CHAPTER 1: REVIEW OF LITERATURE

1.1 INTRODUCTION

Angiogenesis is the process by which new blood vessels are formed from pre-existing vasculature. This is an important natural process occurring in both health and disease. It is controlled and regulated by many molecules including stimulators and inhibitors. Appropriate balance between stimulators and inhibitors are pivotal for maintenance and regulation of angiogenesis in health. Disturbed homeostasis in angiogenesis is associated with many diseases including cancer, heart diseases, stroke, diabetes, blindness, etc. The term angiogenesis was coined over a century ago, but was not fully elucidated until the 1960's when late Dr. Judah Folkman observed that tiny tumours grew to about 1 mm in size and stopped expanding in the absence of neovascularization (Aragon-Ching and Dahut 2009).

1.2 THE PROCESS OF ANGIOGENESIS

Angiogenesis is a multistep process, its initiated by vessel dilation and the release of plasma proteins which changes the endothelial cells (ECs) behaviour (Merks et al. 2008) (Figure 1). The steps involved are as follows:

1. Diseased or injured tissues produce proangiogenic factors.
2. Release of angiogenic growth factors occur that diffuse into the nearby tissues.
3. These angiogenic growth factors then bind to the specific receptors on ECs, which further activates and send signals to the nucleus. The ECs machinery starts to synthesize new molecules including enzymes like matrix metalloproteases (MMPs).
4. These enzymes act on the basement membrane of the surrounding blood vessels that activate the ECs.
5. Adhesion molecules or integrins serve as "grappling hooks" to pull the sprouting new blood vessels forward.
6. Then ECs begin to proliferate, and migrate out towards the diseased tissue. Integrin proteins like αVβ5 facilitate growth and survival of newly formed blood vessels and αVβ3 activate MMP2.
7. Further, MMPs dissolve the tissue and help in the sprouting vessel tip. As the vessel extends, the tissue is remoulded around the vessel. Sprouting endothelial cells roll

up to form a blood vessel tube (tubular morphogenesis) which is modulated by Tie 2 receptors.

8. Individual blood vessel tubes connect to form vascular loops.
9. Finally, newly formed blood vessel tubes are stabilized by smooth muscle cells or pericytes that provide structural support (Chakraborty et al. 2007; Jones et al. 2009).

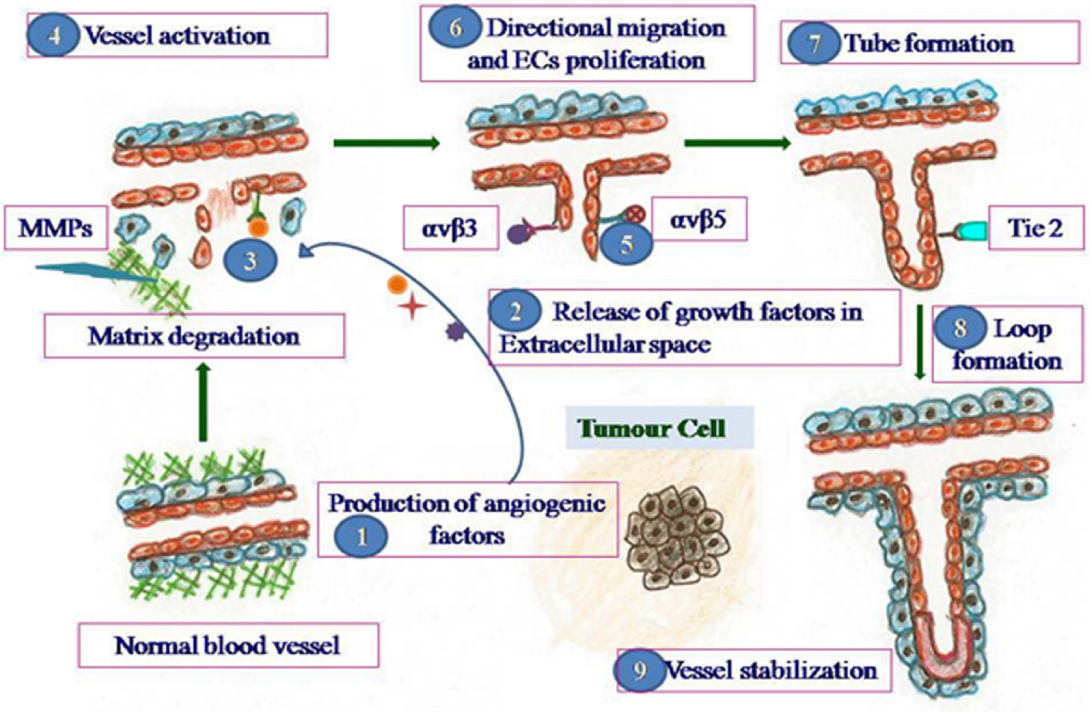


Figure 1: Angiogenesis cascade showing various steps involved in formation of new blood vessel.

Numerous mechanisms are involved in the process of angiogenesis. Signal transduction pathways helps to promote proliferation, metastasis, invasion and tumour survival. The disruption of the balance between pro- and anti-angiogenic growth factors leads to the disease.

1.3 VARIOUS PATHWAYS INVOLVED IN ANGIOGENESIS

Angiogenesis is a tightly regulated process and seems to be under the control of both positive and negative regulatory factors. Although several potential regulators of angiogenesis have been identified, relatively little is known about their role in the physiological regulation of angiogenesis.

1.3.1 Pro-angiogenic molecules:

Pro- angiogenic molecules comprising of growth factors (VEGF, FGF, angiopoietin, PDGF), tyrosine kinase receptors, cell adhesion molecules, hypoxia and axon guidance molecules help in progression of angiogenesis.

1.3.1.1 Growth factors

a) Family of vascular endothelial growth factor and their receptors: Vascular endothelial growth factor (VEGF) is an important signalling protein required for vasculogenesis and angiogenesis. VEGF consist of 4 isoforms from A- D and placental like growth factor. VEGF protein binds to tyrosine kinase receptors on the cell surface. VEGF protein binds to Flt-1 also known as Fms-like tyrosine kinase-1 (VEGFR1), KDR (kinase-insert Domain-containing Receptor) in humans, Flk-1: Fetal-liver kinase-1 in mice), and (VEGFR3 Flt4: Fms-like tyrosine kinase-4)} which transduce signals for tumor angiogenesis (Aragon-Ching and Dahut 2009). Low oxygen tension is known as hypoxia, occurs during embryonic development, ischemia, wound healing, and cancer (detailed description in 1.1.3.3). VEGF-A gene expression is upregulated by hypoxia and activates the angiogenesis cascade by majorly binding to VEGFR-2. The significance of VEGFR-1 is that, it has a dual role in the regulation of angiogenesis. VEGFR-1 can also acts as a 'decoy' receptor that sequesters VEGF and prevents its interaction with VEGFR-2. VEGFR-1 is known to recruit monocytes and other bone marrow- derived cells to promote angiogenesis (Figure 2). In addition, VEGF B binds to VEGFR-1, activates MMPs, and is involved in the paracrine release of growth factors like placental- growth factor from endothelial cells which has important role in embryogenesis (Ferrara and Kerbel 2005).

Anti-VEGF therapies are important in the treatment of certain cancers and in age-related macular degeneration. Numerous monoclonal antibodies have been developed to target VEGF & its receptors which include humanised antibodies such as bevacizumab, ranibizumab etc (Lien and Lowman 2008).

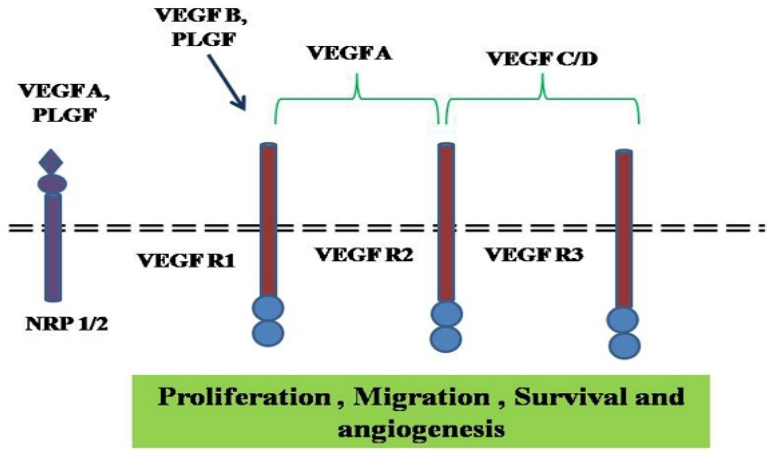


Figure 2: Role of VEGF family and their receptors in angiogenesis.

b) Fibroblast derived growth factor: Fibroblast derived growth factor (FGF) consist of 22 members of structurally related protein. FGF1 is also known as acidic, and FGF2 is also known as basic and it has 4 receptors FGFR1, R2, R3 and R4. FGF binds to heparin and heparin sulphate and is found at the primary sites of vessel branching (Vlodavsky et al. 1987). It exist in 4 different isoforms: Low molecular weight (LMW) - 18 kDa, 22 kDa , 22.5 kDa, and 24-kDa or high molecular weight (HMW) protein (Moscatelli et al. 1986). The biological activity is mediated through FGF-2 which binds to high-affinity, tyrosine kinase receptors and low-affinity heparan sulfate proteoglycans located on the cell surface (Sato et al. 1991). The different forms of FGF-2 have been associated with different cell functions and cellular compartmentalization. LMW FGF-2 is released by the cell and it stimulates cell migration, proliferation, and FGF receptor down regulation through binding to surface receptors; HMW FGF-2 primarily localize to the nucleus and modulates cell proliferation (Bikfalvi et al. 1997). Few drugs are in clinical trials targeting FGFR. Dovitinib is administered orally in patients with melanoma and breast cancer which has progressed to phase II clinical trial (Andre et al. 2013; K. B. Kim et al. 2011). Bono *et al.,* reported oral administration of the pharmacologic agent SSR128129E, inhibited FGFR signaling pathway by binding to the extracellular domain of tyrosine kinase receptor of FGF (Bono et al. 2013).

c) Angiopoietin and its receptors: Vascular ECs express two types of tyrosine kinase receptors namely Tie-1 and Tie-2 receptors. These receptors play a critical role in physiological and pathological angiogenesis (Maisonpierre et al. 1997). Tie-1 is a cell

surface protein present in ECs, platelets and immature hematopoietic cells. Tie -1 up regulates cell adhesion molecules (CAMs), E selectin, vascular cell adhesion molecule (V-CAM1), and Intercellular (I-CAM1) by activating p38 dependent mechanism. Angiopoietin 1-4 (Ang 1-4) has been identified as ligands for the Tie-2 receptor. Ang-1 is important for vessel maturation and it mediates migration, adhesion and survival of ECs. Ang-1 induced Tie 2 signaling, promoted activation of integrin αvβ5 signaling leading to retinal angiogenesis (J. Lee et al. 2013). Ang-2 promotes cell death and vascular regression by disrupting EC – pericytes connections. Felcht *et al*., showed that Ang-2 blocking by antibodies showed reduced focal adhesion kinase (FAK) phosphorylation thereby inhibiting angiogenesis (Felcht et al. 2012). Therefore these dual roles of angiopoietin in tumour and physiological angiogenesis need to be studied further.

d) Platelet derived growth factor: Platelet derived growth factor (PDGF) is a secretory protein which can exist as either homodimer (PDGF A-D) or a heterodimer (PDGF A&B). PDGF is primarily synthesized in platelets and is also expressed in smooth muscle cells, macrophages and endothelial cells. It acts as a potent mitogen for mesenchymal, smooth muscle cell and glial cells (Hannink and Donoghue 1989). The PDGF signalling network consists of four ligands, PDGFA-D, and two receptors: a) PDGFR alpha and b) PDGFR beta (Heidaran et al. 1991). PDGF acts via phosphoinositol 3 kinase/protein kinase (PI3K/AKT) pathway for proliferation. It activates nuclear factor kappa B (NFκB) which further activates proto-oncogene c-myc for proliferation and mediates anti-apoptotic signal (Romashkova and Makarov 1999). Kohno *et al.,* reported that PDGF activates Cu chaperone Atox 1 for ATP7A leading to migration of vascular smooth muscle (Kohno et al. 2013).

1.3.1.2 Cell adhesion molecules:

ECs activation gives rise to a series of events which begin leading the cell to invasive, migratory, and proliferative tendency. Cell adhesion molecules can be classified as calcium independent and dependent molecules. Calcium independent class consist of immunoglobulin superfamily CAMs and addressins molecules. In calcium dependent molecules, comprises of integrins, cadherins and selectins. Integrins are cell surface receptors for ECM having a major role in cell adhesion. Integrins are a family of heterodimer transmembrane glycoproteins consisting of an alpha and beta subunit. The integrins mediate signaling events by activating the integrin-linked kinase (ILK), PKB/Akt, mitogen-activated protein kinase (MAPK), Raf or NF*κ*B pathway, in conjunction with

other growth factor receptors. This results in disruption of cell adhesion, tumor proliferation, migration, and survival (Aragon-Ching and Dahut 2009). MMPs indirectly regulate integrin signalling or cleave VE-cadherins and during cleavage they also release proangiogenic factors thereby, facilitating ECs migration. MMPs support metastasis by inducing an epithelial to mesenchymal transition (EMT) and transendothelial migration (Fink and Boratynski 2012). Vascular adhesion molecules have been identified to localize in between the EC junctions. Cadherin a 135 kDa protein was first identified in EC and mediates calcium dependent homophilic adhesion (Suzuki et al. 1991). The other molecule involved in EC adhesion is platelet endothelial cell adhesion molecule 1 (PECAM 1/CD31). PECAM is a 130-kDa membrane glycoprotein of the immunoglobulin super family that is able to mediate both homophilic and heterophilic adhesion (Newman et al. 1990). Inspite of the close distribution in the cell, cadherin molecules are localised in the sub apical locations associated with adheren junctions (Lampugnani et al. 1992), whereas lateral plasma membrane is occupied by PECAM 1. Both the molecules are associated with the actin cytoskeleton, although the cadherin interaction seems to be stronger. PECAM 1 plays an important role in vascular development, leukocyte migration at sites of inflammation (DeLisser et al. 1994), maintenance of endothelial barrier and angiogenesis (DeLisser et al. 1997). Studies have demonstrated that treatment with blocking anti – PECAM 1 antibodies inhibits cytokine and tumor induced angiogenesis in various animal models (Cao et al. 2002; Zhou et al. 1999). Phase II clinical trial of drugs that antagonize integrin α2β3 (e.g. abciximab), integrin αLβ2 (efalizumab), and integrin α4β1 (natalizumab) are approved for diseases like acute coronary syndromes, psoriasis, and multiple sclerosis (Woodside and Vanderslice 2008).

1.3.1.3 Hypoxia inducible factor 1 alpha:

The relationship between hypoxia and angiogenesis is direct as it has to supply oxygen on demand. Ineffectively, vascularised tumor tissue becomes hypoxic, thereby stimulating angiogenesis to combat the oxygen deficiency. Hypoxia activates several genes that are involved in tumour angiogenesis (Moeller et al. 2004). One of the important mechanisms involved in the regulation of VEGF is via the Von Hippel-Lindau (VHL) protein-induced degradation of the hypoxia-inducible factor 1 (HIF-1α). HIF is a heterodimeric transcription factor consisting of an alpha (oxygen regulated) and beta 1 subunits. It was identified, to regulate erythropoietin protein. The stability and activity of HIF-1 depends on the interaction with various proteins such pVHL, p53 and p300 and also due to the post-

translational modifications such as hydroxylation, acetylation, and phosphorylation (Theodoropoulos et al. 2004). In normoxia, HIF-1α is degraded in 26S proteosomes when bound to VHL protein which recognises E3 ubiquitin ligase. During hypoxia, the hydroxylation of HIF-1α is reduced, thereby allowing the 2 subunits to combine at nuclear hypoxic response elements of target genes that encodes for angiogenesis. In normoxic cells, that are deficient in VHL or functionally inactive VHL leads to inappropriate accumulation of HIF-1α leading to activation of VEGF, PDGF, TGF β thereby triggering angiogenesis (Aragon-Ching and Dahut 2009).

1.3.1.4 Axon guidance molecules:

Ramon and Cajal discovered these molecules in 1800 that act as both attractant and repellent in the patterning of nervous system. There are four main families: the neuropilins (NRP)/semaphorins, the ephrins, Robo/Slit and netrin/Unc5. Although the significance of these molecules in tumorigenesis is still unclear, there is emerging evidence that they have a role in some cancer models and therefore may be potential therapeutic targets (Chedotal et al. 2005). Subsequently, NRP was identified as a receptor for the VEGF-A and its isoforms, on both ECs and tumor cells. NRP was up-regulated in multiple tumor types and correlated with tumor progression (Ellis 2006). The ephrins and their tyrosine kinase Eph receptors were initially studied with neuronal guidance during development and subsequently found to have functions in vascular and other cells. Pandey *et al*., reported that ephrin A1 mediates TNF-α induced angiogenesis *in vivo* in rat model (Pandey et al. 1995). Eph/ephrin plays a major role in tumour neovascularisation and their interactions have been associated with malignant tumor progression and angiogenesis (Ogawa et al. 2000). Netrins are laminin-like secreted proteins involved in angiogenesis and formation of the blood vessel network. The role of netrins as pro or anti-angiogenic is still under debate (Castets and Mehlen 2010).

1.3.2 Anti-angiogenic factors:

Anti-angiogenic property is manifested by various full length proteins or their peptide fragments which are discussed below in detail. These molecules inhibit angiogenesis by inhibiting migration, proliferation or activating apoptotic signals (Figure 3).

1.3.2.1 Proteolytic fragments of protein act as anti-angiogenic molecules:

1.3.2.1.1 Angiostatin:

Angiostatin is a 38 kDa proteolytic fragment derived from plasminogen (O'Reilly et al. 1994). Plasminogen is cleaved by various enzymes such as a) macrophage derived metalloelastase b) MMPs or by c) plasminogen activator enzyme to form angiostatin (Stathakis et al. 1997). It inhibits αVβ3 integrin, ATP synthase and angiomoitin thereby inhibiting angiogenesis (Claesson-Welsh et al. 1998; Moser et al. 1999). Angiostatin inhibits neutrophil, tumor associated macrophage infiltration *in vivo* (Perri et al. 2005). Angiostatin was found to induce cytokine interleukin (IL-12) release from neutrophil thereby mediating anti-inflammatory and anti- angiogenic role (Albini et al. 2009). Clinical trials have progressed to phase II with recombinant angiostatin (NCT00049790) when administered along with carboplatin to patients with non small lung carcinoma.

1.3.2.1.2 Collagen derived peptides:

a) Endostatin:

Endostatin is a 20 kDa C-terminal fragment of collagen XVIII, generated by cathepsin L or by a protease with elastase-like activity (Felbor et al. 2000; O'Reilly et al. 1997). Endostatin inhibits VEGF induced EC migration and induces apoptosis. It also binds to αVβ3 integrins thereby, inhibiting migration (Rehn et al. 2001). Li J *et al.,* showed that endostatin when combined with chemotherapy was found to more effective in mice model which is progressed to phase II clinical trial. Intravenous administration of endostatin of concentration 7.5 mg / m^2 at day 1, 14 and 21 in patients with breast cancer (Li et al. 2008). Intravitreal injections of AAV2 (adenovirus) tagged with the human immunoglobulin for targeting the Flt -1 domain of VEGFR is under phase I/II clinical trial for age related macular degeneration (AMD) (Campochiaro 2012).

b) Tumstatin:

Tumstatin is 25 amino acid anti-angiogenic peptide derived from the α3 chain of collagen IV(Maeshima et al. 2000). This peptide interacts with αVβ3-integrin thereby inhibiting FAK, PI3 kinase, AKT and mTOR signalling pathway (Maeshima et al. 2001). Tumstatin also blocked VEGF induced corneal neovascularisation in mice (Maeshima et al. 2002). Peptides derived from tumstatin were also found to inhibit angiogenesis in HUVEC and primary lung endothelial cells (Grafton et al. 2014).

c) Canstatin:

Canstatin, is another anti-angiogenic peptide of 25 kDa derived from alpha chain of collagen type IV (Magnon et al. 2005). It induces apoptosis through PI3K/AKT pathway (Panka and Mier 2003) and was found to inhibit Tie-2 and VEGFR-3 in HUVEC (Hwang-Bo et al. 2012).

1.3.2.1.3 PEX:

PEX is a proteolytic fragment of MMP-2 containing the C-terminal hemopexin-like domain (Brooks et al. 1998). PEX prevents binding of MMP2 to integrin αVβ3 blocking collagenolytic activity, thereby regulating the invasive behaviour of new blood vessels (Kessler et al. 2002; Pfeifer et al. 2000). It also, further suppresses VEGF secretion and VEGF R-2 protein level thereby, decreasing angiogenesis (Ezhilarasan et al. 2009).

1.3.2.1.4 Prolactin derived peptide:

Prolactin a growth hormone is proangiogenic molecule and is involved in lactation of mammary glands. Full length prolactin is a 23 kDa protein, which on cleavage by cathepsin D yields a 16 kDa peptide fragment. The Prolactin derived peptide 16 kDa peptide was found to be anti-angiogenic (D'Angelo et al. 1995; Kim et al. 2003) by VEGF or FGF proliferation and induced apoptosis in both *in vivo* and *in vitro* studies (Nguyen et al. 2006).

1.3.2.2 Proteins as anti-angiogenic molecules:

1.3.2.2.1 Thrombospondin 1

Thrombospondin 1 (TSP1), a large multifunctional glycoprotein secreted by epithelial cells in the extracellular matrix. It also inhibits angiogenesis associated with tumor growth and metastasis (Good et al. 1990). It performs anti-angiogenic activity by binding to CD36 receptor and activating the MAPK pathway leading to activation of caspase 3 mediating apoptosis (Felbor et al. 2000). TSP-1 expression is up-regulated by the tumor suppressor gene, p53, and down-regulated by oncogenes such as Myc and Ras. Hence, it further inhibits EC migration, proliferation and tumour progression (Ren et al. 2006).

1.3.2.2.2 Pigment epithelium derived factor

Tombran-Tink and Johnson identified Pigment epithelium derived factor (PEDF) as extracellular glycoprotein of 50 kDa belonging to the family of serine protease inhibitor (serpin family) (Tombran-Tink and Johnson 1989). It exhibits a novel role as a neuroprotective agent and also inhibits aberrant neovascularisation (Steele et al. 1993). PEDF activates, fatty acid synthase receptor on immature EC thereby activating caspase- 8 dependent apoptotic pathways (Volpert et al. 2002). Hase *et al*., stably transfected human pancreatic adenocarcinoma cells with PEDF which inhibited proliferation indicating that gene therapy could be used to treat such cancers (Hase et al. 2005). PEDF acts as an anti-sangiogenic molecule by inhibiting VEGF (Cai et al. 2006). Later studies indicated that PEDF also inhibited of HIF -1α induced VEGF dependent angiogenesis (Yang et al. 2009). Campochiaro *et al*., in their phase I clinical trial also showed that single intravitreal injections of AAV vector encoding PEDF was effective in treating AMD (Campochiaro et al. 2006).

