till date ranging from lipoproteins, ECM proteins, protease-inhibitory complexes, viruses, cytokines and growth factors. LRP1 is expressed in liver, lung, brain and in multiple cell types like fibroblasts, VSMCs, neurons, astrocytes, macrophages and cultured brain capillary endothelial cells. The expression of LRP1 in human endothelial cells is somewhat controversial as bovine aortic endothelial cells in culture were found to express LRP1 but not human aortic endothelial cells, human coronary artery endothelial cells and human neonatal dermal micro vascular endothelial cells (Lillis et al., 2008). LRP1 is known to shuttle continuously between the cell surface and cytoplasmic vesicles. LRP1 is essential for normal development in the mouse, as homozygous knockout animals die at day 9 of gestation (Herz et al., 1992).

1.2.1 LRP1 Structure

LRP1 is a 600 KDa protein composed of a 515 KDa α-subunit and an 85 KDa β-subunit. Like all other members of the LDL receptor family, LRP1 structure contains an extracellular transmembrane domain and a cytoplasmic domain which are non-covalently linked. The extracellular domain is mainly composed of clusters of cysteine rich complement type repeats (CR), EGF repeats and β propeller domains. CRs are the ligand binding sites and are present as clusters, termed clusters I - IV. Most ligands including α2m are known to bind to clusters II and IV (Lillis et al., 2008). EGF repeats and β propeller domains are known to function in uncoupling of ligands in endosomal compartments. The cytoplasmic domain encompasses 100 amino acids with two di-leucine motifs and two NPxY motifs, which bind to various adaptor molecules and are known to be involved in cellular traffic and cell signaling. Cell signaling by LRP1 is triggered upon phosphorylation of tyrosine and serine residues present in the cytoplasmic domain (van der Geer P 2002).

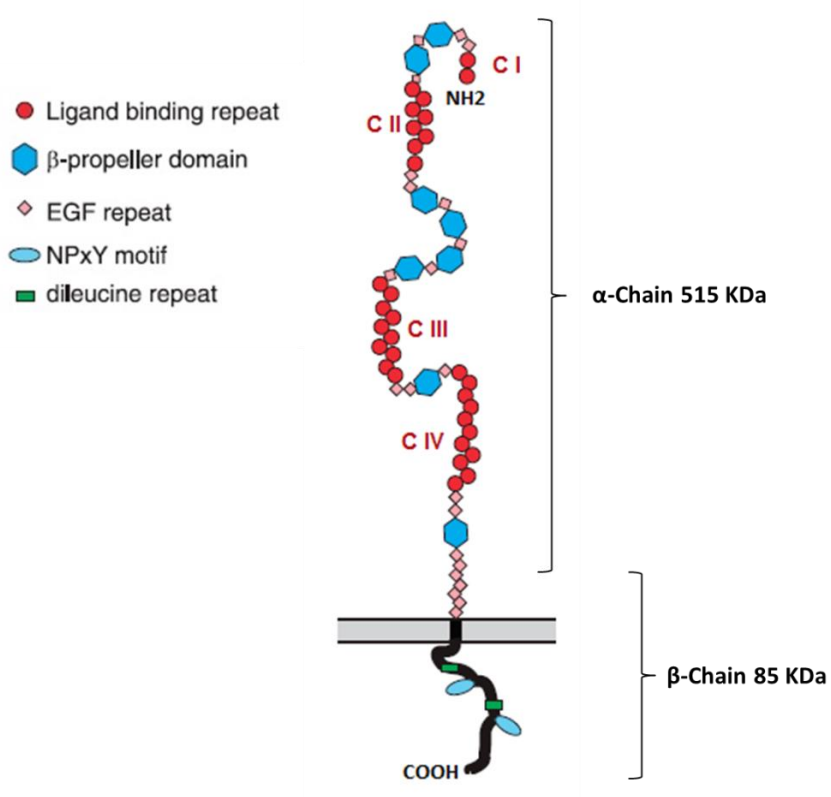


Figure 1.2.1: Representation of LRP1protein structure. CI-CIV are the cluster binding domains within the α-subunit. The β-subunit comprises part of cytoplasmic domain, transmembrane domain and the intracellular domain. (Modified from Lillis et al., 2008)

1.2.2 LRP1 functions

LRP1 upon ligand binding principally has three major functions which are endocytosis, cell signaling and co-receptor activity. Depending on its presence in various tissues and cells, these few actions subsequently get it involved in many physiological activities like inflammation, proliferation, migration, permeability, phagocytosis, immunity, angiogenesis etc. Few of which are described below:

Hepatic LRP1 besides clearing α2m protease complexes from blood plasma also facilitates catabolism of serpin-enzyme complexes like tissue plasminogen activator (tPA) and Urokinase-type plasminogen activator (uPA), the blood coagulation factor VIII and certain Apolipoprotein E rich lipoproteins maintaining normal homeostasis of liver and protection against atherosclerosis as hepatic LRP1 KO mice showed two fold increase in atherosclerotic lesion compared to normal mice (Lillis et al., 2008). LRP1 in the brain is known to regulate the progression of AD by apolipoprotein E mediated amyloid Aβ clearance in the brain (Holtzman et al., 2012).

LRP1 in inflammation: LRP1 modulates lipopolysaccharide signaling in murine macrophages reducing inflammatory responses by proteolytically releasing its intracellular domain to the nucleus where it interacts with IRF3 (Lillis et al., 2008). Also Lactoferrin binding to LRP1 inhibits NFκB activation and production of pro-inflammatory cytokines (Beisiegel et al., 1998). Macrophage uptake of apoptotic cells is mediated by LRP1 by recognizing calreticulin on the target cells with its eventual phagocytosis and degradation thereby resolving inflammation (Nilsson et al., 2012).

LRP1 in growth factor mediated cell signaling: LRP1 is reported to regulate BMP4-mediated endothelial function and vascular development, since LRP1 mouse endothelial knock down cell lines failed to migrate and induce angiogenesis in presence of BMP4 (Pi et al., 2012). LRP1 is involved in signaling processes that involve TGFβ, PDGF, and their receptors in mediating atherosclerosis in VSMC. Deficiency in LRP1 activates PDGF-BB and PDGRβ, phosphorylating SMAD2, a downstream component of the TGFβ pathway promoting atherosclerotic lesion formation (Boucher et al., 2011). Moreover TGFβ receptor TGFR-V was identified to be LRP1 in mediating growth inhibitory response of TGFβ in conjunction with SMAD2/3 signaling. LRP1 was also shown to be associated with PDGF receptor in the endosomal compartment where they form a signaling complex modulating PDGF signaling cascades that involve MAPK and Akt/PI3K pathways. PDGF, a key regulator for cellular proliferation and migration, also mediates the tyrosine phosphorylation of LRP1 at its cytoplasmic domain (Muratoglu et

al., 2010). In VSMC, LRP1 complexes with PDGFR-β to modulate actin organization and migration by activating PDGFR-β-dependent activation of PI3K-PDGF-mediated signaling (Boucher et al., 2002).

1.2.3 LRP1 antagonist, RAP

Receptor associated protein (RAP) a 39 KDa protein was discovered by ligand-affinity chromatography where it was co-purified with LRP1 (Ashcom et al., 1990). It is an LRP1 antagonist which can inhibit binding of all known ligands to LRP1. RAP is an ER-resident protein that binds to multiple sites on LRP1 and also acts as an LRP1 chaperone. Premature association with ligands of newly translated LRP1 is prevented by RAP being transiently bound to LRP1 in the ER until it is successfully delivered to the cytoplasmic membrane. In RAP deficient mice processed LRP1 was found to be less expressed in brain and liver (Lillis et al., 2008).

1.3 GRP78

In addition to the well characterized LRP1 receptor, a second receptor for α2m was reported by Misra et al., in 1994. Binding of activated α2m to GRP78 activates pro-proliferative, anti-apoptotic, and pro-migratory signaling cascades in tumor cells (Misra et al., 2013). GRP78 is a 78 KDa glucose-regulated protein also referred to as the immunoglobulin binding protein, BiP are members of the ER chaperone family discovered in the 1980's as proteins inducible by glucose starvation (Langer et al., 2008). It is widely used as a marker for ER stress as its expression gets highly induced in the ER under stress conditions (Ni et al., 2011). This ER chaperone belongs to the HSP70 family playing a central role in regulating the unfolded protein response (UPR) (Buchkovich et al., 2009). GRP78 function is thought to be critical for eukaryotic cell function as its amino acid sequence is highly evolutionarily conserved from yeast to human (Ting et al., 1988).

GRP78 double knock out mouse embryos exhibited lethality beyond the peri-implantation stage and failed to grow in culture and to exhibit proliferation. Hence GRP78 is essential for embryonic cell growth and pluripotent cell survival (Luo et al., 2006)

GRP78 is expressed on the cell surface of proliferating endothelial cells and prostate cancer cells (Ni et al., 2011). It is primarily located in the ER lumen of non-stressed

cells with a sub fraction detected as transmembrane protein. Beyond the ER mitochondrial, nuclear and secreted forms of GRP78 have been identified and linked to cellular homeostasis. It is also highly expressed in the developing embryo and in the embryonic brain linking its role in neurological disorders (Weng et al., 2011)

1.3.1 GRP78 structure

GRP78 is composed of three domains: the N-terminal ATPase domain, the peptide-binding domain, and a C-terminal domain. The unfolded peptides are bound to the peptide-binding domain and use energy by hydrolyzing ATP to promote proper folding and aggregation of the peptide (Luo et al., 2006). The function of the C-terminal domain is unknown but upon cell surface translocation all three domains are exposed to the cell surface speculating its role in ligand recognition (Ni et al., 2011). Upon α2m binding to GRP78, the N-terminal undergoes auto-phosphorylation triggering pro-proliferative, anti-apoptotic, and pro-migratory signaling cascades in tumor cells (Misra et al., 2013).

1.3.2 GRP78 functions

ER stress signaling: GRP78 regulates UPR signaling by a bind-and-release mechanism with the ER stress sensors and transducers: PERK, ATF6 and IRE1 (Weng et al., 2011). UPR is normally controlled by trans-membrane sensors which initiate the complex UPR signaling where under unstressed conditions GRP78 is believed to bind to these sensors and keep them inactive. However under ER stress, when unfolded proteins accumulate in the ER, GRP78 leaves these sensors to perform its chaperone function, thus allowing the sensors to activate UPR signaling (Buchkovich et al., 2009).

ER stress also induces GRP78 transcription resulting in its upregulation along with a cytosolic form of GRP78, called GRP78va. Cell surface GRP78 emerges as an important receptor in cell signaling, viability and anti-apoptotic signaling pathways (Ni et al., 2011). In the ER lumen GRP78 associates with co-chaperones enhances protein folding and degradation of misfolded proteins whereas GRP78va activates PERK signaling increasing cell survival under ER stress. GRP78 also regulates mitochondrial function especially under ER stress by balancing energy expenditure and maintaining mitochondrial calcium homoeostasis (Ouyang et al., 2011).

Cell proliferation: In cancer cells cell-surface GRP78 interaction with α2m was reported to regulate cell proliferation pathways by activating ERK1/2, MAPK and PI3K and cell survival pathways by activating the Akt and NFκB (Buchkovich et al., 2009). Cell surface GRP78 was also reported to play an important role in endothelial cell

proliferation and in anti-apoptosis signaling. One study reported this receptor to be associated with Glycosyl Phosphatidylinositol (GPI) anchoring T-Cadherin, thereby influencing cell survival in vascular endothelial cells. Another study revealed blocking of GRP78 receptor with recombinant Kringle 5, an angiogenesis inhibitor induced apoptosis of proliferating endothelial cells and tumour cells enhancing Caspase7 activity. Cell surface GRP78 was reported to be elevated in VEGF treated HUVEC cells revealing its active role in endothelial cell proliferation (Ni et al., 2011).

Virus internalization: GRP78 is known to act as a co-receptor with MHC class I molecules on the cell surface of host cells in mediating virus internalization. Viruses like Coxsackie virus A9, dengue virus serotype 2, human cytomegalovirus and Borna disease virus were reported to be bound to GRP78. Antibody blocking against cells expressing GRP78 resulted in reduction of virus infection (Ni et al., 2011).

1.4 Stem cells

Stem cells are undifferentiated or unspecialized cells found in multicellular organisms which have remarkable potential to form any kind of cell in the adult organism (Figure 1.4). Stem cells have two very important characteristics that make them different from other kind of cells; these are its ability of self-renewal and its differentiation capacity (Takahashi et al., 2007). Self-renewal is characterized by its potential to divide mitotically yet remaining in the undifferentiated state. The differentiation capacity is characterized by the ability of stem cells to differentiate into any kind of specialized cell under appropriate stimulation, i.e. types like nerve cell, blood cell, skin cells etc. Other than stem cells, no other cell in the multicellular organism has the ability to generate terminally differentiated cells with specialized functions. The pluripotency of ESCs is governed by expression of the core transcription factors Nanog, Oct4, and Sox2 which are known to down-regulate the expression of developmental genes while regulating transcriptionally active genes involved in pluripotency maintenance (Chen et al., 2008, Saunders et al., 2011).

The potency of stem cells is further classified into totipotent, pluripotent, multipotent, oligopotent and unipotent depending on its differentiation capacity into a whole organism or a specific set of specialized cells. The main sources of stem cells are described below:

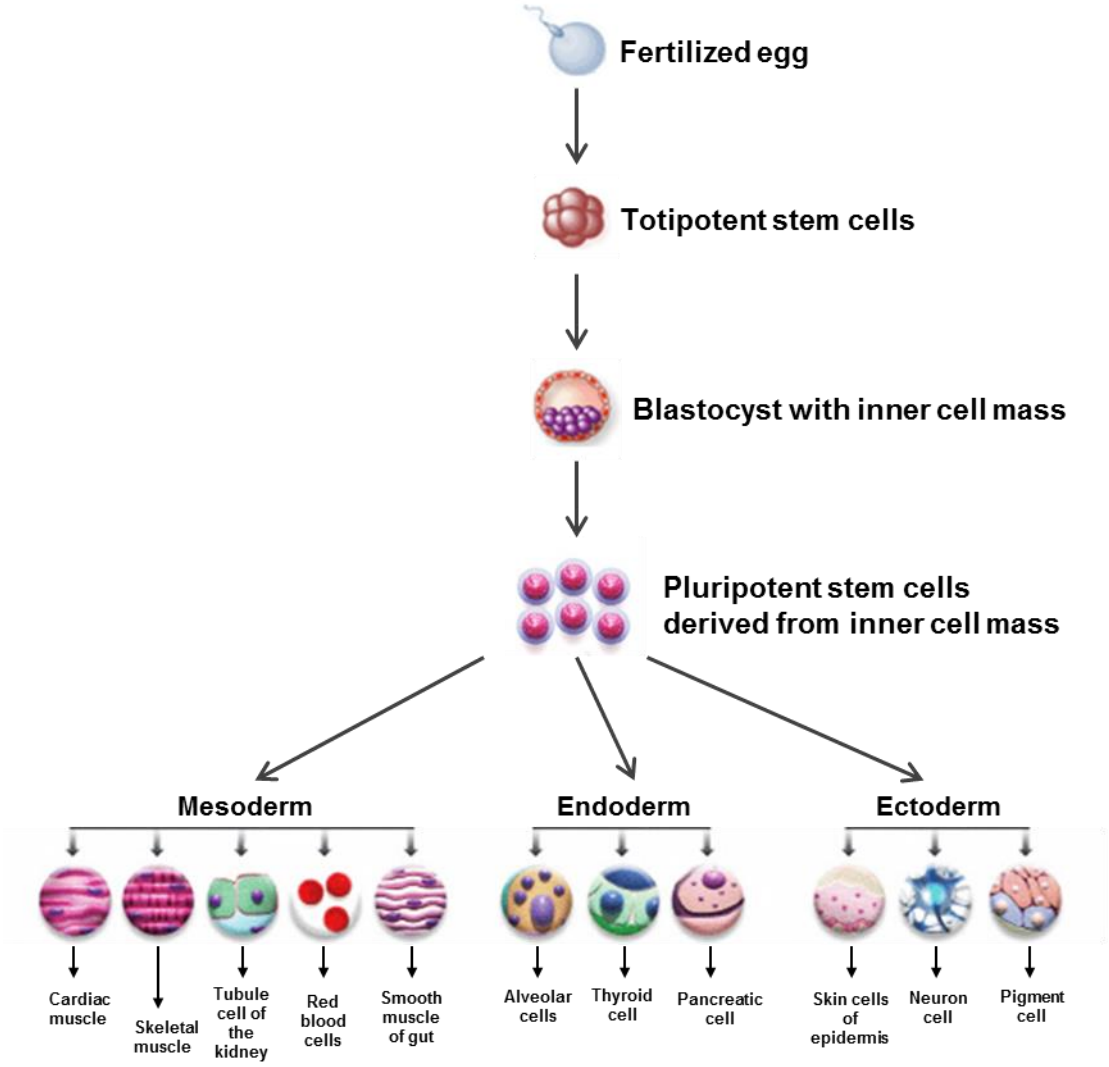


Figure 1.4: Differentiation capacity of pluripotent stem cells. Upon fertilization of an egg, the cells divide to give to totipotent stem cells that form a blastocyst. The differentiation capacity of pluripotent stem cells derived from the inner cell mass of blastocyst to form specialized cells of all three germ layers is illustrated. (Modified from www.sigmaaldrich.com)

Embryonic stem cells: As the name suggests these cells are derived from 3-5 day old embryo from its inner cell mass at the blastocyst phase. These ESCs are totipotent and can differentiate into all three germ layers: ectoderm, endoderm and mesoderm. The isolated ESCs can be cultured in vitro and maintained in undifferentiated state. These cells are a very valuable source for studying early stages of embryonic development.

Adult stem cells: Adult stem cells are multipotent cells found in organisms at the adult stage in various tissues. They have been identified in many organs and tissues throughout the adult body like bone marrow, umbilical cord blood, skin, blood, hair follicle, liver etc. They are thought to reside in specific areas of the tissues in a

quiescent state until they are activated. They are lineage restricted and primarily function to repair, replenish or regenerate the tissue in which they are present. Adult stem cells have already entered into therapeutics with bone marrow transplants performed to treat leukemia.

Induced pluripotent stem cells: These cells also called iPSCs are created by scientists in the laboratory where terminally differentiated cells from the adult organism are engineered or reprogrammed to behave and function like pluripotent stem cells. Mouse iPSCs were first generated by Shinya Yamanaka's team in Japan in 2006 by retroviral transduction of four factors: Oct4, Sox2, c-Myc, and Klf4 into mouse fibroblasts (Takahashi 2006). The iPSC generation led to an important breakthrough in stem cell research as they hold great promise in creating disease specific or patient specific cell lines for therapeutic research purposes (Ma et al., 2013).

1.4.1 Embryonic stem cell lines

Various mouse and human ESC lines were established for research purposes that can be grown and expanded in culture indefinitely in the undifferentiated state. Mouse ESCs were first isolated and cultivated by Evans and Kaufmann in 1981 along with Martin in 1981. Human ESCs were first isolated by Thomson et al., in 1998 (Evans et al., 1981, Martin 1981).

These cells are cultured on cell culture dishes coated with feeder layers of MEFs which provide them a sticky surface for attachment and growth. They proliferate by forming colonies. Mouse ESC lines can be maintained in undifferentiated conditions by culturing them with medium containing LIF which promotes self-renewal by activating PI3K/AKT and JAK/STAT3 signaling pathways. However human ESC lines in culture do not require LIF but the growth factor FGF2 for self-renewal, FGF2 acts by activating MAPK and Activin/Nodal signaling pathways. The ESC lines are characterized routinely by their expression profiles of transcription factors and cell surface markers. They can also be injected into immune suppressed mouse for teratoma formation which is an indication of their differentiation capacity.

Upon withdrawal of self-renewal cytokines from cell culture, the ESCs start to spontaneously differentiate into various kinds of cells. This spontaneous differentiation can be directed towards a specific cell type by altering the composition of the medium by adding various stimulants or inhibitors. EBs are three dimensional aggregates of ESCs which mimic the early development processes of a normal embryo by undergoing differentiation into all three germ layers.

1.4.2 ESC implications

Study developmental processes: ESCs are a remarkable cell source for researchers and scientists for understanding early developmental processes that occur beginning from the fertilization of the egg. The regulation of various genes involved and their molecular cues give insight into the fundamental processes of how organisms grow and develop.

Study diseases and drug screening: The differentiated stem cell lines offer a remarkable source for drug screening and research compared to *in-vivo* studies performed in humans and animals. A more advanced approach is the development of patient-specific or disease-specific stem lines to study human diseases at a much closer level which could replace the use of experimental animal models (Mori et al., 2013).

Regenerative medicine: The most potential application of stem cells is for replacing diseased, aged or damaged tissues, an approach called regenerative medicine. Stem cells which can be directed to differentiate into a particular type of tissue offer the possibility to replace the damaged cells and tissues. Currently stem cells are being used to treat various blood disorders and are proposed to be utilized to treat various diseases like spinal cord injury, burns, heart disease, diabetes, arthritis etc. (Chopra et al., 2013)

1.5 Vasculogenesis

During embryo development, the vascular system is the primary structure that forms within the developing organism for oxygen, nutrient supply and waste removal. The vascular system arises from haemangioblasts which give rise to angioblasts from which blood vessel develops and hematopoietic stem cells that form the blood cells. Formation of blood vessels and blood cells is crucial for the developments of all other organs and any disruption in their formation leads to mortality (Carmeliet 2005). Vasculature which is the formation of blood vessels occurs by two distinct mechanisms: vasculogenesis which is the *de-novo* formation of blood vessels from endothelial precursor cells (EPCs) and subsequently by angiogenesis which is the expansion of the vascular network from the primitive vascular structures by formation of mature blood vessels (Kässmeyer et al., 2009, Velazquez 2007). Blood vessel development plays a key role in human physiology ranging from fetal growth, reproduction, wound healing and tissue repair and also in pathologies like tumor growth.

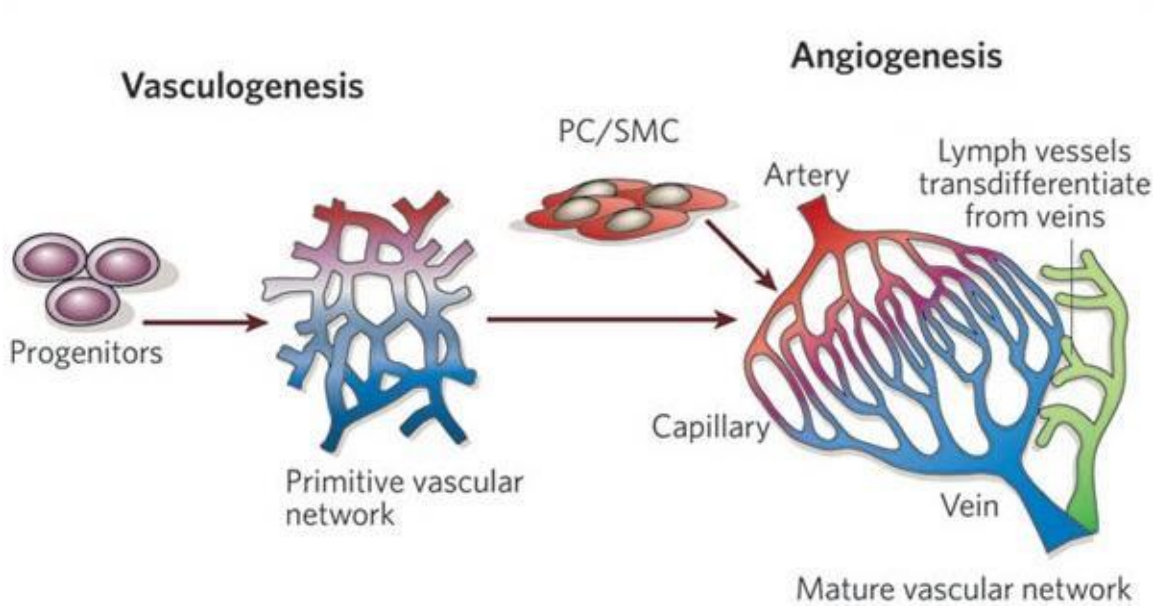


Figure 1.5: Vasculogenesis and angiogenesis of the vascular system. (Coutesy: Carmeliet 2005)

Vasculogenesis is largely restricted to early embryonic development and angiogenesis to late embryogenesis until adult hood. Vasculogenesis is driven by the recruitment of EPCs of angioblasts which are of mesodermal origin and their differentiation into the endothelial cell lineage with *de novo* assembly of these cells into a primitive vascular network. Subsequently angiogenesis is triggered upon angiogenic stimuli; EC permeability of the blood vessel increases with EC cell proliferation and migration, thereby forming a new capillary along with its maturation.

Angiogenesis is a highly regulated process that involves many growth factors and their signal transduction. Angiogenic growth factors, e.g. VEGFs and FGFs stimulate endothelial cells to secrete proteases and plasminogen activators, which result in protein degradation and migration of ECs. These ECs cells migrate, proliferate and eventually differentiate to form a new lumen containing vessel and further secrete the growth factor PDGF that attracts pericytes and SMCs forming a multicellular blood vessel structure and their stabilization (Drake 2003, Tang et al., 2004).

1.5.1 Growth factors and cell signaling pathways involved in vasculogenesis

VEGF: (VEGFs) are secreted proteins which are key promoters of angiogenesis and vascular development. VEGFs are comprised of five members in mammals: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) (Stacker SA et al., 2013). The most widely studied is the VEGF-A with different isoforms of VEGF121, VEGF165, VEGF189, and VEGF206. VEGF binds to receptor tyrosine kinases VEGFR 1-3 and co-receptors neuropilins (NRPs) and heparan sulphate proteoglycans (HSPGs) (Robinson et al., 2001). VEGFs are primarily secreted by endothelial, hematopoietic, and stromal cells upon hypoxia and upon stimulation by growth factors such as TGFβ, interleukins and PDGFs (Stuttfeld et al., 2009). The VEGFRs mainly function in cardiovascular, haematopoietic and lymphatic development (Olsson et al., 2006). A single allele VEGF-A knockout results in lethality at embryonic day 11 due to deficient EC development (Carmeliet et al., 1996). Activation of VEGFR2 regulates the most angiogenic signaling pathways. VEFGR2 gets phosphorylated at Tyr1054 and Tyr1059 thereby activating PLCγ and PI3K. PLCγ activates intracellular calcium and ERK1/2, resulting in the proliferation of ECs. PI3K activation leads to the downstream activation of AKT in survival of ECs and regulates NO production (Olsson et al., 2006).