1.3.2.2.3 Tissue Inhibitor of Matrix metalloproteases:

Tissue inhibitors of matrix metalloproteases (TIMPs) are 21 to 29 kDa proteins, which are endogenous inhibitors and bind MMPs in a 1:1 stoichiometry. TIMPs 1 to 4 isoforms have been identified and their expression is regulated during development and tissue remodelling. The TIMP molecule binds to the active-site cleft of MMP similar to substrate thereby inhibiting MMP activity (Raffetto and Khalil 2008).

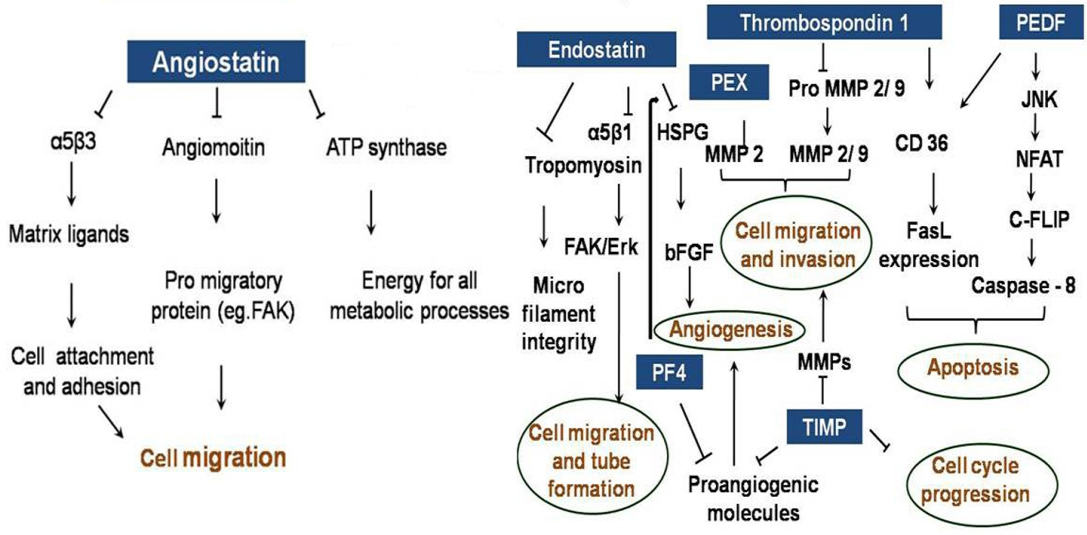


Figure 3: Endogenous inhibitors of angiogenesis targeting various intracellular pathways mediating angiogenesis.

1.3.2.2.4 Adiponectin:

Adiponectin (APN) is a circulating peptide hormone derived from adipocyte tissues. APN exist in oligomeric and globular form to regulate energy homeostasis (Kawano and Arora 2009). APN plays important role in controlling diabetes, inflammation and angiogenesis (Shen et al. 2008). APN binds to ADIPOR1 and ADIPOR2 receptors and activates intracellular molecules such as AMP activated protein kinases, PPARα pathway, glucose uptake and the oxidation of fats in liver and muscles (Kadowaki and Yamauchi 2005). APN suppresses VEGF-stimulated ECs migration via cAMP/PKA dependent signaling pathway (Mahadev et al. 2008). Vidhya *et al.,* showed presence of APN in ocular tissues and was found to be increased in proliferative diabetic retinopathy. Vitreous levels of APN increased with insulin like growth factor (IGF) and VEGF levels indicating the role of APN in ocular angiogenesis (Vidhya S et al. 2013).

1.4 ANGIOGENESIS RELATED DISEASES IN EYE

Ocular diseases also show neovascularisation due to hypoxia, inflammation and respond to systemic changes. Pathological ocular angiogenesis can be classified based on their anatomy (Figure 4).

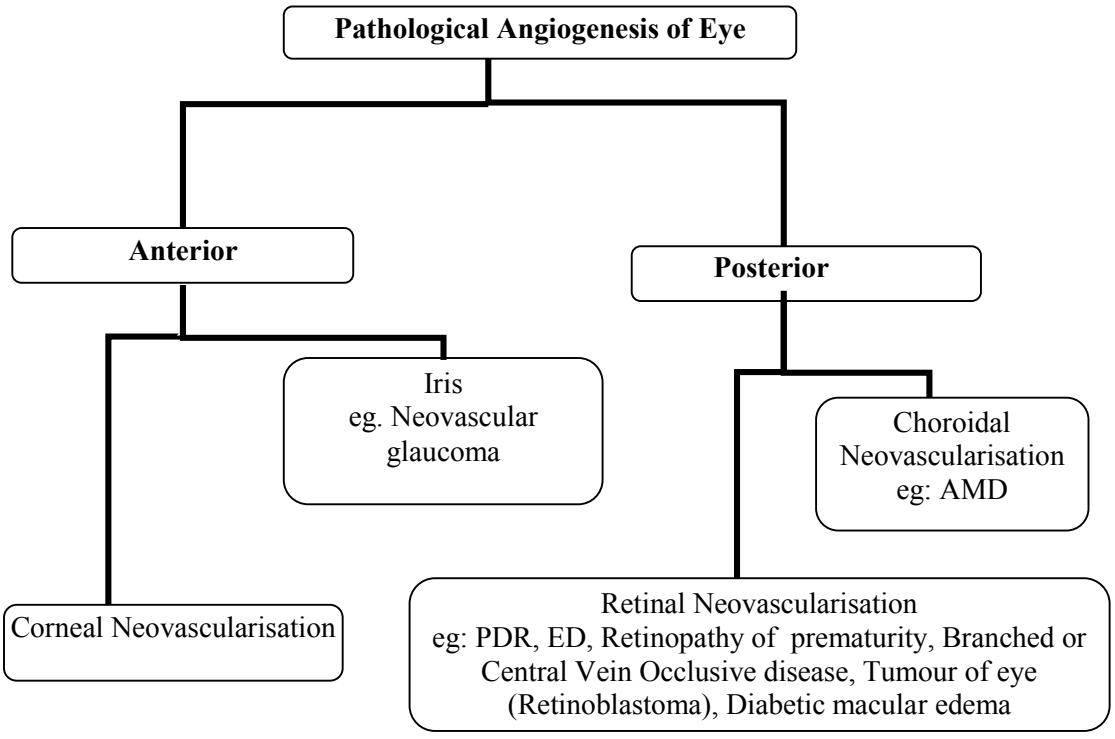


Figure 4: Classification of pathological angiogenesis of the eye based on anatomy of the eye.

Neovascularisation in the eye can be classified as anterior or posterior based on its anatomy. Anterior comprises of corneal neovascularisation seen during infections and neovascularisation in iris seen during glaucoma which occur mainly due to ischemia. Posterior neovascularisation is further classified as retinal and choroidal. Few of these diseases that underlie these conditions are discussed below:

1.4.1 Proliferative diabetic retinopathy:

Diabetic retinopathy (DR) is the leading cause of irreversible blindness. The histological features in DR include capillary basement membrane thickening, loss of pericytes and ECs. Later stage of disease is manifested by excessive neovascularization leading to proliferative DR (PDR). This is represented by vitreous haemorrhage which occurs due leaky blood vessels and break down of inner blood retinal barrier (BRB).

Molecular mechanism in PDR:

Many biochemical mechanisms play an important role in disease progression which is listed below:

1. Polyol pathway - During, diabetes aldose reductase acts on excess glucose to convert it to sorbitol which is further metabolised to fructose by sorbitol dehydrogenase. The polyol accumulation leads to basement thickening, pericyte loss and microaneurysm formation (Gabbay 1975).
2. Hexosamine pathway - Both high glucose and glucosamine treatments lead to increase in UDP-GlcNAc and O-GlcNAc levels, they also can lead to oxidative and endoplasmic reticulum stress which leads to chronic inflammation and insulin resistance (Werstuck et al. 2006).
3. Advanced glycation end products (AGE) pathway- The nonenzymatic reaction of the amino groups peptides or proteins with reducing sugars, result in the formation of complex brown pigments and protein-protein crosslink's. Methylglyoxal (MG) is an important glycolytic intermediates as well as from autoxidation of sugars. In diabetes (both Type I and Type II) the concentration of MG is found to be increased in the lens, blood and kidney. It is a major glycating agent; increased MG level in turn increases macromolecule damage, thereby triggering ROS mediated oxidative stress (Kowluru and Chan 2007).
4. Protein kinase C Pathway - Protein kinase C (PKC), belongs to a family of large group of structurally related enzymes for the activation of diacylglycerol (DAG). Although the activities of multiple PKC isoforms (α, β1, β2, and ε) are increased in vascular

tissues in the diabetic state, studies suggest that the PKC- β 2 isoform preferentially mediates the pathologic complications associated with hyperglycemia (Inoguchi et al. 1992). Moreover, PKC- β has been shown to be an integral component of cellular signaling by VEGF which are important mediators of ocular neovascularization (Xia et al. 1996).

5. Oxidative stress and growth factors - Oxidative damage, cytokine and leukocyte activation. Further, growth factors like VEGF, IGF-1, and FGF-2 increases in intraocular fluid leading EC proliferation, migration and tube formation (Brooks et al. 2004). Whereas the levels of anti-angiogenic factors such as PEDF, TSP, TGF-β in PDR are reduced (Simo et al. 2006).

Treatment: Avastin or Bevacizumab is a humanised antibody synthesized against VEGF and is administered in case of neovascularization of retina. Laser photocoagulation is performed in PDR cases with retinal neovascularization. Vitrectomy is performed in case of vitreous hemorrhage.

1.4.2 Eales' disease

Eales' Disease (ED) was first described by Henry Eales' in the year 1880. ED is an inflammatory idiopathic disease that affects mostly young adult males and is prevalent in the Indian subcontinent (Biswas et al. 2002). Inflammation, non-perfusion in peripheral veins and retinal neovascularization are hallmark of ED. Repeated vitreous hemorrhage leads to loss of vision. Factors such as autoimmunity, oxidative stress and *Mycobacterium tuberculosis* are associated with ED (Saxena et al. 2001).

Molecular mechanism in ED*:* Oxidative stress at the level of systemic circulation and local environment (i.e. vitreous humour and epiretinal membrane) has been found to be related to ED pathology. Elevated levels of thiobarbituric acid reactive substances (TBARS), SOD, GPx, homocysteine and homocysteine thiolactone (Bharathselvi et al. 2013; Bhooma et al. 1997) and decreased level of glutathione have been reported in serum of ED (Selvi et al. 2011). Accumulation of AGE and lipid oxidation end products in epiretinal membranes, were observed in patients with ED. Intracellular levels of iron and copper have been observed in the vitreous and monocytes of ED. Novel 88 kDa iron binding protein has been found to be involved in ED pathology (Sulochana et al. 2001).

Treatment: Anti-inflammatory drugs like prednisone are administered; laser photocoagulation or both are performed during active vasculitis.

1.4.3 Age related macular degeneration

AMD is a disease associated with aging that is characterized by progressive loss of central vision, due to the accumulation of macular phototoxic deposits termed as "drusens" (Cingolani et al. 2006). In early AMD, dry AMD, the accumulation of drusens in RPE leads to hypoxia and retina to atrophy. Drusens can accumulate to a degree wherein they rupture the Bruch's membrane and trigger neovascularization of the choroid that leads to late AMD or "wet-AMD", and contributes to blindness (Coleman and Chew 2007).

Molecular mechanism in AMD:

Oxidative stress:

Although the vision loss in AMD results from photoreceptor damage in the central retina, the initial pathogenesis involves degeneration of RPE cells. The retina is particularly susceptible to oxidative stress because of its high utilization of oxygen, its high proportion of polyunsaturated fatty acids and its exposure to visible light. Improper diet, genetic predisposition and environmental factors play an important role in AMD. Exposure to heavy metals, air pollutants, and cigarette smoke correlate with susceptibility to AMD. Studies have shown that the cytotoxic effects of pollutants is largely due to the oxidative stress induced by these agents (Coleman and Chew 2007). The link between oxidative stress and development of AMD is supported by human clinical studies. Superoxide dismutase (SOD), the key enzyme required for removal of superoxide radical showed a significant increase in human donor eyes with late stage AMD. The Age-Related Eye Disease Study (AREDS) found, that an antioxidant formulation (vitamins C, E, beta-carotene and zinc) is successful in preventing the development of advanced age-related macular degeneration by 25 % (Decanini et al. 2007).

Treatment: Avastin or Bevacizumab is administered in case of neovascularization of retina. Laser photocoagulation is performed in case of peripheral choroidal neovascularization.

1.5 ROLE OF COPPER IN ANGIOGENESIS

The effects of copper (Cu) on neovascularization and metastasis were not studied in detail because early research into angiogenesis focused more on growth factors and cell signaling agents. *In vivo* experiments on rats performed by MacAuslan and Gole showed that micro molar concentration of Cu induced intraocular neovascularisation. Later, they also studied that Cu is not just a chemotaxic substance but is involved in the synthesis of fibronectin which is important for cell adhesion (Gole and McAuslan 1981; Hannan and McAuslan

1982). Similar studies on proinflammatory compounds, such as prostaglandin E-1 (PGE-1) and interleukins induced neovascularisation in cornea of rabbits and Cu concentrations also were found to be increased in cornea. It was also seen that Cu deficient diets in rabbit lead to suppression in neovascularisation (Ziche et al. 1982). Cu levels were increased in angiogenic and metastatic tumour cells (Fuchs and de Lustig 1989). Cu induced collagenase secretion from leukocytes in PGE-1 mediated corneal neovascularization (Lin and Chen 1992). Hu *et al*., demonstrated Cu 500 μM induced proliferation of HUVEC in culture in serum free medium (Hu 1998). Also, Cu stimulates various factors such as VEGF, FGF1 and angiogenin (Finney et al. 2009). Cu is required for the activation of HIF-1 α, a major transcription factor regulating the expression of VEGF. Copper chaperone for superoxide dismutase (SOD) CCS transports Cu into the nucleus and binds to HIF-1 α further activating VEGF signalling pathway (Xie and Kang 2009).

1.6 COPPER

Cu is an essential micronutrient and is the third abundant trace element required for various biological activities other than iron and zinc (Goodman et al. 2004). Cu in free form is potentially harmful hence Cu homeostasis plays an important role in cellular function. Cu is transported by ceruloplasmin (CP) to the cells where copper transporter 1 (CTR1), a transmembrane protein, imports Cu inside the cell. Chaperones of Cu such as ATOX for cytochrome-*c* oxidase (CCO), further carries Cu inside the cell and distributes to other proteins for their function. ATP 7A&7B exports Cu from the cell helping in maintaining the Cu homeostasis (Banci et al. 2010). Metallothioneins (MT) are low molecular weight intracellular metal binding proteins rich in cysteine and exist in four forms (MT1-4). MTs are also known to play a protective role in Cu overload (Evering et al. 1991). Cu is involved in various biological processes listed in Table 1:

Table 1: List of few copper binding proteins and their biological roles (Pena et al. 1999)

Common Name	Biological function	Consequence of deficiency or defect
Cu /Zn (SOD)	Free radical detoxification	Oxidative damage
CCO	Electron transport in mitochondria	Deficiency of ATP: myopathy , ataxia , seizures
Lysyl oxidase (LOX)	Cross linking of collagen and elastin	Connective tissue disorder
Dopamine β hydroxylase (DBH)	Catecholamine production	Hypothalamic imbalance
Tryosinase (TYR)	Melanin production	Depigmentation
Peptidyl -α-amidating enzyme (PAM)	Bio activation of peptide hormones	Malfunction of peptide hormones
CP	Ferroxidase , Cu transport	Anemia
Clotting factor V,VIII	Blood clotting	Bleeding disorder
Metallothionein (MT)	Cu sequestration	Cu toxicity
Hephaestin	Iron transport	Sex linked anemia

1.6.1 Mechanism of action of copper:

Cu during its homeostasis binds to plasma proteins such as CP and albumin. It occurs in Cu (II) or Cu (I) form and is interconverted by the action of oxidoreductase enzymes. Cu is released from the loosely bound protein due to change in pH. In conditions of acidosis, trauma, ischemia, sepsis or shock there is more Cu entry in the cell due to low pH (Bar-Or et al. 2003).

1.6.2 Sources of copper:

Cu is supplemented through dietary intake. When Cu is less than 1 mg/day, more than 50% of it is absorbed, whereas if intake increases to 5 mg/day, then only 20% is absorbed. Plants act as a direct source of Cu for higher organisms. Rich dietary sources of Cu are seafood, organ meats, nuts, and seeds. The “recommended dietary allowance” for adults is 0.9 mg Cu/day and the “tolerable upper intake level” has been set at 10 mg/day (Bertinato and L'Abbe 2004).

1.6.3 Copper distribution and homeostasis:

Cu in free form is toxic and is generally found bound to proteins. Dietary Cu is absorbed into the body through the intestinal mucosa and transported via the portal blood to the liver. Much of the Cu taken up by the liver is incorporated into CP, released into the blood, and delivered to tissues. Cu is absorbed in the stomach and duodenum, stored in the liver and excreted through the bile. When hepatic Cu levels increases, biliary excretion increases to prevent Cu overload. As Cu is only excreted through bile any interference leads to hepatic Cu accumulation (Figure 5).

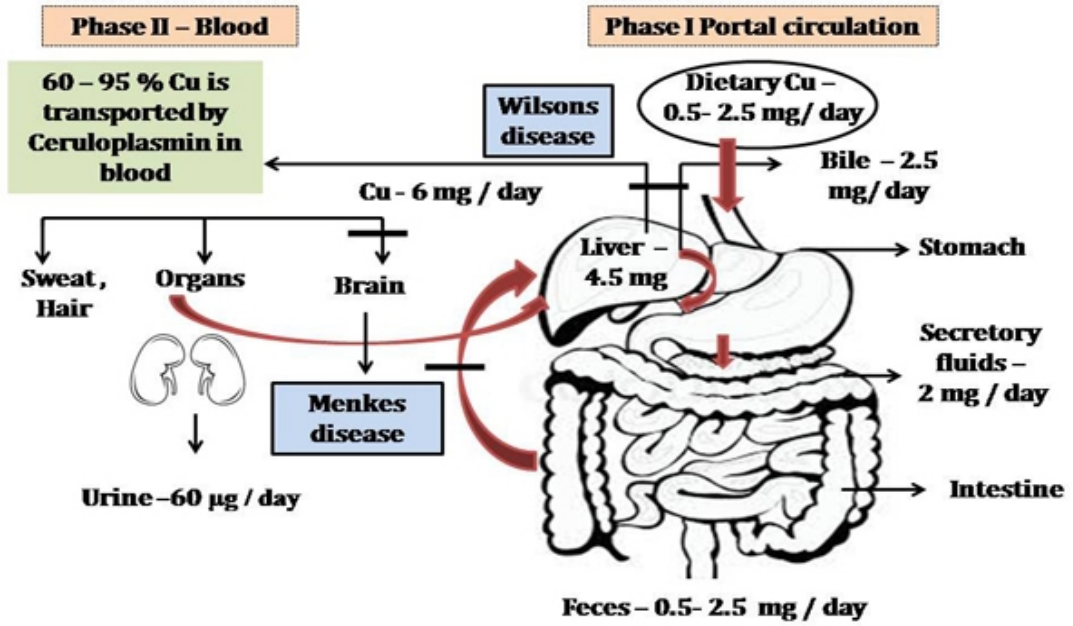


Figure 5: Cu homeostasis in human body.

Cu is supplemented through dietary source is stored in the liver. Accumulation of Cu leads to Menke's and Wilson's disease. Normal levels of Cu in various tissues in the body tissues as listed in (Table 2):

Table 2: Distribution of Cu in adult human tissues (Linder et al. 1998)

Tissues	Amount (mg)
Total body	110
Liver	10
Brain	8.8
Blood	6
Skeletal Muscle	26

Cu bound to CP is converted from cupric (Cu II) to cuprous ions (Cu I) by oxido - reductases enzymes. CTR1 imports, Cu as Cu I inside the cell by forming a pore. In cytoplasm, Cu is bound to GSH, MT, or Cu chaperones like COX, CCS, and ATOX. COX17 transports Cu to mitochondria and CCS delivers Cu to Zn-SOD by forming a heterodimer. ATOX1 interacts with trans-Golgi network (TGN) membrane copper transporting P-type ATPases to deliver Cu into the lumen of the secretory compartments. ATP7A is a Cu exporter that delivers Cu to PAM, DBH, TYR, LOX, and extracellular SOD 3 (Barry et al. 2010; Lee et al. 2002a; Lutsenko et al. 2007). The human Cu-ATPases ATP7A and ATP7B in liver are essential for intracellular Cu homeostasis and energy from ATP hydrolysis is used to transport Cu from cytosol to extracellular space (Figure 6). At low Cu concentrations, the protein localization is at the TGN, but at high concentrations it is relocated to the plasma membrane (Tumer and Moller 2010).

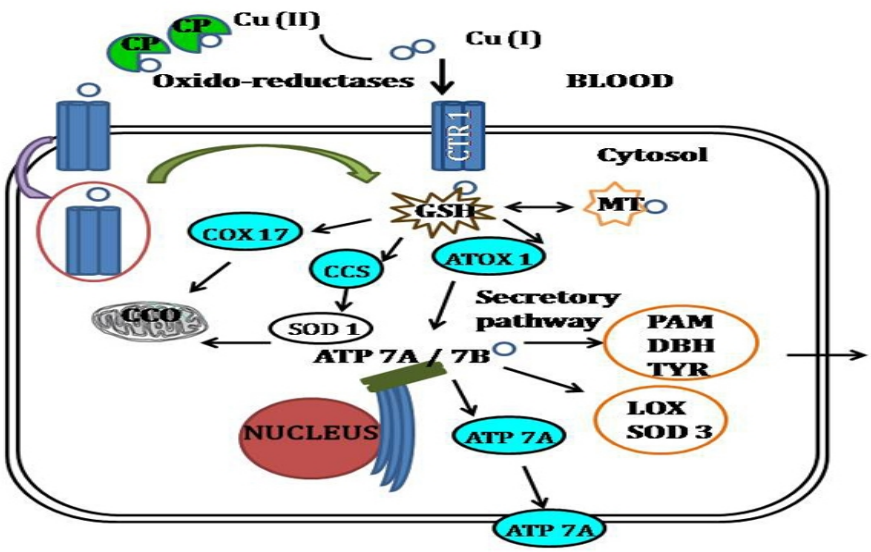


Figure 6: Copper homeostasis in cell which is imported by CTR1 and exported by ATP7 A&B.

1.6.4 Diseases related with copper imbalance:

Homeostasis of Cu is an important process and is tightly regulated by CTR1 and Cu chaperones. The two well studied diseases of Cu metabolism are Menke's disease where Cu is deficient and Wilson's disease where Cu accumulation is seen (Lutsenko 2010).

1.6.4.1 Menke's Disease:

Menke's disease (MD) is an X-linked recessive multisystemic lethal disorder characterized by a Cu deficiency. Most patients exhibit a severe classical form, while about 9 % of the

patients exhibit a milder form of MD known as occipital horn syndrome (OHS) (Yasmeen et al. 2013). Typical features of MD include neurological disturbances, connective tissue disorders and hair abnormalities that are due to abnormally low activity of Cu-dependent enzymes. The current treatment of MD is parenteral administration of Cu-histidine. New born babies should be immediately treated with Cu-histidine to prevent neurological symptom but even then connective tissue damage cannot be reversed by this treatment (Kodama et al. 2011; Tumer and Moller 2010).