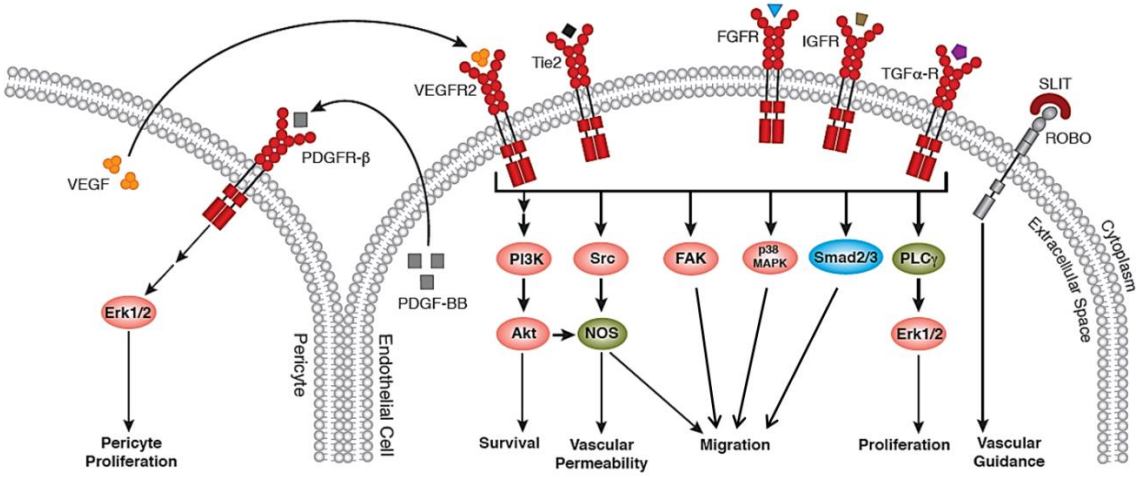


Figure 1.5.1: Angiogenesis signaling pathways (Courtesy: www.cellsignal.com)

FGF2: Fibroblast growth factor 2 is a potent angiogenic growth factor that induces endothelial migration and proliferation (Kottakis et al., 2011). It is one of the 23 members of FGF family which are involved in angiogenesis, wound healing, embryonic development and tumor growth. FGFs bind to receptor tyrosine kinases FGFR 1-5. The FGFs are heparin binding proteins and heparan sulfate proteoglycans (HSPGs) regulate FGF activity by acting as a co-receptor. FGFR gets activated upon auto-phosphorylation of the tyrosine residues present in the intracellular region of the FGF receptor. The binding of FGF2 to its receptors leads to the activation of downstream signaling pathways such as JAK/STAT, PLCγ, PI3K/Akt and MAPK/ERK, thus stimulating cell proliferation and differentiation (Lau et al., 2013, Lanner et al., 2010).

PDGF-B: Platelet-derived growth factor (PDGFs) comprises of four ligands PDGF A until D and two tyrosine kinase receptors PDGFRα and PDGFRβ which are involved in cell growth and proliferation. They also play a significant role in angiogenesis. PDGF-B and PDGFR-β double KO mouse are lethal leading to mortality due to hemorrhaging and edema formation (Stratman et al., 2010 and Meinecke et al., 2012). PDGF-B exists as the dimer PDGF-BB, which is produced by ECs and functions in recruitment of mural cells to stabilize the newly formed vessels (Wijk et al., 2013). PDGFRβ gets phosphorylated at Tyr-857 and activates MAPK, PLCγ and PI3K pathways. ROS-dependent STAT3 activation has been reported to be a downstream mediator of PDGFR signaling in VSMCs (Blazevic et al., 2013).

Besides above mentioned growth factors various other factors are also involved in the process of angiogenesis. Growth factors like TGFβ1 function in embryonic vascular

assembly by promoting cellular differentiation into pericytes and VSMCs and in the establishment and maintenance of vessel wall integrity. Hepatocyte growth factors (HGF) are involved in regulating expression of VEGF, VEGFR2 and thrombospondin-1 (TSP1). Regulation of angiogenesis also depends on the balance between pro-angiogenic factors and anti-angiogenic factors like TSP1, platelet factor 4 (PF4) and endostatin. TSP1 and PF4 modulates angiogenesis by binding to VEGF165 and FGF2 respectively preventing their binding to their receptors. SHH plays role by up regulating angiogenic growth factors. Notch family of proteins and their activation regulates EC sprouting with formation of tip cells and stalk cells (Wijk et al., 2013). The Slit proteins and their Robo receptors function in axon guidance and are implicated in permeability and migration (Yuen et al., 2013).

Clinical applications of angiogenesis:

Abnormal angiogenesis causes numerous diseases. Ischemic diseases are caused due to decrease in angiogenesis; in diseases like cancer there is excessive angiogenesis. Angiogenesis can be manipulated and utilized for clinical application where angiogenesis stimulator can be used to induce therapeutic angiogenesis. Regranex, a recombinant human PDGF-BB is used for diabetic foot ulcer disease. Anti angiogenic factors like Avastin, an anti-VEGF monoclonal antibody, are attenuating excessive angiogenesis and are used for treatment of cancers (Carmeliet et al 2011). Besides acceleration and inhibition of angiogenesis, the quantification of angiogenesis in a biopsy specimen can be utilized for diagnostic and prognostic applications to assess the risk of metastasis or recurrence.

1.6 Aim of the study

The acute phase protein α2m which is a protease inhibitor and a growth factor carrier protein is speculated to be involved in tissue remodeling processes under normal and inflammatory conditions. In a developing embryo, various signaling pathways are activated in a highly coordinated manner to ensure proper development and morphogenesis. A differentiating mouse EB is a replica of a developing embryo and administration of α2m during its development is hypothesized to activate various signaling pathways pertaining to differentiation. α2m exerts its effects through its receptors LRP1 and GRP78 which are known to activate various growth factor cell signaling pathways. The aim of this research project was to investigate the effects of α2m on vasculogenesis in the differentiating mouse EB.

The specific aims of this study were as follows:

1. Study endogenous expression of α2m and its receptors in differentiating mEBs
2. Investigate the effects of exogenous application of α2m on differentiating mEBs and examine its impact on vasculogenesis
3. Determine the activity of the α2m receptors by blocking them with specific antibodies or known inhibitors
4. Investigate the regulation of growth factors in differentiating mEBs upon α2m treatment
5. Elucidate the signaling pathways activated upon α2m treatment in mEBs
6. Access the functional activity of α2m on sprouting assay of mESC spheroids
7. Analyze the anti-inflammatory role of α2m and expression of α2m and its receptors upon induction of inflammation in mEBs with TNFα

2. Materials

2.1 General laboratory materials

4 well cell culture plate	Nunc, Thermo Scientific, USA
6 well cell culture plate	BD falcon, USA
Bacterial culture dish	Greiner bio-one, Germany
Blotting chamber	Xcell II Blot module, Life Technologies, Germany
Cell scrapper	Corning, USA
Conical flasks	Fischerbrand, Fischer Scientific, Germany
Conical tubes 15ml, 50ml	Greiner bio-one, Germany
Cover slips	Deckgläser, Menzel-Gläser, Germany
Cryovial	Sarstedt, Germany
Electrophoresis chamber	Xcell SureLock Mini-Cell, Life Technologies, Germany
Filter paper for blotting	Universal, Biotech Fischer, Germany
Filter	Stericup & Steritop, Millipore, Germany
Glass slides	R.Langenbrinck, Germany
Microcentrifuge tubes	Sarstedt, Germany
Multichannel pipette	Transferpette S8/12, Brandtech Scientific, USA
Nalgene Mr.Frosty	Thermo Scientific, USA
Nitrile gloves	Ansell Healthcare Europe, Belgium
Nitrocellulose membrane	Whatman™ GE Healthcare, Germany
NUPAGE® 4-12% Bis-Tris Gel	Life Technologies, Germany
Parafilm	Bemis Flexible Packaging, USA
Pipette filter tips	Biozym Scientific, Germany
Pipette tips	Sarstedt, Germany
Serological glass pipettes	ISO Lab, Germany
Serological plastic pipettes	BD falcon, USA
Spinner flask	Cellspin 250, Integra biosciences, Germany
Sponge Pad for blotting	Life Technologies, Germany
Storage bottles	Schott Duran, UK
Tissue culture treated dish	BD falcon, USA

2.2 Instruments

-80ºC Freezer	HERAFreeze, Heraeus, Germany
Aspirator	HLC, Kobe, Germany
Autoclave	Holzner GmbH, Germany
Cell culture bench	HERAsafe, Heraeus, Germany
Cell culture incubators	HERAcell 240, Heraeus, Germany HERAcell 150, Heraeus, Germany
Centrifuges	Eppendorf 5417C, Germany Multifuge 1S-R centrifuge, Heraeus, Germany Labofuge 300 centrifuge, Heraeus, Germany
Chemiluminescence imaging system	Peqlab Biotechnologie, Germany
Confocal microscope	Leica SP2 AOBS, Leica Microsystems, Germany
ELISA reader	Magellan Infinite M200, Tecan, Switzerland
Gel electrophoresis power station	PowerEase®500, Life Technologies, Germany
Heating block	TB2 Thermoblock, Biometra, Germany
Homogenisor	Pellet Pestle Motor, Sigma, Germany
Ice machine	Icematic F200, Castel MAC, Italy
Light microscope	Axiovert 40C, Zeiss, Germany
Liquid nitrogen tank	Air Liquide, France
Magnetic stirrer for spinner flask	Integra biosciences, Germany
Magnetic stirrer	RH-KT/C, IKA, Germany
pH meter	pH 211, HANNA Instruments, Germany
Pipettes	Gilson, USA
Pipette filler	Pipetus, Hirschmann Laborgerate, Germany
Power station for WB	PowerEase 500, Life Technologies, Germany
Safety Bunsen burner	Fireboy, Integra biosciences, Germany
Shakers/Rockers	Mini see-saw-rocker SSM4, Stuart, UK
Vortexer	Vortex genie 2, VWR, Germany

Waterbath	Hirschmann, Germany
Weighing machines	TE153S Sartorius, Germany AB265S Mettler Toledo, USA

2.3 Proteins and Enzymes

α2-macroglobulin, human plasma	Calbiochem, USA (Cat no. 441251)
Aprotinin, bovine lung	Calbiochem, USA (Cat no. 616370)
Bovine serum albumin	Gentaur, Germany (Cat no.BAH65)
Collagenase B	Roche, Germany (Cat no. 11088815001)
ESGRO Leukemia Inhibitory Factor	Millipore, Germany (Cat no.ESG1107)
FGF2, recombinant human	Sigma-Aldrich, Germany (Cat no. F0291)
Fibrinogen, bovine plasma	Calbiochem, USA (Cat no. 341573)
Nonfat dried milk powder	AppliChem, Germany (Cat no. A0830)
PDGF-BB, recombinant human	R&D Systems, UK (Cat no. 220-BB)
Phosphatase inhibitor cocktail 3	Sigma-Aldrich, Germany (Cat no. P0044)
Protease inhibitor cocktail	BioVision, USA (Cat no. K271-500)
RAP, recombinant	R&D Systems, UK (Cat no. 4480-LR)
Thrombin, bovine plasma	Sigma-Aldrich, Germany (Cat no. T7513)
TNF-α, recombinant mouse	Millipore, Germany (Cat no.GF023)
Trypsin EDTA 1X	Life Technologies, Germany (Cat no. 25300-062)
VEGF 165, recombinant human	R&D Systems, UK (Cat no. 293-VE)

2.4 Inhibitors

AG1296 (PDGFR inhibitor)	Calbiochem, USA (Cat no. 658551)
AG490 (JAK2 inhibitor)	Calbiochem, USA (Cat no. 658401)
Compound C (AMPK inhibitor)	Calbiochem, USA (Cat no. 171260)
LY294002 (PI3K inhibitor)	Calbiochem, USA (Cat no. 440202)
PD98059 (ERK1/2 inhibitor)	Calbiochem, USA (Cat no. 513000)
SU5402 (FGFR2 inhibitor)	Calbiochem, USA (Cat no. 572630)
SU5614 (FLK1 inhibitor)	Calbiochem, USA (Cat no. 572632)
Wortmannin (PI3K inhibitor)	Calbiochem, USA (Cat no. 681675)

2.5 Cell lines

MEF: Derived from C57BL/6 mouse embryos at 12.5 day gestation

MES: CCE S103, 129/Sv/Ev-derived ES cell line (Robertson, 1987)

2.6 Cell culture media components

Basal Iscove's Medium	Biochrom, Germany
Dulbecco's 1X PBS with Ca & Mg ions	PAA, Germany
Dulbecco's 1X PBS without Ca & Mg ions	PAA, Germany
Dulbecco's Modified Eagle Medium	Merck Millipore, Germany
EmbryoMax® 0.1% Gelatin Solution	Millipore, Germany
ESGRO® LIF	Millipore, Germany
Fetal calf serum (Not USA origin)	Sigma-Aldrich, Germany
L-Glutamine 200mM (100X)	PAA, Germany
Mitomycin-C	Calbiochem, USA
Non-essential amino acids (100X)	Biochrom, Germany
Penicillin/Streptomycin (100X)	PAA, Germany
Plasmocin™	InvivoGen, USA
Sodium pyruvate (100mM)	Biochrom, Germany
KnockOut™ Serum replacement	Life Technologies, Germany
β-Mercaptoethanol	Sigma-Aldrich, Germany

2.7 Media compositions

MEF Medium	
1X	DMEM
24%	Heat inactivated fetal calf serum
2mM	L-Glutamine
100µM	Non-essential amino acids
50µg/ml	L- Penicillin/Streptomycin

MESC Medium	
1X	Basal Iscove's Medium
8%	Fetal calf serum
7.6%	Heat inactivated fetal calf serum
2mM	L-Glutamine
1mM	Sodium pyruvate
100µM	Non-essential amino acids
50µg/ml	L- Penicillin/Streptomycin
100µM	β-Mercaptoethanol
1000U/ml	ESGRO® LIF
2.5µg/ml	Plasmocin™

Knock out serum medium	
1X	Basal Iscove's Medium
18.9%	KnockOut™ Serum replacement
2mM	L-Glutamine
1mM	Sodium pyruvate
100µM	Non-essential amino acids
100µM	β-Mercaptoethanol
50µg/ml	Penicillin/Streptomycin

MESC differentiation medium:	
1X	Basal Iscove's Medium
15.6%	Fetal calf serum
2mM	L-Glutamine
1mM	Sodium pyruvate
100µM	Non-essential amino acids
100µM	β-Mercaptoethanol
50µg/ml	Penicillin/Streptomycin

Serum free medium:	
1X	Basal Iscove's Medium
2mM	L-Glutamine
1mM	Sodium pyruvate
100µM	Non-essential amino acids
100µM	β-Mercaptoethanol
50µg/ml	Penicillin/Streptomycin

Freezing medium	
72%	MESC Medium
20%	Heat inactivated fetal calf serum
8%	Dimethyl sulfoxide

Fibrin matrix solution:	
1X	PBS with Ca & Mg
1.8mg/ml	Fibrinogen
200U/ml	Aprotinin

Thrombin solution:	
1X	PBS with Ca & Mg
0.65U/ml	Thrombin

Collagenase solution:	
1X	PBS
1%	Collagenase B (w/v)

2.8 Chemicals

Ammonium bicarbonate	Sigma-Aldrich, Germany
Copper sulphate	Sigma-Aldrich, Germany
Disodium hydrogen phosphate	Roth, Germany
Folin-Ciocalteu phenol reagent	Sigma-Aldrich, Germany
Luminol	Sigma-Aldrich, Germany
Methyl cellulose	Sigma-Aldrich, Germany
Monopotassium phosphate	Roth, Germany
Paraformaldehyde	Roth, Germany
p-Coumaric acid	Sigma-Aldrich, Germany
Ponceau S	AppliChem, Germany
Potassium chloride	Roth, Germany
Sodium carbonate	Roth, Germany
Sodium chloride	Roth, Germany
Sodium deoxycholate	Sigma-Aldrich, Germany
Sodium dodecyl sulfate	Roth, Germany
Sodium hydroxide	Roth, Germany
Sodium potassium tartrate	Merck, Germany
Tris	Roth, Germany

2.9 Solutions

Ethanol	Roth, Germany
Dimethyl sulfoxide	Merck, Germany
Fluoromount-G	Southern Biotech, USA
Folin - Ciocalteau reagent	Sigma-Aldrich, Germany
Hydrogen peroxide Solution (30%)	Sigma-Aldrich, Germany
Methanol	Roth, Germany
Nonidet P-40	AppliChem, Germany
Novex Sharp Pre-Stained Protein standard	Life Technologies, Germany
NUPAGE® Antioxidant	Life Technologies, Germany
NUPAGE® LDS Sample buffer (4X)	Life Technologies, Germany
NUPAGE® MOPS SDS Running Buffer (20X)	Life Technologies, Germany
NUPAGE® MOPS SDS Running Buffer	Life Technologies, Germany
NUPAGE® Reducing Agent (10X)	Life Technologies, Germany
NUPAGE® Transfer Buffer (20X)	Life Technologies, Germany
Restore Western Blot Stripping buffer	Thermo Fisher Scientific, Germany
Sigmacote	Sigma-Aldrich, Germany
Triton X-100	Sigma-Aldrich, Germany
Tween-20	Sigma-Aldrich, Germany

2.10 Buffer compositions

Lowry Solution
Solution A, Solution B, Solution C mixed in the ratio 100:1:1
Solution A: 2% Sodium carbonate (w/v) in 0.1N Sodium hydroxide solution
Solution B: 1.25 % Copper sulphate (w/v)
Solution C: 3.37 % Sodium potassium tartrate (w/v)

RIPA buffer	
50mM	Tris pH 7.4
150mM	Sodium chloride
1%	Nonidet P-40 (v/v)
0.01%	Sodium dodecyl sulfate (w/v)
0.05%	Sodium deoxycholate (w/v)

Protein extraction buffer	
1X	RIPA buffer
1X	Protease inhibitor cocktail

Phospho-protein extraction buffer	
1X	RIPA buffer
1X	Protease inhibitor cocktail
1X	Phosphatase inhibitor cocktail 3

Ponceau staining solution	
0.01%	Ponceau S (w/v)
5%	Acetic acid (v/v)

Blotting buffer	
1X	NUPAGE® Transfer buffer
0.01%	NUPAGE® Antioxidant (v/v)
20%	Methanol

IX Tris buffered saline (1XTBS) pH 7.4	
50mM	Tris
150mM	Sodium chloride

Membrane washing solution (1X TBST)	
1X	TBS
0.05%	Tween 20

Membrane blocking Solution (5%BSA)	
1X	TBST
5%	BSA (w/v)

1X Phosphate buffered saline (1XPBS)	
137mM	Sodium chloride
2.7mM	Potassium chloride
8mM	Disodium hydrogen phosphate
2mM	Monopotassium phosphate

Permeabilisation Buffer for IF (1% PBST)	
1X	PBS
1%	Triton X-100

Blocking buffer for IF (10%BSA)	
1X	PBST
10%	BSA (w/v)

Washing solution for IF (0.01% PBST)	
1X	PBS
0.01%	Triton X-100

Paraformaldehyde fixing solution	
1X	PBS
4%	PFA (w/v)

ECL detection substrate	
625µM	Luminol
450µM	Cumaric acid
0.03%	Hydrogen Peroxide Solution (30%) (v/v)
100mM	Tris HCl

2.11 Antibodies

For α2m and its receptors

Mouse anti-α2 macroglobulin antibody	Abcam, Cambridge, UK (Cat no. ab52651)
Rabbit anti-LRP1 antibody	Abcam, Cambridge, UK (Cat no. ab92544)
Rabbit anti-GRP78 antibody	Abcam, Cambridge, UK (Cat no. ab21685)

For endothelial markers

Rat anti-PECAM-1 antibody	Millipore, Germany (Cat no. CBL1337)
Rabbit anti-VE Cadherin antibody	Abcam, Cambridge, UK (Cat no. ab33168)
Rat anti-CD144 antibody	BD Biosciences, USA (Cat no. 555289)
Rabbit anti-VEGF Receptor 2 antibody	Cell Signaling Technology, USA (Cat no.2479)
Rat anti-Eph receptor B4 antibody	Abcam, Cambridge, UK (Cat no. ab106130)
Rabbit anti-Ephrin-B2 Antibody	Santa Cruz Biotechnology, USA (Cat no. sc1010)

For mesodermal markers

Rabbit anti-Isl1 antibody	Millipore, Germany (Cat no. AB5754)
Rabbit anti-Brachyury antibody	Abcam, Cambridge, UK (Cat no. ab20680)
Rabbit anti-Nkx2.5 antibody	Abcam, Cambridge, UK (Cat no. ab35842)
Mouse anti-Smooth Muscle actin	Thermo Fisher Scientific, USA (Cat no MS-113-P1)

For growth factors

Mouse anti-FGF-2 antibody	Millipore, Germany (Cat no. 05-118)
Rabbit anti-VEGF165 Antibody	Millipore, Germany (Cat no. 07-1419)
Rabbit anti-PDGF-BB Antibody,	BioVision, USA (Cat no. 5489-100)

For house keeping

Rabbit anti-GAPDH antibody	Abcam, Cambridge, UK (Cat no. ab9485)
Mouse anti-Vinculin antibody	Sigma-Aldrich, Germany (Cat no. V9131)
Rabbit anti β-Actin antibody	Cell Signaling Technology, USA (Cat no.4970)

For phospho proteins

Rabbit anti-p-PI3K (Tyr458/199) antibody	Cell Signaling Technology, USA (Cat no.4228)
Rabbit anti-p-Akt (Ser473) antibody	Cell Signaling Technology, USA (Cat no.9271)
Rabbit anti-p-p44/42 ERK1/2 antibody	Cell Signaling Technology, USA (Cat no.9107)
Rabbit anti-p-Stat3 (Ser727) antibody	Cell Signaling Technology, USA (Cat no.9136)
Rabbit anti-p-AMPKα (Thr172) antibody	Cell Signaling Technology, USA (Cat no.2535)
Rabbit anti-p-mTOR (Ser2448) antibody	Cell Signaling Technology, USA (Cat no.2976)

Secondary antibodies for IF

DyLight® 650 goat anti-rat IgG	Abcam, Cambridge, UK (Cat no. ab98408)
Alexa Fluor® 488 donkey anti-rat IgG	Life Technologies, Germany (Cat no. A-21208)

Secondary antibodies for WB

HRP-linked donkey anti-goat IgG	Abnova, Germany (Cat no. PAB10570)
HRP-linked goat anti-rabbit IgG	Cell Signaling Technology, USA (Cat no. 7074)
HRP-linked goat anti-rat IgG	Cell Signaling Technology, USA (Cat no. 7077S)
HRP-linked horse anti-mouse IgG	Cell Signaling Technology, USA (Cat no. 7076S)

2.12 Softwares

Chemicapt 500 - Western blot imaging software	Peqlab Biotechnologie, Germany
GraphPad Instat - Statistical analysis software	GraphPad Software, USA
Image J - Western blot analysis software	National Institutes of Health, USA
Leica Application Suite Advanced Fluorescence	Leica Microsystems, Germany
Microsoft Office - Data documentation	Microsoft, USA
Tecan Magellan™ - ELISA Software	Tecan, Switzerland

3. Methods

3.1 Mouse embryonic stem cell culture

mESCs in undifferentiated state were cultured on mitotically inactivated MEFs which serve as the feeder layer and with medium supplemented with LIF. The mESCs grew by dividing and spreading over the surface of these MEFs forming undifferentiated colonies. Medium was changed every 24 h with mESCs passaged every 2-3 days when they were semi confluent. The cell culture procedures were always performed under sterile conditions under the cell culture bench. The benches were always wiped with 70% alcohol prior to and upon completion of all culturing procedures to avoid contamination. The cell culture medium was also supplemented with antibiotics to prevent contamination. The incubator was always maintained at 37°C with humidified atmosphere of 5% CO_2. The detailed mESC culture and maintenance procedure are described below.

Thawing MEF: MEF vial was thawed two days prior to plating mESCs. The cryovial containing MEFs were carefully taken out from the liquid nitrogen storage tank and gently swirled in water bath at 37ºC to thaw for a min. When only a small ice crystal remained, it was removed from the water bath, wiped clean with 70% alcohol and taken under the cell culture bench. The vial was carefully opened, flamed and 1ml of the thawed cells was pipetted out very slowly into a 50ml conical tube containing 25ml warm MEF medium. This tube was centrifuged at 1000rpm for 5 min at RT. The supernatant was aspirated and pellet was resuspended in appropriate amount of warm MEF medium. The MEFs were plated at a density of 5 x 10^5 cells onto a 60mm cell culture dish with 5ml MEF medium. Upon adding the MEF cell suspension, the dish was labeled, placed in the incubator and moved in several quick back-and-forth and side-to-side motions to disperse the cells evenly across the cell culture plate. Since these MEFs were already expanded, they usually get 70% confluent after 24 h of plating as shown in **Figure 3.1 A**.

Mitotic inactivation of MEFs: Mitomycin C was used to chemically inactivate MEF cell division. Treating actively dividing cells with Mitomycin C can crosslink complementary strands of the DNA double helix thereby inhibiting cell division. Confluent MEF culture dish was removed from the incubator, media aspirated and 3ml of MEF medium with Mitomycin C at a concentration of 10µg/ml was added and incubated for 3 hours in the incubator. After incubation, the dish was removed from incubator, medium aspirated and cells washed thrice with 3ml MEF medium to remove all traces of Mitomycin C.