1.6.4.2 Wilsons Disease:

Wilson's disease (WD) is a rare autosomal recessive disorder caused by mutations in ATP7B. It maintains the biliary copper excretory pathway. Cu slowly accumulates in affected patients with subsequent liver and brain damage (Goodman et al. 2004). Symptoms vary among patient hence leading to difficulty in diagnosis. Chelating agents such as tetrathiomolybdate and zinc are effective for the treatment of WD (Kodama et al. 2011).

1.6.4.3 Alzheimer's Disease:

Alzheimer's disease (AD) is characterised by accumulation of beta amyloid peptide (βAP) triggered by increased levels of Cu and Zn. Subsequent treatment with Cu – Zn chelators in transgenic mice showed effective decrease in the accumulation of βAP (Cherny et al. 2001). Later work done by Brewer GJ *et al.,* also indicated that free Cu levels are increased in AD, and Cu may be involved in disease pathogenesis, opening the way to possible therapy anticopper therapy for neurodegenerative diseases (Brewer 2008).

1.6.4.4 Cardio Vascular Disease:

Heart and vessel abnormalities are common in Cu deficient animals and humans. ''Falling disease'' that occurred in cattle in Western Australia can be attributed to severe Cu deficiency characterized by myocardial atrophy and replacement fibrosis leading to sudden death, that could be prevented by maternal Cu supplementation. Cardiomyopathy induced by Cu deficiency during pre, peri and postnatal periods is well established in experimental animals. Cu deficient hearts exhibit concentric hypertrophy, fibrosis, myofibril derangement, contractile and electrophysiological dysfunction, aneurysms, and eventual heart failure. Offspring from dams fed with Cu deficient diets from pregnancy through lactation have low cardiac CCO activity, high hydrogen peroxide levels, cardiomyocyte

structural damage, that persists despite long-term Cu repletion initiated at weaning (Uriu-Adams et al. 2010).

1.6.4.5 Other Diseases:

Low Cu levels are linked with bone malformation during development, and also contribute to the risk of developing osteoporosis later in life. A decrease in collagen stability and impaired melanin synthesis can also be consequences of low Cu status. Cu deficiency has also been associated with a weakened immune system and an increase in infections, with a decline in cardiovascular health and adverse alterations in cholesterol metabolism. Cu deficiency leads to oxidative stress and also affects the metabolism of other trace elements; most notably it perturbs Fe mobilisation and causing secondary Fe deficiency (Danzeisen et al. 2007).

1.7 COPPER TRANSPORTING PROTEINS

The molecular basis of Cu transport was a mystery and it was thought that CP was responsible for Cu transport in the cell. However patients with aceruloplasminemia showed Cu transport indicating a role of another protein in Cu homeostasis (Zhou and Gitschier 1997). CTR1 is a member of a family of proteins that provide Cu to the copper chaperone to specific intracellular proteins like Cu, Zn superoxide dismutase or compartments such as the secretory machinery or mitochondria. Two CTR1 dependent Cu transports are possible: 1) direct Cu transport mediated by CTR1 across the plasma membrane, and 2) via endocytosis of the Cu-CTR1 complex. The second mechanism is thought to be meant for Cu storage or supply of Cu to specific metabolic processes in intracellular organelles. During Cu deficiency, excess of CTR1 present in physiological conditions for Cu uptake and during excess degradation of CTR1 protein occurs leading to feed back inhibition, thus maintaining Cu homeostasis (Petris et al. 2003). Mouse embryonic fibroblasts lacking CTR1 showed Cu accumulation indicating its important role in physiological process and embryo development (Liang et al. 2009; Nose et al. 2006). Another low affinity Cu transporter CTR2 is also present in the cell. Blair *et al*., showed mouse embryonic cells with CTR2 knockdown showed increased uptake of Cu due to presence of CTR1 but also induced uptake of cisplatin an anticancer drug which is transported through CTR1. Thus CTR 2 knockdown helps in increased sensitivity to platinum drugs (Blair et al. 2009).

1.7.1 Structure of copper transporter1:

Lee J *et al*., had shown that Cu transport is directed through methionine rich motifs present in CTR1. Also these conserved motifs play an important role in the co-ordination of Cu in CTR1 for transport (Lee et al. 2002b). Later work done by Aller and Unger demonstrated that CTR1 protein has projection which forms a symmetrical trimer that is <40 Å wide. Translocation of Cu occurs at threefold axis of each trimer due to low electron density. Resolution of 6 Å showed a ~9 Å diameter pore for metal ions at the interface of three identical subunits. The structure of CTR1 was more closely related to channel protein and not transporters. The three dimensional structure of CTR1 was then determined from the same system to an in plane resolution of 7 Å. From this structure, transmembrane (TM) 2 was identified as the pore lining helix, and functionally important methionine residues at its extracellular end were shown to be in close proximity within the trimer. Complementary metal analysis and extended X-ray absorption fine structure data indicate the presence of two Cu (I) binding sites, ligated by three sulphur donors (Aller and Unger 2006). Three highly conserved amino acid sequences in CTR1 are involved in Cu binding (MxMxxM, MMMMxM, and GxxxG). The first two methionine-rich motifs Met150 and Met154 are located at the extracellular N terminus, whereas the last glycine- rich motif located at Gly167 and Gly171 of the GxxxG motif was proposed to mediate a tight interface between TM3 and TM1 (Figure 7).

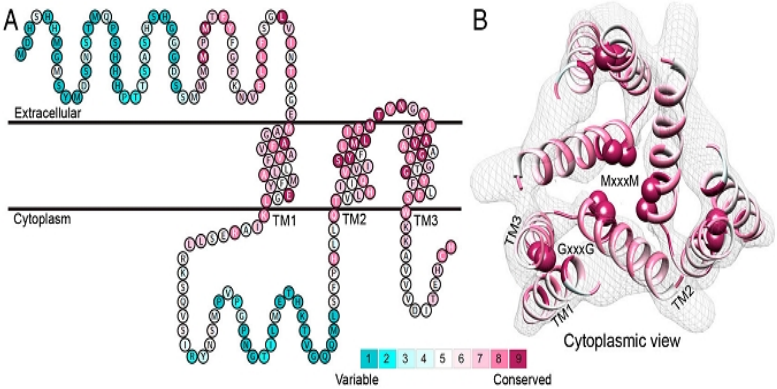


Figure 7: CTR1 full sequence and its transmembrane model. A) Topology of human CTR1. B) Intracellular view of the transmembrane domains and the MXXXM and GXXXG motifs are depicted (Schushan et al. 2010).

By studying the C α model it was observed that, there was a symmetry in the methionine and cysteine derived from N-terminal MPM motifs, MX3M motifs in TM2, and C-terminal HCH motifs. When the cysteine residues were removed it did not affect the Cu (I)

stoichiometry, but resulted in nitrogen coordination, presumably from the flanking histidines (Liang et al. 2009; Schushan et al. 2010).

Electron crystallography revealed that CTR1 exists as a channel-like trimer in a phospholipid bilayer and for the first time provided visual evidence of a pore located in the centre of the trimer. Structure of CTR2 is almost same when compared to CTR1, except that CTR2 lacks the extended N-terminal domain that provides Cu access to the pore and to the Cu chaperones. When Cu binds to CTR2, it leads to conformational changes thereby internalising Cu by the endocytotic machinery (Figure 8) (Lutsenko 2010; van den Berghe and Klomp 2010).

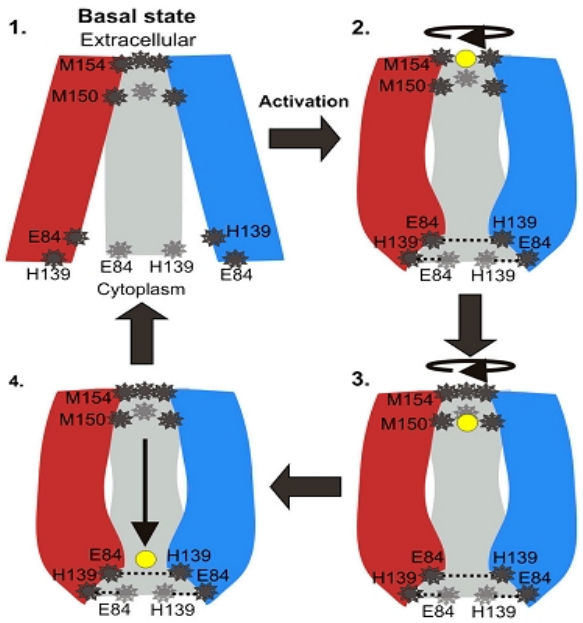


Figure 8: Transport mechanism of Cu ions at the transmembrane regions. 1) Cu ions do not bind to any amino acid residues and thus M154 and 150 serve as gate keepers. 2) Cu ion binding or pH change leads a 1st conformational change and protonated state of the M154 thereby leading to the interaction of histidine and glutamine. 3) The 2nd conformational change leads to inward movement of Cu ion from M154 to M150 and M154 remain closed to facilitate entry of one ion at a time. 4) Cu ion freely passes through the polar pore (Schushan et al. 2010).

1.7.2 CTR1 role in transport of metal ions:

1.7.2.1 Transport of silver:

Silver (Ag) is a non-essential, toxic metal. Mouse embryonic fibroblast cells lacking CTR1 showed approximately a 50% reduction in silver content when incubated in silver-supplemented medium compared to a wild-type isogenic cell line (Lee et al. 2002a). Cells transfected to express a CTR1-green fluorescent protein (CTR1-GFP) accumulated Ag when incubated in medium supplemented with 2.5-10 µmol/L of $AgNO_3$. Whereas the CTR1 mutant for M150 and M154 were replaced by leucine showed defects in Ag transport indicating Cu and Ag choose same mechanism of entry (Bertinato et al. 2010).

1.7.2.2 Transport of platinum:

Platinum based drugs have been used for cancer chemotherapy such as tumours in ovary, testes, and the head and neck. Platinum enters the cells and forms adducts with DNA leading to cell death (Sherman and Lippard 1987). Lin *et al*., first demonstrated that platinum drugs given for treatment are transported through CTR1. They showed that when CTR1 was knockdown in cells, platinum entry was reduced by 8 fold and Cu entry by 16 fold at the end of 1 h (X. Lin et al. 2002). Transport of platinum-based antitumor agents like cisplatin occurred through NH_2 terminal of CTR1 and methionine rich motifs (Sinani et al. 2007; Song et al. 2004). Peptides from methionine and histidine rich domain of CTR1 showed that NH_2 terminal 1- 20 amino acid was identified as cisplatin binding site using the tandem MS method (Wu et al. 2009). Deletion of either the first 45 amino acids or just the (40) MXXM (45) motif in the N-terminal domain affected the Cu uptake property, but it did not alter its subcellular distribution or the amount of protein in the plasma membrane. Neither of these structural changes prevented cisplatin from triggering the rapid degradation of CTR1 but altered the potency indicating the transport of cisplatin is by both pore formation and endocytosis (Larson et al. 2010).

1.8 ANTI- ANGIOGENIC THERAPIES

Drugs that target angiogenesis are called angiogenesis inhibitors or anti-angiogenesis drugs.

1.8.1 Angiogenesis inhibitors

These are designed to target growth of new blood vessels thereby reducing nutrients and oxygen supply for tumour growth. For e.g. Lenalidomide and thalidomide are used.

1.8.2 Cancer growth inhibitors

Cancer cells communicate using chemical signals to grow and divide. These inhibitors interfere with the signalling molecules thereby inhibiting with further growth of tumour development. For e.g. Everolimus, bortezomib, dasatinib, erlotinib, gefitinib, imatinib, lapatinib, pazopanib, sorafenib, sunitinib temsirolimus, and vemurafenib are approved for clinical use.

1.8.3 Monoclonal antibodies

Antibodies targeting specific proteins that are overexpressed in cancer cells are targeted for therapy. For e.g. bevacizumab, rituximab, Trastuzumab and alemtuzumab are used clinically (Table 3).

Table 3: List of anti-angiogenic therapies their mechanism of action and limitations.

S.No	Drug	Disease	Action	Limitation
1.	Thalidomide (Contergan)	Melanoma	It inhibits NFκB, and TNF –α at the mRNA level.	Blood clots, reduction in blood cells, constipation, dizziness, numbness, nausea and loss of appetite (Eleutherakis-Papaiakovou et al. 2004).
2.	Lenalidomide (Revlimid)	Myeloma	Immunomodulator exact mechanism is not known.	Neutropenia, increased blood clot, decreases platelet count, nausea and vomiting (Galustian et al. 2009).
3.	Bevacizumab (Avastin)	Metastatic cancer like colorectal, breast, renal cell carcinoma, non squamous non-small cell lung cancer (NSCLC)	Humanised monoclonal antibody targeting VEGF.	Gastrointestinal perforation, non healing wounds, stroke, high blood pressure, severe nervous and kidney problem (Nalluri et al. 2008).
4.	Sorafenib Tosylate (Nexavar)	Thyroid cancer, liver cancer and kidney cancer	RAF/RAS signalling pathway is inhibited. It is also known to inhibit various tyrosine kinases receptor (VEGFR1 &R2).	Heart attack, bleeding problem, high blood pressure, wound healing problem, drug induced hepatitis (Strumberg 2005).
5.	Sunitinib (Sutent)	Kidney, renal carcinoma, gastrointestinal stromal tumours and non endocrine tumour.	Multi-kinase inhibitor.	Fatigue, thyroid problem, bleeding problem, blood clots, slow wound healing, diarrhoea, nausea.
6.	Aldesleukin (Proleukin)	Renal cell carcinoma	It is also known as IL-2, which stimulates body's immune system to fight against tumour.	Flu- like symptoms, loss of appetite, Fluid retention and low blood pressure (Yang et al. 2003).

1.8.4 Anti copper therapy for cancer treatment:

Cu has a role in angiogenesis thus Cu regulation can be used as a tool to regulate angiogenesis. Chelator reduces the affinity of Cu for protein and allows it to be more efficiently chelated. Penicillamine, tetrathiomolybdate and trientine are widely used Cu chelators (Goodman et al. 2004).

Elevated Cu levels have been reported in various types of human cancers including prostate, breast, colon, lung and brain. The important role of Cu targeted therapy is that it reduces VEGF levels thereby making them anti-angiogenic in nature. Cu chelation is also used in treating various neurodegenerative diseases, MD and WD. But, there are some limitations targeting angiogenesis as suppression leads to cardiac hypertrophy and that may lead to heart failure. In such cases Cu supplementation is useful in improving the disease condition. Therefore, a comprehensive understanding of a patient's condition before the implementation of Cu manipulation therapy must be done for different diseases (Xie and Kang 2009). Platinum (II) based drugs cisplatin, carboplatin are widely used as cancer drugs. Platinum accumulates inside the cell leading to intracellular toxicity and apoptosis (Tisato et al. 2010). Inspite so many new drug and novel approaches, targeting angiogenesis has been a big challenge. Newer approaches such as RNAi mediated therapy and peptide based approach are being tested in clinical trials so that they can be further developed into drugs.

1.9 RNA INTERFERENCE MEDIATED THERAPHY FOR ANGIOGENESIS

RNA interference (RNAi) is a mechanism of posttranscriptional gene silencing mediated by small interfering RNA (siRNA) that are chemically synthesised.

1.9.1 Processing and production of si RNA:

Silencing of the gene expression by siRNA involves homology-dependent suppression of the cognate mRNA. The string of 21 nucleotide double stranded siRNA combines with multiprotein RISC complex (RNA-induced silencing complex) (Baranova et al. 2011). The double stranded siRNA separates in such a way that the antisense strand remains attached with the RISC complex. This RISC complex further identifies the target mRNA in the cytoplasm thereby cleaving the mRNA and preventing the process of translation (Figure 9) (Li et al. 2006).

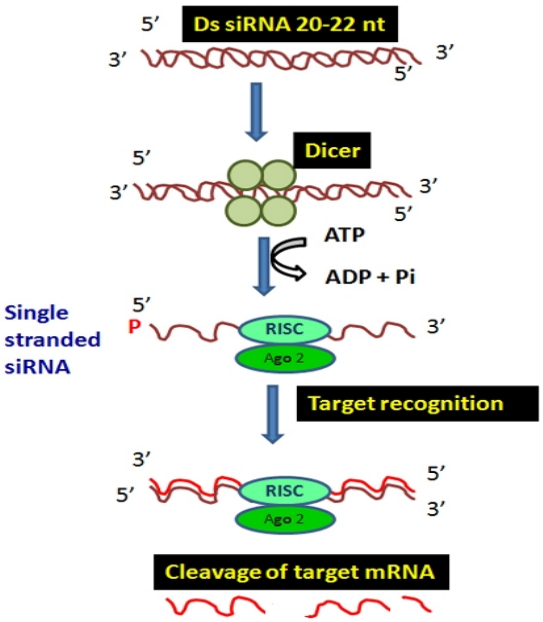


Figure 9: Schematic representation of siRNA interference technology.

1.9.2 Delivery of si RNA:

Delivery of siRNA remains a main challenge because of various physiological barriers. The half-life of unmodified siRNAs *in vivo* is short (seconds to minutes) and is due to their rapid elimination by kidney filtration (~ 7 kDa). Endogenously RNase degrades siRNA with a half-life of ~ 5 – 60 min. The siRNA half-life can be extended to days by complexing it with other molecules to bypass renal filtration (Figure 10) by chemical modification such as 2' -O-Methyl or by capping the ends of the siRNA to make them resistant to digestion and stabilise their action (Dykxhoorn et al. 2006).

Intravenous administration of siRNA
↓
Blood
Degraded by nucleases
↓
It is also cleared by the reticuloendothelial system (RES) comprising of liver and spleen (upto 200 nm)
↓
Further smaller sized (upto 5 nm) are removed by the kidney macrophages.
↓
Excreted in the urine

Figure 10: Schematic illustration of the limitations involved in systemic delivery of siRNA by intravenous administration are i) degradation in blood by action of nucleases, ii) uptake by reticuloendothelial system, iii) excretion from the kidney (Kawakami and Hashida 2007).

1.9.3 Different strategies used to deliver and achieve RNAi-mediated silencing *in vivo.*

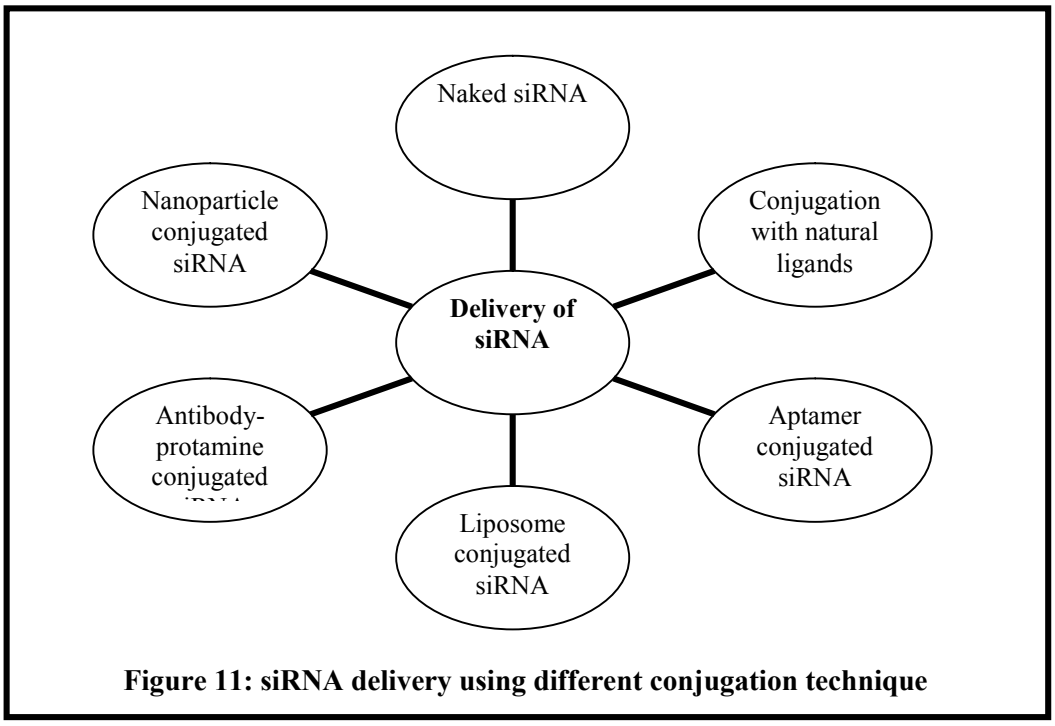


Figure 11: siRNA delivery using different conjugation technique

a) Direct injection of naked siRNA (unmodified or chemically modified) b) Direct conjugation to natural ligands e.g. Cholesterol and siRNA c) Aptamer–siRNA conjugation d) Liposome- Cationic lipids help in cellular uptake and endosomal release and also shield surface charges. Cholesterol and PEG are widely used in different ratios formulations. e) Antibody–Protamine fusion proteins have been used to non-covalently bind siRNAs through charge interactions and to deliver siRNA specifically to cells that express the surface receptor that is recognized by the antibody (de Fougerolles et al. 2007). f) Nanoparticle delivery of siRNA has improved the pharmacokinetics, pharmacodynamics, biodistribution. Particles ranging from 10–100 nm or as large as 200 nm, are the optimal size for a non-viral vector because they are large enough to be retained by the body yet small enough to access the cell surface receptors and pass through the cell membrane by receptor-mediated endocytosis. However, nanoparticles with a diameter greater than 100 nm are recognised by the reticulo-endothelium system (RES) and thus will be internalised and degraded (Figure11) (Guo et al. 2010).

1.9.4 Different modes of siRNA synthesis:

The next step for mammalian RNAi experiments is to produce the siRNA. Till date, various methods listed for production of siRNA are as under:

1. Chemical Synthesis,
2. In Vitro Transcription,

3. RNase II/ Dicer digestion of long ds RNAs,
4. Expressions from plasmids,
5. Expressions from PCR cassettes and
6. Expressions from viral vectors

The first two methods involve production of individual siRNAs either by chemical synthesis or by *in vitro* transcription. The major benefit of using chemically designed siRNAs is the ability to obtain large quantities of highly pure siRNA but the only drawback is its cost of synthesis. The siRNAs are generated by *in vitro* transcription from DNA templates that encode the two siRNA strands. The individual strands are then purified and hybridized to generate dsRNA molecules.

Plasmid vectors are also used for the continuous expression of siRNA and few of these also synthesize small hair pin RNA (shRNA). These vectors have polymerase III promoters site which help in siRNA expression *in vivo* (Scherr et al. 2007). There are various disadvantages for using plasmid vectors viz: difficult to introduce into tissues and certain cell types, the linearized plasmids do not readily integrate. Thus, viral vectors were developed as they were capable of infecting dividing cells as well as non dividing cells. Stable transfections are done using coxsackie, adeno and retro viruses (Spranger et al. 2001).

1.9.5 Different methods of siRNA detection:

Efficient intracellular delivery of siRNA is a significant hurdle to its therapeutic success. For biological studies on the efficiency of carrier-mediated uptake of siRNA, quantitative determination of the amount of internalized siRNA is required. There are various methods for intracellular detection of siRNA listed below:

1.9.5.1 Radioactive siRNA detection:

This method employs the labelling of the phosphate group with ^{32}P-labelled probe which is protected by nucleases activity. This helps in quantifying the internalised siRNA using liquid scintillation counter in mammalian cell extracts. This method is suitable for both double stranded and antisense siRNA (Overhoff et al. 2004).