After washing fresh 5ml MEF medium was added and placed in the incubator. This Mitomycin C inactivated MEF served as the template for mESCs

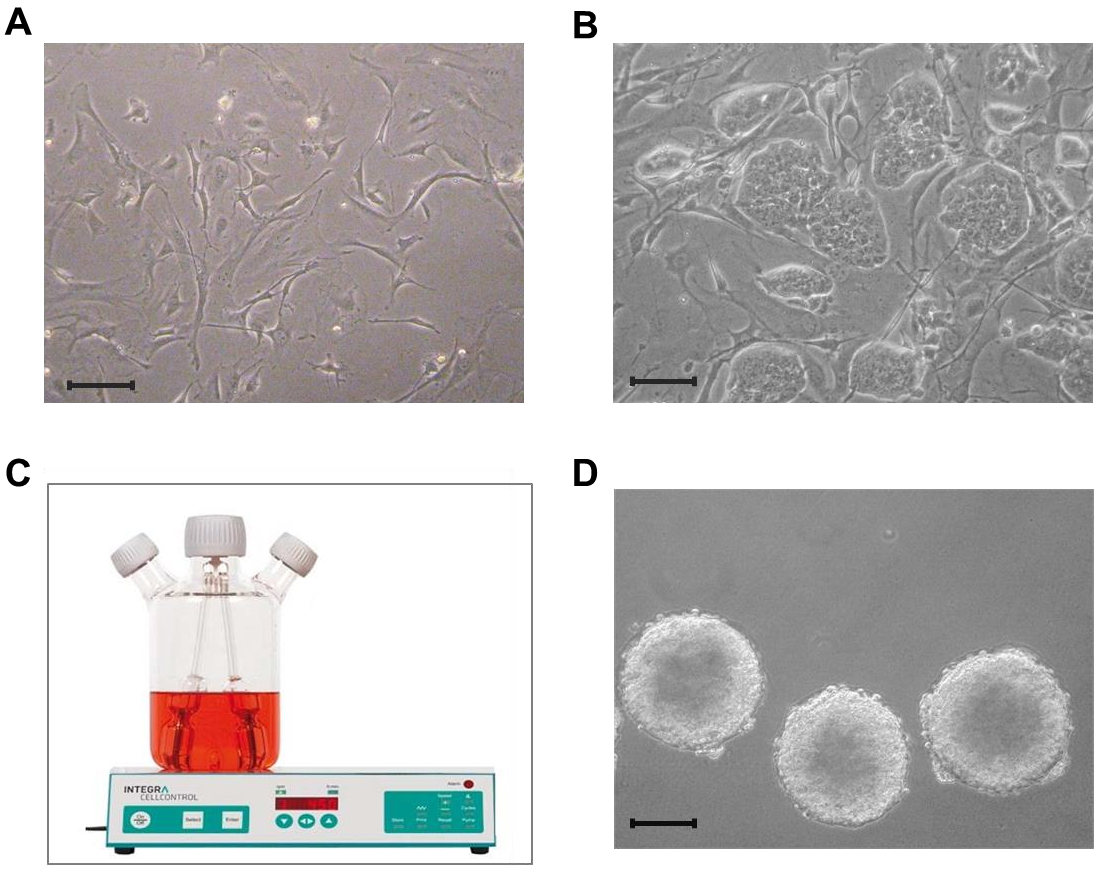


Figure 3.1: mESC culture. A: MEF **B:** mESC colonies growing on mitotically inactivated MEFs**. C:** Spinner flask rotor system **D**: 3-day-old EBs generated in the spinner flask system. The bar represents 100µm.

Thawing mESCs: mESCs were thawed when the MEF dish was ensured to be confluent and mitotically inactivated. The MEF cell dish was removed from the incubator, MEF media aspirated and warm 5ml mESC medium was added prior to mESC plating and placed back in the incubator. The mESC cryovial was thawed similar to the MEF vial. The mESCs were plated at a density of 7 x 10^4 cells per 60mm dish onto the MEF cells. Upon adding the mESC suspension, the dish was moved in several quick back-and-forth and side-to-side motions to evenly disperse the cells across the cell culture dish. The dish was then labeled with the passage number and date and placed in the incubator.

Passaging and maintenance of mESCs: Confluent mESC plates were passaged every 2-3 days. Prior to passaging confluent and Mitomycin-inactivated MEF dish was ensured to be prepared as described above. For passaging, both MEF and mESC culture dishes were removed from the incubator. The MEF media from the MEF dish was aspirated, warm 5ml MESC medium was added and placed back into the incubator. The medium from the mESC dish was aspirated; warm 2ml trypsin was added to wash the cells of the medium and serum. Trypsin was immediately aspirated and fresh warm 2ml trypsin was added and the dish placed in the incubator to lyse the cells. Approximately after 30 s the both the mESC and MEF dishes were removed from incubator, the dislodged mESCs could be observed in the mESC dish. These dissociated mESCs were pipetted up and down using a 1ml pipette to get single cell suspension. Approx. 7×10^4 cells from this cell suspension was added to the MEF dish. The dish was then labeled with the passage number and date and placed into the incubator. After placing into the incubator, the dish was moved in several quick back-and-forth and side-to-side motions to evenly disperse the cells across the cell culture dish. Fresh mESC medium was added every 24 h and dish was observed for colony formation under the microscope every day. Confluent mESCs are shown in **Figure 3.1 B**.

Freezing mESCs: Confluent mESC culture dish was removed from incubator and trypsinised with 2ml trypsin as described above for passaging. After inactivating with warm mESC medium, they were centrifuged at 1000rpm for 5 min. The pellet was resuspended in 1ml freezing medium and transferred into a cryovial. This cryovial was placed into Nalgene Mr. Frosty, placed in -80ºC overnight and transferred to liquid nitrogen tank the next day.

3.2 Generation of EBs

EBs are three-dimensional aggregates of differentiating mESCs generated in suspension cultures. mESCs when grown in the absence of anti-differentiation factor LIF and feeder layer, tend to differentiate.

Spinner flask system: Spinner flasks were used for generating EBs in suspension as shown in **Figure 3.1 C**. These flasks are fitted with hanging magnetic stirring bars. The inner walls of the spinner flask and the magnetic bars were coated with Sigmacote, a silicone solution. Sigmacote forms a thin film on glass surface. As it is water repellent it prevents cells from attaching to glass surface and promotes cell-cell adhesion in order to form 3D aggregates.

EB generation: Sigmacote-coated and autoclaved spinner flask was ensured to be prepared. This spinner flask was taken into the cell culture hood and 125ml warm mESC differentiation medium was added. The mESC dish from which EBs were to be generated was taken, media aspirated and washed with warm 2ml trypsin. Fresh warm 2ml trypsin was added and the dish placed in the incubator to lyse the cells. Approximately after 30 s the mESC dish was removed from incubator, the dislodged mESCs could be observed. These dissociated mESCs were pipetted up and down using a 1ml pipette to get single cell suspension. To this 8ml warm mESC media was added to inactivate trypsin. Approx. 1.5 to 2 x 10^6 cells from this cell suspension were added to the spinner flask containing medium. The spinner flask was labeled with the passage number and date and then placed into the incubator on the magnetic stirring plate. The flask was stirred clockwise and anti-clockwise at a speed of 20rpm. The following day fresh 125ml medium was added to make a total of 250ml medium. On the consecutive days, partial medium was changed, where 125ml medium was aspirated out and fresh 125ml medium was added. On the desired end day of EB formation usually on day 3, the spinner flask was removed from the incubator and the EBs were collected using a wide open 10ml pipette onto a 100mm non-adherent bacteriological dish, placed into the incubator on the shaker until further use. Formation of 3-day-old EBs is represented in **Figure 3.1 D**.

3.3 Treatment of EBs with α2m and other factors

For each experimental sample approx. 20-25 3-day-old EBs were taken in each 60mm bacterial culture dish containing 5ml Knock out serum medium. Knock out serum medium was used to rule out the presence of α2m or any other growth factors present in FCS. On day 4, the EBs were treated with appropriate factors in fresh 5ml Knock out serum medium and the dish was placed on the shaker inside the incubator. Medium was changed every 24 h with fresh factors added each time. On day 9 the experiment was terminated with treated EBs collected for protein extraction or for immunofluorescence studies. The EBs were treated with the factors mentioned in **Table 3.3** along with their concentration used for this research work.

Factors	Concentration
α2m	4µg/ml
RAP	10nM
anti-LRP1 antibody	0.2µg/ml
anti-GRP78 antibody	0.2µg/ml
AG1296 (PDGFR inhibitor)	800nM
SU5402 (FGFR2 inhibitor)	15µM
SU5614 (FLK1 inhibitor)	1.5µM
AG490 (JAK2 inhibitor)	50µM
Compound C (AMPK inhibitor)	235nM
LY294002 (PI3K inhibitor)	20µM
PD98089 (ERK1/2 inhibitor)	20µM
Wortmannin (PI3K inhibitor)	2.5µM

Table 3.3: Concentrations of all α2m and other inhibitors used for experiments

3.4 PECAM1/ VE-Cadherin Immunohistochemistry

To visualize three dimensional vascular network formations, the EBs were stained for the endothelial markers, PECAM1 and VE-Cadherin. Vasculature could be observed in a differentiating EBs from day 7 onwards. For all IHC staining experiments the EBs were treated until day 9 of differentiation to look for well-established vascular structures.

Sample preparation: On day 9 after treatment with different factors the plates containing EBs were removed from the incubator and the EBs were collected into microcentrifuge tubes and labeled. Cell culture medium was aspirated and EBs were washed twice with PBS in order to remove traces of the cell culture medium.

Fixation: Ice cold methanol was used to precipitate proteins. Methanol with a temperature of -20ºC was added to each of the tubes until the EBs were completely immersed for fixation and put at -20ºC for 30 min until further use.

Permeabilization and blocking: After fixation EBs were washed twice with PBS and permeabilized using 1% PBST and placed on shaker for 15 min, consequently blocked with blocking buffer (10% BSA) for 1 h to block nonspecific protein binding sites.

Antibody staining: After blocking, the EBs were washed once with 0.01% PBST and incubated with the primary antibody against PECAM1 or VE-Cadherin at a dilution of 1:100 in blocking buffer for 2 h on the shaker at RT. After this they were washed three times with 0.01% PBST and incubated with the secondary antibody anti-rat Alexa 488 at a dilution of 1:100 in blocking buffer for 1 h in dark on the shaker at RT. Then they were washed three times, 5 min each with 0.01% PBST and taken for imaging or stored at 4ºC until further use.

Imaging: The stained EBs were taken, transferred onto a chamber slide and observed for vascularization under the Leica SP2 AOBS confocal microscope. Using 10X objective, Z-series images, measuring 50µm sections were taken from the top to the bottom of each EB that may have a diameter ranging from 600-1000µm. Around 10-15 Z-series images were captured for each EB and then merged to obtain a single 2D image. This 2D image was further utilized for analysis.

Evaluation: From the image acquired on the confocal microscope, vascularization was determined manually by counting the number of branching points in an EB divided by the total area of that EB in μm^2. The area of the EB was measured using Leica LCS imaging software. These values were tabulated on an excel sheet and graph plotted. Approximately 10-15 EBs of each experimental sample were analysed. Untreated sample served as the control for each experiment.

3.5 Western blotting

Protein extraction: RIPA buffer was used for whole cell lysate and membrane bound protein extraction. For total protein isolation the treated EB samples were removed from the incubator, cell culture media aspirated and after washing the EBs twice with cold 1X PBS, they were collected into a microcentrifuge tube and equal amount of ice cold protein extraction buffer was added and sonicated using a homogenisor for 30 s. This was then centrifuged at 15000rpm for 10 min at 4ºC. The supernatant containing the extracted protein was collected into a new microcentrifuge tube, labeled and stored at -80ºC. Phospho-protein isolation was performed in the same manner as total protein extraction except that phospho-protein extraction buffer was used instead of protein extraction buffer.

Quantification: Protein concentration was determined using Lowry's method. BSA protein standards were prepared. 10µl of the extracted protein taken in a microcentrifuge tube was mixed with 700µl of Lowry Solution and incubated for 10 min at RT. 200µl of 1N Folin - Ciocalteau reagent solution was added and incubated for additional 30 min at 37ºC. This was then centrifuged at 15000rpm for 30 min at RT. The colorimetric absorbance was read at 578nm using ELISA reader and values were obtained using the Magellan software. Absorbance was plotted against the known BSA protein standard to obtain the calibration curve from which the unknown protein concentrations of all samples were determined.

Sample preparation for gel electrophoresis: Samples for electrophoresis were prepared by mixing 20µg of protein sample with 1X NUPAGE® LDS Sample buffer, 1X NUPAGE® reducing agent and volume made up to 20µl with water. This mixture was heated for 10 min on the heating block at 75 ºC.

Gel electrophoresis: Samples were loaded onto NuPAGE® 4-12% Bis-Tris precast gels. 3µl Novex Sharp Pre-Stained Protein standard was also loaded to one of the wells to determine protein size. 200ml of 1X MOPS SDS Running Buffer was added onto the upper buffer chamber and 300ml added to the lower buffer chamber. 500µl of NUPAGE® antioxidant was added to the upper buffer chamber. The electrophoresis apparatus was closed, connected to the power supply and run at 180V for 1 h till the dye reached the bottom of the gel.

Western blotting: Blotting was performed by wet transfer method using Xcell II™ Blot module. Blotting sponges and filter paper were soaked in transfer buffer. The gel

cassette was opened and the gel was carefully removed into a tray. A sandwich was prepared by layering 3 blotting sponges, a filter paper, gel, nitrocellulose membrane, a filter paper and 3 blotting sponges. This sandwich was placed on top of the cathode core of the blot module and closed with the anode core. This blotting module was slid into the Xcell SureLock™ Mini-Cell and Blotting buffer was poured into it. The outer chamber was filled with 650ml water. The blotting apparatus was closed, connected to the power supply and run at 25V for 1 h.

Ponceau staining: To check the success of blotted proteins on the nitrocellulose membrane, the membrane was incubated in Ponceau staining solution for 2 min. The membrane was then destained in water to visualize the resolved protein bands.

Immuno-staining: The blotted membrane was cut based on the protein of interest referring to the protein standard. The membrane was blocked in membrane blocking solution for 1 h with agitation. After washing once with 1X TBST, the membrane was incubated with appropriate primary antibody diluted in 5% BSA in 1X TBST overnight at 4ºC with agitation. The primary antibody was taken directly from the original stock solution as supplied from the company and diluted with the dilution factor shown in **Table 3.5**. The membrane was washed thrice with 1X TBST for 5 min and incubated with HRP-conjugated secondary antibody diluted in 5% BSA in 1X TBS for 1 h with agitation. The membrane was washed thrice with 1X TBST for 5 min each. The concentrations of the primary and secondary antibodies used for staining for all the WB experiments are listed in **Table 3.5**.

Chemiluminescence detection and imaging: The membrane was incubated for 1 min in ECL detection substrate and placed into the chemiluminescence imaging system. Using the camera fitted to this imaging system the emitted chemiluminescence was captured and converted this into a digital image using the Chemicapt 500 Western blot imaging software.

Analysis: From the images obtained, densitometric quantification was performed using the ImageJ software. The final value of each band was obtained after normalizing with their respective loading control.

Stripping membranes: In order to reprobe for another protein on a chemiluminescent membrane, the membrane was washed in 1X TBST and incubated in Restore Western Blot Stripping buffer for 15 min on RT, washed in 1X TBST, blocked and stained for primary and secondary antibody as described above.

Primary antibody	Dilution	Protein size (KDa)
Anti-alpha 2 macroglobulin antibody	1:1000	163
Anti-LRP1 antibody	1:5000	85
Anti-GRP78 antibody	1:2000	75
Anti-PECAM-1 antibody	1:1000	130
Anti-VE Cadherin antibody	1:1000	115
Anti-VEGF Receptor 2 antibody	1:1000	230
Anti-Isl1 antibody	1:1000	40
Anti-Brachyury antibody	1:1000	53
Anti-Nkx2.5 antibody	1:1000	72
Anti-Smooth Muscle actin	1:5000	42
Anti-FGF-2 antibody	1:1000	18
Anti-VEGF165 Antibody	1:1000	16
Anti-PDGF-BB Antibody,	1:500	18
Anti β-Actin antibody	1:2000	42
Anti-GAPDH antibody	1:2000	36
Anti-Vinculin antibody	1:1000	117
Anti-Phospho-PI3K (Tyr458/199) antibody	1:1000	60 & 85
Anti-Phospho-Akt (Ser473) antibody	1:1000	60
Anti-Phospho-p44/42 ERK1/2 antibody	1:1000	42 & 44
Anti-Phospho-Stat3 (Ser727) antibody	1:1000	86
Anti-Phospho-AMPKα (Thr172) antibody	1:1000	62
Anti-Phospho-mTOR (Ser2448) antibody	1:1000	289

Secondary HRP linked antibody	Dilution
HRP-linked donkey anti-goat IgG	1:1000
HRP-linked goat anti-rabbit IgG	1:1000
HRP-linked goat anti-rat IgG	1:1000
HRP-linked horse anti-mouse IgG	1:1000

Table 3.5: Primary antibody and secondary antibody dilution used for WB and protein band size observed.

3.6 Sprout assay

Sprout assay is an *in-vitro* angiogenesis assay developed by Korff et al., 1998. It is based on the principle that aggregates of ECs can form capillary structures when embedded onto a 3D matrix that mimic *in-vivo* microenvironment. 5-day-old EBs were dissociated into single cells and spheroids of defined cell number were generated in methyl cellulose medium by hanging drop method. These spheroids were embedded onto fibrin matrix polymerised with thrombin to monitor induction or inhibition of capillary formation by adding α2m macroglobulin and other exogenous factors.

Methyl cellulose stock preparation: 6g methyl cellulose was autoclaved in 500ml storage bottle along with a magnetic stirrer. Under the sterile bench 250ml of warm basal Iscove's DMEM was added to dissolve methyl cellulose and put on the magnetic stirrer at 60°C for 20 min. Another 250ml basal medium was added and put on the magnetic stirrer for 1 h at room temperature. This was then put at 4°C overnight on stirrer to further dissolve methyl cellulose (methyl cellulose sometimes does not dissolve completely). This solution was aliquoted in 50ml tubes and centrifuged at 4000*g* for 2 h at RT. the supernatant was transferred to a new 50ml tube and stored at 4°C till use.

Dissociating EBs: 5-day-old EBs were removed from the incubator and placed into a sterile bacterial dish, washed twice with 1X PBS and warm 5ml collagenase B solution was added and placed on the shaker inside the incubator for 5 min. After incubation, the dish was removed and using a 1ml pipette, the EBs were pipetted up and down to get a single cell suspension. This cell suspension was collected into a 15ml conical tube and warm MESC medium was added to inactivate collagenase activity. The tube was centrifuged at 300*g* for 5 min at RT to pellet the cells. The cells were resuspended in 10ml MESC medium and cell number determined using haemocytometer.

Spheroid Generation by Hanging Drop Method: After determining the cell number, 0.34 million cells were suspended in 10ml 20% methyl cellulose (v/v) in MESC medium to get approx. 1000cells/spheroid in every 30µl of the medium. Using a multi-channel pipette, several 30µl drops of this cell suspension were dispensed on to the inside of 100mm bacterial dish lid. The bottom dish was placed onto this lid containing the many drops and the plate was inverted to original posture and placed into the incubator for 24 h as hanging drops. Approx. 15-20 spheroids are generated for each experimental sample.

Spheroid collection: After 24 h, the plate was assessed under the microscope for well-formed spheroids. The plates were then taken under the bench, inverted and 10ml medium was slowly added to the inside of the lid containing spheroids. These spheroids were then carefully removed into a conical tube and centrifuged at 300*g* for 3 min at RT to pellet the spheroids. The spheroids were then washed once in 1X PBS to remove traces of medium by centrifuging at 300*g* for 3 min at RT and re-suspended in 1ml fresh 1X PBS.

Spheroid embedding: 250µl of fibrin matrix solution was added to each well on a 4 well plate. To this 50µl of spheroid suspension in PBS was added and the plate swirled well in order to disperse the spheroids evenly. Approx. 15-20 spheroids were ensured to be present in each well. To polymerize the fibrin matrix, 10µl of thrombin at a final concentration of 0.65 NIH U/ml was added, rapidly mixed and placed in the incubator for 30 min.

Sprout stimulation with various factors: Once the fibrin matrix was polymerized, 750µl basal MESC medium (without fetal calf serum) with appropriate stimulant was added to each well and placed back into the incubator. A well with no stimulant added served as the control. 24 h after stimulation, the plate was observed under the light microscope for sprout formation. **Figure 3.6** represents the formation of sprouts.

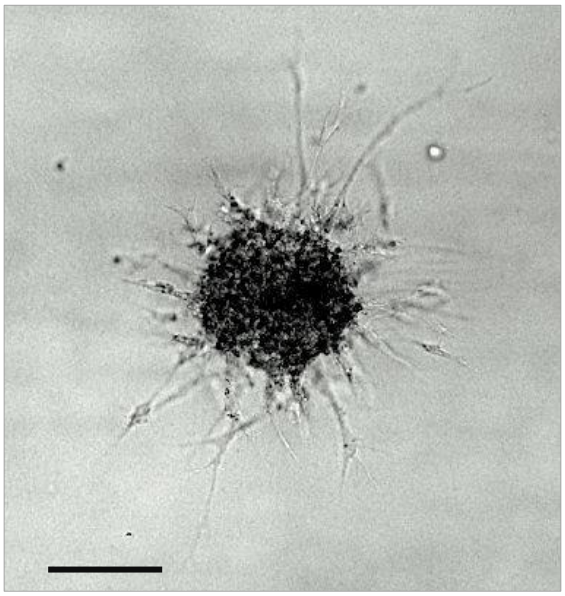


Figure 3.6: Sprout formation from 5-day-old mEB-derived spheroid within 24 h of embedding on fibrin matrix. The bar represents 100µm.

Fixation: The plate was removed from incubator; media was aspirated and washed once with PBS. To this cold 4% Paraformaldehyde solution was added to fix the sprouted spheroids. The plate was stored at 4 ºC until imaging.

Imaging: The plate was placed under the Leica SP2 AOBS confocal microscope for imaging of sprouted spheroids. As observed in **Figure 3.6** each spheroid could be observed with numerous sprouts of varying lengths originating from its surface under the 10X objective lens. 2D images were taken from 10-15 spheroids for each experimental sample. This image was further utilized for analysis.

Analysis: The image acquired from the confocal microscope was taken for analysis. Using Leica LCS imaging software the sprout length of each sprout of a spheroid was measured in μm^2. The branches in the each sprout were also measured. The cumulative sprout length for each spheroid was calculated. On average 10-15 spheroids of each experimental sample were analysed. These values were tabulated on an Excel sheet and graph plotted. Untreated sample served as the control for each experiment.

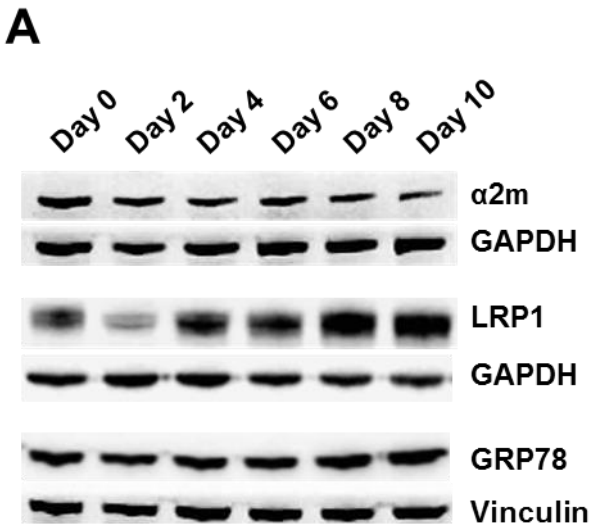
3.7 Statistical analysis

Each experiment was performed minimum 3 times on independent experiments. Data were represented as percentage of control with percentage of standard deviation (%SD). Data was analysed using the statistical analysis software GraphPad Instat using one-way analysis of variance (ANOVA) with Tukey post-tests for multiple comparisons and Student's t-test for single measurements. Differences were considered statistically significant when *P* value was calculated to be lesser than or equal to 0.05.

4. Results

4.1 Endogenous expression of α2m and its receptors in differentiating mEBs

To examine the effects of exogenous α2m on differentiating mESCs, the endogenous expression of α2m and both its receptors were investigated. Total protein was collected from EBs every alternate day from day 0 until day 10 where day 0 samples were the samples cultivated in the presence of LIF and are in the undifferentiated state. The results revealed that in undifferentiated and differentiating state mEBs expressed α2m, its expression was down-regulated upon differentiation **(Figure 4.1)**. Undifferentiated mEBs express LRP1. During the onset of differentiation i.e. on day 2 LRP1 was down-regulated and progressively upregulated throughout differentiation. This expression pattern supports previous findings that LRP1 is involved in various cell signaling pathway pertaining to cell proliferation and differentiation (Hennen et al., 2013). The relative GRP78 expression was also progressively upregulated upon differentiation suggesting its active role in folding and assembly of proteins in the endoplasmic reticulum thus sustaining homeostasis in the developing EB, as GRP78 was reported to be essential for pluripotent cell proliferation (Boucher et al., 2011).



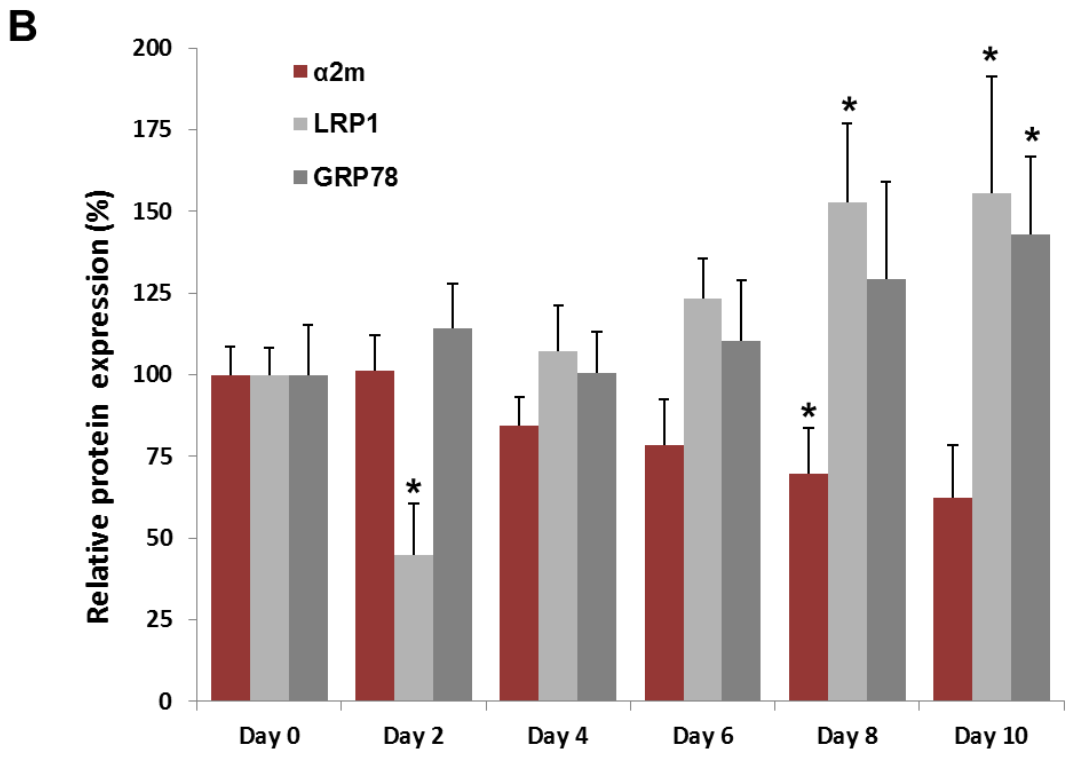
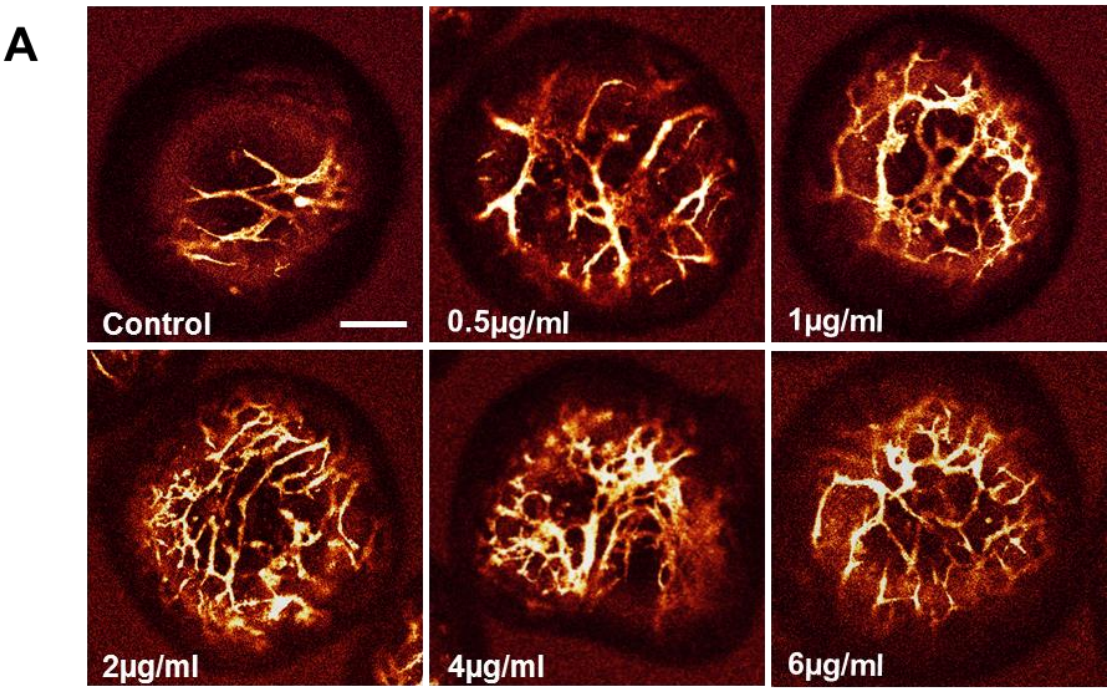
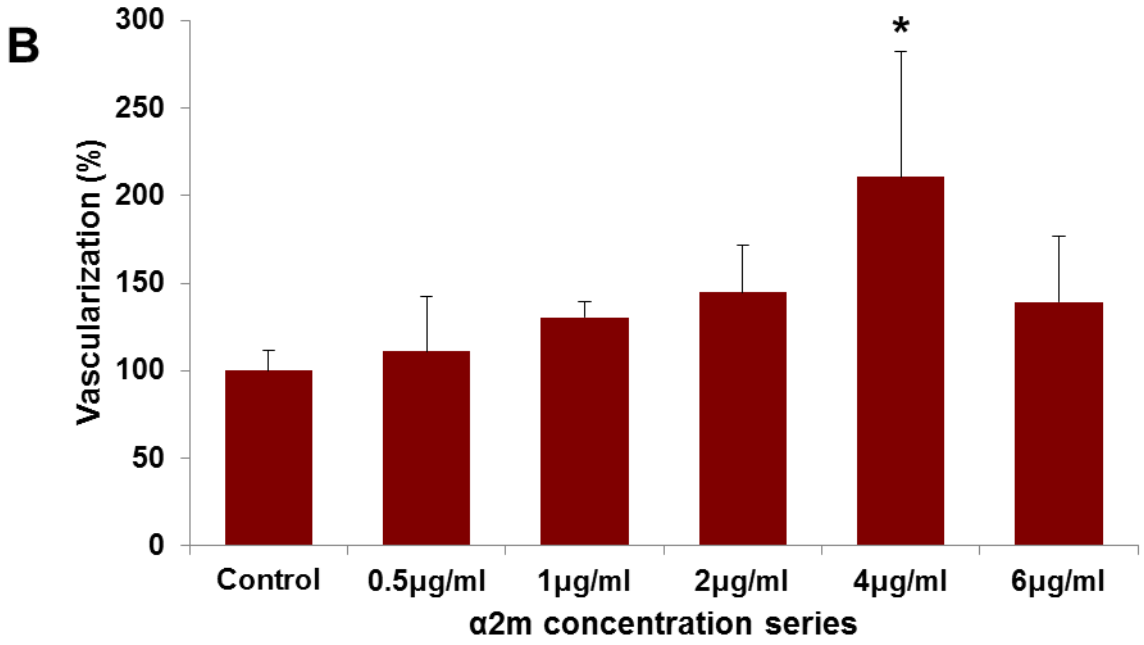


Figure 4.1: Endogenous expression of α2m, LRP1 and GRP78 in differentiating mESCs. A: Western blot analysis α2m, LRP1 and GRP78 expression from day 0 until day 10 B: Graphical representation of the relative protein expression of A. n=3 (α2m), n=4 (LRP1), n=6 (GRP78). *: $P \leq 0.05$

4.2 Enhancement of vasculogenesis in differentiating mEBs upon α2m treatment

4-day-old mEBs treated for 6 days with increasing concentration of α2m ranging from 0.5µg/ml to 6µg/ml concentration were assessed for vascularization by staining the mEBs against the endothelial marker, CD31/ PECAM1 which is expressed at the intercellular junctions of endothelial cells. The vascular network formed in the differentiating EBs was quantified as the number of branching points per micrometer square. As shown in **Figure 4.2**, a dose-dependent increase in vascularization with increasing concentrations of α2m could be observed. Maximum vascularization was obtained with α2m concentration of 4µg/ml. In order to reconfirm the vascularization, the mEBs were stained against VE-Cadherin, an endothelial intracellular junctional protein responsible for EC permeability. A similar increase in branching points was observed with 4µg/ml α2m treated mEBs.


A
Control
0.5µg/ml
1µg/ml
2µg/ml
4µg/ml
6µg/ml


B
300
250
200
150
100
50
0
Vascularization (%)
*
Control
0.5µg/ml
1µg/ml
2µg/ml
4µg/ml
6µg/ml
α2m concentration series

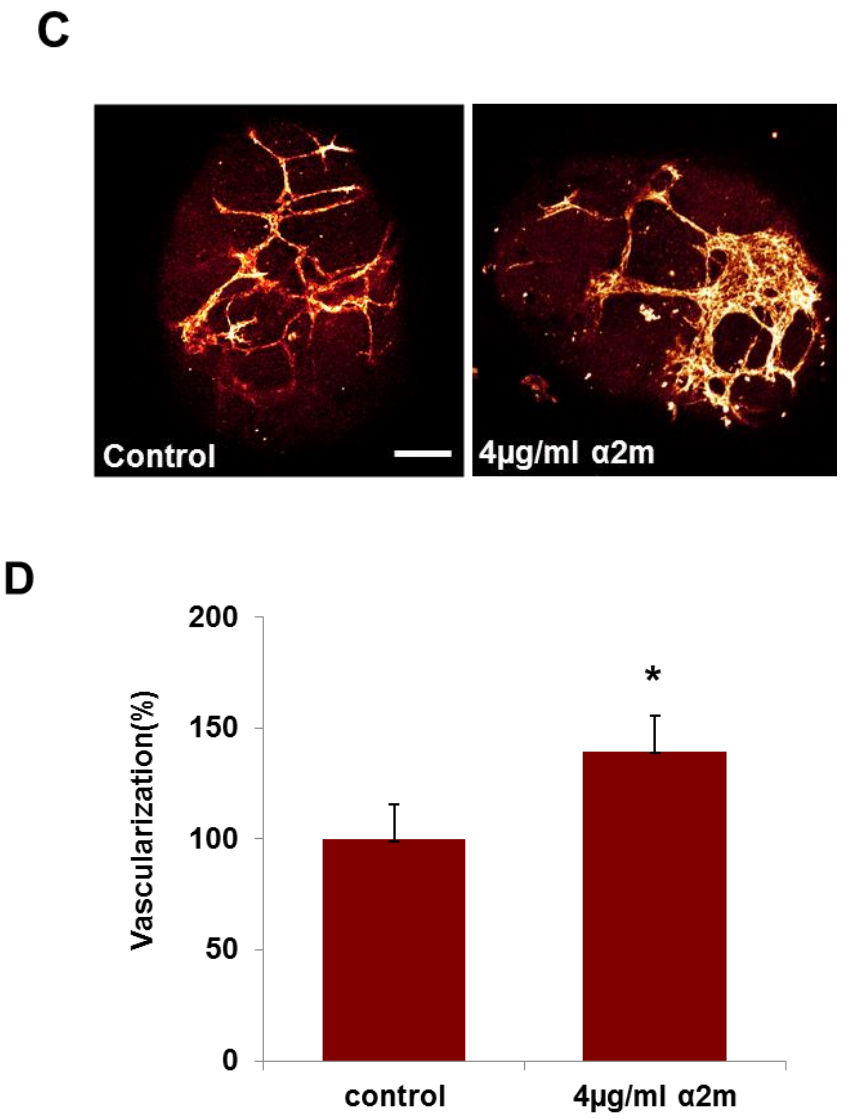


Figure 4.2: Vascularization upon α2m treatment in mEBs. A: Confocal images of EBs stained against PECAM1 with increasing concentrations of α2m. The bar represents 100µm. **B:** Quantification of A representing the vascularization induced by α2m treatment. n=4, *: $P \leq 0.05$ **C:** Confocal images of EBs stained against VE-Cadherin with 4µg/ml α2m. The bar represents 100µm**. D:** Quantification of C representing the vascularization induced by α2m treatment. n=4, *: $P \leq 0.05$

4.3 α2m-induced vasculogenesis upregulates expression of angiogenic growth factors FGF2, VEGF165 and PDGF-BB

In order to investigate whether the vasculogenesis induced by α2m is regulated by angiogenic growth factors, 4-day-old EBs treated with increasing concentrations of α2m for 6 days were analysed for the expression of FGF2, VEGF 165 and PDGF-BB. As shown in **Figure 4.3**, the protein expression of all growth factors showed dose-dependent increase with α2m with 4ug/ml concentration exerting maximal upregulation.

The relative expression of VEGF165 and FGF2 was upregulated 2.5 fold compared to control. Comparatively PDGF-BB expression was only slightly upregulated and could be attributed to the fact PDGF-BB plays active role in the remodeling of the vascular structure.

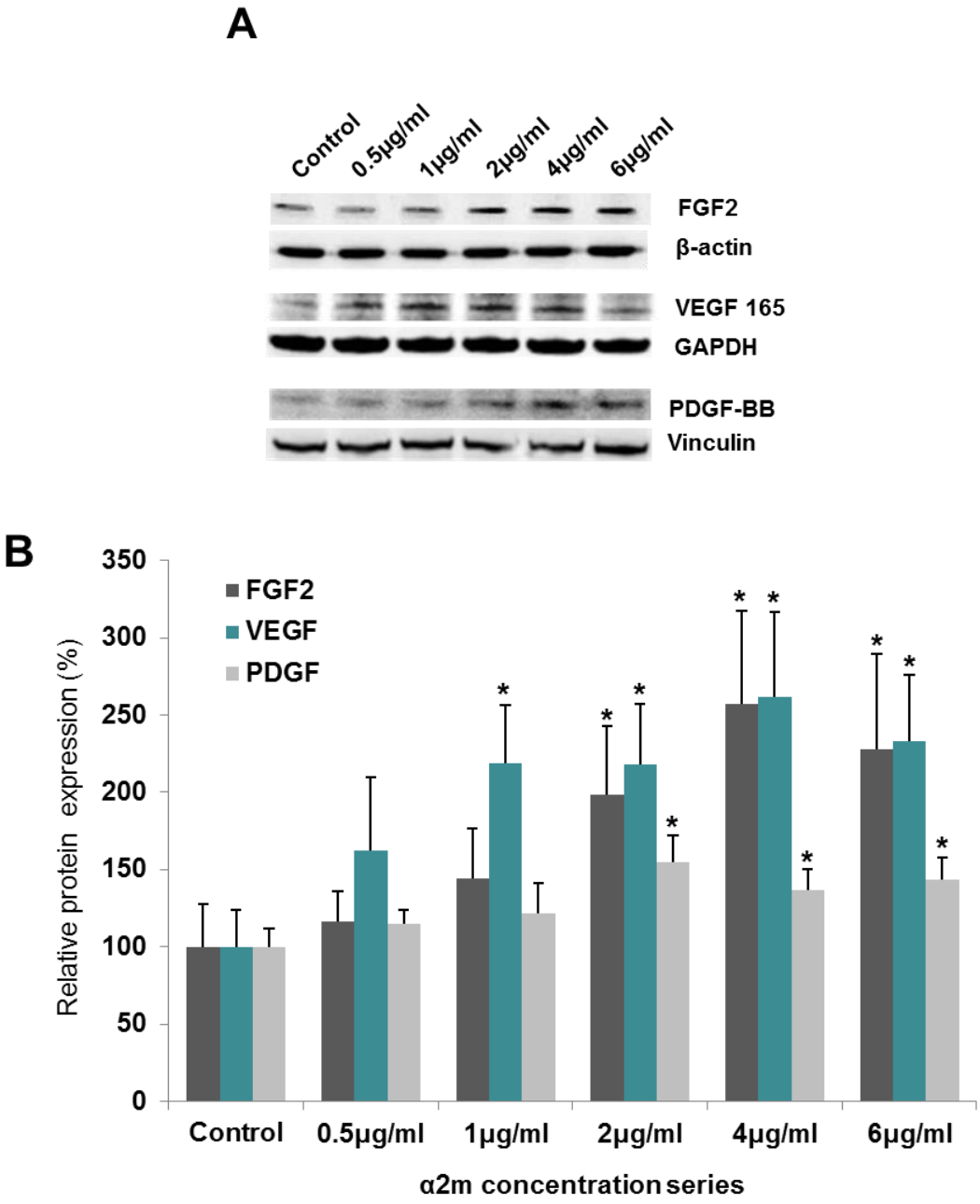
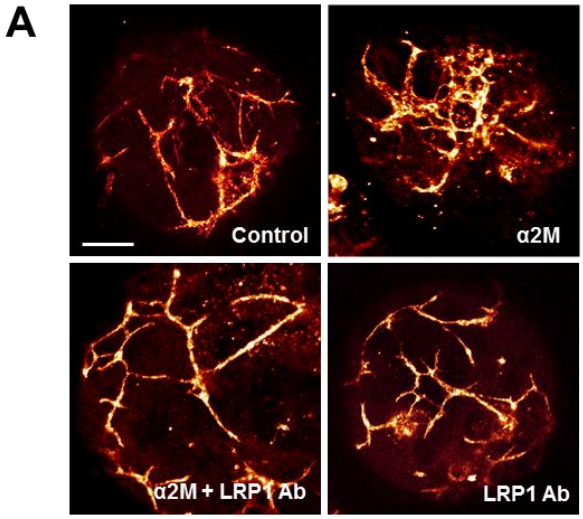
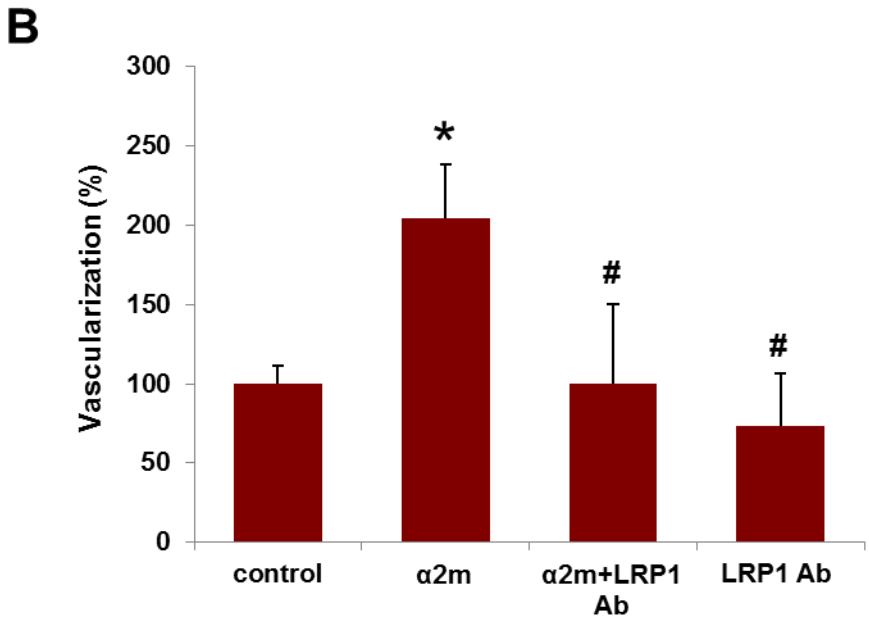


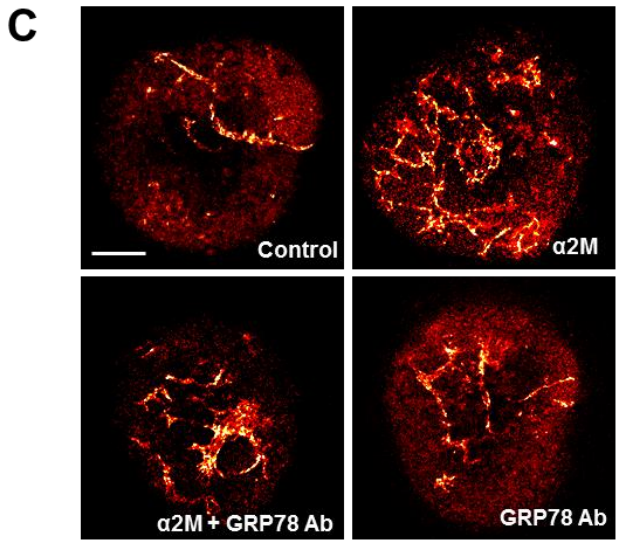
Figure 4.3: Upregulation of the angiogenic growth factors FGF2, VEGF and PDGF-BB on α2m-induced vasculogenesis. A: Western blot analysis of FGF2, VEGF165 and PDGF-BB expression in mEBs treated with increasing concentrations of α2m **B:** Graphical representation of the relative protein expression of A. n=6 (FGF2), n=5 (VEGF165), n=4 (PDGF-BB), *****: $P \leq 0.05$.

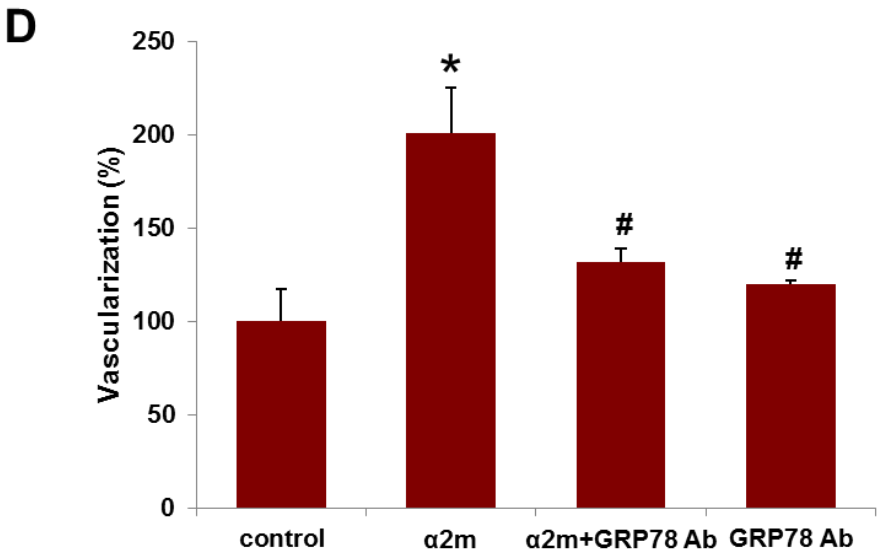
4.4 Impact on the activity of LRP1 and GRP78 receptors for α2m- induced vasculogenesis

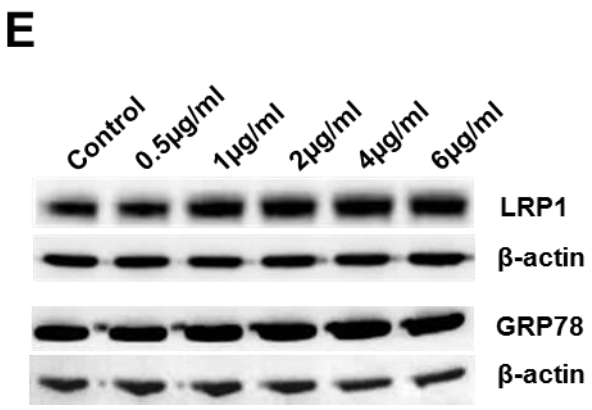
To determine the impact of LRP1 and GRP78 for α2m-induced vasculogenesis, 4-day-old EBs were treated for 6 days with antibodies against the receptors LRP1 and GRP78 and were analysed for vascularization by staining against PECAM1. As illustrated in **Figure 4.4 A-D**, the treatment with anti-LRP1 antibody and anti-GRP78 antibody and co-treatment with α2m abolished vascularization observed with α2m alone, confirming that α2m exerts its angiogenic effects by acting on both its receptors LRP1 and GRP78. The protein expression of LRP1 and GRP78 receptors showed a dose-dependent upregulation on increasing α2m concentrations **Figure 4.4 C, D**.