1.9.5.2 Fluorescent siRNA detection:

Fluorescent labelling of siRNA with various fluorescent dyes can be performed and detected using confocal microscopy or FACS. **Cy 3, Cy 5 dye, Alexa and FITC** are

labelled at the 5'-end of the sense strand and cellular uptake and localisation studies are performed. Plasmid vectors, expressing green fluorescent protein tagged with siRNA are used to identify gene silencing (Grunweller et al. 2003). These labelling techniques help in identifying siRNA delivery internalisation , quantification and subcellular localisation for both *in vitro* and *in vivo* (Lingor et al. 2005).

1.9.5.3 PCR based approaches:

PCR-based siRNA quantification method is based on isolation of small RNA and converted to a cDNA. This target is further amplified using a specific primer and a universal primer. This can be done using Taqman specific probes and thus helping in absolute quantification of siRNA inside the cell. This technique unlike fluorescent method is highly specific as any mismatch or truncated siRNAs fail to amplify confirming sequence specificity and an ability to discriminate between degraded and non-degraded siRNA sequences (Pabla et al. 2009).

1.9.6 Disease targeted by siRNA delivery

Various diseases have been targeted for siRNA based therapy. These drugs are now being tested in clinical trials in combination with other drugs or standalone basis. Few of the siRNA that are being developed for therapy are listed in the (Table 4) below:

Table 4: List of siRNA in development for therapy

S.No	Disease	Target	Model	Mode of delivery	Drug name	Reference
1.	Dominant disorders- Pulmonary fibrosis	TGF-β1s	Mice model	Intra pulmonary delivery	-	(D'Alessandro-Gabazza et al. 2011)
2.	Neurological disorder - AD	β –secretase (BACE 1)	Mouse model	Injection in the bilateral hemispheres	-	(Singer et al. 2005)
3.	AMD	VEGF-R	Phase II	Intravitreal injection	siRNA-027	(Kaiser et al. 2010)
4.	Autosomal dominant- Pachyonychia congenita (PC)	Keratin 6a	Phase I	Injected into the bottom of the patient feet	TD101	(Leachman et al. 2010)
5.	Primary and secondary liver cancer	Serine/threonine- Polo like protein kinase 1(Plk1)	Phase I	Hepatic Intra-Arterial Administration	BI 2536	(Steegmaier et al. 2007)

1.9.7 Obstacles to Effective siRNA Therapeutics:

Inspite of its high specificity siRNA therapy faces major obstacles with the mode of delivery and stability. Sometimes siRNA induces innate responses through interferon's thus designing si RNA less than 30 nucleotides is suggested and sh RNA is shown to have less of innate responses (Shi et al. 2009). The other major limitation of siRNA is poor cellular uptake due to the strong anionic charge of the phosphate backbone and therefore, leading to electrostatic repulsion from the anionic cell membrane surface of naked siRNA (Reischl and Zimmer 2009). The physiological barriers also affect siRNA efficacy due to rapid excretion and low stability in serum (Shim and Kwon 2010).

Nevertheless, prospects for siRNA are substantially better than antibodies, due to their availability in plasma. Therapeutic targets for siRNA are many, as more than one mode of intervention can be used (transcription factors and intracellular molecules) or even in the absence on the structural knowledge of the target protein (Schiffelers et al. 2004; Seyhan 2011).

1.10 PEPTIDE BASED APPROACH TO TARGET ANGIOGENESIS RELATED DISEASES

Most of the anti-angiogenic peptides are derived from endogenous proteins. On the other hand, designing peptides by combinatorial approach followed by activity screening is also a feasible approach to obtain anti-angiogenic peptides. Such peptides can represent complete or partial protein functional modules as well as possible protein-protein interaction sites (Sulochana et al. 2005). Any polymer of two-or-more amino acids linked by amides can be termed as a peptide. Conjugation of small molecules, to a peptide leads to potential application of these molecules in the growing area of targeted cytotoxic agents for antitumor therapy. Small peptides have the advantage of easy production by synthesis using automatic peptide synthesizers (solid phase synthesis). They also express low antigenicity, high solubility in water, and improved bioavailability due to which they are potential drug targets and also developed as vaccines (Lien and Lowman 2003; Sulochana and Ge 2007). Since peptides are made up of amino acids they can be degraded by endogenous proteases. Thus, to increase the half-life of a peptide modifications such as glycosylation, conjugation with polyethylene glycol (PEG), substituting D-amino acids for L-amino acids to reduce susceptibility to certain proteases, or engineering the peptide to associate with serum albumin to limit proteolysis and filtration clearance from the body are done. PEG polymers can be readily conjugated to free amino group in a peptide, thereby

shielding the peptide from proteolysis by steric hindrance, and improving peptide solubility while reducing immunogenicity. Peptides are prepared by solid-phase or solution phase synthetic techniques. The purity of each peptide can be confirmed by high performance liquid chromatography (HPLC) and can be identified by mass spectrometry. An advantage of such peptide molecules is that they can be easily synthesized chemically and modified molecularly according to pharmacokinetic requirements.

1.10.1 Peptide based diagnosis:

Many tumors overexpress a variety of receptors for various peptides and these peptides can be used for diagnosis of tumours. For e.g. in patients with tumour, a radiotracer is injected that binds to somatostatin receptor which is specifically overexpressed in tumour tissues. The rapid internalisation of this helps in identifying tumour of even few mm size with the help of positron emission tomography (Reubi and Maecke 2008).

1.10.2 Anti-angiogenic peptides

Proteins functions through interacting with other molecules. Molecular modelling of protein structure has resulted in the rational design of peptides from proteins that control cell proliferation and proteins that block the growth of cancer cells selectively. Peptides containing arginine–glycine–aspartate (RGD) motif are the integrin recognition motif present in many proteins, which inhibit cell adhesion, migration, growth and differentiation (Zitzmann et al. 2002). Proteins such as fibronectin, fibrinogen, vitronectin and osteopontin contain these RGD motifs which further interact with intergrins to induce angiogensis. Cilengitide (Merck) is a RGD derivative cyclized pentapeptide cyclo-Arg-Gly-Asp-DPhe-(NMeVal) that is currently under phase II trial for the treatment of glioblastoma and refractory brain tumors in children (Mas-Moruno et al. 2010). Peptides from the ras-p21 protein block uncontrolled cell growth induced by oncogenic ras-p21 but leave the normal functioning of the wild-type protein intact. These peptides induced necrosis in different types of human tumors and blocked the growth of highly metastatic pancreatic tumor in nude mice. The translational implication of these results is that, both sets of peptides may be effective in the treatment of a variety of human cancers (Bowne et al. 2007). These proteins provide a rich source to design and derive small peptides to inhibit angiogenesis. By exploiting these properties, several small peptides have been designed synthetically and tested for therapeutic efficacy for the treatment of angiogenesis (Table 5).

Table 5: List of peptide developed to target angiogenesis

S. No	Disease	Target	Model	Delivery	Drug name	Reference
1.	Prostate cancer	Sequesters Ang I & II thereby inhibiting interaction with Tie 2 receptors	Phase II	Intravenous	AMG 386	(Berthold et al. 2005)
2.	Metastatic melanoma	ABT 510 derived from TSP1which activates caspase activity, inhibits fatty acid uptake, VEGF & FGF dependent neovascularisation.	Phase II	Subcutaneous	ABT-510	(Markovic et al. 2007)
3.	Anti-angiogenic	Suppressed migration and tube formation by targeting c-Src/ERK signaling pathways	HUVEC	-	KV11 Apolipoprotein - α	(Shi et al. 2009).
4.	Glioblastoma	Tyrosin kinase inhibitor	Phase II	Oral	AZD2171	(Batchelor et al. 2007)
5.	Anti-angiogenic	Designed peptide from the β - sheet domains of anti-angiogenic proteins, inhibits VEGF and induces apoptosis	Mice model	-	Angiex	(Griffioen et al. 2001)
6.	Anti-angiogenic	Decorin derived LRR5 ppetides inhibits VEGF levels	HUVEC	-	-	(Fan et al. 2008)

1.10.3 Peptides as vaccines:

Cancer treatment involving synthesis of vaccines identified from proteins expressed in specific tumour cells is now emerging. These peptide vaccines are given targeted to single protein or multiple proteins thus increasing the efficacy. Peptide vaccines targeting HER1 in tumours are helpful in inhibiting breast, lung, colon, head and neck carcinoma (Foy et al. 2013). Multi peptides in phase 1 clinical trial for NSCLC patients derived from VEGFR1, R2 receptors, lymphocyte antigen 6 complex locus K (LY6K), TTK protein kinase or cell division associated protein 1 in lung cancer were administered along with Freund's adjuvant (Suzuki et al. 2013). Not only cancers, but infections such as HIV can also be targeted using vaccines. HIV-1 therapeutic vaccine targeting conserved domains on p24Gag, in adults infected with HIV-1 are in phase 2 clinical trial (Pollard et al. 2014). However, peptide vaccines face few challenges like they have low immunogenicity. Use of adjuvants and epitope enhancements are being done to improve the use of peptides as vaccine (Thundimadathil 2012).

1.10.4 Metal binding peptides:

The amino terminal Cu (II) - and Ni (II) binding (ATCUN) motifs are found to be involved in DNA cleavage thereby having antitumor activity. The ATCUN motif in proteins constitute of histidine as a third residue. Four nitrogen atoms from these three residues act as metal ligands (Sankararamakrishnan et al. 2005). A histidine-rich peptide HSHRDFQPVLHL–NH2, similar to the N- terminal region of endostatin binding to Zn/Cu showed antitumor effect in 65 different tumour types (Kolozsi et al. 2009). Cu binding tripeptide glycine- histidine-lysine (GHK) functions as an antiaging, anti-inflammatory, anti oxidant and anti cancer activity (Pickart et al. 2012). These peptides bind to TNF α dependent IL-6 secretion thereby mediating an anti-inflammatory peptide (Gruchlik et al. 2012). Anti cancer property of GHK was due its potential to activate p63 a protein from the tumour suppressor family which lead to apoptosis.

1.10.5 Peptides as drug carriers:

Cellular internalization of large molecules such as proteins or nucleic acids is still a challenging task. The presence of plasma membrane acts as an impermeable barrier for such molecules. Peptides derived from membrane interacting proteins, such as fusion proteins, signal peptides, transmembrane domains and antimicrobial peptides that help in cellular delivery of macromolecules (Deshayes et al. 2005). These cell penetrating peptides (CPPs) promote the delivery and correct intracellular localization of a wide variety of peptides, proteins, and antibodies in their native, biologically active form into a broad spectrum of cell lines, without the need for prior chemical covalent coupling or denaturation steps. Few of the cell penetrating peptides have been listed below (Table 6):

Table 6: List of CPP used as cargos for delivering drugs

S.No	Peptide	Size	Biological function	Reference
1.	Penetratin family pAnt pISl	60- aa	Inhibits cell cycle progression and induction of apoptosis	(Lin et al. 2000; Terrone et al. 2003)
2.	Tat	101 aa	Macromolecules such as fusion proteins and nucleic acids are transferred by this peptide.	(Astriab-Fisher et al. 2002)
3.	Transportan	27 aa	Transfer of proteins, siRNA , double stranded oligopeptides and cross epithelial layers more efficiently than penetratin	(Morris et al. 2001)
4.	Amphipathic peptides(secondary and primary) These peptides consist of 2 domains: a hydrophilic (polar) and hydrophobic (non polar) domain. Most of secondary Amphipathic peptides adopt an alpha helical structure. Primary amphipathic peptides are the sequential assembly of hydrophobic and hydrophilic domains			
	i) secondary Model Amphipathic Peptides a. KALA	30 aa	promotes internalization of cargo	(Magzoub and Graslund 2004)
	ii)Primary a. MPG b.Pep – 1	27 aa 21 aa	DNA encoding β-galactosidase, luciferase or antisense DNA, are covalently linked to the primary amphipathic carrier.	(Simeoni et al. 2003)
5.	Poly Arg-	Arg_7 and Arg_9	for *in vitro* transfer of peptides	(Mitchell et al. 2000)
6.	Loligomer	21- 25 aa	transfer of cytotoxic compounds and genes	(Singh et al. 1999)
7.	hCT	(9-32) aa	transfer of green fluorescent protein through cellular membrane via a carrier cargo linkage	(Rennert et al. 2008)

1.10.6 Methods of evaluation:

Peptide delivery and entry of these drugs remains a crucial challenge in therapy thus monitoring the evaluating mode of delivery is critical. Morris *et al.,* and Richards *et al.,* have used **biophysical methods** such as NMR and CD spectra for membrane-interacting protein and peptide structures (Morris et al. 2008; Mueller et al. 2008). In **fluorescent based** approaches FRET (Forster resonance energy transfer) and fluorescent microscopy has been widely employed. Fluorescence spectroscopy is used for studying lipid–peptide and/or peptide–peptide interactions. The use of either intrinsic fluorescence of peptides based on amino acid residues like tyrosine and tryptophan can also be used for studying peptide based interactions. Fluorescently

labelled peptides allow the determination of the thermodynamic and kinetic parameters of peptide–membrane interactions. This also helps in the localisation of the peptide complex for effective delivery. However, there are few drawbacks such as fluorescent by cleaved fluorochrome might also emit florescence thus indicating false positivity. To overcome this drawback FRET is used (Deshayes et al. 2005). FRET has been used to quantify and monitor the entry of peptides into the cell and has the ability to resolve, molecular interactions, beyond the diffraction limit of conventional microscopy. When FRET occurs, a donor fluorophore excites an acceptor via a nonradiative dipole–dipole interaction if they are sufficiently close (within ~10 nm). This so-called 'molecular ruler' can be used to determine distances between labelled molecules inside cells, including gene carriers and nucleic acids. This further helps in studying the nanocomplex stability and dissociation (Grigsby et al. 2012). Other methods involving direct analysis of peptides are HPLC and Mass spectroscopy. CPP quantification by **HPLC** also helps in estimating the internalised fraction alone excluding the plasma membrane bound form (Oehlke et al. 1998). **Mass spectroscopy** protocols allow the accurate quantification of CPP cellular uptake by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) or LC MS. These methods allow determination of the amount of intact internalized peptide and the identification of potential intracellular digests. These methods also can be used to compare between various cargos used for peptide delivery and their uptake by the cell (Burlina et al. 2006; Jiao et al. 2009). Fluorescent probes may alter the biological activity of peptide, thus HPLC coupled with Mass Spectroscopy has an upper hand in quantification of CPP internalization.

1.11 GAP IN EXISTING LITERATURE

Angiogenesis is a complex process involving a tight molecular balance between pro and anti-angiogenic molecules in health and disease. Understanding the molecular mechanism in angiogenesis and interplay of transient interactive molecules are the current area of research to discover new drug targets. During angiogenesis Cu acts as a cofactor for many of these molecules such as VEGF, LOX and fibronectin and several other proangiogenic molecules are involved. In pathological condition like cancer and abnormal neovascularisation drugs are mainly targeted towards VEGF and its receptors. Inspite of so many drugs available in market, treatment remains a major challenge in

angiogenesis related diseases. Drugs such as avastin, sunitinib, sorafenib, thalidomide, Lenalidomide and aldesleukin have lead to resistance to therapy, side effects such as anemia and delay in wound healing problem. Cu chelators such as penicillamine and tetrathiomolybdate are widely used in cancer to remove excess Cu. But, even these compounds have limitations such as liver toxicity and also decrease antioxidant capacity.

The present work focuses on the role of CTR1 in angiogenesis. Therefore, siRNA and peptides were designed to target Cu mediated angiogenesis. Understanding the molecular mechanism is critical as several drugs have failed in clinic because of lack of complete knowledge. This thesis attempts to answer the following three questions

a) The role of CTR1 in angiogenesis?

b) Whether CTR1 siRNA / peptides can modulate angiogenesis?

c) If so what are the molecular events involved in CTR1 mediated angiogenesis?

Hitherto, siRNA for CTR1 have been targeted to understand its role in embryonic growth and drug transport. But CTR1 siRNA in Cu induced angiogenesis have not been studied so far. Crystal structure of CTR1 is not available in protein databank. Hence, to understand the structure – function of the protein, smaller peptides which have functional motif (Cu binding domain) were tested in HUVEC. These domains were identified from CTR1 – CP interacting regions. Studies in endostatin derived peptide rich histidine residues have been reported to be anti-angiogenic by binding to Zn. In this study, peptides rich in histidine and methionine were screened for Cu binding function and anti-angiogenic property.

CTR1 has been reported to be interacting with FGF, PDGF and Insulin soluble receptor 4 indicating its role in angiogenesis. In this report some new interacting partners have been identified which will help in understanding the interplay of molecules and how these molecules can also be used as therapeutic target to control angiogenesis.

CHAPTER 2: OUTLINE OF THE WORK

Angiogenesis is a process of development of new blood vessel from the existing vasculature. The balance between pro and anti-angiogenic molecules is very important to maintain homeostasis. Diseases such as cancer, diabetes, heart disease etc. are associated with angiogenesis. Cu is micronutrient required for various metabolic functions. Ceruloplasmin transports Cu from the liver to the cell. CTR1, a transmembrane protein, and functions as a major Cu importer at the plasma membrane. CTR1 transports Cu by forming a homotrimer which creates a central region of low electron density facilitating transport of one Cu atom at a time. This Cu is further distributed to other Cu chaperones like CCO, ATOX and CCS for cellular function and ATP 7 A&B exports Cu, thus maintaining cellular homeostasis.

In this study, first isolation of primary endothelial cells from umbilical vein was done. The method was established, cultured and used as a model for angiogenesis. HUVEC were exposed to Cu at different doses and time points to check at which concentration influenced angiogenesis in cells. The mRNA levels of Cu dependent proteins, involved in angiogenesis which are ceruloplasmin, CTR1, SPARC and LOX and their isoforms were screened. Out of 4 proteins, CTR1 showed greater response to Cu. Thus, further experiments were designed to study the role of CTR 1 in angiogenesis using HUVEC model.

Concentration of Cu at 100 μM increased migration, tube formation, proliferation and adhesion of HUVEC. Cu concentration of 100 μM increased CTR1 expression at the level of mRNA and protein. Since, there was no report of CTR1 mRNA or protein in ocular tissues it was confirmed by real time PCR and western blot. Protein expression of CTR1 was done by ELISA, western blot and confocal microscope showed presence of CTR1 in all ocular tissues. Eales' disease was selected as a model since it is associated with peripheral neovascularisation besides excess Cu accumulates in vitreous of Eales' patient has been reported.

To silence CTR1, siRNA targeting CTR1 were designed using Ambion web tool. Three siRNA were selected based on the score (Reynolds *et al*) and tested in HUVEC in the presence of Cu. Out of the three siRNA (si 1) showed better, inhibition in terms of reproducibility when compared to others. CTR1 siRNA decreased mRNA, protein

expression and also inhibited tube formation, migration, proliferation and intracellular Cu levels. Its anti-angiogenic effect was also confirmed *in vivo* CAM assay model and by corneal pocket assay in rabbit.

Further, peptides were designed from N-terminal region of CTR1 interacting with ceruloplasmin. Three peptides were designed and screened in HUVEC model. Rhodamine labelled peptides showed no entry of the peptides into the cell. These peptides bound to Cu and chelated extracellular Cu thereby inhibiting angiogenesis. Further, these peptides were found to be anti-angiogenic when screened *in vivo* using CAM model.

These data indicated that CTR1 had an important role in angiogenesis. The mechanism behind how CTR1 plays a role in angiogenesis through its interacting, molecules is still unclear thus immunoprecipitation (IP) for CTR1 was done. IP results indicated gelsolin and caldesmon as important proteins involved in CTR1 dependent angiogenesis.

OBJECTIVES:

1. Establishment of HUVEC cell and screening for Cu responsive molecules related to angiogenesis in HUVEC model.
   a) Establishment of HUVEC culture.
   b) Screening of copper responsive molecules.
   c) Selection of Cu concentration.
   d) Validation of Cu responsive molecule.

2. Standardisation of copper concentrations for angiogenesis in HUVEC model.
   a) Cytotoxicity assay
   b) Angiogenesis assay – migration and tube formation assay

3. Relevance's of increased Cu and CTR1 protein levels in ocular disease (Eales' disease).
   a) To identify the CTR1 mRNA and protein expression in ocular tissues.
   b) Its relevance in angiogenesis related disease (Eales' disease).

4. To design siRNA targeting copper transporter 1 (CTR1) and screen them *in vitro* using HUVEC cells and *in vivo* by rabbit corneal packet assay for their anti-angiogenicity and measure the intracellular copper level.

a) Design of siRNA to target CTR1.
b) Selection of siRNA and its concentration.
c) Standardization of different time points for optimum mRNA expression of CTR1.
d) Bioavailability of CTR1 siRNA in HUVEC.
e) CTR1 silencing inhibits protein synthesis.
f) Effect of CTR1 siRNA on other copper modulators.
g) CTR1 siRNA reduces intracellular Cu level.
h) *In - vitro* angiogenesis assays to test the effect of CTR1 silencing on the inhibition of angiogenesis.
i) *In vivo* assay to test the efficacy of the developed antisense si RNA.

5. To design short peptides using bioinformatics tools from ceruloplasmin CTR1 binding region and to test their anti-angiogenic effect *in vitro* using HUVEC.
   a) To design short peptides using bioinformatics tools from ceruloplasmin CTR1 binding region and to test their effect in inhibiting angiogenesis.
   b) Cytotoxicity assay of CTR1 derived peptides. Standardisation of peptide concentration for angiogenesis assay.
   c) *In vitro* angiogenesis assay.
   d) Copper binding function of CTR1 derived peptides.

6. To identify the interacting partners for further understanding the mechanism.

CHAPTER 3: MATERIALS AND METHODS

3.1 ISOLATION OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECs)

Materials required:

- T25 vented flask (Nunc)
- Fibronectin: Commercially available 1 mg/ml (Sigma) was diluted to 10 µg/ml concentration in PBS and used for coating culture dishes.
- Collagenase: Collagenase (Sigma) 2 mg was dissolved in 10 ml of 1X PBS and filter sterilized. Aliquots of 1 ml were stored at -20 °C until use. Working collagenase concentration of 0.2 mg/ml was made upto 10 ml and used for cord processing.
- Antibiotic solution: Commercially available antibiotic mixture (Gibco) 100 X was diluted to 1X in PBS.
- Artery forceps - 2 nos.
- Drape - 1 no.
- Glass petriplate - 2 nos.
- Scissor - 1 no.
- 10 ml syringes - 2 nos.
- 21 gauge needle - 1 no.
- Butterfly needle - 1 no.
- Glass beaker - 1000 ml
- Pair of surgical gloves - 2 no.
- 50 ml Tarson tubes - 1 no.
- Phosphate buffered saline: NaCl- 8 g, Na_2HPO_4- 1.15 g, KH_2PO_4- 0.2 g, KCl- 0.2 g. Adjust to pH between 7.2- 7.4.
- Endothelial growth medium 2 {EGM- 2 medium (Lonza)}

Procedure:

Ethics statement: All the protocols involving the collection and processing of human samples were strictly adhered to the tenets of Helsinki declarations, and were approved by the Institutional Review Board of Vision Research foundation (IRB – VRF) where the study was conducted. Reference number -150-2009-P is the IRB-VRF approval number

for this work. Written consents were obtained from mothers who volunteered to donate their umbilical cord for research.

This method was modified from Baudin et al., (Baudin et al. 2007)

1. Cords were procured and processed within 3 h. All the reagents were kept in 37°C water bath before use.
2. To 500 ml of prewarmed PBS, 500 µl of antibiotic solution was added.
3. The cords were washed externally to remove RBCs.
4. A butterfly needle was inserted into one end of the vein and clamped using artery forceps. The cord was internally washed with PBS until the wash appeared to be colorless. The cord was also checked for any clots or leaks while washing. If the cord were very long they were cut into pieces of suitable length and then processed.
5. Collagenase solution was added through the veins after clamping the cord on both sides and then gently massaged.
6. The cord was then incubated for 20 min at 37°C water bath in a beaker containing PBS.
7. After incubation the cord was gently massaged to dislodge the cells from the vein.
8. The clamp was removed from one end, then 10 ml PBS was allowed to flow through the cord gently thereby collecting the dislodged cells. Cells were collected in 5 ml of EGM medium in a 50 ml falcon tube. Centrifuged for 10 min at 1500 rpm.
9. The supernatant was discarded and the cell pellets were resuspended in 5 ml of EGM2 medium.
10. The cells were seeded in fibronectin coated flask and incubated at 37°C in 5 % CO_2.
11. After 2-3 h, change of medium was done to remove unattached cells and incubated till the cells were grown to confluency.

3.2 TRYPSINISATION OF HUVECs

Materials required: (filter sterilized)

- Trypsin – 100 mg
- EDTA- 30 mg
- Glucose – 500 mg
- PBS – 100 ml

Principle: Cells were grown to 80-90 % confluency in tissue culture flask containing 5 ml of EGM2 medium. The process of trypsinisation helps in disaggregating the cells into a single cell from a monolayer. Thus, removing cells from the primary culture and transferring them to secondary cultures constitutes a passage, or subculture. Endothelial cells of passages 3- 5 were used for all experiments.

Procedure:

1. Medium from the T25 flask was removed and cells were washed with PBS and 0.5 ml of trypsin was added. Cells were incubated at 37°C incubator for 2-3 min.
2. The entire solution from the flask was transferred to a 15 ml falcon tube and centrifuged for 5 min at 1500 rpm.
3. The supernatant was removed and the pellet was resuspended with 1 ml of EGM medium.
4. Cells were counted using haemocytometer.
5. The required number of cells were seeded onto 0.1 % gelatin coated dishes for further experiments.

3.3 GELATIN PREPARATION

Gelatin 100 mg (Sigma) was dissolved in 100 ml of PBS and sterilized at 110°C in 110 lbs pressure for 15 min. This was used for coating tissue culture dishes / flask for HUVECs from passage 2- 5.

3.4 CRYOPRESERVATION OF HUVECs

A cryo protective agent, dimethyl sulfoxide (DMSO), was used in conjunction with complete medium for cryopreserving cells. DMSO acts to reduce the freezing point and allows a slower cooling rate. Gradual freezing reduces the risk of ice crystal formation and cell damage. Cells grown to confluency were trypsinised and pellet were resuspended in EGM medium with 20 % FBS and 10 % DMSO. Cells were kept in one degree step down coolant and later stored in liquid nitrogen. The number of cells were counted using hemocytometer and diluted with accordingly to obtain 10^6 - 10^7 cells / ml.

3.5 HUVECs RETRIEVAL

When the cryopreserved cells were needed for study, each vial was taken out, thawed rapidly to prevent formation of ice crystals and plated at high density to optimize recovery.

Cells taken from liquid nitrogen were thawed at 37°C. Fresh 5 ml of EGM medium was added and centrifuged at 1500 rpm for 10 min. The cell pellet was resuspended in EGM medium and seeded onto gelatin coated culture dishes.

3.6 CELL COUNTING

Materials Required:

- Trypan Blue (0.4 % in PBS)
- Haemocytometer

Principle: It is a dye exclusion method where the membrane of the live cells excludes the trypan blue and the cell remains unstained. Dead cell membrane is permeable to the dye and stains blue.

Procedure:

1. Cell suspension of 20 µl was equally mixed with dye and 10 µl of it was loaded.
2. The total count of cells in the four corners (WBC counting squares) was counted.
3. The numbers of dead and live cells were counted separately to calculate the % viability.

Calculation

No of cells counted = *X*

Therefore cells /ml Y= *X**2 (dilution factor) *10^4

Total no. of cells = *Y* * volume of the total medium trypsin inactivation.

10^4- 1000*10 (i.e. 1 µl - 1000 µl conversion, depth factor)

3.7 SERUM STARVATION OF HUVECs

Cells were grown to required confluency in 6 well plates; the medium was removed and 2 ml of 1 % EBM medium was added. Serum starvation was performed for 4 h at 37°C in 5 % CO_2 incubator.

3.8 PREPARATION OF COPPER AND PENICILLAMINE

Copper chloride (sigma) 1 mg was weighed and dissolved in serum free medium to make upto 1 mM stock. The stock was filter sterilised and then diluted to 10 nM - 500 µM concentrations in 1 % EBM medium. Penicillamine, a known Cu chelator was used as a

positive control. Penicillamine (P) stock of 1 mM was prepared in PBS and specified concentrations were used in the cell culture experiments.

3.9 MTT ASSAY

Materials Required:

- 96 well plate
- MTT- 5 mg/ml in PBS {(3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), Invitrogen}
- DMSO (Merck)

Principle: The MTT assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystal by metabolically active cells. After exposure cells were treated with MTT and the formazan crystal formed were dissolved in DMSO and read at 570 nm in spectra max fluorescent plate reader (Molecular devices, USA) (Mosmann 1983).

Procedure:

1. Cells were plated into 96-well plates. Cell densities of 5,000 to10,000 cells/ well were seeded and appropriate conditions were exposed.
2. After the exposure, 20 µl solution of MTT with 180 µl of medium was added to wells and incubated for 4 h at 37°C.
3. The medium was removed and 200 µl DMSO was added into each well to dissolve the formazan crystals. Absorbance was measured on plate reader (Spectra max M2^{e}) with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain sample signal (OD 570 – OD 630).

3.10 MIGRATION ASSAY

3.10.1 Wound healing assay:

Materials required:

- 0.1 % gelatin
- 6-well plates
- HUVECs
- EGM medium
- 1 % EBM medium

- 4 % Para formaldehyde – 0.4 g of paraformaldehyde (Merck) dissolved in 10 ml of 1 X PBS at 60°C for 30 min was freshly prepared.
- PBS

Principle: Cell migration is a process induced by various growth factors and chemokines that are associated with complex signalling. Cell migration occurs in normal condition of wound healing, cell differentiation and embryonic development and pathological condition like tumour and metastasis.

Procedure:

1. A wound was created in monolayer of cells with a sterile 200 µl tip in a 6-well plate.
2. Cells were exposed to the appropriate conditions and kept at 37°C incubator for overnight.
3. The medium was removed and 500 µl of 4 % paraformaldehyde was added to all wells and kept at room temperature for 30 min for fixation.
4. Cells were washed with PBS twice gently. Photographs were taken from 3 different fields and analyzed (Liang et al. 2007).

3.10.2 Transwell migration assay:

Materials required:

- Transwell inserts- Millipore
- 24 well tissue culture dishes
- HUVECs
- Gelatin (0.1 % solution)
- PBS
- Paraformaldehyde (4 % solution)
- Giemsa stain - Stock solution
  - Dissolve 3.8 g of Giemsa powder into 250 ml of methanol
  - Heat the solution from step 1 to ~ 60^{o}C
  - Slowly add in 250 ml of glycerol to the solution from step 2 and filter the solution from step 3.
  - The solution needs to stand a period of time prior to use.

Working solution: Add 10 ml of stock solution to 80 ml of distilled H_2O and 10 ml of methanol. Nucleus is stained from blue to violet and cytoplasm appears pale blue in colour.

Principle: Transwell migration assay is routinely used for studying the motility of different types of cells including metastatic cancer cells. The assay helps in screening compounds that are chemoattractant or inhibitors of chemotaxis. The micro-porous membrane usually acts as a support. Cells on one side of the membrane migrate to other side in the presence of chemoattractant. Migrated cells can be quantified by fixing, staining and counting them under the microscope (Zarrabi et al. 2011).

Procedure:

1. The cover sheet was peeled off from the hanging multicell inserts. Sterile forceps were used to remove the insert from the pack and placed it into a culture plate well.
2. Wells were coated with 0.1 % gelatin and then the cells treated with appropriate conditions were added to the upper chamber of the insert and were incubated at 37°C for 8 h.
3. The medium was removed and cells were fixed with paraformaldehyde for 30 min at room temperature.
4. Cells were stained with working giemsa for 30 min. The upper part of the transwell was cleaned to remove the unmigrated cells. The migrated cells in the lower part of the transwell were photographed at 5 different fields and counted using image J software (NIH).

3.11 TUBE FORMATION ASSAY

Materials required:

- 96-well culture plates- kept at 4 °C
- ECM matrix- Millipore - kept at 4 °C
- Microtip - kept at 4 °C
- EGM medium
- HUVECs

Principle: The assay measures the ability of endothelial cells to form capillary (tube) like structure in the presence of extracellular matrix support when plated at sub confluent densities. This assay helps in screening pro and anti-angiogenic molecules. Upon plating, endothelial cells attach and generate mechanical forces on the surrounding extracellular

support matrix to create tracks or guidance pathways that facilitate cellular migration. The resulting cords of cells will eventually form hollow lumens. The comparisons between cells with and without treatment are measured in parameters such as tube length, size and number of junctions.

Procedure:

1. All the plates and consumables were pre cooled at 4°C. The ECM matrix 1 ml was mixed with 100 µl of 10X ECM buffer.
2. ECM gel solution of 50 µl was added to each well and incubated for 30 min at 37°C.
3. Cells were trypsinised and counted. Cells ranging from 15000-30000 cells / well were incubated in desired culture medium along with molecules to be tested for 30 min at 37°C.
4. Then 150 µl of the culture medium with cells were added to the well and incubated at 37°C for 8 h. The plates were examined for endothelial tubes under light microscope.
5. The medium was carefully removed without disturbing the tubes and fixed it with 4 % paraformaldehyde.

3.12 PROLIFERATION ASSAY

Materials required:

- 6-well plates
- Tritiated thymidine – Tritiated thymidine (H^3) was dissolved in 10 ml of serum free medium (EBM) and kept for boiling till 10 min, the mixture was then filter sterilized and appropriate concentration was added to cell culture.
- EBM medium
- HUVECs
- Lysis buffer- NaOH (0.2 N) was dissolved in 1 % SDS
- Scintillant preparation:
    - Toluene- 666 ml (Merck)
    - Triton X 100- 322 ml (Merck)
    - POP (2, 5-diphenyloxazole) - 5 g
    - POPOP (1, 4-bis (5-phenyloxazol-2-yl) benzene) - 0.15 g

Principle: The process of liquid scintillation counter relies on the transfer of kinetic energy from the beta particle to the solution components through a series of energy transformations. The released ultraviolet light excites fluor molecules in the solvent. The fluor molecules will then release the energy in the UV-Visible range of energy of 300-450

nm. Sometimes, the primary scintillators (POP) are not sensitive in the region of fluorescence emission. Thus, a wavelength shifter (secondary scintillator - POPOP) was introduced which absorbs energy from the primary scintillator and emits light at a wavelength more suitable to the photomultiplier tubes.

Proliferation assay: Cell proliferation is measured with the amount of radioactive thymidine uptake by the cell which indicates the newly synthesized DNA.

Procedure:

1. Cells were seeded onto 24 well plates (3,000 / well). Cells were grown till 60 % confluency and serum starved with 1 % EBM for 4 h.
2. Then cells were exposed to appropriate conditions; H^3 100 nCi was added to the medium and grown in 5 % EBM medium.
3. Cells were kept at 37°C in 5 % CO_2 incubator and covered with aluminum foil.
4. After 48 h, cells were washed in PBS twice and 0.2 ml of lysis buffer was added to each well.
5. The lysate was transferred to a vial and heated for 1 h at 60 °C.
6. Scintillation liquid 3 ml each was added to the tubes and 0.1 ml of lysate was added.

3.13 ATTACHMENT ASSAY

Materials required:

- 0.1% gelatin
- 6-well plates
- HUVECs
- EGM medium
- 1 % EBM medium
- Para formaldehyde (4 %) PBS

Principle: Cell attachment is a process involved in angiogenesis. During tumour or wound healing, cells express a lot of molecules with adhesive property thereby influencing tumorogenic property.

Procedure:

1. Cells were trypsinised and 50,000 cells were taken in each vial.

2. Cells were exposed to the appropriate conditions and kept at 37°C incubator for 30 min.
3. After incubation, cells were allowed to attach for 15 min in 0.1 % gelatin coated 24 well plates.
4. Unattached cells were washed carefully with PBS. Attached cells were fixed with 4 % paraformaldehyde and stained with giemsa.
5. Photographs were taken from 3 different fields and analyzed using Image J cell counter (NIH).

3.14 INTRACELLULAR COPPER ESTIMATION

3.14.1 Phen Green staining: Fluorescent Activated Cell Sorter Method:

Materials required:

- Cells
- 6 well plate
- Vials
- EGM
- 4 % Paraformaldehyde

Phen green FL (Invitrogen) – 1 mg / ml was dissolved in DMSO. Required concentration was diluted in serum free medium.

Principle: Fluorescence-activated cell sorting (FACS) (BD) is a specialized type of flow cytometry which is modified coulter principle. It is used for sorting cells from heterogeneous mixture based upon the light scattering and fluorescent characteristics of each cell. The technique was modified by Len Herzenberg, and coined the term FACS. This instrument uses a laser light of single wavelength which is directed onto hydrodynamically focused stream of liquid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescence detectors.

Cu estimation using Phen green: Phen green is a fluorescent dye, which quenches fluorescence when it's bound to Cu (Lou et al. 2010). This was measured as median fluorescent index at excitation / emission – 490 nm / 528 nm.

Procedure:

1. Cells grown were serum starved for 4 h in 6-well plates.

2. Appropriate dosage and exposure were given to cells.
3. After exposure, cells were trypsinised and taken in suitable vials which were incubated with 5 µM of Phen green dye (binds to Cu) for 30 min at 37°C with intermittent mixing.
4. Cells were washed twice in PBS and resuspended in PBS, 1×10^4 cells were gated and analyzed by FACS calibur (BD, USA).

3.14.2 Atomic absorption spectroscopy :

Materials required:

- HUVECs
- 6 well plate
- Vials
- EGM
- Nitric acid - 0.2 % HNO_3 prepared in MQ H_2O.
- Lysis buffer – Nitric acid: Perchloric acid (5:1) (Merck)

Principle: Atomic Absorption Spectroscopy (AAS) (Perkin Elmer AA700) is used principally for the quantitative determination of metal elements. This technique employs absorption spectrometry which follows Beer- Lambert law to assess the concentration of an analyte in a sample by plotting against a known standard curve. Every element has a specific number of electrons associated with its nucleus. They normally remain in the ground state but when energy is applied to an atom electrons of the atoms reach higher orbital or excited state. Since it is highly unstable electrons liberate energy and return to the ground state. These wavelengths are unique for each element and thus act as a specific determination of each element.

Procedure:

1. Cells grown were serum starved for 4 h in 6-well plates.
2. Appropriate dosage and exposure were given to cells.
3. After exposure, cells were washed twice in PBS.
4. Cells were lysed in 500 µl of perchloric acid and nitric acid.
5. Samples were completely ashed and then the ashed material was mixed with 1 ml of 0.2 % nitric acid. Then samples were centrifuged at 5000 rpm for 10 min to remove debris and the supernatant was taken for analysis.

6. Cu was estimated at 324.8 nm using a hallow cathode lamp. The slit was maintained at 0.7 nm, Cu was atomized at 2300°C and detected using graphite furnace system.
7. System was calibrated with standards prepared in 0.2 % nitric acid.

3.15 APOPTOSIS ASSAY- CELL DEATH ELISA

Materials required:

- HUVECs – Sample was prepared by adding the lysis buffer to cell grown on 6 well plates. Cells were incubated with 500 µl of lysis buffer for 30 min at room temperature. The sample was centrifuged at 14000 rpm for 10 min. The supernatant was carefully taken and used for the assay.
- Cell death ELISA kit (Roche)

Principle: A programmed cell death occurs usually by necrosis (osmotic lysis) or apoptosis (zeiosis). Apoptosis is characterized by membrane blebbing, where cytoplasm condenses and activation of endogenous nucleases occurs. The endogenous nucleases are dependent on Ca^{2+} and Mg^{2+} ions which act upon intracellular DNA to form mono and oligo nucleosomes. Apoptosis assay is based on sandwich ELISA technique using mouse monoclonal antibody towards DNA and histones. This assay allows specific determination of mono and oligo nucleosomes of the cytoplasmic fraction of the cell lysate.

Procedure:

1. Coating solution (100 µl) anti - histone antibody was added to the 96 well plates. The plate was then covered with foil and incubated for overnight at 2-8°C or 2 h at 37 °C.
2. After removing the coating solution, 200 µl of incubation buffer was added and kept for 30 min at room temperature.
3. Three washes with 250 - 300 µl of wash buffer were done.
4. Sample solution of 50 µl was added to appropriate wells and incubated for 90 min at room temperature.
5. The washing step was repeated and 100 µl of substrate was added to the wells and incubated for 20 min in dark at room temperature.
6. Colour developed was recorded using a plate reader at 405 nm and 490 nm.

3.16 RNA EXTRACTION

Materials required

- TRIzol (Sigma)
- DNA RNAase free H_2O, tips and vials

- Cooling centrifuge
- Chloroform (Merck)
- Iso propanol (Merck)
- Ethanol (Merck) – 70 % made in DEPC treated H_2O

Principle: TRI Reagent has phenol and guanidine thiocyanate in a monophase solution to facilitate the immediate RNAase activity. Biological samples are homogenized or lysed in TRI Reagent; the subsequent addition of chloroform results in the separation of the homogenate into aqueous and organic phase. RNA partitions to the aqueous phase, DNA to the interphase, and protein to the organic phase. The RNA can then be precipitated from the aqueous phase with the addition of iso propanol. The isolated RNA is suitable for any downstream application, including RT-PCR.

Procedure:

1. Cells were trypsinised and centrifuged at 1500 rpm. To the pellet, 1 ml of TRIzol reagent was added and incubated at room temperature for 5 min.
2. Chloroform 200 µl was added and mixed well for 15 sec and incubated at room temperature for 3 min.
3. It was then centrifuged at 12000 rpm for 15 min.
4. The aqueous layer was transferred to a new vial without disturbing the protein layer.
5. Iso-propanol (2- propanol -500 µl) was added and mixed well by inverting and incubated at room temperature for 10 min and step 4 was repeated.
6. The supernatant was discarded and 1 ml of 70 % alcohol was added to the pellet and incubated at room temperature for 2-3 min.
7. It was then centrifuged at 14000 rpm for 5 min and supernatant was discarded air dried for 3 min.
8. Finally, 20 µl of RNAase free H_2O was added and stored at - 80°C (2 µl was taken for quantification and gel electrophoresis)

3.17 CONVERSION OF RNA TO CDNA

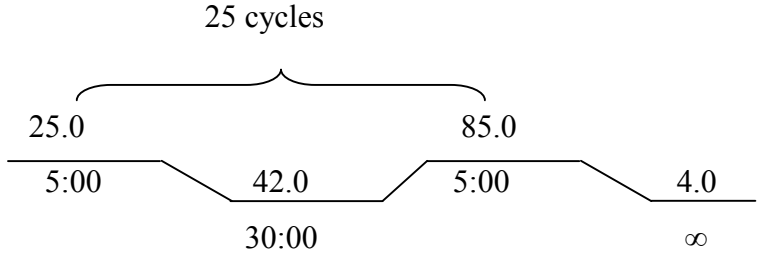
RNA was converted to cDNA using iScript RT-PCR Kit (Bio-Rad) following the manufacturer's protocol using the reagents provided in the kit.

Procedure

Step 1: The following reaction mix was prepared

RNA Template	-	2 µl (1 µg)
Nuclease free H_2O	-	13 µl
5x I script Reaction Mix	-	4 µl
Reverse Transcriptase	-	2 µl
		20 µl

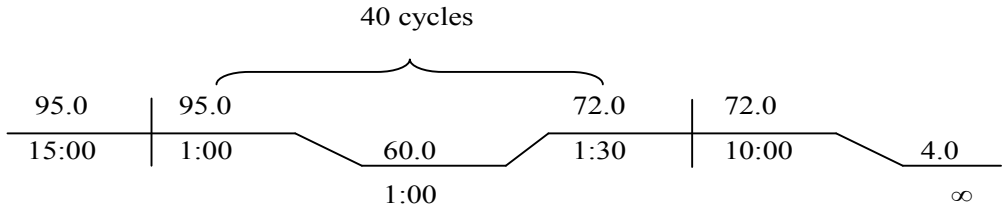
Step 2: The following protocol was followed for cDNA conversion



3.18 POLYMERASE CHAIN REACTION

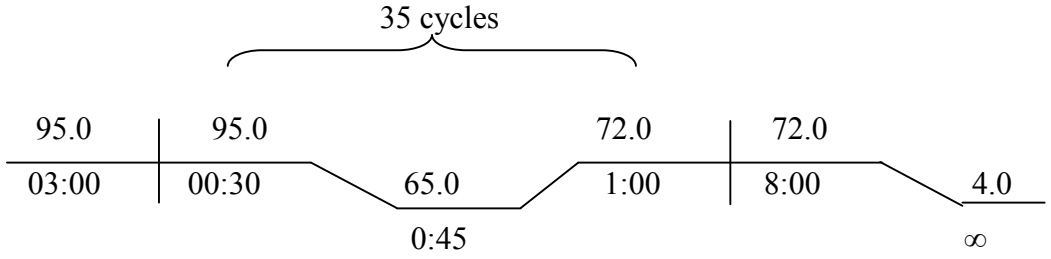
The polymerase chain reaction (PCR) is a biochemical technology in molecular biology used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

PCR protocol for LOX, LOX L1 and LOX L2



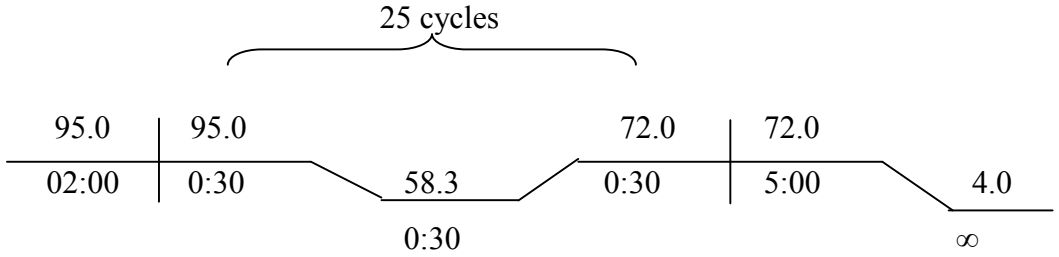
Product size: 158 bp

PCR protocol for SPARC



Product size: 216 bp

PCR Protocol for CTR1 & CP



Product size: 237 bp

Product size of CP: 110 bp

3.19 REAL TIME PCR

Quantification of RNA was done using nanodrop. Quantitative real-time PCRs were performed using Applied Biosystems 7300 with SYBR Green chemistry (Eurogentech, Belgium). Real-time PCR cycle conditions included the following steps: denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 25 sec. Each sample was run in triplicate and C_t was determined for the target transcripts. Real time calculations were done using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Table 7: List of primers used for this study.