C
Control
α2M
α2M + GRP78 Ab
GRP78 Ab


D
Vascularization (%)
250
200
150
100
50
0
*
#
#
control
α2m
α2m+GRP78 Ab
GRP78 Ab


E
Control
0.5μg/ml
1μg/ml
2μg/ml
4μg/ml
6μg/ml
LRP1
β-actin
GRP78
β-actin

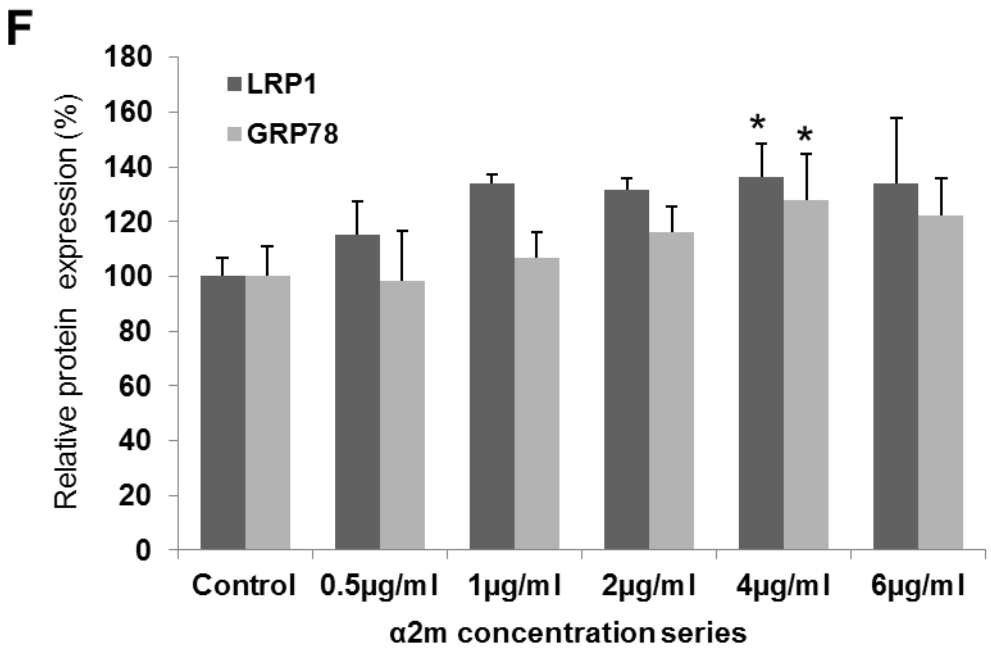
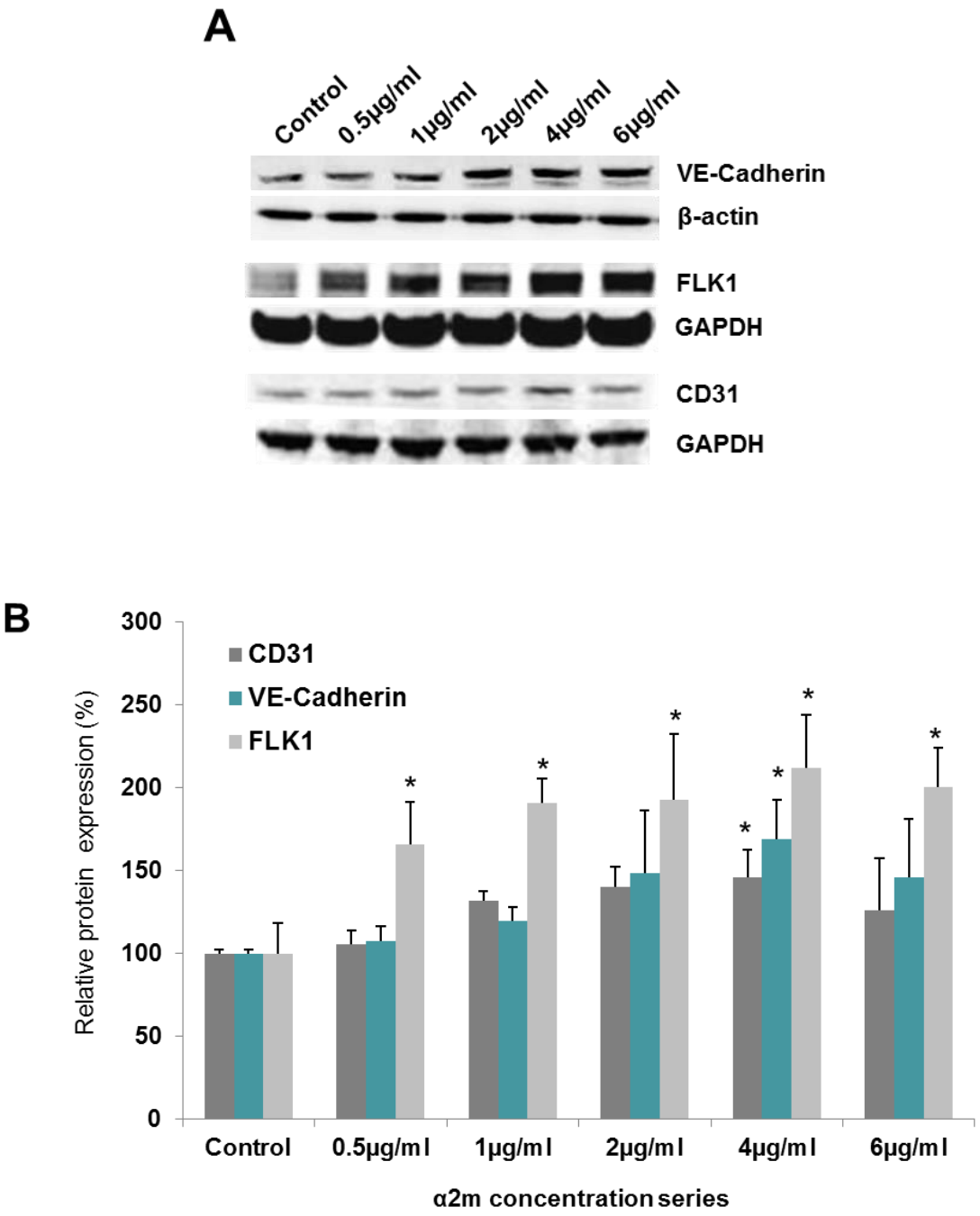


Figure 4.4: Impact on the activity of LRP1 and GRP78 receptors for α2m-induced vasculogenesis. A: Confocal images of EBs stained against PECAM1 on blocking the receptor LRP1 with anti-LRP1 antibody and its co treatment with α2m. The bar represents 100µm **B:** Quantification of A representing the vascularization inhibited upon anti-LRP1 treatment (n=3), *****: $P \leq 0.05$, significant compared to the untreated control; $^{\#}$: $P \leq 0.05$ significant compared to α2m treatment alone **C:** Confocal images of EBs stained against PECAM1 on blocking the receptor GRP78 with anti-GRP78 antibody. The bar represents 100µm **D:** Quantification of C representing the vascularization inhibited upon anti-GRP78 treatment (n=3), *****: $P \leq 0.05$, significant compared to the untreated control; $^{\#}$: $P \leq 0.05$ significant compared to α2m treatment alone. **E:** Western blot analysis of LRP1 and GRP78 expression in mEBs treated with increasing concentrations of α2m **F:** Graphical representation of the relative protein expression of A, n=3 (LRP1), n=6 (GRP78), *****: $P \leq 0.05$

4.5 Upregulation of endothelial and mesodermal markers on α2m treatment of mEBs

Besides PECAM1 immunofluorescence studies on vascularization, protein expressions of PECAM1/CD31, VE-Cadherin and FLK1/VEGFR2 were analysed and a similar dose-dependent upregulation with increasing concentrations of α2m was observed **(Figure 4.5 A,B)** which can be correlated with the expression pattern of angiogenic growth factors. Expression of mesodermal markers Nkx2.5, Isl1, Brachyury and αSMA revealed an upregulation upon 4µg/ml α2m treatment compared to control **(Figure 4.5 C,D)**.


A
Control
0.5μg/ml
1μg/ml
2μg/ml
4μg/ml
6μg/ml
VE-Cadherin
β-actin
FLK1
GAPDH
CD31
GAPDH
B
CD31
VE-Cadherin
FLK1
Relative protein expression (%)
300
250
200
150
100
50
0
Control
0.5μg/ml
1μg/ml
2μg/ml
4μg/ml
6μg/ml
α2m concentration series

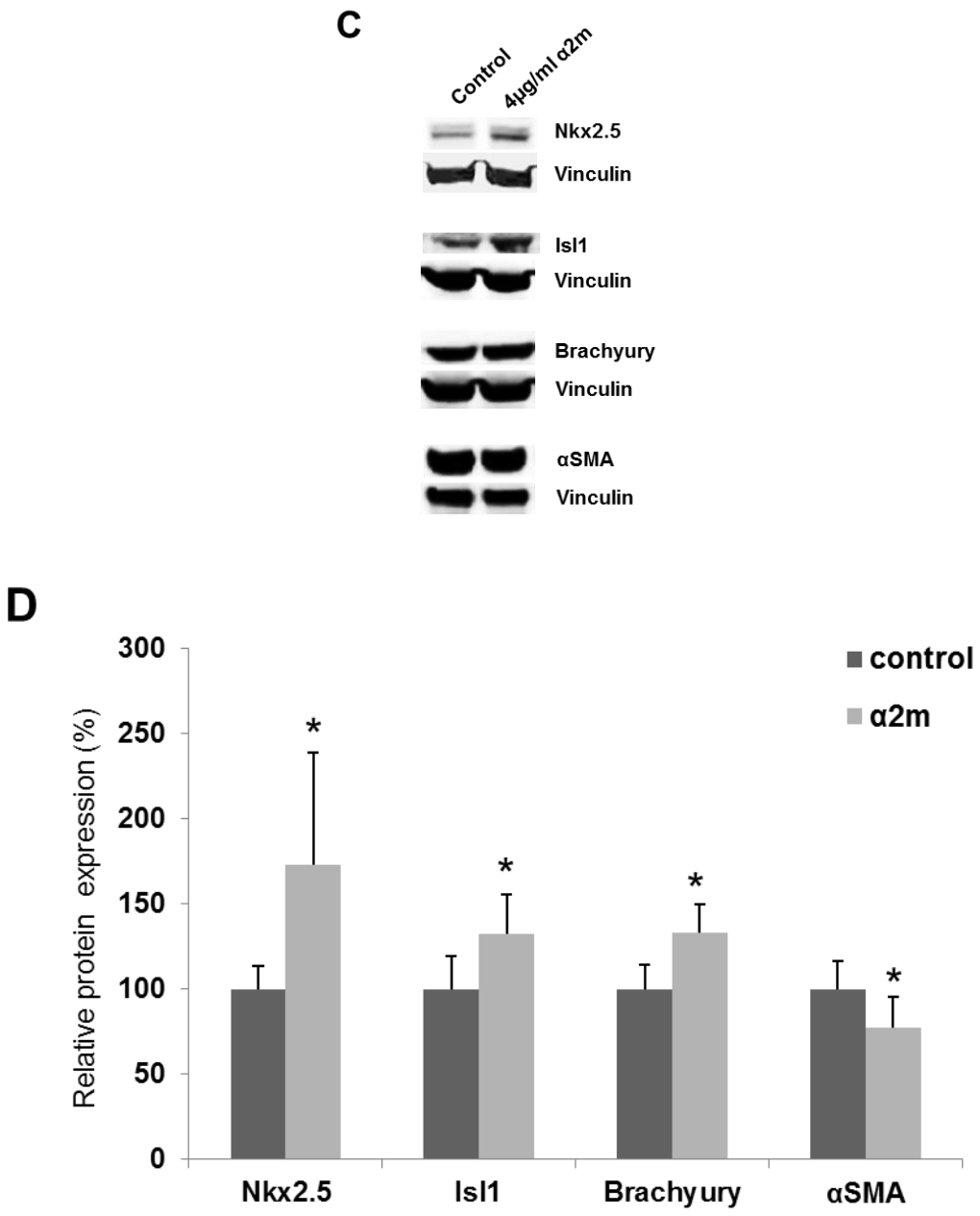
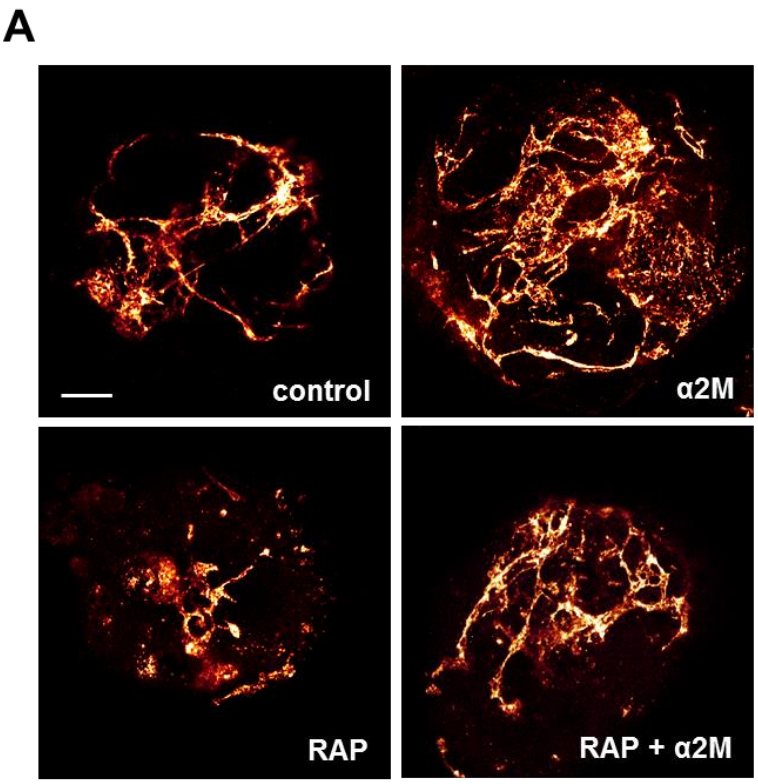


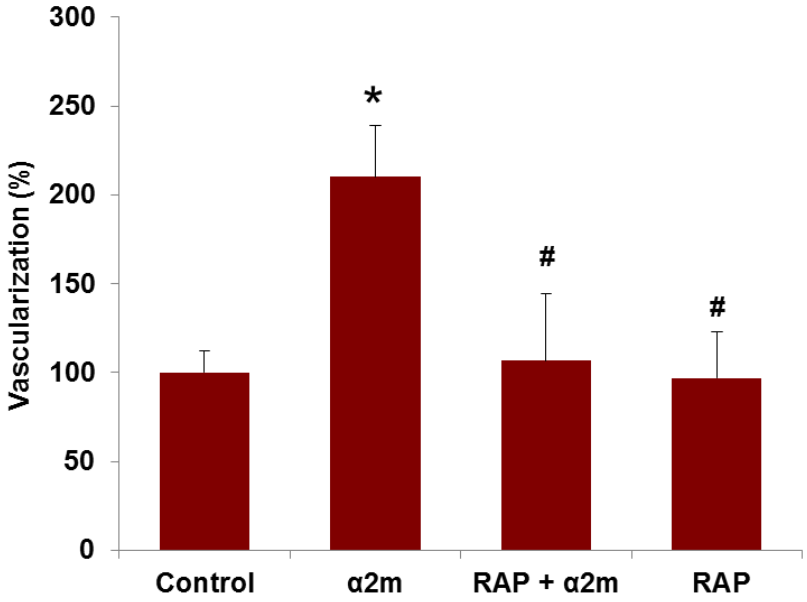
Figure 4.5: Upregulation of endothelial and mesodermal markers on α2m treatment of mEBs. A: Western blot analysis of VE-Cadherin, FLK1 and CD31 expression on increasing concentrations of α2m. **B:** Graphical representation of the relative protein expression of A, n=3 (VE-Cadherin), n=6 (FLK1), n=3 (CD31), *****: $P \leq 0.05$, significant as compared to untreated control **C:** Western blot analysis of Nkx2.5, Isl1, Brachyury and αSMA expression in mEBs treated with increasing concentrations of α2m. **D:** Graphical representation of the relative protein expression of A: n=3 (Nkx2.5), n=7 (Isl1), n=7 (Brachyury), n=8 (αSMA), *****: $P \leq 0.05$ significant as compared to untreated control.

4.6 LRP1 antagonist RAP abolishes α2m-induced vasculogenesis with down-regulation of growth factors

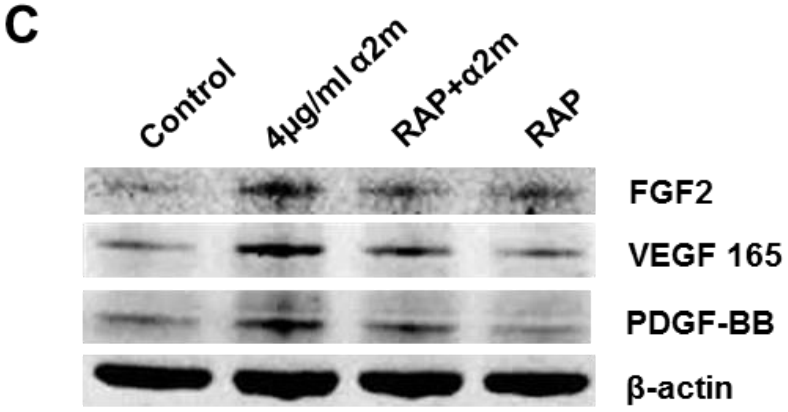
RAP, an LRP1 antagonist can inhibit binding of all ligands to LRP1. In order to further investigate the effects of blocking LRP1, EBs were treated with RAP from day 4 onwards for 6 days along with co treatment of α2m. This study validates the previous experiment with LRP1 antibody since blocking LRP1 with RAP treatment abolished α2m-induced vasculogenesis **(Figure 4.6 A,B)**. For further investigations, western blot analysis for angiogenic growth factors FGF2, VEGF 165 and PDGF-BB was performed. Our experiments revealed that their increase in expression upon α2m treatment was blunted upon blocking the LRP1 receptor with RAP, thereby proving that growth factor expression is regulated downstream of the LRP1 signaling pathway **(Figure 4.6 C,D)**.



B


300
250
200
150
100
50
0
Vascularization (%)
*
#
#
Control
α2m
RAP + α2m
RAP

C


Control
4μg/ml α2m
RAP+α2m
RAP
FGF2
VEGF 165
PDGF-BB
β-actin

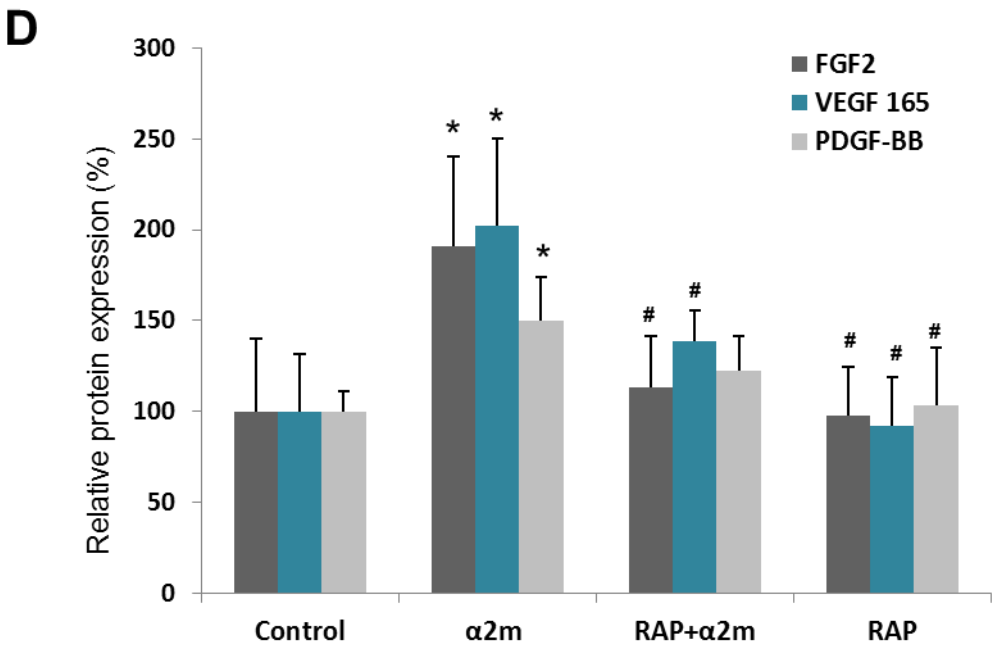
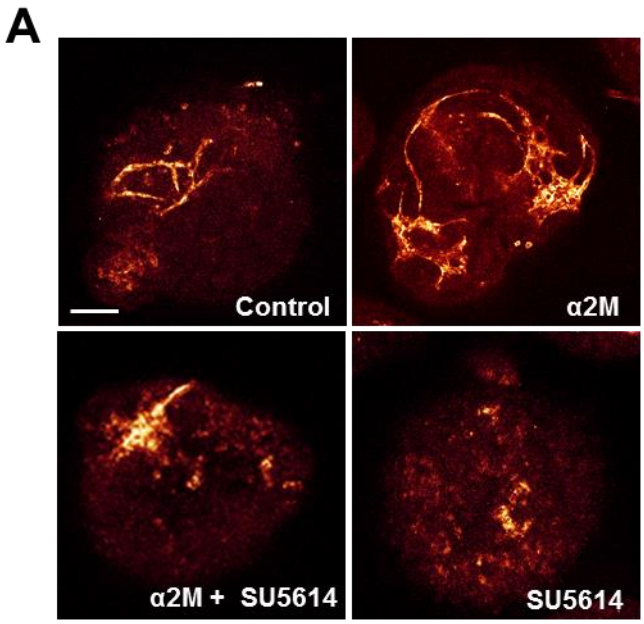
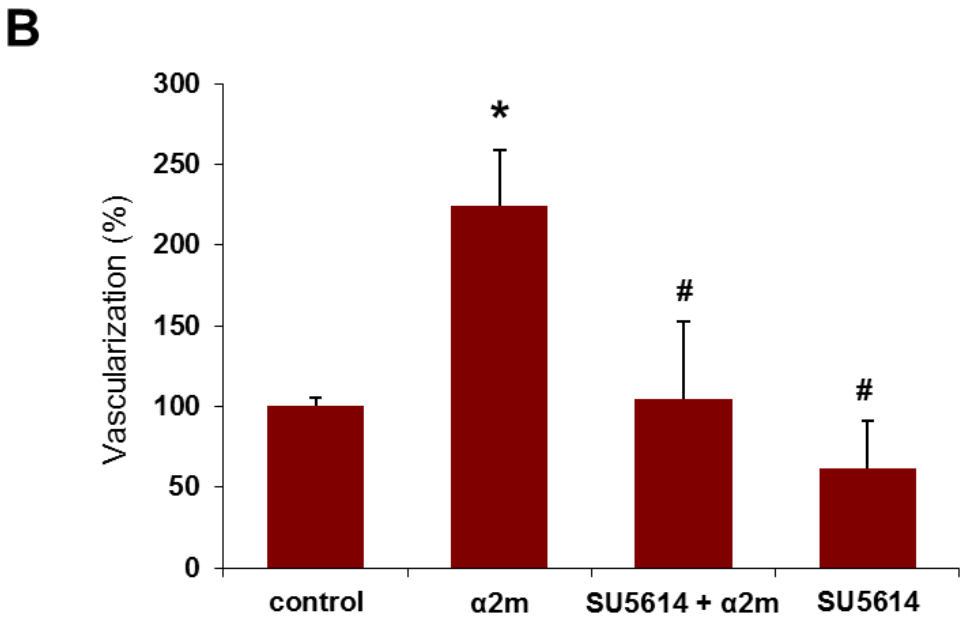


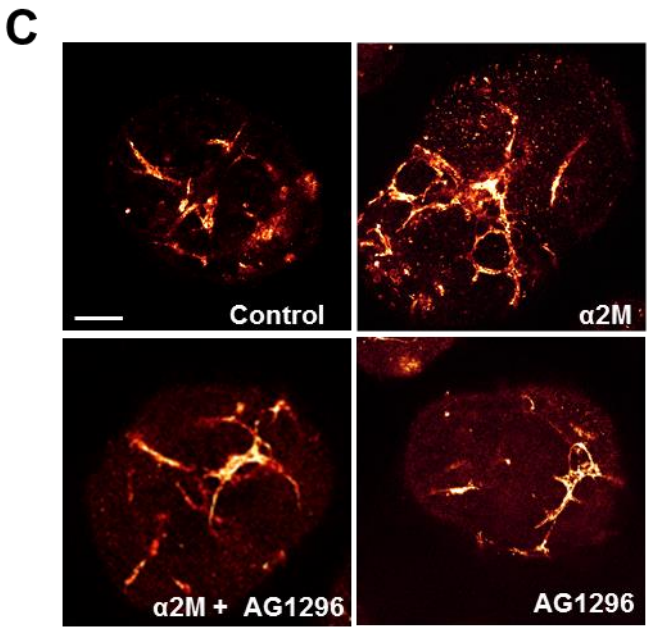
Figure 4.6: RAP abolishes α2m-induced vasculogenesis with down regulation of FGF2, VEGF165 and PDGF-BB. A: Confocal images of EBs stained against PECAM1 on treatment with RAP and co-treatment with α2m. The bar represents 100µm **B:** Quantification of A representing inhibition of vascularization upon RAP treatment. n=7, *: $P \leq 0.05$, $^{\#}$: significant compared to α2m treatment alone with $P \leq 0.05$ **C:** Western blot analysis of FGF2, VEGF165 and PDGF-BB expression in mEBs treated with RAP along and co-treatment with α2m **D:** Graphical representation of the relative protein expression of C. n=7 (FGF2), n=5 (VEGF165), n=7 (PDGF-BB), *: $P \leq 0.05$ as compared to the untreated control; #: $P \leq 0.05$, significant compared to α2m treatment alone.

4.7 Inhibitors of VEGFR2 and PDGFR abolish α2m-induced vasculogenesis

To investigate further the downstream signaling pathways of LRP1 activation, the growth factor receptors VEGFR2 and PDGFRβ were blocked with SU5614 and AG1296 respectively and assessed for vascularization by staining against PECAM1. EBs were treated with either 1.5µM SU5614 or 800nM AG1296 for 6 days along with co treatment of α2m from day 4 onwards. It was observed that blocking the receptors alone or with co treatment of α2m inhibited vasculogenesis suggesting that α2m is activating VEGFR2 as well as PDGF mediated signaling pathways **(Figure 4.7)**.


A
Control
α2M
α2M + SU5614
SU5614


B
Vascularization (%)
300
250
200
150
100
50
0
*
#
#
control
α2m
SU5614 + α2m
SU5614



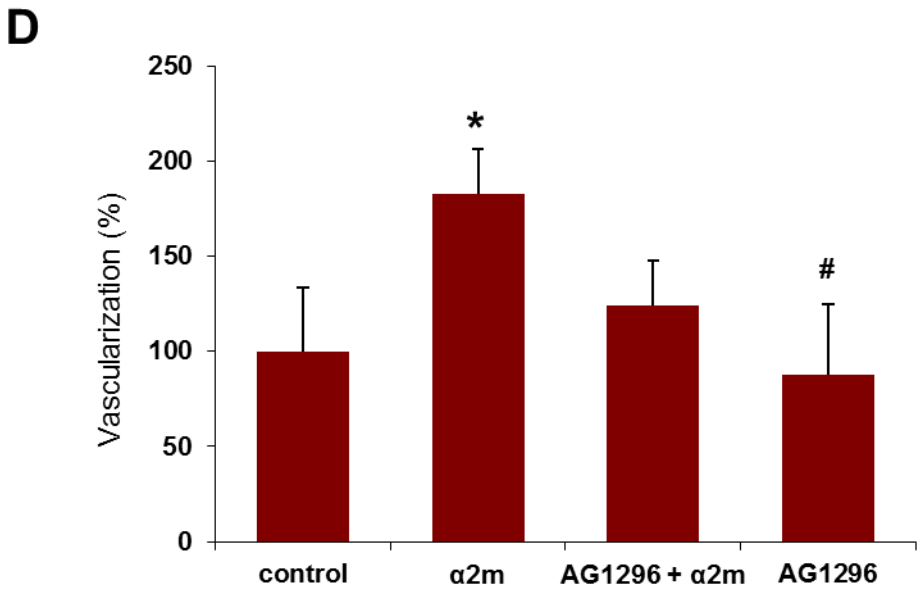


Figure 4.7: Inhibitors of VEGFR2 and PDGFR abolish α2m-induced vasculogenesis. A: Confocal images of EBs stained against PECAM1 on blocking VEGFR2 with 1.5µM SU5614 in either presence or absence of α2m. **B:** Quantification of A representing the vascularization inhibited with SU5614 (n=3), *: $P \leq 0.05$, significant compared to the untreated control; $^{\#}$: $P \leq 0.05$, significant compared to α2m treatment alone **C:** Confocal images of EBs stained against PECAM1 on blocking PDGFR (α and β) with 800nM AG1296 in either presence or absence of α2m. **D:** Quantification of C representing the vascularization inhibited with AG1296 (n=3), *: $P \leq 0.05$, significant compared to the untreated control; $^{\#}$: $P \leq 0.05$, significant compared to α2m treatment alone. The bar represents 100µm.