S.No	Target gene	Forward Primer	Reverse primer
1.	CTR1 (RT primer) (NM_001859.3)	5'- CTT AGA CTG GCT GCC AAA GG- 3'	5'- AGA GTA AGG GGGGGC CAA AGA A- 3'
2.	CTR1 (NM_001859.3)	5'- GCG TAA GTC ACA AGT CAG CAT TC- 3'	5'- GCG TAA GTC ACA AGT CAG CAT TC- 3'
3.	CP (NM_000096.3)	5'- ATC CGT GGG AAG CAT GTT AG-3'	5'- CTT CAG GGC CTC TCT CCT TT- 3'
4.	VEGF (NM_0003376.5)	5'- CGG TAT AAG TCC TGG AGC GTT C-3'	5'- GCC TCG GCT TGT CAC ATC TG- 3',
5.	SPARC (NM_003118.3)	5'- GCT GGA TGA GAA CAA CAC- 3'	5'- AAG AAG TCG CAG GAA GAG- 3'
6.	LOX (NM_002317)	5'- ACG GCA CTG GCT ACT TCC AGT A - 3'	5'- TCT GAC ATC TGC CCT GTA TGC T - 3'
7.	LOX L1 (NM_005576)	5'-TGT ACC GGC CCA ACC AGA A- 3'	5'AGA CAC TTC TCC TCC GCA GCA 3'
8.	LOX L2 (NM_002318)	5'-ACT GCA AGC ACA CGG AGG A- 3'	5'-AGG TTG AGA GGA TGG CTC GA- 3'
9.	GAPDH (NM_002046)	5'- GAA CAT CAT CCC TGC CTC TAC TG - 3'	5'- CGC CTG CTT CAC CAC CTT C - 3'.
10.	ATP 7A (NM_000052.6)	5'- CCU UCU ACU UUG GCU UUA A - 3'	5'- UUA AAG CCA UAG AAG G- 3'

3.20 PROTEIN ESTIMATION

3.20.1 *Bradford method*: Brilliant Blue G-250 dye exists in three forms: cationic (red), neutral (green), and anionic (blue). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (λ= 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (λ= 595 nm) which is detected using a spectrophotometer or microplate reader.

Materials:

- Bradford- thermo scientific
- Sample
- Normal saline – 0.9 % NaCl in distilled H_2O

Procedure:

1. To a 96 well plate, 10 µl of the sample, lysis buffer and standards were added and 90 µl of normal saline was added.
2. Bradford reagent 100 µl was added and kept for incubation for 10 min at room temperature and read at 595 nm.
3. Standard curve was plotted against known standard concentration.
4. Samples were extrapolated onto the standard curve to calculate the protein concentration.

3.20.2 *Bicinchoninic acid assay*:

Materials:

- BCA kit- thermo
- Sample

Principle:

This assay is based on the principle reduction of Cu^{2+} ion to Cu^{+} by the peptide bonds in proteins which are dependent on temperature. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Further the two molecules of BCA chelate with each Cu^{+} ion, forming a purple-colored product that absorbs light at a wavelength of 562 nm.

Procedure:

1. Solution A of 5ml was mixed with 100 µl of solution B and 200 µl were added to 96 well plates.
2. Standards (125 – 1000 µg / ml) and samples 5 µl volume were added to the well. Normal saline 5 µl was added to blank well.
3. The plate was kept for incubation for 30 min at 37°C and read in plate reader at 562 nm.
4. The standard curve was plotted and the unknown samples protein concentrations were calculated by extrapolating the values in the standard graph.

3.21 WESTERN BLOT

It is an analytical method wherein a protein sample is electrophoresed on an SDS-PAGE and electro transferred onto nitrocellulose membrane. The transferred protein is detected using specific primary antibody and secondary enzyme labelled antibody (HRP) and chemiluminescent substrate.

Materials required:

- Acrylamide (30 %): Acrylamide - 29.2 g, Bisacrylamide - 0.8 g, H_2O - 100 ml.
- TRIS-HCl (pH 8.8) - 18 g of Tris dissolved in 50 ml of MQ H_2O and made upto 100 ml (adjust pH with concentrated HCl)
- TRIS-HCl (pH 6.8) - 6 g of TRIS dissolved in 100 ml of MQ H_2O; pH was adjusted using concentrated HCl.
- Electrophoresis buffer (pH 8.6) - 3 g of Tris, 15 g of Glycine and 1 g of SDS was added in 1000 ml of MQ H_2O.
- Laemmli buffer (2X): 10 % SDS – 4 ml, Glycerol – 2 ml, 1M Tris (pH 6.8) – 1.2 ml, H_2O – 2.8 ml and (0.02 %) bromophenol blue.
- 10 % Ammonium per sulphate (APS) in distilled H_2O
- 10 % Sodium dodecyl sulphate (SDS) in distilled H_2O
- TEMED
- TBS (Tris buffered saline)
- Transfer buffer – Same composition as running buffer except addition of 200 ml of methanol made up to 1000 ml using DH_2O. Stored at -20°C.
- Blocking buffer - 5 % skimmed milk (non fat dry milk) prepared with 1X TBST.
- Washing buffer-TBST: 0.1 % tween-20 in 1X TBS
- Secondary antibody
- Luminescent Mixture – Equal volume of HRP substrate peroxide solution and HRP substrate luminol reagent were mixed and added just before use.
- Nitrocellulose membrane
- Whatmann filter paper no.1
- Mini transfer western blot- Bio Rad

Protocol

Table 8: Preparation of 10 % PAGE

S. No.	Reagents	Stacking gel (4 %)	Separating gel (10 %)
1.	Acrylamide 30 % (ml)	1.33	2.5
2.	TRIS HCl (ml)	2.5 (pH 6.8)	3.33 (pH 8.8)
3.	MQ H_20 (ml)	6.1	4.17
4.	10 % APS (µl)	50	50
5.	10 % SDS (µl)	100	100
6.	TEMED (µl)	5	10

SDS-PAGE

1. Cleaned glass plates with 1.5 mm spacer were clamped together
2. The bottom was sealed with molten agarose gel
3. Separating gel was poured up to 70 % of the plate, overlayed with H_2O and allowed to polymerise.
4. Then stacking gel was poured and comb was placed and allowed to polymerise.
5. After polymerisation comb was removed and washed twice with H_2O
6. The sample containing 50 µg of protein equal part of 2X SDS sample buffer was added
7. Electrophoresis was performed at 100V current till the run was over.

Transfer

1. The gel was cut and incubated in the transfer buffer for 20 min.
2. Nitrocellulose membrane of required size was cut and incubated in the transfer buffer for 20 min.
3. The gel and nitrocellulose membrane were sandwiched in the mini transfer western blot without any air bubble.
4. Transfer was performed under 100V electric field for 1 h in ice cold buffer.
5. Then the membrane was taken and washed thrice in TBST each for 5 min
6. The membrane was kept in blocking buffer for 1 h at room temperature under mild rocking
7. Washed thrice in TBST (5 times each).

8. Blot was incubated with primary antibody at 4°C for overnight.
9. Washed in TBST (5 times).
10. Incubated in secondary antibody for 1 h at room temperature.
11. Membrane was washed in TBST thrice and twice with TBS, developed using chemiluminol and documented using fluorchem FC3 chemidoc instrument.

3.22 INDIRECT IMMUNOFLUORESCENCE/ IMMUNOHISTOCHEMISTRY STAINING PROTOCOL

Materials required:

- HUVECs
- Chamber slide – Nunc
- TBS- Tris- 2.8 g, NaCl - 0.8 g pH (7.2-7.4) in 100 ml of H_2O. For TBST add 100 µl of Tween - 20 to 100 ml of TBS.
- Citrate buffer (0.01 M) - Trisodium citrate of 294 mg was weighed, dissolved and adjust the pH to 6.0 with citric acid and made to 100 ml with H_2O. This was freshly prepared.
- Peroxidase block - 0.3 % hydrogen peroxide (H_2O_2) (Fischer scientific)
- Protein block - 3 % BSA in TBST
- Membrane permeabilization - Triton X- 100 – 0.1 % in 5 % BSA
- DAPI - The stock (1 mg/ml) 100 µl was made upto 100 ml in H_2O.
- Diaminobenzidine (DAB) preparation: 40 mg/ml in DH2O (freshly prepared).
- Cobalt chloride- 80 mg/ml in DH_2O (freshly prepared).
- Citrate buffer: Citric acid – 0.09 M, disodium hydrogen phosphate- 0.16 M
- Developing solution: To 10 ml of citrate buffer, 200 µl of DAB, 50 µl of cobalt chloride and 30 µl of H_2O_2 was added and mixed just before use.

Principle: Indirect labelling requires two incubation steps; the first with a primary antibody followed by a compatible secondary antibody. The secondary antibodies have the fluorescent dye (FITC) conjugated or tagged to horse radish peroxidase (HRP) which was developed using DAB.

Procedure:

1. Cells were grown to 80 % confluency on 0.1 % gelatin coated chamber slides.
2. Cells were exposed to appropriate exposure conditions and fixed with 4% paraformaldehyde.
3. Cells were permeabilised with 0.1 % triton X- 100 and incubated for 10 min at room temperature.
4. Blocking was done in 5 % BSA and incubated for 30 min at room temperature.
5. Primary antibody of concentration 0.1-10 µg/ml in TBST was added onto each well, kept for overnight rocking at 4°C.
6. Cells were washed with TBST thrice (5 min each).
7. The FITC-labeled or HRP labeled secondary antibody was diluted in TBST at the optimal dilution (according to the manufacturer's instructions)
8. Cells were incubated for 30 min at room temperature. This incubation must be done in the dark for FITC labeled antibody.
9. Then cells were washed thrice with TBST and 2 washes of TBS were performed. Incase, of HRP tagged antibody cells were incubated with DAB, a chromogenic substrate to develop brown colour.
10. Cells were kept for drying and mounted in glycerol. For FITC labeled cells were stained with DAPI for 30 s and then mounted.
11. Document using a microscope (Carl Zeiss) attached to the computer.

Immunohistochemistry procedure for ocular tissues:

Control eye balls from donor eye were collected from CU- SHAH eye bank. The tissues were formalin fixed and embedded in wax. Sections of 5 µm thickness were taken in charged slides (leica), then deparaffinised, followed by staining.

Procedure: (Microwave oven)

1. Antigen retrieval was performed in microwave oven for 2 min for 4 cycles each lasting for 30 s in citrate buffer.
2. Once antigen retrieval was performed tissues were kept in moist chamber.
3. Endogenous peroxidase activity was blocked using H_2O_2.
4. Washed with TBS 2X5 min.
5. Next, the sections were incubated with BSA for 5 min and then washed with TBS 2X5 min.

6. The steps from 5 to 9 were followed as mentioned above for immunofluorescence staining.
7. Unstained sections were used for background elimination for confocal microscopy.

3.23 ELISA- CTR1

Materials required:

- ELISA kit (E94493Hu , USCN, USA)
- Cell lysate – Cells were lysed in MPER (Pierce).

Principle: CTR1 protein was detected with a biotin – conjugated antibody. Avidin conjugated to HRP was added to which TMB substrate exhibited change in colour. The reaction was terminated with sulphuric acid and colour was read at 450 nm.

Procedure:

1. Standards and samples were prepared as per manufacturer's instruction. Sample and standard of 100 µl were added to each well and incubated for 2 h at 37°C.
2. The liquids were removed and without washing 100 µl of diluted detection A reagent was added and incubated for 1 h at 37°C.
3. Three washes with 350 µl of wash buffer were performed.
4. Detection Reagent B working solution 100 µl was added to each well and incubated for 30 min at 37°C.
5. The aspiration/wash processes were repeated for total 5 times as conducted in step 4.
6. Substrate Solution of 90 µl was added to each well. Plate was covered with a sealer and incubated for 10 - 15 min at 37°C in dark. Substrate solution colour changed to blue, and then stop solution of 50 µl was added to each well. Yellow colour developed at the end of the reaction.
7. Mixed well and read at 450 nm using an ELISA plate reader (Septramax2e).

3.24 CO IMMUNOPRECIPITATION- IP

Materials required:

- Co – IP kit – Pierce (26149)
- Peripheral blood mononuclear cell (PBMC) – 5 ml of heparanised blood was collected. Histopaque (sigma) a density gradient was used to separate mononuclear

cells. 5 ml of histopaque was added to 15 ml falcon tube which was carefully overlayed with 5 ml of blood. Tubes were centrifuged at 5000 rpm for 30 min and the mononuclear cells were immediately separated to a different tube and washed with PBS thrice (5mins each).

- Cell lysate – Prepared in MPER in the presence 10 µl of proteinase inhibitor cocktail (1 mg/ ml)

Principle: Co- immunoprecipitation (Co-IP) approach is used for studying protein: protein interactions, using a specific antibody to immunoprecipitate the antigen (bait protein) and co-immunoprecipitate any interacting proteins (prey proteins). This method allows pull down of interacting proteins under native condition without the antibodies as their light and heavy chain mask the protein of interest. Unlike tradition pull down assay where antibody is coupled to an agarose resin, in this method the antibody is cross linked covalently to an amine reactive bead thus making this technique more efficient and easy.
Further, after pull down assay these proteins are subjected to dithiothreitol (DTT) treatment to reduce the disulphide bridges and alkylated with Iodoacetamide (IAA). Next step involves tryptic digestion of proteins into smaller peptides which are submitted to mass spectrometer. The mass spectrometer identifies the peptides based on their Mass/charge (M/Z) ratio. These peptides sequences are analyzed by software which helps in unraveling the new protein based on their unique peptide matches.

Procedure:

Step 1: Lysis of Cell

1. PBMC were washed with PBS carefully.
2. Ice-cold IP lysis/wash buffer was added to the cells and incubated on ice for 5 min with periodic mixing.
3. The beads were washed with ice-cold 1 X coupling buffer and then coupled with antibody (10-75 µg). Antibody coupling with beads was done using sodium cyanoborohydride solution for 90- 120 min.
4. The reaction was quenched with quenching buffer.
5. The beads were washed once with coupling buffer and then with IP lysis/wash buffer.
6. Cell lysate were incubated with antibody-bound beads for 2 h at room temperature or overnight at 4ºC.

7. The beads were washed twice with IP lysis/wash Buffer to remove the unbound proteins. Bound proteins were eluted using elution buffer (50 µl) after 5 min of incubation.
8. Protein estimation was performed using BCA kit (Thermo). Eluted proteins were loaded onto gel and run in 10 % SDS PAGE and silver staining was performed for in gel tryptic digestion. Elutes were also subjected to insol tryptic digestion.

Step 2 - Silver staining:

Materials Required:

- Sodium thiosulphate -120 mg/ 100 ml of MQ H_2O.
- Stain - Silver nitrate - 250 mg/ 125 ml of MQ H_2O with 50 µl of formalin
- Developer -Sodium carbonate - 2 % of sodium carbonate with 50 µl of formalin
- Stopping solution- 7 % acetic acid

Procedure:

1. Sodium thiosulphate was added to the gel and rocked for 1 min.
2. Silver nitrate was added, and incubated for 20 min.
3. Three washes of MQ H_2O were done
4. Sodium carbonate was added cold to the gel was used for developing the gel.
5. The reaction was stopped using acetic acid and gel was kept in MQ H_2O.
6. Silver stained gel protein bands were cut into smaller pieces and subjected to tryptic digestion for mass spectroscopy to identify the interacting partners.

Step 3- Tryptic digestion:

Materials required:

- Destain: 30 mM Potassium ferricyanide, 100 mM Sodium thiosulfate prepared in deionised water.
- Ammonium bicarbonate buffer (NH_4HCO_3) (100 mM) - 7.90 mg/ ml in MQ water.
- NH_4HCO_3 (50 mM) - 3.95 mg/ ml in MQ water.
- DTT 100 mM - 1.5 mg/ 100 µl of 0.1 M NH_4HCO_3
- IAA 55 mM - 3.7 mg/ 100 µl in 0.1 M NH_4HCO_3

Step 3a: In-gel Procedure:

1. Gel bands were cut into cubes, and then transferred into a 1.5 ml micro centrifuge tube.
2. Gels were soaked with 30 µl MQ water for 15 min and washed with MQ H_2O (5 X 10 min).
3. The gel was destained, after which the gel was washed with MilliQ H_2O (5 X 1 min) till it turned colourless.
4. Acetonitrile 35 µl was added to the gel and kept for 15 min at room temperature.
5. The supernatant was removed and 50 µl of 0.1 M NH_4HCO_3 was added to the gel and kept at room temperature for 5 min.
6. Acetonitrile 35 µl was added and mixed for 15 min (2 X 15min). The supernatant was removed and dried completely using a Speed Vac (15 min)
7. Then 150 µl of 10 mM DTT was added and incubate at 56°C for 45 min.
8. The supernatant was removed and 150 µl of IAA was added, incubated in the dark for 30 min 4°C and then repeated steps 5-8 for once.
9. Trypsin (12.5 ng/ µl) was added to the gel incubated in ice for 30 min for it to be reswollen.
10. The excess solution was removed, 20 µl of 50 mM NH_4HCO_3 was added and incubated at 37°C for 16 h.
11. The supernatant was collected after centrifugation at 14000 rpm for 1 min. Next, 50 µl of 0.1 % formic acid in 50 % acetonitrile was added to the gel pieces and was incubated for 15 min at room temperature. This step was repeated thrice to extract the peptides from the gel.
12. The extracts were pooled, centrifuged at 14000 rpm for 10 min to remove the debris if any.
13. Oasis column were used for desalting the samples. Column was equilibrated with 200 µl of acetonitrile / methanol. Samples were loaded on to the column (maximum column capacity – 500 µl) and washed with 200 µl of 0.1% TFA. A second wash with 800 µl of MQ water was performed.
14. The peptides were eluted with 50 µl of 70% acetonitrile and the digestion solution was completely dried using speed vac. The samples were then reconstituted with 2 % acetonitrile containing 0.1 % formic acid. Before transferring the sample into Mass spec, they were centrifuged at 14000 rpm for 10 min.

Step 3b: Insol Tryptic digestion

1. Protein concentration of 1 µg was used for digestion and 50 µl of 50 mM NH_4HCO_3 was added.
2. DTT (1 µl) was added and incubated at 56°C for 15 min. Next, 20 mM IAA 1 µl added and incubated at RT for 30 min in dark.
3. Trypsin (50 ng) was added and incubated at 37°C for overnight.
4. Trypsin action was arrested by adding formic acid (2 µl) and incubated at 37°C for 20 min.
5. Vortexed and centrifuged the samples at 10000 rpm for 5 min.
6. Transferred the supernatant to clean fresh vials and steps 12- 15 were performed as mentioned above for in-gel procedure above and submitted to mass spectrometer.

Step 4: Analysis by Mass spectrometer

Mass spectrometer parameters:

Buffer A: 0.1 % formic acid in water (Sigma).

Buffer B: 0.1 % formic acid in acetonitrile (Sigma).

(Nano ESI LC MS Xevo G2S Qtof, Waters) was used for the experiments. Samples were run using linear gradient system from (0-100%) for 2 h. Flow rate of 0.3 µl/ ml was set and 3 µl of the samples were injected. Column of 0.75 µm pore size were used. The experiments were performed in a positive ion mode. All spectra were measured at a scan rate of 0.014 s with the capillary voltage and cone voltage set at 3.0 kV and 30 V respectively. The source temperature and the desolvation temperature were 80 ºC and 150 ºC respectively and the flow rates were maintained at 50.0 l/h for cone gas flow and 600.0 l/h for desolvation gas flow. The acquired spectra (ranged between 50 to 2000 m/z) were analyzed as centroid data for establishing the purity of the peptides. Samples were acquired in resolution mode (30,000).

3.25 DESIGN OF siRNA

The si RNAs was designed using Ambion web tools, and the rules described by Reynolds *et al.,* for siRNA designing were followed (Table 9). Scrambled siRNA (Ssi), was designed using scrambled siRNA software.

Table 9: List of siRNA for CTR1 using Ambion web tool

S. No	Sense Sequence	Antisense Sequence	GC%	R1	R2	R3	R4	R5	R6	R7	R8	Points
1	**CAGUACCAU GCAACCUUCU UU**	**AGAAGGUU GCAUGGUA CUGUU**	42.9	✓	✓	61.28						**4**
2	CCUUCUCACC AUCACCCAAU U	UUGGGUGAU GGUGAGAAG GUU	47.6	✓		63.33	✓					4
3	GAAUGUGGAA CUACUGUUUU U	AAACAGUAG UUCCACAUU CUU	33.3	✓	✓	55.13		✓				5
4	UGUGGAACUA CUGUUUUCCU U	GGAAAACAG UAGUUCCAC AUU	38.1	✓	✓	60.07				✓	✓	0
5	CUACUGUUUU CCGGUUUGGU U	CCAAACCGG AAAACAGUA GUU	42.9	✓	✓	60.69		✓		✓	✓	1
6	UACAGCUGGA GAAAUGGCUU U	AGCCAUUUC UCCAGCUGU AUU	42.9	✓		62.64						3
7	AUGGCUGGAG CUUUUGUGGU U	CCACAAAAG CUCCAGCCA UUU	47.6	✓		65.03				✓		2
8	UGUUCUAUGA AGGACUCAAU U	UUGAGUCCU UCAUAGAAC AUU	33.3	✓	✓	56.4	✓				✓	4
9	GUCACAAGUC AGCAUUCGCU U	GCGAAUGCU GACUUGUGA CUU	47.6	✓		62.46				✓		1
10	GUCAGCAUUC GCUACAAUUU U	AAUUGUAGC GAAUGCUGA CUU	38.1	✓	✓	56.95						4
11	AUGGAACCAU CCUUAUGGAU U	UCCAUAAGG AUGGUUCCA UUU	38.1	✓	✓	59.16	✓		✓			7
12	CCAUCCUUAU GGAGACACAU U	UGUGUCUCC AUAAGGAUG GUU	42.9	✓	✓	60.77	✓	✓	✓			7
13	AACUGUUGGG CAACAGAUGU U	CAUCUGUUG CCCAACAGU UUU	42.9	✓	✓	62.22				✓		4
14	CUGUUGGGCA ACAGAUGCUU U	AGCAUCUGU UGCCCAACA GUU	47.6	✓	✓	63.55						4
15	CAGAUGCUGA GCUUUCCUCU U	GAGGAAAGC UCAGCAUCU GUU	47.6	✓		63.16				✓		2
16	ACAGUGCUGC ACAUCAUCCU U	GGAUGAUGU GCAGCACUG UUU	47.6	✓		64.53		✓		✓		3
17	GCUACUUCCU CAUGCUCAUU U	**AUGAGCAU GAGGAAGU AGCUU**	42.9	✓	✓	60.86			✓			**4**
18	GAAGGCAGUG GUAGUGGAUU U	**AUCCACUAC CACUGCCU UCUU**	47.6	✓	✓	64.21		✓				**5**
19	GGCAGUGGUA GUGGAUAUCU U	GAUAUCCAC UACCACUGC CUU	47.6	✓	✓	64.5				✓	✓	2

Rule 1 – GC content (33 – 52 %)

Rule 2 - 15 – 19 (AAA or UUU) residues.

Rule 3 – Melting point (nearest neighbour) less than 60.

Rule 4 – 19th base pair (A)

Rule 5 – 3rd residue (A)

Rule 6 - 10th position "U"

Rule 7 – 19th position (G or C)

Rule 8 – 13th position G

Rule 7 & Rule 8 are negative points G or C residues thus should be avoided.

Three highlighted sequences of sense strand having score of more than 5 were taken and the antisense strands of the selected sense si RNA were further given for synthesis. These three (si 1, si 2, si 3) were screened for the silencing effect on HUVECs – CTR1 expression.

3.26 ISOLATION OF siRNA AND cDNA CONVERSION:

Overall procedure of siRNA quantification using real time PCR

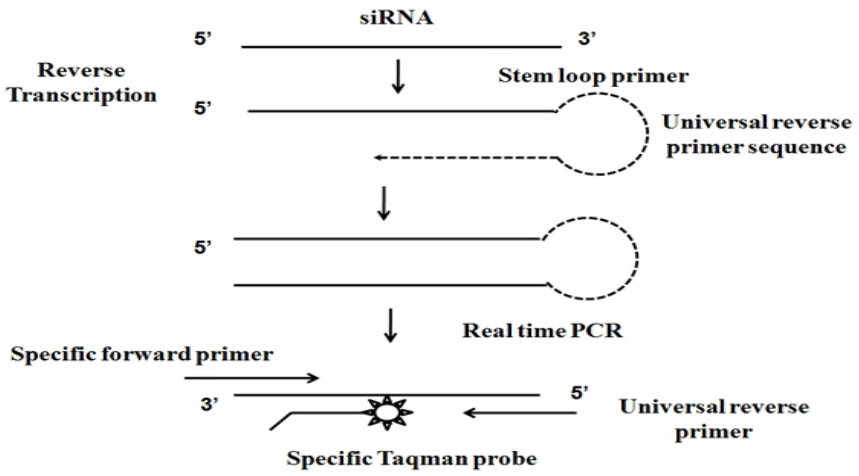


Figure 12: Step involved in siRNA quantification by Taqman probes (Chen et al. 2005).

a) Isolation of siRNA: mirVana (life technologies) isolation kit was used for siRNA isolation.

Materials required:

- HUVECs
- Isolation kit - siRNA

Principle: RNA extraction was performed in combination of the 2 methods of chemical extraction followed by immobilization onto a glass (solid phase extraction). Chemical extraction using chaotrophic agents in the presence of RNAase provide highly pure RNA. Next the RNA is isolated in high salt and alcohol mixture which enhances its affinity towards the solid phase. Further desalting and elution yields help in enrichment of small RNA fragments.

Procedure:

1. Cultured HUVECs were trypsinised and samples were lysed using 300 µl of lysis buffer. Vortexed or pipetted vigorously to obtain a homogeneous lysate.
2. Added 1/10th the volume of miRNA homogenate additive was added to the cells for purification of small RNA.
3. The mixture was incubated on ice for 10 min.
4. Added equal volume of acid phenol: chloroform to the original lysate volume.
5. The mixture was vortexed for 30- 60 s to mix.
6. It was then, centrifuged for 5 min at 10,000 g at room temperature to separate the aqueous and organic phase.
7. The upper aqueous layer was carefully removed without disturbing the lower phase and transferred to a fresh tube.
8. Next, for each sample, placed a filter cartridge into a collection tube.
9. The lysate or ethanol mixture upto 700 µl was added onto the first cartridge.
10. This mixture was centrifuged at 10,000 g for 15 s.
11. The flow through was collected and 2/3rd of the volume was transferred to the next cartridge.
12. Wash solution I (miRNA) 700 µl was added and step 10 was repeated.
13. Next, 500 µl of wash solution II and III were performed twice.
14. Finally, the small RNA samples were eluted in 100 µl of preheated (95°C) elution buffer.

b) Conversion of cDNA from small RNA (Reverse transcription)

Materials required:

a) Small RNA
b) Applied biosystems small RNA cDNA conversion kit
c) Thermal cycler

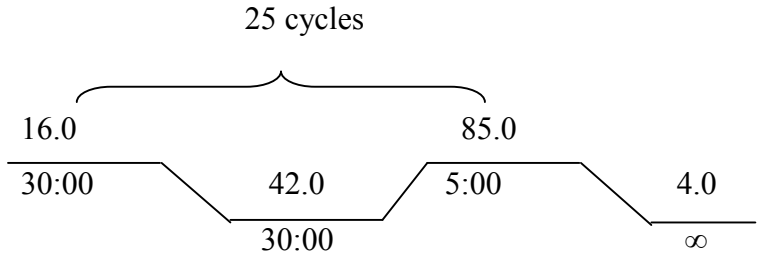
Principle: TaqMan microRNA assay employs a new method of identifying small mature RNA (miRNA & siRNA). Target specific stem loop reverse transcription method is employed for microRNA assay. The primer extends the 3' end of the target to produce a template that can be used in standard TaqMan assay-based real-time PCR. Further, specific primers toward the target gene will specifically amplify the target.

Procedure: All the kit contents were brought to 4°C.

Table 10: Procedure of PCR reaction for reverse transcriptase

Reagents	**Volume (µl)**
dNTP	0.15
RT enzyme	1.00
10X RT buffer	1.5
RNAase inhibitor	0.19
H_2O	4.16
Primer (5X)	3
RNA sample (1- 10 ng)	5
Total volume	15

Thermal cycler profile for siRNA cDNA conversion



Experimental conditions for CTR1 siRNA:

Control, Cu 100 µM, Cu+ si 10 nM, Cu+Ssi 10 nM, Cu+ P800 µM

Table 11: Experimental condition for CTR1 siRNA in HUVEC

S. No.	Experiment name	Time point	Dosage of siRNA
1.	mRNA expression	1 h	1 dose
2.	Protein expression by western blot and ELISA	6 h	3 doses (2 h once)
3.	Bioavailability of siRNA	1 h, 2 h and 4 h	Single dose
4.	Migration assay	16 h (Scratch) 8 h (Transwell)	Single dose
5.	Proliferation assay	48 h	Single dose
6.	Tube formation	6- 8 h	Single dose
7.	Attachment assay	30 min	Single dose
8.	Cu estimation	1 h	Single dose
9.	Apoptosis assay	24 h	Single dose
10.	CAM assay	16 h	Single dose
11.	Rabbit corneal packet assay	11 days	CTR1 O methyl siRNA 100 ng was administered twice a day topically from day 3 to day 11

Table 12: List of Peptides used for screening in HUVEC

Peptides	Peptide sequence	Residues
Peptide 1 (Pep 1)	QPSHHHPTD	19-26
Peptide 2 (Pep 2)	DSMMMMPMTD	39-46
Peptide 3 (Pep 3)	MDHSHHMGMSYD	1-11
Scrambled peptide (Scp)	KAYNDADPP	Random sequence

3.27 PEPTIDE DESIGN

Based on the *in silico* work the interacting regions of CTR1 were taken for peptide designing. The regions will be studied further to understand structure function relationship. Three peptides were designed based on their interactions.

3.28 PURITY CHECK USING HPLC

Peptides were synthetically manufactured. These peptides of 1 mg/ ml concentration were dissolved in H_2O and injected into Agilent HPLC. Buffer A consisted of 0.1 % trifluoroacetic acid in H_2O and buffer B 0.1 % trifluoroacetic acid in acetonitrile were prepared. Buffers were filtered using 0.22 µ filter and degassed by sonicating for 15 min twice. Linear gradient elution from 0 to 90 % was performed. HPLC reverse phase C_{18} column (Jupiter) of length 250 mm×4.6 mm, 300 A° pore size, 5 µm thickness, flow rate of 0.5 ml/ min was used for peptide detection at 214 nm for 1 h time point.

3.29 RHODAMINE LABELED PEPTIDE FOR INTRACELLULAR ENTRY OF CTR1 DERIVED PEPTIDES

CTR1 derived peptides were synthesized with rhodamine dye conjugated at the N- terminal residue. The peptides of 1 mM concentration were prepared in stock. HUVECs were grown in cover slips coated with gelatin. Cells were serum starved and exposed to appropriate conditions. Cells were washed with PBS twice and fixed with 4 % paraformaldehyde and photographs were taken using fluorescent microscope.

3.30 *In vivo-* RABBIT CORNEAL PACKET ASSAY

Ethics statement: Animal research protocols were in accordance with NIH guidelines for responsible animal care and use. The study was approved by Sri Ramachandra Medical College – Innovis committee on animal care and use of laboratory animals where the study was conducted. Reference number IAEC/XXVIII/SRU/2 12/2012 is the approval number from this committee for this animal work.

Procedure: Normal 4 healthy rabbits were taken in the study, which were distributed in two groups. Group 1– matrigel implanted and PBS treated were used as vehicle controls and Group 2– matrigel implanted si 1 100 ng with 2' – O – methyl modification treated were used in the study. All the animals in the study were operated in the right eye to implant the matrigel (BD, USA), whereas the left eye was taken as untreated control. New Zealand white male rabbits (2 kg) were subcutaneously injected with ketamine (50 mg/kg) and xylazine (5 mg/kg) to anesthetize. Using a central intrastromal linear keratomy (~2.5 mm in length) was made with a surgical knife. A lamellar micro pocket was dissected to 2 mm near the limbus and 50 µl of matrigel (BD) was implanted. Antibiotic ointment (erythromycin) was applied once to the surgical eye to prevent infection and to decrease

irritation of the irregular ocular surface. On postoperative days 3, 5, 7, 9, and 11 after gel implantation 100 ng of si 1 was topically dispensed in the form of drops twice a day. At the end of 11th day, the animals eyes were photographed using stereomicroscope SMZ1000 (Nikon, Japan) and euthanized.

3.31 *In vivo* - CHICK CHORIO ALLANTOIC MEMBRANE ASSAY

Closed model: Eggs were purchased. On day 3, cleaned with 70% ethanol and the orifice were created. After implantation, the filter disc with different conditions like (Cu alone, Cu+siRNA) was placed inside the egg on the smaller capillaries and observed for blood vessel formation every 2 h till the end of 18 h (Lokman et al. 2012).

Open model: The day 4 fertilized white leghorn chicken eggs were procured from the poultry house and incubated for 1 day with constant humidity of 60- 70 % at 37°C. On day 5 the eggs were rinsed with 70 % methanol, the egg was broken and kept on a petriplate and transferred to the incubator. The peptides were dissolved in H_2O (5 µl) and were placed on a sterile disc (0.5 mm in diameter). The disc was kept on the branching blood and monitored periodically. Photographs were taken immediately after placing the disc and after 6 h in a stereomicroscope (Nikon, Japan) (Lokman et al. 2012).

3.32 COPPER BINDING TO PEPTIDE USING MASS SPECTROSCOPY

Cu binding experiments were performed using Xevo G2S Q-Tof mass spectrometer (Waters). Peptides (10 µM) were dissolved in 0.1 % formic acid: 50 % acetonitrile (deionized H_2O) and infused at a flow rate of 2.0 µl/min. The experiments were performed in a positive ion mode. All spectra were measured at a scan rate of 0.014 s with the capillary voltage and cone voltage set at 3.0 kV and 30 V respectively. The source temperature and the desolvation temperature were 80 ºC and 150 ºC respectively and the flow rates were maintained at 50.0 l/h for cone gas flow and 600.0 l/h for desolvation gas flow. The acquired spectra (ranged between 50 to 2000 m/z) were analyzed as centroid data for establishing the purity of the peptides and for studying copper binding properties. Samples were acquired in resolution mode (30,000).

3.33 OCULAR TISSUES SAMPLES

Patient sample: Human ocular tissues were obtained from the CU Shah eye bank. All materials procured were used under the protocols approved by the Institutional Review Board (IRB) of Vision Research foundation and in accordance to the Declaration of

Helsinki. PBMC were isolated from heparinised blood of ED patient and healthy control male volunteers with informed consents. Fresh Eales' patient (n= 4), and control (n= 5). Inclusion criteria consisted of patients with acute vasculitis, males were included in the study. Patients suffering from any other ocular diseases, smokers, alcoholics and any other metabolic diseases were excluded from the study.

CHAPTER 4: RESULTS

4.1 SCREENING FOR COPPER RESPONSIVE PROTEIN MOLECULES RELATED TO ANGIOGENESIS IN HUVEC CELL MODEL

4.1.1 Establishment of HUVEC culture:

Isolation of HUVEC was standardised as given in methods (3.1) and this was used as an experimental model for *in vitro* angiogenesis assays (Figure 13 A). The established cells were confirmed by RT PCR and immunofluorescence for the presence of PECAM (CD31) marker which is specific for endothelial cells. PCR showed band for PECAM at 110 bp for HUVEC whereas HEpG2 did not express CD31 confirming that the isolated cells were endothelial in origin (Figure 13 B). Immunofluorescence of HUVEC showed positive for PECAM stained in red colour, nucleus was stained blue with DAPI (Figure 13C).

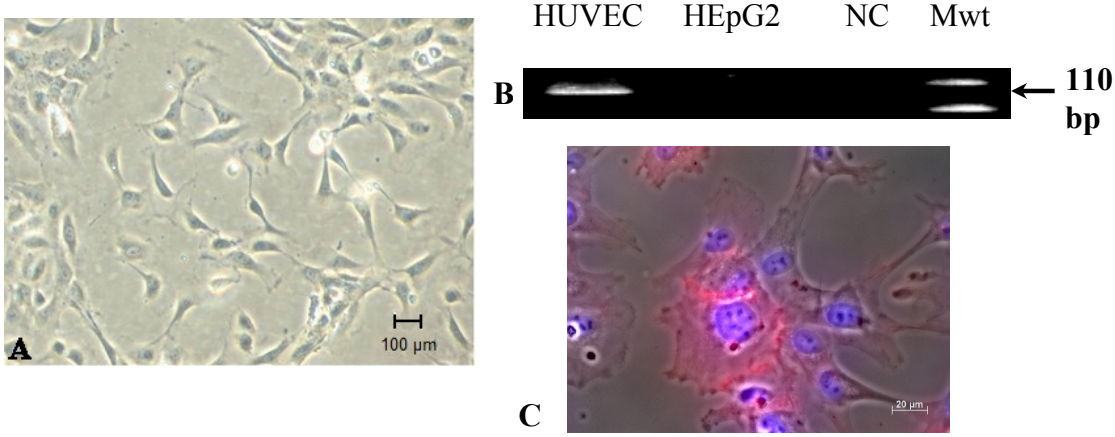


Figure 13: Establishment of HUVEC model A): Phase contrast (10X) photograph of isolated endothelial cell from umbilical vein. B) RT PCR for PECAM using 50 ng cDNA C) Immunofluorescence image of TRITC labelled CD31 HUVEC, nucleus is stained with DAPI.

4.1.2 Selection of Cu responsive molecules:

The Cu responsive angiogenic molecules were listed as CP, CTR1, LOX, and SPARC from literature search (Holzer et al. 2006; Ros-Bullon et al. 2001; Senra Varela et al. 1997; Tai and Tang 2008). These molecules were screened for their mRNA expression in HUVEC. PCR Data showed presence of CTR1, LOX and SPARC expression in HUVEC

(Figure 14. A, B&C). On the other hand cell types such as ARPE 19, HeLa, HEpG2, A549, and retinal tissue showed presence of CP mRNA. CP was absent in HUVEC (Figure 14 D).

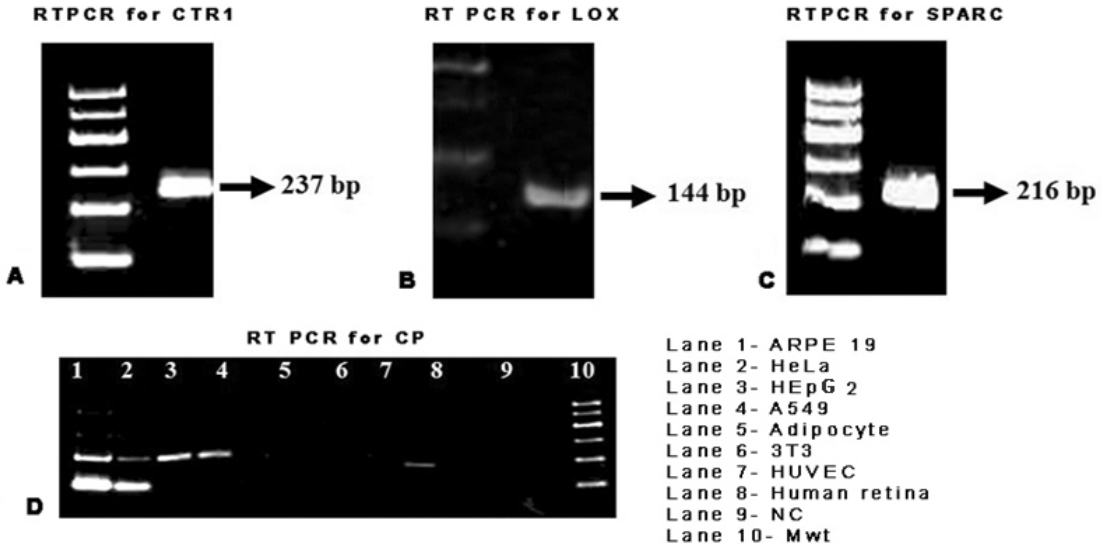


Figure 14: Standardisation of RT PCR in HUVEC for Cu responsive molecules.

A) CTR1 mRNA expression was seen at a product size of 237 bp. B) LOX mRNA expression was seen at a product size of 144 bp. C) SPARC mRNA expression was seen at a product size of 216 bp. D) CP RT PCR in different cell lines showed presence of CP mRNA expression in ARPE19, HeLa, HEpG2, A549 and Human retina but was absent in HUVEC. PCR products were loaded onto 2% agarose gel and electrophoresed.

Since CP is also an acute phase protein. To check whether CP is expressed with stimulation, HUVEC were treated with 100 ng of bacterial lipopolysaccharide (LPS) for 1 h and RT PCR was performed. The results showed absence of CP mRNA expression. Even after LPS treatment. ARPE 19 was used as positive control in this study (Figure 15).

Lane 1 – ARPE19
Lane 2 – HUVEC
Lane 3 – LPS activated HUVEC
Lane 4 – NC
Lane 5 – Mwt

Figure 15: RT PCR in HUVEC with LPS treatment for CP mRNA: 100 ng of LPS treatment did not show CP mRNA expression in HUVECS after 1 h time point.

4.1.3 Standardization of optimum Cu concentration in HUVEC for Cu responsive proteins

The objective was to find out the optimum Cu concentration for Cu responsive proteins. Cells were treated with Cu concentration from 1 µM to 300 µM for 1 hr and

then mRNA expression studies for CTR1, SPARC and LOX were performed. **CTR1 mRNA expression** showed a **dose dependent increase** with its highest expression at **Cu concentration of 100 µM**. SPARC showed increased mRNA expression in all concentrations of Cu from 1 µM to 100 µM and decreased at 200 µM to 300 µM concentrations. LOX mRNA expression did not increase considerably with the exposed Cu concentration (Figure 16).

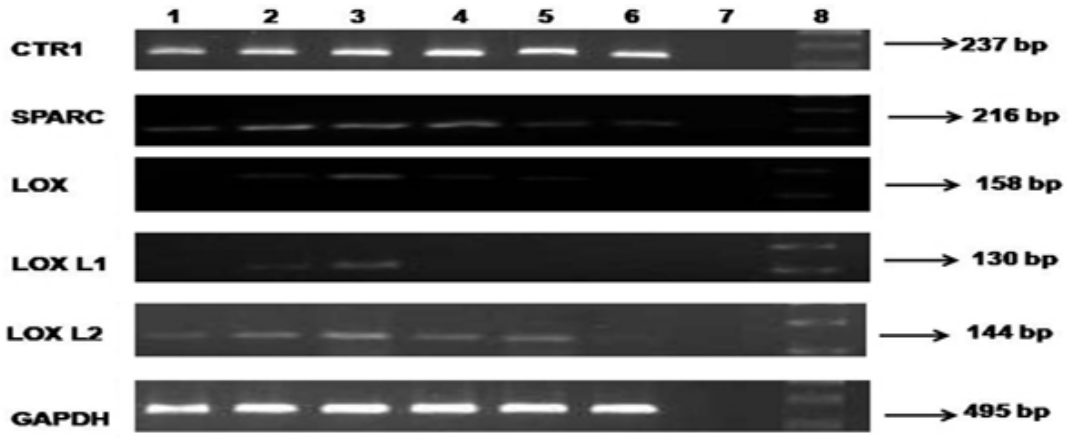


Figure 16: Varying mRNA expression of CTR1, SPARC, LOX, LOXL1 and LOXL2 with different Cu concentration:
A. RT PCR for CTR1with varying Cu concentration : lane 1 – control, lane 2- 1 µM Cu, lane 3- 10 µM Cu, lane 4 – 100 µM Cu, lane 5 – 200 µM Cu, lane 6 - 300 µM Cu, lane 7- NC, Lane 8- molecular weight ladder. RT PCR showed increased CTR1 expression in 100 µM Cu with a product size of 237 bp length. All the samples in the concentration range of 50 ng were normalised to GAPDH which had a product size of 495 bp.

Thus, CTR1 the Cu importer was chosen for further studies as a therapeutic target for angiogenesis.

4.1.4 Standardization of penicillamine concentration in HUVEC for optimal Cu chelation

Penicillamine (P) a known Cu chelator was used as a positive control in the study. Since Cu 100 µM showed the increase in CTR1 mRNA expression, whether Cu chelation with penicillamine could affect CTR1 mRNA was studied. Thus, concentration of 400 µM and 800 µM P were tested in HUVEC in the presence of Cu. Decrease in CTR1 mRNA expression was seen at 800 µM with Cu 100 µM concentration (Figure 17) and this concentration was used for all the experiments.

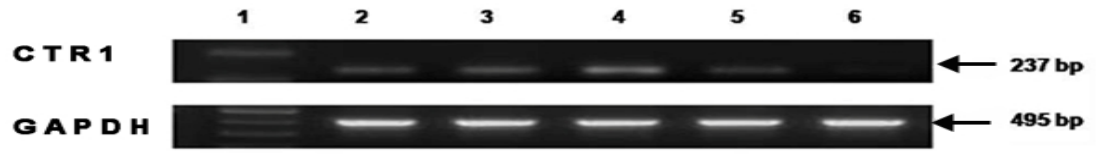


Figure 17: RT PCR for CTR1 with varying P concentrations, lane 1 – molecular weight ladder, lane 2-control, lane 3- 1 µM Cu, lane 4- 100 µM Cu, lane 5 – 100 µM Cu + 400 µM P, lane 6 – 100 µM Cu + 800 µM. PCR showed decreased CTR1 expression in a dose dependent manner in the presence of 400 µM P and 800 µM P with Cu showed product size of 237 bp length. All the samples of 50 ng cDNA concentration were normalised to GAPDH which had a product size of 495 bp.

4.2 COPPER IS PROANGIOGENIC

The first hypothesis that was laid by Hu *et al.,* when tested showed, increased proliferation of EC treated with Cu 500 µM in serum free medium. In this study, Cu concentration that will increase migration and tube formation for HUVEC were screened.