4.8 Inhibiting FGFR, VEGFR2 and PDGFR down-regulates α2m-mediated VE-Cadherin, VEGF165 and PDGF-BB expression

Blocking the receptors of VEGF165 and PDGF-BB blunted vasculogenesis inhibiting downstream angiogenic signaling cascades. The expression of the growth factors FGF2, VEGF165 and PDGF-BB and endothelial marker VE-Cadherin were investigated upon blocking the receptors for FGF2, VEGF165 and PDGF-BB with 15μM SU5402, 1.5μM SU5614 and 800nM AG1296, respectively. Upon treatment of 4-day-old EBs with these inhibitors alone and in combination with α2m for 6 days the expression of growth factors and VE-Cadherin was abolished as analysed using western blot technique **(Figure 4.8)**. It was also revealed that blocking VEGFR2 and PDGFRs, did not abolish FGF2 expression, but blocking FGF2 receptor decreased the expression of both VEGF165 and PDGF-BB suggesting the activation of VEGF and PDGF signaling to be down-stream of FGF signaling. Furthermore the angiogenic differentiation occurred only after the activation of growth factor signaling as revealed by expression analysis of VE-Cadherin.

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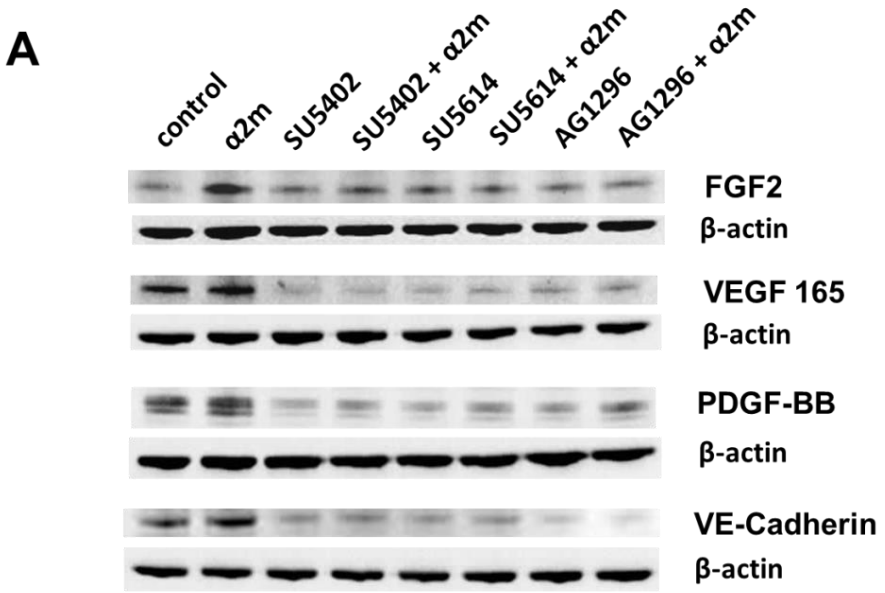


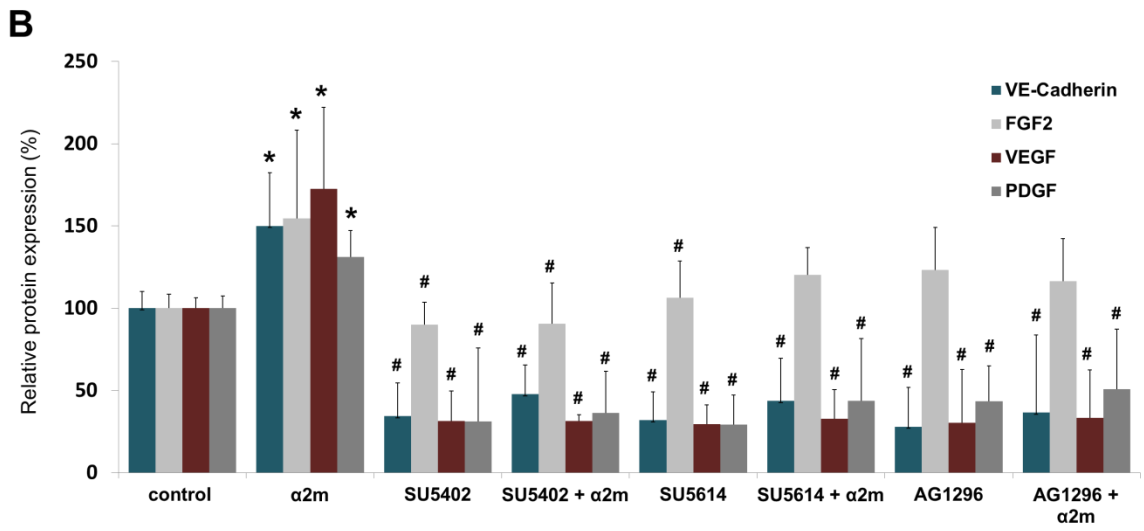
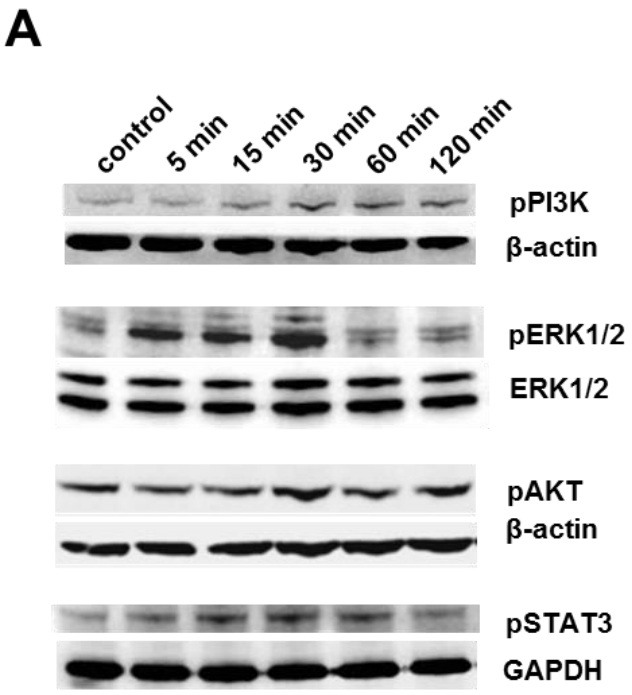
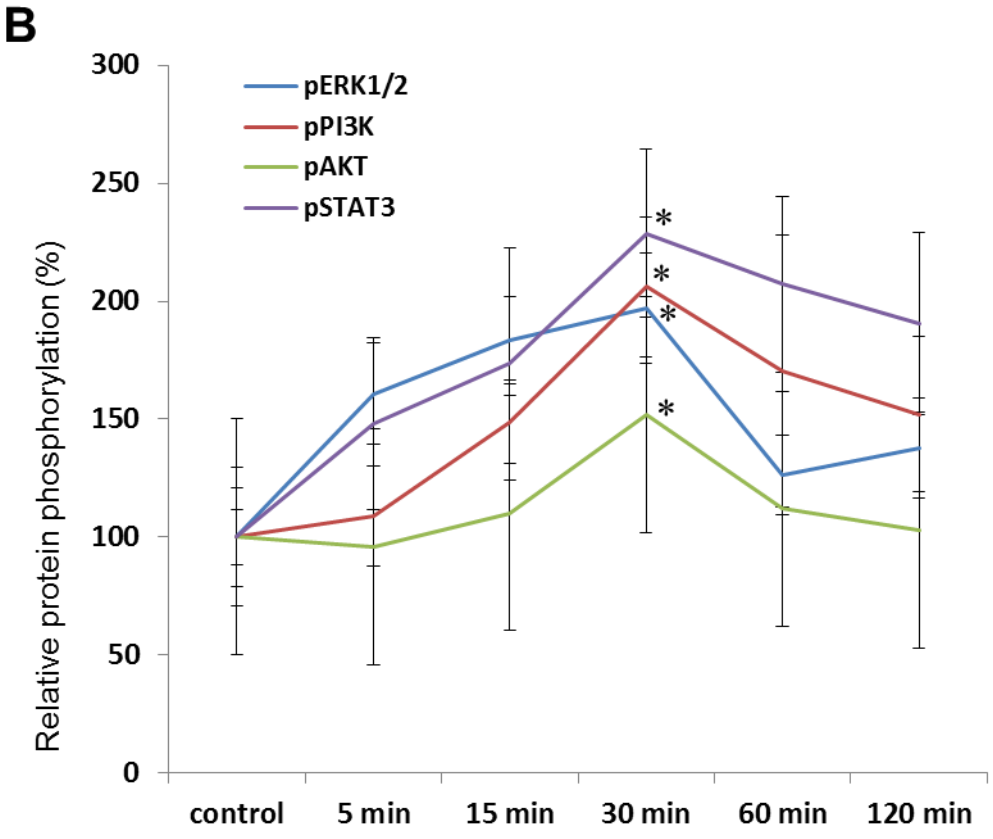


Figure 4.8: Blocking growth factor receptors down regulates expression of VE-Cadherin, FGF2, VEGF165 and PDGF-BB. A: western blot analysis of VE-Cadherin, FGF2, VEGF165 and PDGF-BB expression in mEBs treated with either 15µM SU5402 (FGF2R inhibitor), 1.5µM SU5614 (VEGFR2 inhibitor) and 800nM AG1296 (PDGFR inhibitor) alone or in combination with α2m. **B:** Graphical representation of the relative protein expression of A, n=6 (VE-Cadherin) n=7 (FGF2), n=6 (VEGF165), n=5 (PDGF-BB), *: $P \leq 0.05$, significant to the untreated control; $^{\#}$: $P \leq 0.05$ significant compared to α2m treatment alone.

4.9 Activation of ERK1/2, PI3K, AKT, AMPK and STAT3 upon treatment of mEBs with α2m

To further elucidate the signaling pathways involved in α2m-induced vasculogenesis, the immediate effects of α2m on phosphorylation of various signaling proteins were analysed. To achieve this aim 5-day-old EBs were treated with α2m and proteins were collected at intervals of 5, 15, 30, 60 and 120 min and analysed by western blot for protein phosphorylation. As shown in **Figure 4.9 A, B** administration of α2m activated ERK1/2, PI3K, AKT and STAT3 in EBs with maximum activation achieved after 30min. ERK1/2 functions in cellular proliferation, differentiation, and survival (Roskoski 2012). The phosphorylation of STAT3 indicates activation in transcription playing a role in cell proliferation, differentiation and migration (Teng et al., 2009). Activation of PI3K and its downstream activator AKT suggests involvement of α2m in the regulation of cell growth, survival, and proliferation. **Figure 4.9 C, D** illustrates that AMPKα was activated upon α2m indicating the need for energy in the cells to drive endothelial differentiation programs as AMPKα was reported to be activated under hypoxic conditions on endothelial cells promoting angiogenesis (Ouchi et al 2005). The downstream effector of AMPK, mTOR was observed to be inhibited.


A
control
5 min
15 min
30 min
60 min
120 min
pPI3K
β-actin
pERK1/2
ERK1/2
pAKT
β-actin
pSTAT3
GAPDH


B
Relative protein phosphorylation (%)
300
250
200
150
100
50
0
pERK1/2
pPI3K
pAKT
pSTAT3
*
*
*
*
control
5 min
15 min
30 min
60 min
120 min

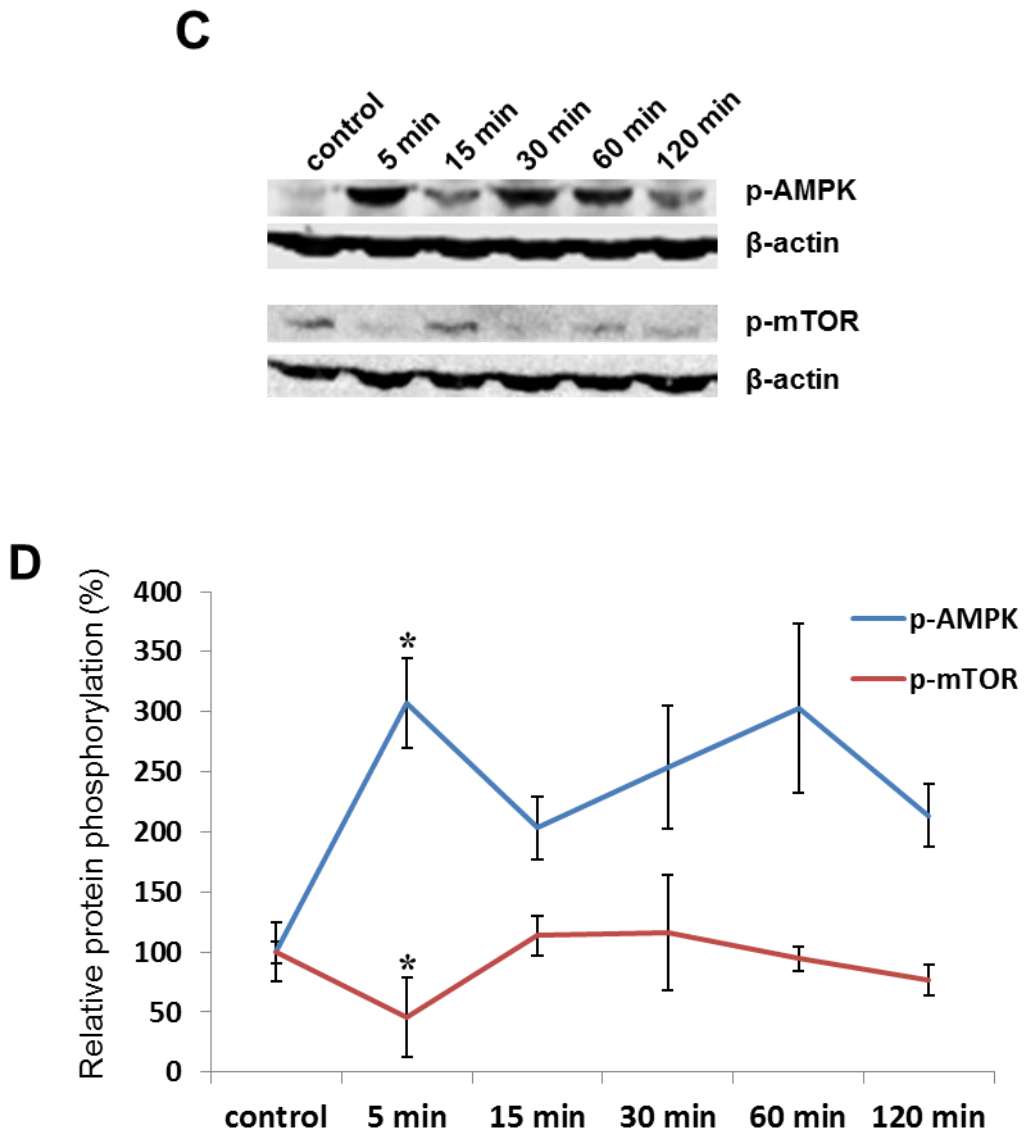


Figure 4.9: Protein phosphorylation upon α2m. A: Western blot analysis of phosphorylation of ERK1/2, PI3K, AKT and STAT3 **B:** Graphical representation of A p-PI3K (n=6), p-ERK1/2 (n=3), p-AKT (n=5), p-STAT3 (n=5), **C:** Western blot analysis of phosphorylation of AMPK and mTOR. **D:** Graphical representation of C with p-AMPK (n=5) and p-mTOR (n=5) *: $P \leq 0.05$, significant as compared to the untreated control.

4.10 Inhibition of signaling cascades blocks α2m-induced vasculogenesis

Administration of α2m on mEBs activated ERK1/2, PI3K, AKT, STAT3 and AMPK. To investigate further the downstream targets in the α2m-induced vasculogenesis, these phospho-proteins were blocked with their respective inhibitors and EBs were assessed for vascularization. The 4-day-old EBs were treated either with 50μM AG490 (JAK2

inhibitor), 235nM Compound C (AMPK inhibitor), 20µM PD98059 (ERK1/2 inhibitor), 2.5µM Wortmannin (PI3K inhibitor) alone or in combination with α2m and vascularization determined by staining against PECAM1. As represented in **Figure 4.10,** the treatment with inhibitors against the phospho-proteins attenuated endothelial differentiation leading to abolishment of vascularization, suggesting activation of phospho-proteins downstream of the LRP1 signaling cascade. This experiment also signifies the importance of ERK1/2, PI3K, STAT3 and AMPK in modulating and inducing differentiation programs within mEBs.

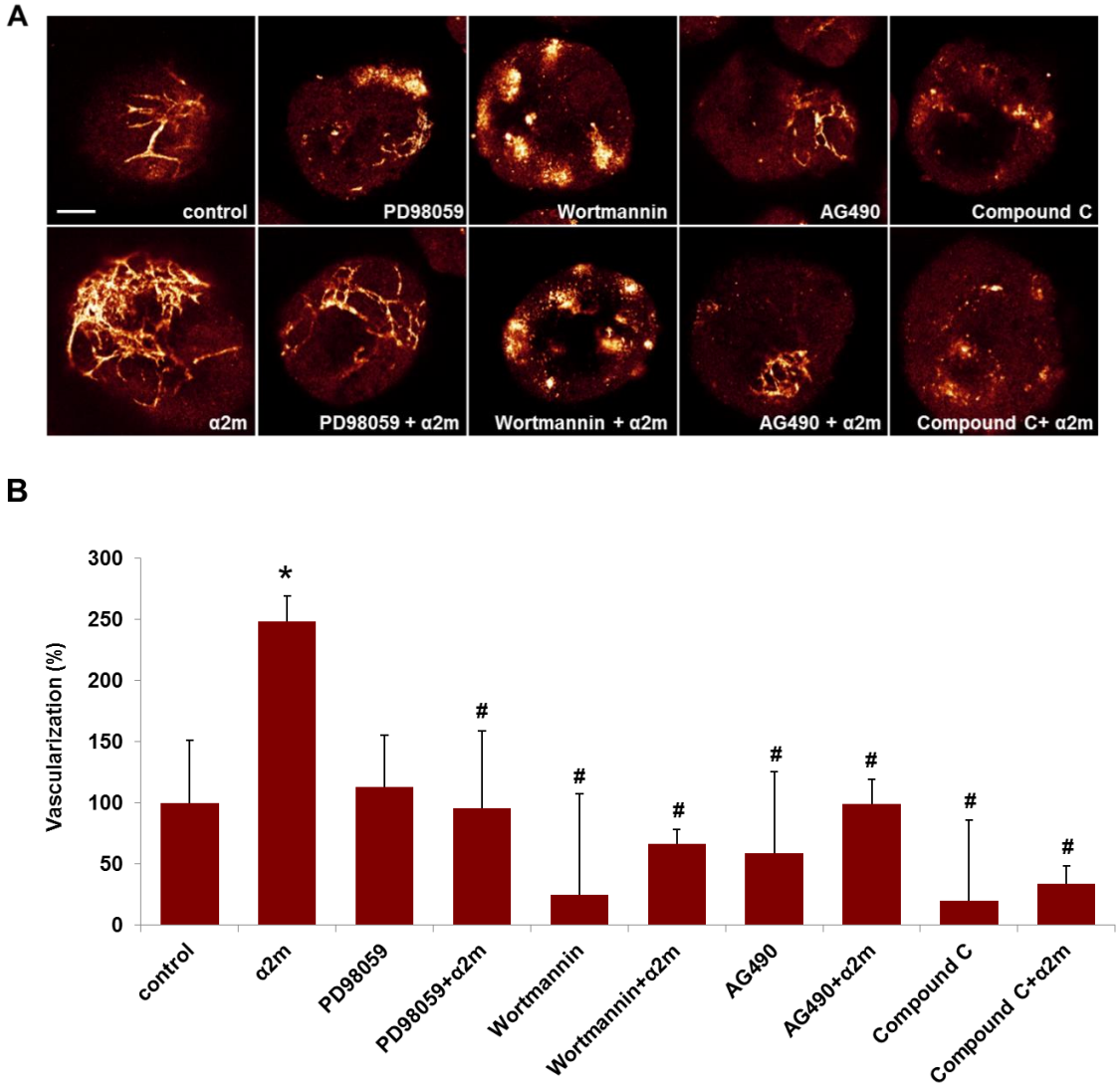


Figure 4.10: Inhibiting protein phosphorylation of signaling molecules blocks α2m-induced vasculogenesis. A: Confocal images of EBs stained against PECAM1 under AG490 (JAK2 inhibitor), Compound C (AMPK inhibitor), PD98089 (ERK1/2 inhibitor), Wortmannin (PI3K inhibitor) and cotreatment with α2m. The bar represents 100µm. **B:** Quantification of A representing abolishment of vascularization upon inhibition of JAK2, AMPK, ERK1/2 and PI3K protein phosphorylation with their respective inhibitors. n=3, *, $P \leq 0.05$, significant compared to the untreated control; $^{\#}$: $P \leq 0.05$, significant compared to α2m treatment alone.

4.11 Inhibiting growth factors blocks ERK1/2 phosphorylation

To understand the relation among upregulation of angiogenic growth factors and protein phosphorylation of signaling proteins in α2m-induced vasculogenesis, 5-day-old EBs were incubated either with 15µM SU5402 (FGF2R inhibitor), 1.5µM SU5614 (VEGFR2 inhibitor) and 800nM AG1296 (PDGFR inhibitor) alone or in combination with α2m for 60 min and protein extracted. As shown in **Figure 4.11** phosphorylation of ERK1/2 was inhibited upon blocking the angiogenic growth factor receptors, suggesting the signal transduction pathways induced by growth factors and pERK1/2 activation are interconnected and are modulated by each other.

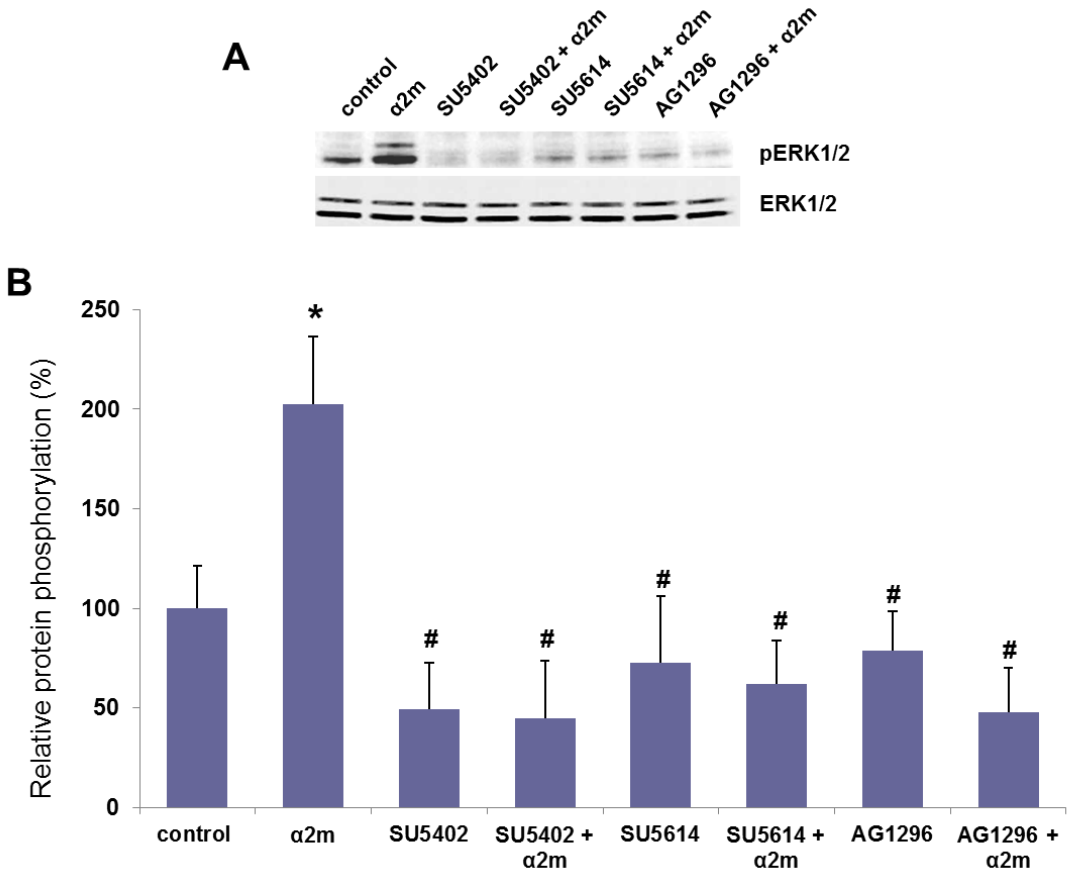


Figure 4.11: Blocking growth factor receptors inhibits ERK1/2 phosphorylation. A: western blot analysis of ERK1/2 phosphorylation in EBs treated either with SU5402, SU5614, AG1296 or with α2m. **B:** Graphical representation of the relative protein expression of A, n=4 *: $P \leq 0.05$, significant compared to the untreated control; #: $P \leq 0.05$, significant compared to α2m treatment alone.

4.12 Functional assay of α2m-induced vasculogenesis

Endothelial cell proliferation, migration and tube formation are central to angiogenesis. In this experiment sprouting assay was performed which aims to measure the outgrowth of endothelial cell structures from EBs. Here spheroids were derived from 5-day-old mEB and embedded onto fibrin matrix which was polymerized using thrombin. Subsequently sprout formation was assessed by light microscopy. As shown in **Figure 4.12**, spheroids treated with α2m produced longer sprouts and individual sprouts showed increased branching compared to the untreated control. Spheroids treated with 10ng/ml FGF2 served as positive control.

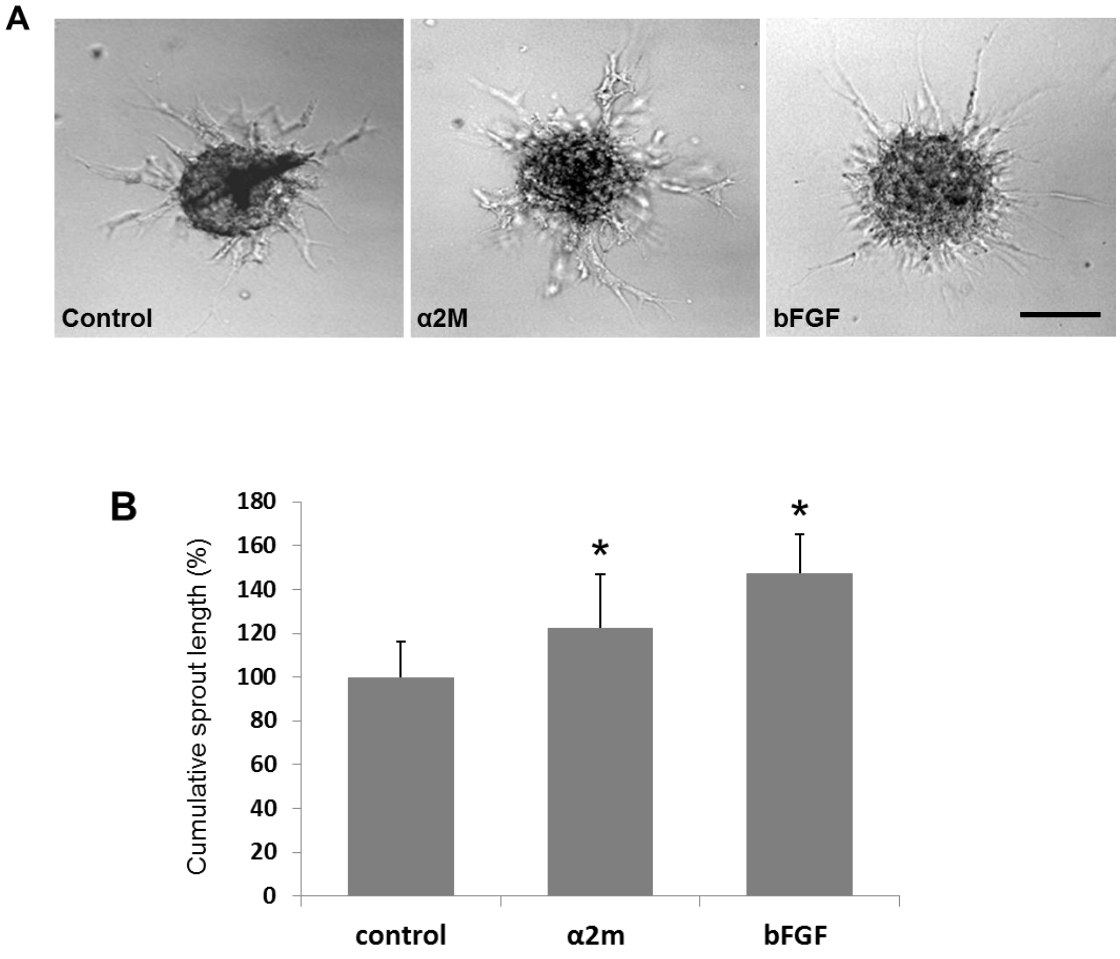
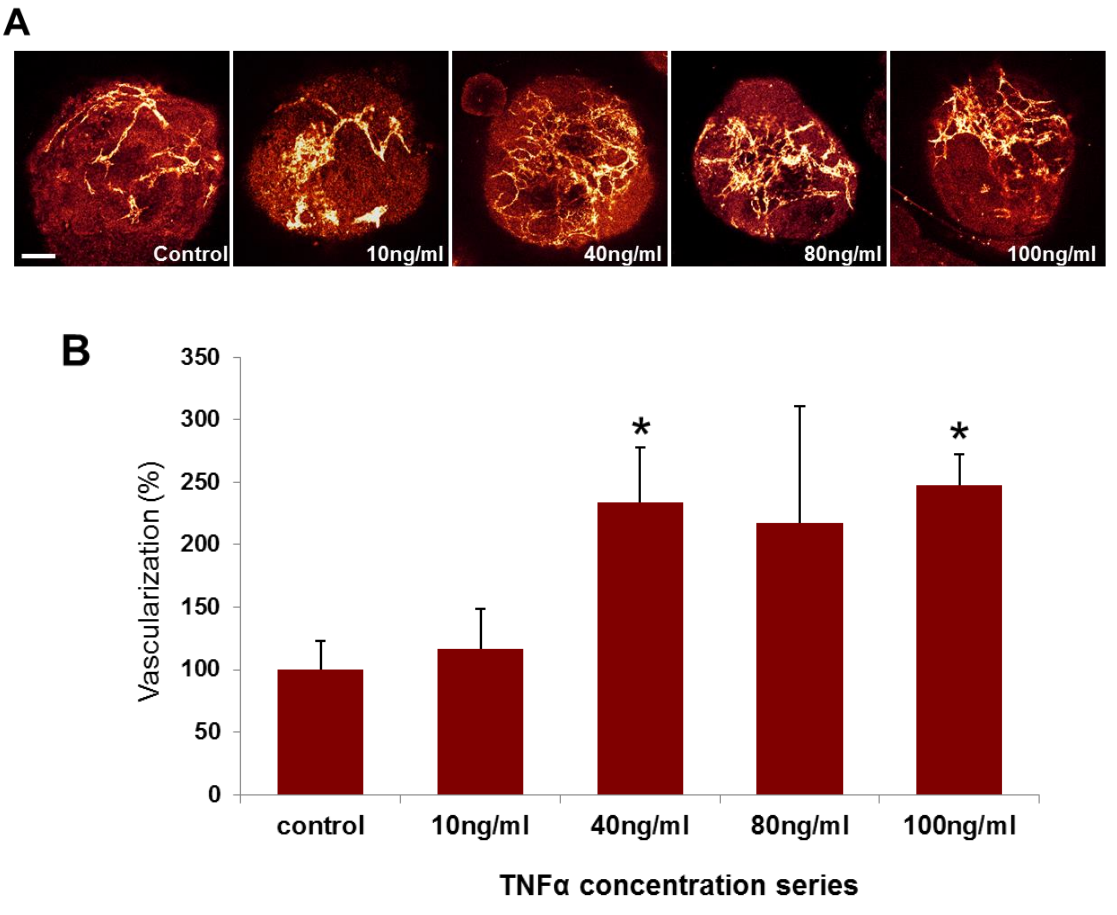


Figure 4.12: Sprout assay of MESC spheroids upon α2m treatment. A: Sprouting of mESC spheroids upon α2m and bFGF. The bar represents 100µm. **B:** Graphical representation of A with n=5 *: $P \leq 0.05$, significant compared to the untreated control.

4.13 Treatment of mEBs with TNFα induces vasculogenesis with upregulation of α2m and LRP1

TNFα is an inflammatory cytokine that can stimulate an acute phase reaction. Stimulating an acute phase reaction in mEBs theoretically should induce an upregulation of α2m as it is considered to be an acute phase protein. To investigate the regulation of α2m under inflammatory conditions in mEBs, 4-day-old mEBs were subjected to increasing concentrations of TNFα ranging from 10ng/ml to 100ng/ml for 6 days. Subsequently proteins were extracted and analysed for the expression of α2m and its receptor LRP1. As illustrated in **Figure 4.13 A, B** the protein expression of α2m along with its receptor LRP1 was increased 2-fold suggesting that α2m gets upregulated even in early developmental stages of a differentiating mEB under inflammatory conditions exerting its acute phase protein property. Besides triggering inflammatory signaling cascades, TNFα may be also implicated in angiogenesis. In order to study the angiogenic effects of TNFα the same samples were examined for vascularization by staining the EBs against PECAM1. As observed in **Figure 4.13 C, D**, TNFα increased vascularization in mEBs from 40ng/ml to 100ng/ml.



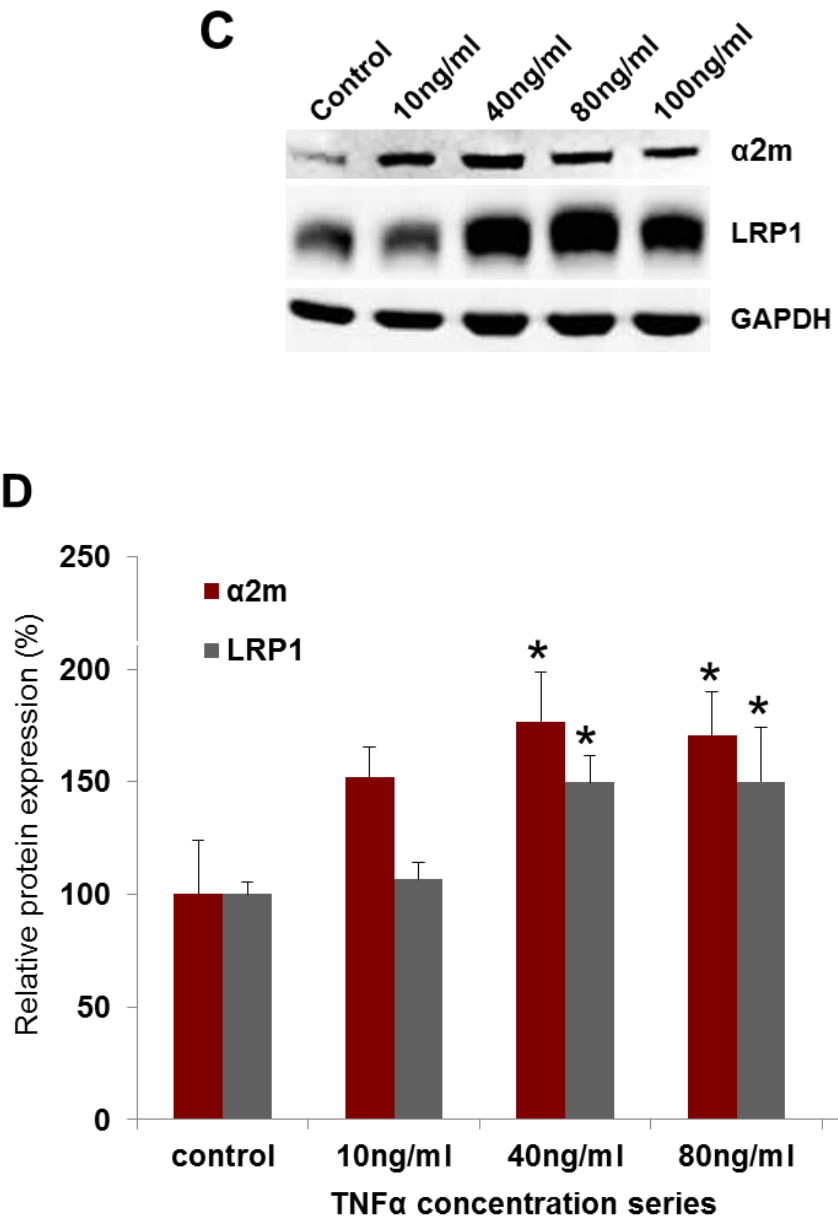


Figure 4.13: Treatment of mEBs with TNFα induces vasculogenesis with upregulation of α2m and LRP1 protein expression. A: Confocal images of EBs stained against PECAM1 upon treatment with TNFα. The bar represents 100µm **B:** Quantification of A representing the vascularization upon increasing concentrations of TNFα (n=4). *: $P \leq 0.05$, significant to the untreated control. **C:** Western blot analysis of α2m and LRP1 expression in mEBs treated with increasing concentrations of TNFα **D:** Graphical representation of the relative protein expression of C, α2m (n=3), LRP1 (n=3). *: $P \leq 0.05$, significant compared to the untreated control.

4.14 Antibody blocking against LRP1 receptor impairs TNFα-induced vasculogenesis

To determine if the receptor LRP1 is involved in TNFα-induced vasculogenesis 4-day-old mEBs were treated for 6 days with TNFα (40ng/ml), TNFα (40ng/ml) plus LRP1 antibody (0.2µg/ml) and LRP1 antibody (0.2µg/ml) alone and were assessed for vascularization. As illustrated in **Figure 4.14,** vascularization induced by TNFα in mEBs was abolished upon blocking the LRP1 receptor suggesting LRP1 to be involved in the modulation of TNFα-induced vascularization.

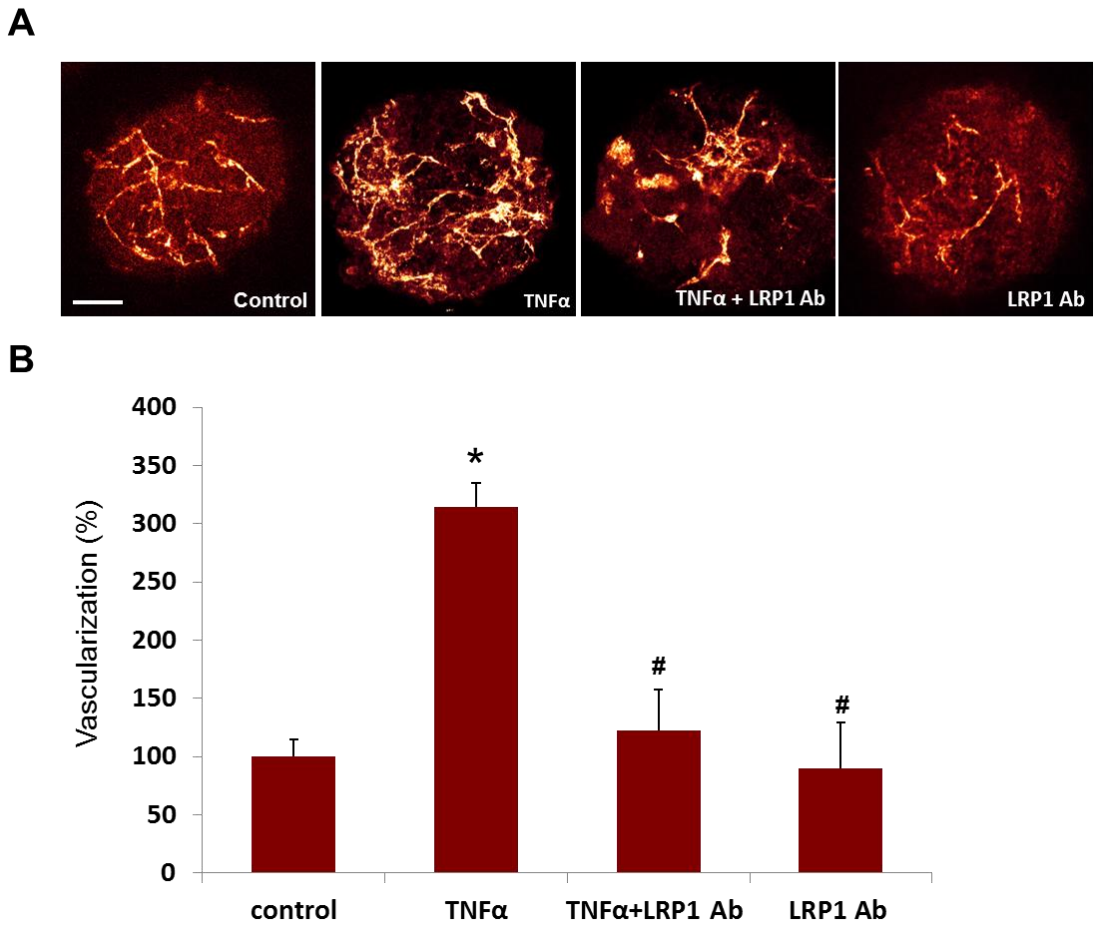


Figure 4.14: Antibody blocking against LRP1 receptor blocks TNFα-induced vasculogenesis A: Confocal images of EBs stained against PECAM1 upon treatment with TNFα and on blocking the receptor LRP1 with anti-LRP1 antibody upon co-treatment with TNFα. The bar represents 100µm **B:** Quantification of A representing the impairment of vascularization upon blocking LRP1 receptor (n=3). *: $P<0.001$, significant compared to the untreated control; $^{\#}$: $P<0.001$ significant compared to TNFα treatment alone.

4.15 α2m dose-dependently down-regulates endogenous expression of TNFα in differentiating mESCs

To investigate the regulatory mechanism of α2m on the endogenous expression of TNFα, 4-day-old mEBs treated for 6 days with increasing concentrations of α2m ranging from 0.5µg/ml to 6µg/ml were assessed for the endogenous protein expression of TNFα. The endogenous expression of TNFα was observed to be dose-dependently down regulated as illustrated in **Figure 4.15**, suggesting α2m may exert anti-inflammatory effects by clearing TNFα.

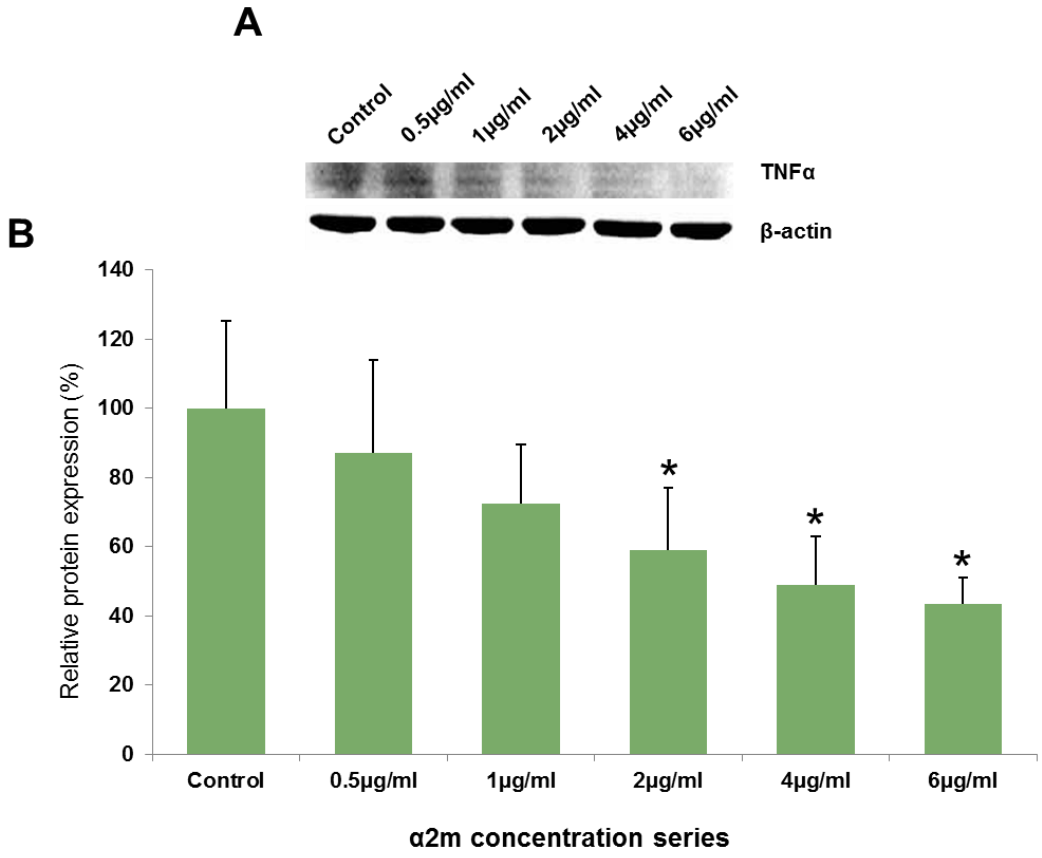


Figure 4.15: α2m down-regulates endogenous TNFα expression in mEBs A: Western blot analysis of TNFα expression in mEBs upon increasing concentrations of α2m **B:** Graphical representation of the relative protein expression of A, n=4 *: $P \leq 0.05$, significant as compared to the untreated control.

5. Discussion

The vascular system consists of the body's network of blood vessels that include arteries, veins and capillaries. Vascular diseases like atherosclerosis are caused due to obstruction in the blood vessels which lead to ischemia and consequently myocardial infarction. Under such conditions, replacement of the damaged blood vessel can restore blood flow and improve the diseased state. Vascular tissue engineering can address this challenge by regeneration of blood vessels, vascular tissue transplantation, stem cell technology etc. All these processes require generation of new blood vessels where angiogenesis is a critical step. Understanding vascular regeneration or repair mechanisms will enable the development of new strategies for innovative therapies.

Release of acute phase proteins is a defense mechanism as to how the body reacts in getting back to normal homeostasis after infection, tissue injury, inflammation, trauma or immunological disorders. At the site of the injury, local inflammatory cells release pro-inflammatory cytokines like interleukins: IL-1, IL-6 and IL-8 and TNF-α, this release in turn further activates the production of more cytokines and inflammatory mediators which diffuse into the extracellular fluid compartment and circulate in the blood (Gruys et al., 2005). One such acute phase protein is the plasma protein α2m which is released by the liver during inflammatory conditions and functions mainly in the clearance of proteases by binding to them and in their subsequent elimination. Besides protease clearance, α2m functions in transportation and clearance of cytokines, protection against toxic cytokines, protection of cytokines against degradation and targeting the cytokines to cells expressing LRP1 or GRP78 receptors for induction of various cell signaling pathways (Esadeg et al., 2003).

In adult tissues, a cardiac specific α2m has also been reported to be an early marker in cardiac hypertrophy in humans, but its physiological function is obscure (Annapoorani et al., 2006). Our previous study also reported α2m to induce hypertrophy in rat ventricular cardiomyocytes activating ERK1/2 and PI3K/Akt signaling cascades along with improvement in cardiac cell function (Padmasekar et al., 2007). Moreover, α2m was detected to be highly elevated in rats following induction of thermal injury (Kataranovski et al., 1999) and accumulation of α2m in wounds and skin burns has also been reported (Oehmichen et al., 1989, Grinnell et al., 1998), suggesting α2m could be involved in healing or repairing programs thereby restoring homeostasis. Interestingly, characterization of *in-vitro* cultured cord blood derived CD34+ cells differentiated into

early EPCs revealed upregulation of α2m suggesting its role in endothelial cell maturation (Ahrens et al., 2011).

5.1 Induction of vasculogenesis by α2m:

Vasculogenesis begins with the process of angioblasts forming a primitive vascular network through the action of FGFs. The assembly of this primordial vascular structure is then mediated by the action of VEGFs. The further transition from vasculogenesis to angiogenesis is the result of the activation of FGF and VEGF receptors (Wang et al. 2010). The cell adhesion molecules, VE-Cadherin and PECAM1 are further involved in the subsequent endothelial cell differentiation (Risau et al., 1995).

In this study, administration of native α2m to 3-day-old differentiating mEBs for 6 days stimulated endothelial differentiation programs leading to increased vascularization in the developing EB. The EB system enables investigation of vasculogenesis virtually as it occurs *in vivo*. EBs were stained against PECAM1 for quantification of vascularization and the increase in branching points was evaluated.

α2m is known to bind to two receptors LRP1 and GRP78. Homozygous LRP1 KO in mouse leads to mortality, likewise homozygous GRP78 KO die at peri-implantation site suggesting both these receptors are very critical during embryonic development (Herz et al., 1992, Luo et al., 2006). LRP1 is involved in various cellular processes that involves cell signaling, lipid homeostasis and anti-inflammation whereas GRP78 is highly expressed in tumor cells and is implicated in tumor cell proliferation, protection against apoptosis, and promotion of tumor angiogenesis (Dong et al 2008). In this study both LRP1 and GRP78 were found to be highly expressed in differentiating mEBs with their expression upregulated steadily until day 10 of differentiation. The expression pattern of LRP1 was distinct as it was down-regulated and re-expressed at day-2 of differentiation where vasculogenesis/angiogenesis begins to mEBs suggesting its active role in this process.

In contrast, the expression of intrinsic α2m was observed to be down-regulated throughout differentiation. The treatment with α2m could be responsible for the additional activation of the receptors LRP1 and GRP78 which resulted in the enhancement of vascularization. The expression of LRP1 and GRP78 was also dose-dependently upregulated upon α2m treatment. Moreover, blocking the receptors LRP1 and GRP78 with their respective antibodies abolished vascularization which suggests that these receptors are crucial for the development of the EB. Additionally, the

treatment with RAP, an LRP1 antagonist also provided identical results in the abolishment of vascularization induced by α2m. Thus our data suggested that the effects of α2m on vascularization of EBs followed signaling pathways in sequence of LRP1 and GRP78 receptor activation.

α2m stimulated a dose-dependent increase in vasculogenesis accompanied by a similar dose-dependent upregulation in protein expression of angiogenic growth factors, i.e. FGF2, VEGF165 and PDGF-BB as well as endothelial markers, i.e. PECAM1, VE-Cadherin and FLK1.

A strong upregulation in FGF2 was observed upon α2m treatment which suggests a pronounced induction of angioblasts from the mesodermal cells in the differentiating EB. The isoform of VEGF-A, VEGF165 was also equally upregulated confirming activation of endothelial differentiation pathways. VEGF165 binding to its receptor VEGFR2/FLK1 results in receptor kinase activity, auto-phosphorylation and activates its downstream signaling cascades. VEGFR2 plays a major role in EC differentiation, migration, proliferation and survival (Patel-Hett et al., 2011). PDGF is involved in vessel maturation by recruiting pericytes. PDGF-BB is secreted by proliferating ECs and undifferentiated mesenchymal cells expressing its receptor PDGFRβ are recruited to form pericytes and SMCs (Wijk et al., 2013). In this study PDGF-BB was upregulated upon α2m further confirming the activity of angiogenic growth factors in α2m-induced vasculogenesis.

FLK1 expressing cells can differentiate into both endothelial and mural cells. The VEGF stimulates endothelial cell differentiation and PDGF-BB stimulates mural cells which are pericytes and smooth muscle cells. Further the activation of PDGFRβ results in the growth and migration of these pericytes, which along with ECs form vessel-like three-dimensional vascular network (Yamashita et al., 2000). In this study FLK1 expression was strongly upregulated which suggests that α2m stimulated vascularization through the differentiation of FLK1 expressing cells. FLK1 is highly expressed in tip cells and stalk cells that are responsible in branching of vessels (Gerhardt et al., 2003). The protein expression of mesodermal lineage markers Nkx2.5, Isl1 and Brachyury was also upregulated which indicates that α2m initiated differentiation programs for endothelial progenitor cells rather than the proliferation of mature endothelial cell. Surprisingly the expression of αSMA, the major component of SMCs was down-regulated suggesting the tunica media of the blood vessels specifically arterial vessels lacks SMCs which could lead to loss of contractility of the vessels (Hinz et al., 2001).

The expression of the angiogenic growth factors was abolished when treated with RAP suggesting LRP1 receptor activation in the induction of FGF2, VEGF165 and PDGF-BB. The involvement of the GRP78 receptor in the stimulation of these growth factors still needs to be investigated. Blocking the receptors of VEGF165 and PDGF-BB with SU5614 and AG1296 respectively, abolished vascularization in mEBs, signifying the importance of these growth factors in the endogenous vascularization process. Blocking FGF2 receptor with SU5402 also abolished vascularization which was shown in our previous study (Sauer et al., 2013). Additionally, blocking FGF2 receptor with SU5402 down-regulated expression of VEGF165 and PDGF-BB suggesting, FGF2-signaling to be upstream of VEGF and PDGF-signaling pathways in the induction of vasculogenesis. Thus, α2m stimulated LRP1 and GRP78 in the upregulation of angiogenic growth factors that led to increase in the vascularization.

PI3K/AKT pathway is involved in a number of cellular functions like proliferation, adhesion, migration, metabolism and survival (Bader et al., 2005). The inactivation of the p110α subunit of PI3K led to embryonic lethality with severe defects in angiogenic sprouting and vascular remodeling which implies that PI3K pathway plays a key role in the normal development of blood vessels and moreover PI3K also regulates EC migration (Graupera et al., 2008). In this study administration of α2m on mEBs stimulated this pathway as PI3K/AKT was observed to be activated.

Previous studies have demonstrated that ERK1/2 is involved in numerous signaling pathways that regulate survival, proliferation, migration, metabolism, differentiation and transcription; furthermore it has been shown that vasculogenesis of ES cells can be efficiently inhibited by ERK1/2 antagonists (Na et al., 2010). ERK1/2 was shown to be activated upon α2m. This activation in ERK1/2 was strongly abolished in presence of SU5402 (FGF2R inhibitor), SU5614 (VEGFR2 inhibitor) and AG1296 (PDGFR inhibitor) which suggest this signaling to be interconnected with growth factor activation in the modulation of vasculogenesis which needs to be further elucidated.

α2m activated STAT3, a pathway involved in gene transcription in response to cytokines and growth factor stimulation. It is known to play an important role at the transcriptional level in angiogenesis besides cell survival, proliferation, differentiation, and onco-genesis (Chen et al., 2008). Studies have shown that VEGF upregulation is a result of STAT3 activation with STAT3 binding to the promoter region of VEGF resulting in its transcription (Niu et al., 2002).

The metabolite-sensing protein kinase, AMPK was observed to be activated upon α2m administration indicating the need for energy requirement to drive differentiation programs. AMPK gets activated upon sensing a decrease in ATP levels where it drives β-oxidation of fatty acid promoting ATP production. AMPK has been implicated as a regulator in angiogenesis required for migration and differentiation under hypoxic condition (Nagata et. al 2003).

The downstream target of AMPK is mTOR which is a central controller of cell growth and proliferation by regulating protein synthesis and transcription (Hay et al., 2004). Upon sensing ATP deprivation, AMPK gets activated to phosphorylate effectors that inhibit mTOR activity (Hay et al., 2004). In this study a similar effect has been shown where upon activation of AMPK, an opposite inactivation of mTOR is observed upon α2m treatment. This suggests that mTOR may repress autophagy in order to sustain protein synthesis and energy production.

Treating mEBs with PD98089 (ERK1/2 inhibitor), Wortmannin (PI3K inhibitor), AG490 (JAK2 inhibitor) and Compound C (AMPK inhibitor) alone completely abolished vascularization. In combination with α2m these inhibitors significantly reduced the stimulation of vascularization observed upon treatment with α2m alone. This study proves that these signaling cascades are also intrinsically involved in vasculogenesis pathways.

eNOS plays a critical role in angiogenesis and vascular permeability through VEGF mediated stimulation (Fukumura et al., 2001). In our previous study we have reported activation of eNOS along with NO generation upon α2m treatment in mEBs in stimulating vascularization (Sauer et al., 2013). These findings further prove the angiogenic potential of α2m in the differentiating mEBs.

An *in-vitro* sprout formation assay was performed to test the efficacy of α2m to mediate vascular outgrowth. Aggregates of single celled mESCs were made to form spheroids by hanging drop method. These mESC-spheroids embedded onto fibrin matrix and treated with α2m exhibited increased sprout length compared to the untreated control sample. Pronounced vascular outgrowth was observed in mESC-spheroids treated with FGF2 alone which served as positive control. Interestingly, in the mESC-spheroids treated with α2m, individual vascular sprouts were branched which complements the α2m-induced vascularization in mEB and suggests that α2m could be specifically activating the tip and stalk cells. This study thereby confirms the angiogenic role of α2m in inducing vasculogenesis playing role in migration, proliferation and tube formation.

Taken together α2m activates various signaling cascades within the mEB by directing differentiation towards the endothelial lineage acting through LRP1 and GRP78 signaling pathways. **Figure 5.1** illustrates the activity of α2m in the enhancement of vascularization in differentiating mEB.

The physiological function of the acute phase protein α2m is still very obscure. It is known that its plasma concentration increases upon various challenges occurring in adult organisms and that this property can be utilized as a possible biomarker for different diseases. In our study, α2m is shown to play a role in endothelial differentiation in early developmental stages of mouse. This angiogenic potential of α2m could be utilized for therapeutic purposes involving stem cell technologies in vascular injuries.

The exact mechanism as to how native α2m gets activated and induces vasculogenesis of mESCs is yet to be determined. It might be hypothesized that the administered native α2m gets activated by endogenous proteases or nucleophiles that are produced by the differentiating mEBs. The activated α2m could then bind to LRP1 and GRP78 mediating stimulation of vascular differentiation pathways. Further studies have to be performed to investigate the underlying mechanism for this activation of native α2m, about the oxidation state of α2m and the growth factors that could be bound to α2m in triggering this differentiation. It is clear that α2m binds to both the receptors GRP78 and LRP1 but the further downstream signaling targets of GRP78 and if any interaction among GRP78 and LRP1 also needs to be investigated.

5.2 Interaction of α2m and TNFα in induction of vasculogenesis:

TNFα was identified due to its anti-angiogenic property but it was also reported to exert pro-angiogenic activity since injection of TNFα in rabbit corneas induced neovascularization (Fràter-Schröder et al., 1987, Rosenbaum et al., 1987). Moreover subcutaneous implantation of a pellet containing TNFα stimulated angiogenesis in mice (Fajardo et al., 1992). The angiogenic activity of TNFα could be attributed to its activation of NFκB and MAPK signaling cascades with stimulation of angiogenic growth factors. In response to TNFα vascular ECs have shown to initiate pro-inflammatory pathways leading to leukocyte adhesion, trans-endothelial migration and vascular leak promoting thrombosis (Bradley 2008).

Various studies have reported the interaction of eNOS and TNFα. TNFα induced cell death was prevented by endogenous production of NO upon activation of eNOS in HeLa cells (Bulotta et al., 2001, Barsacchi et al., 2003). In another study, in human ECs

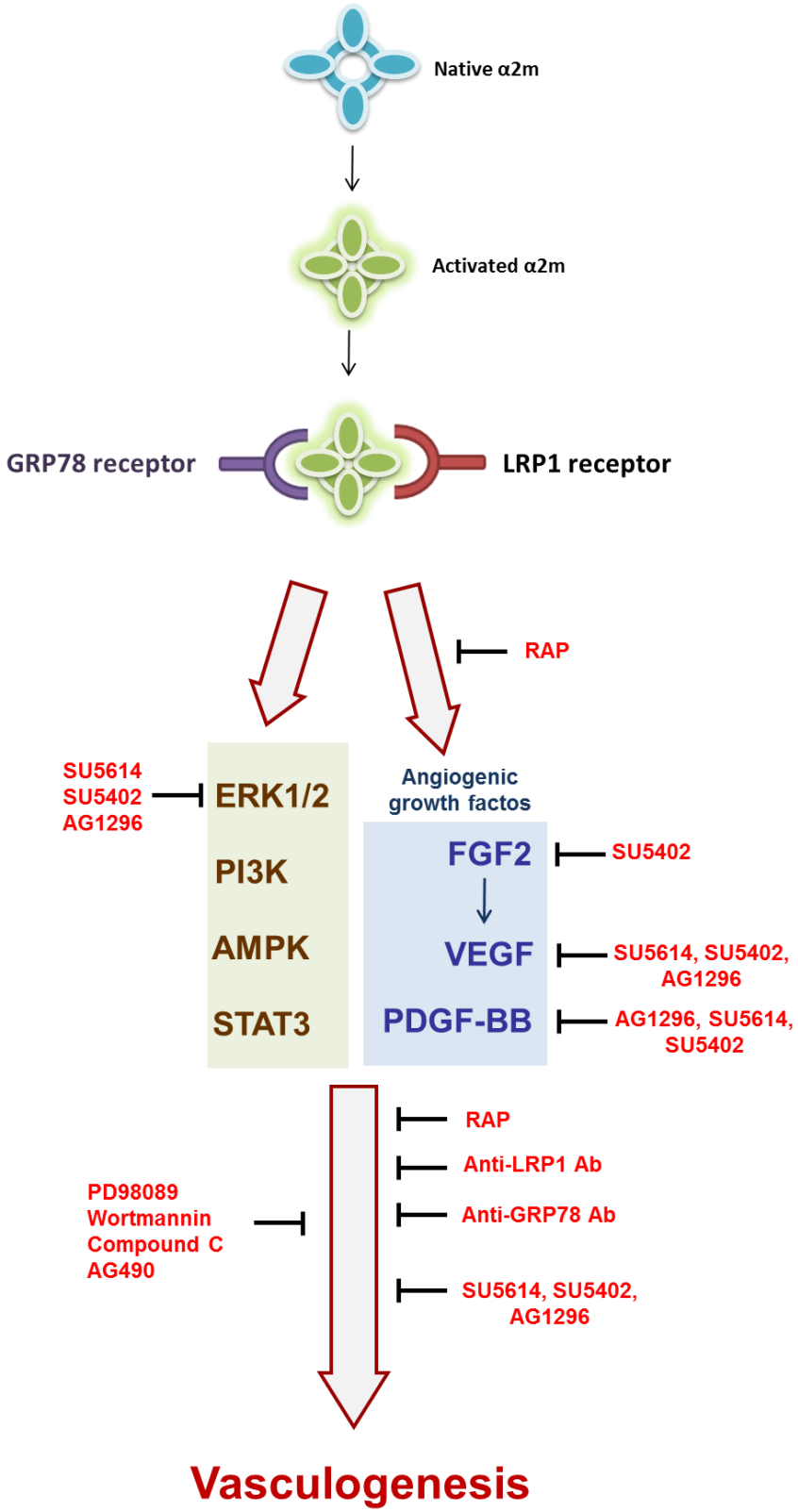


Figure 5.1: Schematic representation of α2m triggering vasculogenesis in mEBs

TNFα activated eNOS and produced NO through the AKT pathway which resulted in the inhibition of adhesion molecules to the endothelium. eNOS KO mouse, under inflammatory stimuli, displayed poor wound healing with high susceptibility to inflammation. These results suggest a significant anti-inflammatory role of eNOS in TNFα activity (De Palma et al., 2006).

We hypothesized, since α2m is an acute phase protein, that induction of inflammation in mEB could stimulate its expression. Inflammation in mEB could be stimulated upon treatment with TNFα. To test this hypothesis, mEBs treated with varying concentrations of TNFα resulted in strong increase of α2m protein expression along with its receptor LRP1. Surprisingly the EBs did not undergo apoptosis under the effect of TNFα but survived. It could be hypothesized that TNFα triggered pro-inflammatory cues which resulted in the up-regulation of α2m. This study also signifies the acute phase property and protective role of α2m even in early developmental stages of mouse embryo.

To understand the effects of this endogenous production of α2m, the mEBs treated with varying concentrations of TNFα were analysed for vascularization. Interestingly, TNFα concentration of 40ng/ml and above displayed increased vascularization in mEBs. This effect could be a result of TNFα stimulating endothelial differentiation pathways through the TNFα receptors or through the α2m-LRP1 stimulatory pathway since endogenous α2m was strongly upregulated. But another study has reported that mESCs and early vascular cells have relatively low expression of TNF receptors and its expression increases during differentiation (Zampetaki et al., 2007). This could be one reason why TNFα does not induce apoptosis upon its treatment in mEBs but rather plays a role in differentiation. To test if LRP1 receptor plays a role in this TNFα mediated vascularization, mEBs were treated with TNFα co-treated with anti-LRP1 antibody, TNFα alone and anti-LRP1 antibody alone. This resulted in the abolishment of TNFα induced vascularization suggesting TNFα may act via the LRP1 receptor.

TNFα was reported to strongly bind non-covalently to both native and activated forms of α2m (Wollenberg et al., 1991). It was also reported that activated form of α2m injected onto LPS-treated mice prevented expression of iNOS in liver, kidneys and heart leading to their survival compared to untreated controls suggesting therapeutic potential of α2m in reversing LPS toxicity induced by TNFα (Webb et al., 1998). To understand this we investigated the expression of TNFα in mEBs treated with increasing concentrations of α2m. This resulted in dose-dependent down-regulation of the endogenous expression of TNFα, suggesting that α2m may bind to TNFα and mediates its degradation or gets activated upon binding to TNFα and triggers the LRP1

signaling cascade to induce vasculogenesis. This study also signifies the presence of endogenous TNFα in differentiating EBs playing a role in early differentiation pathways.

In summary, the acute phase protein α2m which also was earlier reported to be expressed during endothelial differentiation in adult tissues (Sayegh et al., 1997), indeed plays a pro-angiogenic role in the developing mEB. α2m treatment in mEBs resulted in dose-dependent increase in vascularization through LRP1 and GRP78 activity as well as dose-dependent decrease in endogenous TNFα expression. These results suggest that α2m exerts angiogenic properties in mEBs as well as presumably exerts an anti-inflammatory role with TNFα down-regulation.

Stem cell therapy has long been implicated for vascular regeneration due to the vascular differentiation capacity of stem cells. This pro-angiogenic potency of α2m could be utilized for clinical therapies to regenerate blood vessels in ischemic vascular injury diseases.

Inflammation is necessary for normal wound healing, but persistent and chronic inflammation leads to non-healing wounds. Treatments aimed at immune suppression under such conditions can improve healing process. The anti-inflammatory role of α2m along with its angiogenic property may be a potential strategy for treatment of non-healing wounds. α2m could reduce inflammation induced by TNFα which would aid in natural healing processes as well as in the generation of new blood vessels to patch up the wound in a regenerative way. Whether α2m could be injected directly onto an injured wound is still questionable because the effects of α2m on terminally differentiated cells still needs to be investigated. Our study demonstrates the angiogenic capacity of α2m in early ESCs. Hence we hypothesize that stem cell treatment with addition of α2m could contribute to better blood vessel regeneration along with lowering the adverse effects of inflammation.

6. Summary

α2m the largest non-immunoglobulin protein present in blood plasma of mammals is an acute phase protein with its serum level elevated upon tissue damage and inflammation. In this study α2m administration in mEBs during early differentiation processes resulted in an enhancement of vascularization as assessed with PECAM1 and VE-Cadherin immunofluorescence studies. α2m stimulated a dose-dependent increase in protein expression of the endothelial specific markers PECAM1, FLK1 and VE-Cadherin along with up-regulation of the angiogenic growth factors FGF2, VEGF-165 and PDGF-BB. α2m modulates it activity upon binding to and activating its receptors LRP1 and GRP78. Upon blocking with specific antibodies α2m mediated vascularization was abolished. Moreover the LRP1 antagonist RAP and inhibitors of the angiogenic growth factors blunted the expression of the growth factors FGF2, VEGF-165 and PDGF-BB along with abolishment of vascularization, suggesting the role of LRP1 receptor in triggering endothelial differentiation signaling. α2m treatment also resulted in the activation of ERK1/2, PI3K, AKT and STAT3 which are all implicated in angiogenic signaling cascades. Their subsequent blocking with their respective inhibitors resulted in the abolishment of vascularization. Investigating the functional characteristic of α2m under systematic *in-vitro* analysis of sprout formation revealed that α2m stimulates endothelial cell proliferation and migration inducing vessel formation with longer and branched sprouts.

Induction of inflammation in mEBs upon treatment with the inflammatory cytokine TNFα stimulated upregulation of α2m and its receptor LRP1 along with increase in vascularization. The vascularization induced by TNFα was abolished upon antibody blocking of the receptor LRP1 suggesting its activation in triggering increased vascularization. In contrast α2m treatment resulted in a dose-dependent decrease in the expression of intrinsic TNFα along with increase in vascularization, suggesting α2m mediates TNFα removal by activating LRP1 signaling which could also be responsible for enhanced vascularization in mEBs.

In summary this study reports that α2m stimulates vasculogenesis in mESCs with upregulation of growth factors and activation of angiogenic signaling pathways. Additionally α2m exerts anti-inflammatory effects in mESCs through inhibition of TNFα expression.

7. Summary (German)

α2M, das größte nicht-immunglobuläre Protein im Blutplasma von Säugetieren, ist ein Akutphase Protein, dessen Serumwerte im Zuge von Gewebsverletzungen und Entzündungen ansteigen. In dieser Studie konnte gezeigt werden, dass die Gabe von α2M in der frühen Phase der Differenzierung von mEBs eine verstärkte Vaskularisierung zur Folge hatte, was durch Immunfluoreszenz-Untersuchungen für PECAM1 und VE-Cadherin nachgewiesen werden konnte. Dabei ist die Stimulierung der Expression der endothelspezifischen Marker PECAM1, FLK1 und VE-Cadherin sowie die Expression der pro-angiogenen Wachstumsfaktoren FGF2, VEGF165 und PDGF-BB durch α2M dosisabhängig. Mittels Aktivierung und Bindung seiner Rezeptoren LRP1 und GRP78 wird die Aktivität von α2M moduliert. Dabei hat die Blockade der Rezeptoren durch spezifische Antikörper zur Folge, dass der zuvor beobachtete Effekt der Vaskularisierung aufgehoben wird. Eine Behandlung mit dem LRP Antagonisten RAP und Inhibitoren der pro-angiogenen Wachstumsfaktoren zeigte, dass die Expression von FGF2, VEGF165 und PDGF-BB verringert und die Vaskularisierung aufgehoben werden konnte. Dies lässt den Schluss zu, dass der Rezeptor LRP1 eine steuernde Wirkung im Verlauf der Signalübertragung für die endotheliale Differenzierung besitzt. Außerdem zeigte die Behandlung mit α2M eine Aktivierung der an der angiogenen Signalkaskade beteiligten Proteine ERK1/2, PI3K, AKT und STAT3. Eine Blockade dieser Proteine mit den jeweiligen Inhibitoren hatte ebenfalls zur Folge, dass der beobachtete Effekt der Vaskularisierung aufgehoben werden konnte. Eine in-vitro Untersuchung des funktionellen Charakters von α2M durch einen „sprout-formation assay“ zeigte, dass α2M die Proliferation und Migration endothelialer Zellen stimuliert und so die Bildung von langen und verzweigen Gefäßen fördert.

Eine durch die Behandlung mit TNFα induzierte Entzündungsreaktion in den mEBs hatte eine Stimulierung der α2M Bildung und einen Anstieg der LRP1 Expression sowie eine Zunahme der Vaskularisierung zur Folge. Die TNFα induzierte Vaskularisierung konnte durch eine Antikörper vermittelte Blockierung des LRP1 Rezeptors aufgehoben werden . Dagegen zeigten sich durch die Behandlung mit α2M eine Verringerung der Expression von intrinsischem TNFα sowie Dosis-abhängig ein Anstieg der Vaskularisierung. Dies lässt vermuten, dass α2M die Herunterregulation von TNFα durch die Aktivierung des LRP1 Rezeptors vermittelt, was auch für die verstärkte Vaskularisierung der mEBs verantwortlich sein könnte.

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass α2M die Vaskularisierung in mESCs durch eine verstärkte Expression von Wachstumsfaktoren und die Aktivierung von pro-angiogenen Signalwegen stimuliert. Zudem zeigt α2M durch die Inhibierung der TNFα Expression einen anti-inflammatorischen Effekt in mESCs.

8. Index of abbreviations

α2m	Alpha 2 macroglobulin
αSMA	Alpha Smooth muscle actin
Ab	Antibody
AD	Alzheimer's disease
AMPK	Adenosine monophosphate-activated protein kinase
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BMP	Bone morphogenic protein
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CO2	Carbon dioxide
CR	Complement type repeats
EB	Embryoid body
ECL	Enhanced chemi luminescence
ECM	Extra cellular matrix
ECs	Endothelial cells
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinases 1/2
ESCs	Embryonic stem cells
FGF2	Fibroblast growth factor 2
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GRP78	Glucose-regulated protein 78
h	Hours
HRP	Horse radish peroxide
HSP	Heat shock protein
HUVEC	Human umbilical vein endothelial cells
IF	Immuno-fluorescence
IFNɣ	Interferon gamma
iNOS	Inducible nitric oxide synthase
iPSCs	Induced pluripotent stem cells
IRE1	Inositol-requiring enzyme 1

ISL1	Insulin gene enhancer protein 1
JAK	Janus kinase
KDa	Kilo Daltons
Klf4	Kruppel-like factor 4
KO	Knock-out
LDL	Low-density lipoprotein
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LRP1	Low density lipoprotein receptor-related protein 1
LTβ	Lymphotoxin-beta
MAPK	Mitogen-activated protein kinases
mEB	Mouse embryoid body
MEF	Mouse embryonic feeders
mESCs	Mouse embryonic stem cells
MHC	Major histocompatibility complex
min	Minute
MOPS	3-(N-morpholino)propane sulfonic acid
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NIH Unit	National Institute of Health unit
NO	Nitric Oxide
ºC	Degree Celcius
Oct4	Octamer-binding transcription factor 4
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PECAM1	Platelet endothelial cell adhesion molecule 1
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PI3K	Phosphatidyl inositide 3-kinase
PKB/AKT	Protein kinase B
PLCγ	Phosphoinositide phospholipase C
PZP	Pregnancy zone protein
RAP	Receptor associated protein
RIPA	Radio immune precipitation assay
Robo	Roundabout homolog 1
rpm	Revolutions per min
RT	Room temperature
s	Second

SDS	Sodium dodecyl sulfate
SHH	Sonic hedgehog
SMAD2	Mothers against decapentaplegic homolog 2
SOX2	Sex determining region Y Box 2
STAT3	Signal transducer and activator of transcription 3
TBS	Tris buffered saline
TGFβ	Transforming growth factor beta
TNFR	Tumor necrosis factor receptor
TNFα	Tumor necrosis factor alpha
uPA	Urokinase-type plasminogen activator
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells
WB	Western blot
(v/v)	volume/volume
(w/v)	weight/volume

9. List of diagrams, figures and tables

Figures

Tables

10. References

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Publications

1. Sauer H, **Ravindran F**, Beldoch M, Sharifpanah F, Jedelská J, Strehlow B, Wartenberg M (2013) α2-Macroglobulin enhances vasculogenesis/angiogenesis of mouse embryonic stem cells by stimulation of nitric oxide generation and induction of fibroblast growth factor-2 expression. Stem Cells Dev. 22, 1443-54

POSTER PRESENTATIONS

1. **Febina Ravindran**, Fatemeh Sharifpanah, Heinrich Sauer. Induction of vasculogenesis in mouse embryonic stem cells by alpha 2 macroglobulin activating LRP1 signaling pathway. 7th Fraunhofer Life Science Symposium 2012, Leipzig, Germany

2. **Febina Ravindran**, Fatemeh Sharifpanah, Heinrich Sauer. Signaling mechanisms of the stimulation of vasculogenesis of mouse embryonic stem cells by α2- macroglobulin. 92nd Annual Meeting of the German Physiological Society 2013, Heidelberg, Germany

3. **Febina Ravindran**, Fatemeh Sharifpanah, Heinrich Sauer. Induction of vasculogenesis in mouse embryonic stem cells by alpha 2 macroglobulin activating LRP1 signaling pathway. Cardiovascular Network Retreat 2013, Rauischholzhausen, Germany

ORAL PRESENTATIONS

1. Impact of α2 macroglobulin and LRP1 mediated signaling pathways for vascular differentiation in mouse embryonic stem cells. PROMISE Barcelona Retreat 2012, Barcelona, Spain

2. Impact of α2 macroglobulin and LRP1 mediated signaling pathways for cardiovascular differentiation in mouse embryonic stem cells. Cardiovascular Network Retreat 2012, Rauischholzhausen, Germany

3. Impact of α2 macroglobulin and LRP1 mediated signaling pathways for cardiovascular differentiation in mouse embryonic stem cells. PROMISE Barcelona Retreat 2011, Barcelona, Spain

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Giessen, 25 June 2015 **Febina Ravindran**

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