4.2.1 Cytotoxicity assay:

Cu concentrations from 10 nM to 1 mM were tested for its cytotoxic effect in HUVEC when exposed for 1 h. The IC_{50} for Cu was found to be 500 µM (Figure. 18).

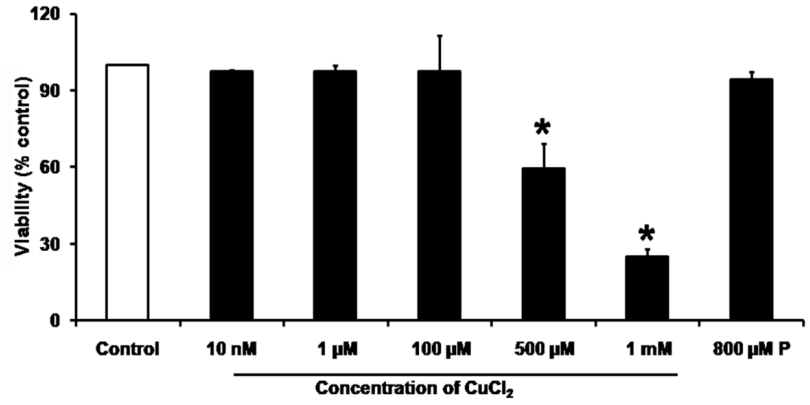


Figure 18: Cytotoxicity assay to check the effect of different concentration of Cu on HUVEC. Cu concentration of 500 µM and 1 mM showed significant decrease in viability whereas, penicillamine did not show any cytotoxic effect on EC.

4.2.2 Scratch assay

Cu concentrations from 100 nM to 100 µM were tested for its influence in migration at 16 h time point in HUVEC. Increase in Cu concentration increased migration indicating that

Cu induces migration. There was a dose dependent increase in migration, whereas cells exposed to 800 µM P showed inhibition in migration (Figure 19).

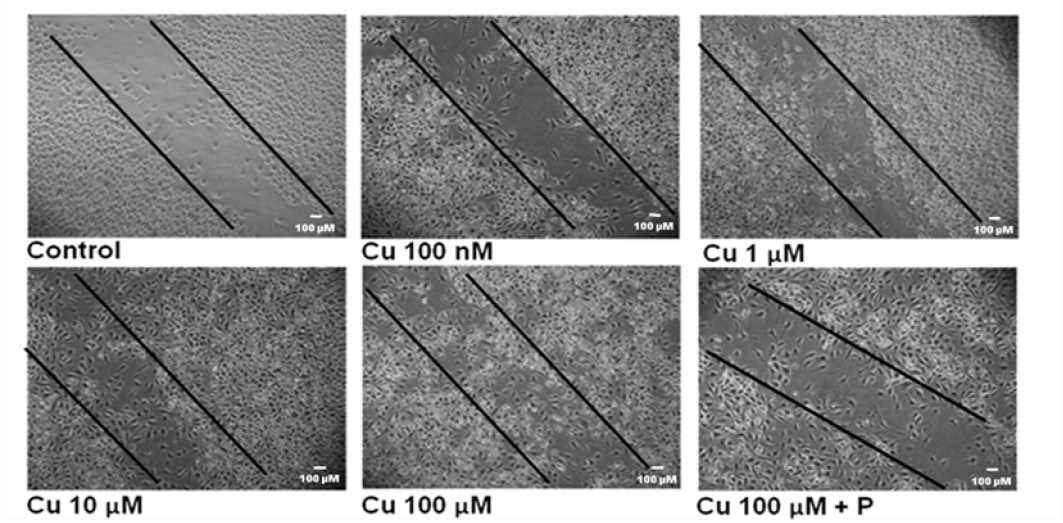


Figure 19: Migration assay in HUVEC exposed to different Cu concentration.

4.2.3 Tube formation

Cu increased migration at 100 µM concentration of HUVEC while penicillamine inhibited Cu induced migration. Cu concentration of 1 µM and 100 µM were tested in HUVEC for tube formation assay. HUVEC showed increased tube formation in the presence of Cu concentration of 100 µM when compared to Cu concentration of 1 µM and control (Figure 20).

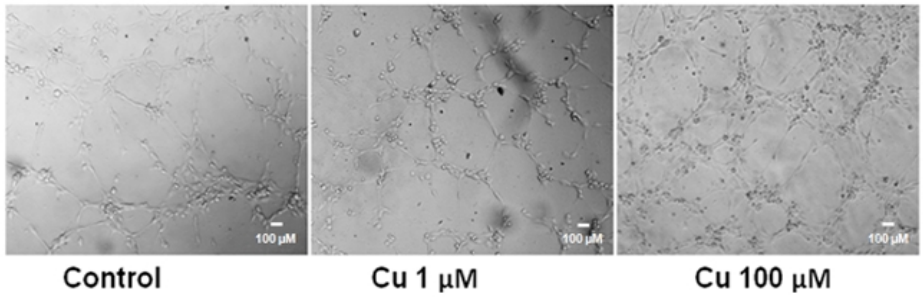


Figure 20: Tube formation assay in HUVEC exposed to different Cu concentration.

4.3 RELEVANCE OF INCREASED CU AND CTR1 PROTEIN LEVELS IN OCULAR DISEASE

Earlier studies by Sulochana *et al.,* (Konerirajapuram et al. 2004) have shown increased Cu (0.128 µg/ml ± 0.04) levels in the vitreous of patients with ED when compared to control (0.08 µg/ml ±0.02). Since Cu levels were reported in vitreous, the role of CTR1 in ocular tissues was to be studied. Real time PCR for CTR1 mRNA was performed in ocular tissues which showed higher levels of CTR1 in optic nerve head, choroid, iris whereas ciliary showed lower levels than retina (Figure 21).

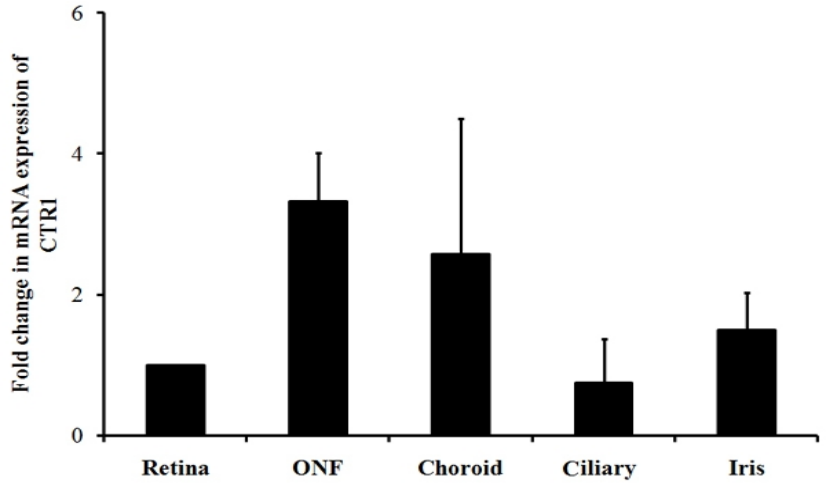


Figure 21: Real time PCR of CTR1 in human ocular tissues from donor eye ball.

CTR1 protein was expressed in all ocular tissues. CTR1 ELISA showed protein expression in all ocular tissues. The protein levels were found to retina (0.29 ng/ µg ± 0.18), iris (0.23 ng/ µg ± 0.027), ciliary (0.14 ng/ µg ± 0.06), ONF (0.13 ng/ µg ± 0.038) and choroid (0.08 ng/ µg ± 0.013) respectively (Figure .22 A). Western blot analysis also showed the presence of CTR1 in ocular tissues (Figure. 22 B). Retina showed 2 bands for CTR1 indicating multimeric form of CTR1 protein in western blot at 50 and 75 kD regions similar to what was also reported by others (Y. Guo et al. 2004; Pabla et al. 2009). Confocal microscopy image showed presence of CTR1 in all the layers of Retina especially in the inner and outer nuclear cell layer (Figure. 22 C).

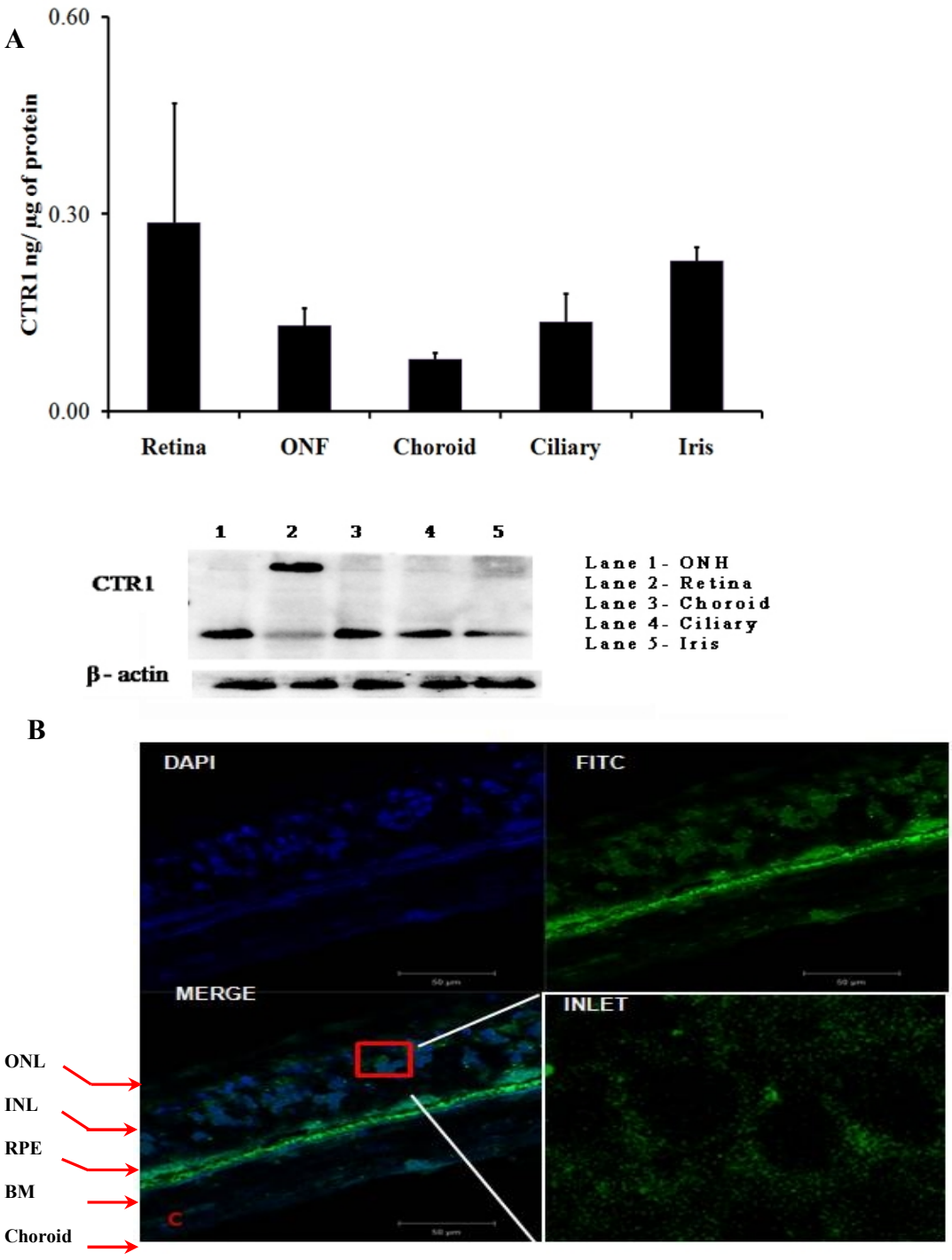


Figure 22: Protein expression of CTR1 in ocular tissues from donor eye ball by A) ELISA and B) Western blot analysis also showed presence of CTR1 in ocular tissues (panel 1) and β actin was used as a loading control (panel 2). C) Confocal microscopy showed presence of CTR1 in all the layers of the retina which are outer nuclear layer (ONL), inner nucler layer (INL), retinal pigment epethelium (RPE), bruschs membrane (BM) and choroid.

Since, all the tissues showed the presence of CTR1 at the protein level, it was intriguing to look for disease which had increase Cu concentration and in ocular angiogenesis. Eales' disease is one such disease wherein both excess Cu and peripheral neovascularisation in the eye was reported. Since ocular tissues cannot be obtained from ED patients PBMC was used to study CTR1expression. CTR1 mRNA expression was compared in PBMC of healthy control and Eales' disease subjects. Data showed increased mRNA expression in ED by 2.4 fold when compared to control (Figure. 23A). CTR1 ELISA in PBMC showed significant increase in protein level in ED (92.56 pg/µg ± 15.35) significantly (p= 0.0001) when compared to control (27.77 pg/µg ± 11.3) (Figure 23 B).

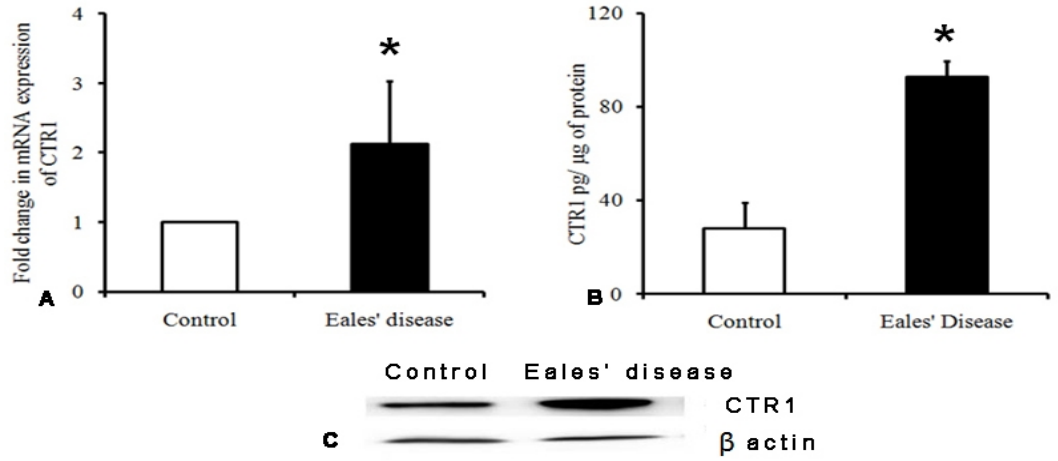


Figure 23: CTR1mRNA and protein expression in PBMC of control and Eales' disease A) Real time PCR CTR1 mRNA expression. B) CTR1 ELISA and C) Western blot in PBMC from control and ED: panel a) CTR1 b) β actin.

Since, over expression of CTR1 was found in ED; decreasing CTR1 expression could be beneficial in treating Cu related diseases. Two strategies were chosen a) design of siRNA targeting CTR1 mRNA b) design of small peptide from Cu binding regions of CTR1 thereby competing for extracellular Cu.

4.4 DESIGN OF siRNA TO TARGET CTR1

4.4.1 Selection of siRNA and its concentration:

Designing of CTR1 siRNA has been already mentioned in methods section. These siRNA were tested for the cytotoxic effect on HUVEC. Three different siRNAs si 1, si 2, si 3 (1 nM – 100 nM) targeting CTR1 and Ssi (1 nM – 100 nM) were tested and found to be non cytotoxic in all tested concentrations (Figure 24).

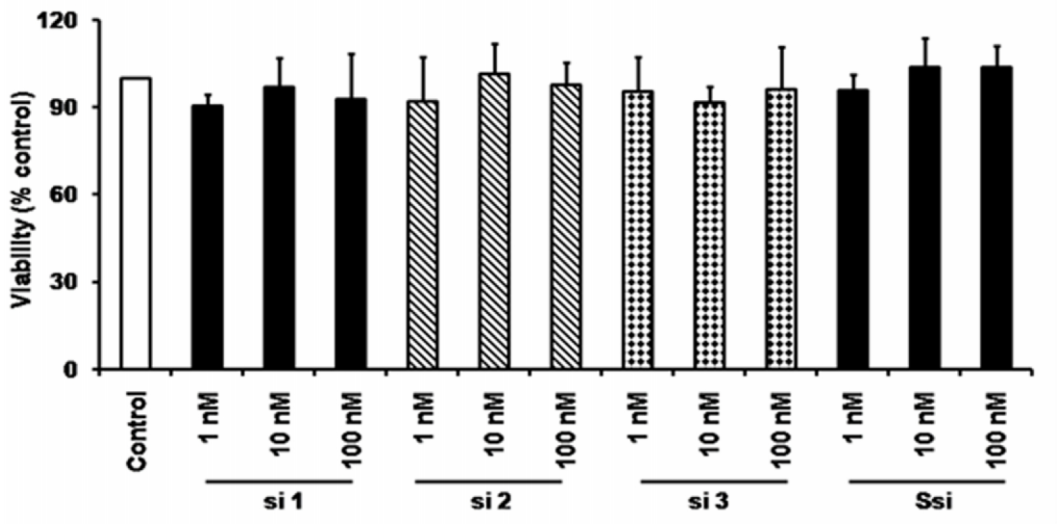


Figure 24: Cytotoxicity assay of CTR1 siRNA si 1, si 2, si 3 and Ssi with varying concentrations on HUVEC.

Real time data Cu concentration of 100 µM showed an increase by 45 % in CTR1 mRNA expression ($p = 0.01$) compared with control. CTR1 mRNA silencing effect of all the three siRNAs in the presence of Cu 100 µM was performed. Real time data showed that 10 nM concentration of si 1 decreased CTR1 expression by 71 %, whereas si 2 and si 3 did not show any significant effect. Cells treated with 800 µM P also showed decrease in CTR1 mRNA expression by 0.3 fold when compared to Cu treated cells. Ssi 10 nM did not show any significant decrease in mRNA expression when compared with Cu treated cells. GAPDH was used as the house keeping gene (Figure 25) to normalise CTR1 expression. Since si 1 showed a significant decrease in CTR1 expression it was chosen for further experiments.

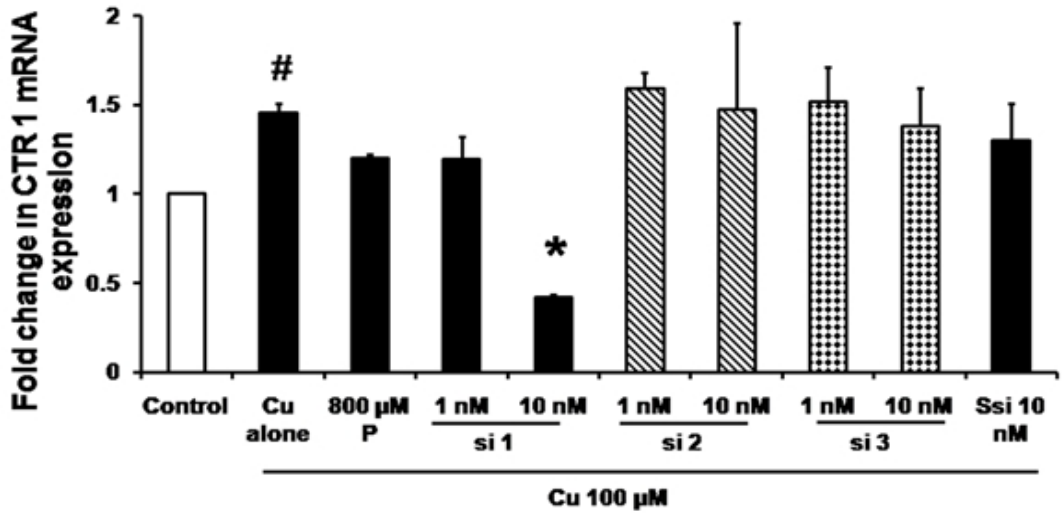


Figure 25: Effect of designed siRNAs and 800 µM P in the presence of Cu on CTR1 expression in HUVEC at 4 h time point. CTR1 si 1 at 10 nM concentration showed significant silencing effect when compared to other siRNAs (si 2 and si 3) respectively. Comparison between control and Cu treatment is represented as # furthermore Cu treatment and siRNA comparison is represented as *.

Based on the results shown in (Figure 25) si 1 at 10 nM, concentration was chosen for further experiments.

4.4.2 Standardization of different time points for optimum mRNA expression of CTR1:

CTR1 mRNA expression was tested at 1 h, 4 h and 16 h time points. CTR1 mRNA expression increased at 1 h and 4 h time point and si 1 (10 nM) significantly decreased its expression by 50%, whereas, Ssi (10 nM) did not show any change. CTR1 mRNA expression was also reduced in the presence of P 800 µM (Figure 26). It was noted at 16 h time points CTR1 mRNA expression did not show considerable difference between cells treated with Cu and siRNA. These results indicate that the Cu induced mRNA expression within 1 h time point.

Optimisation of exposure time was done, with si 1 – 10 nM at various time points from 1, 4 and 16 h.

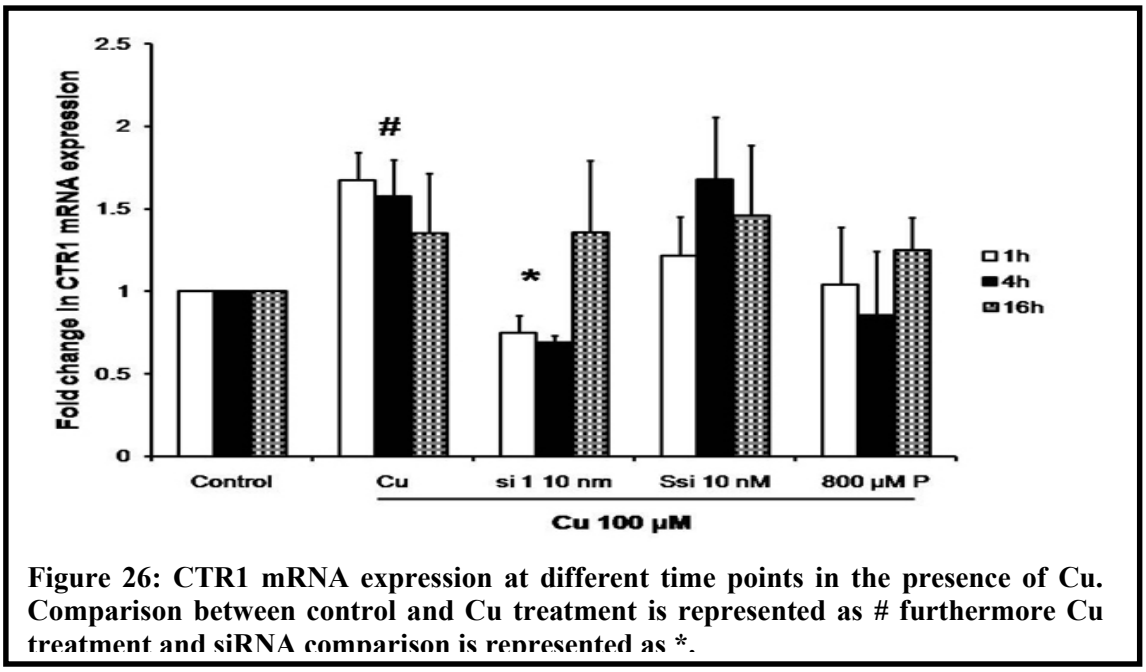
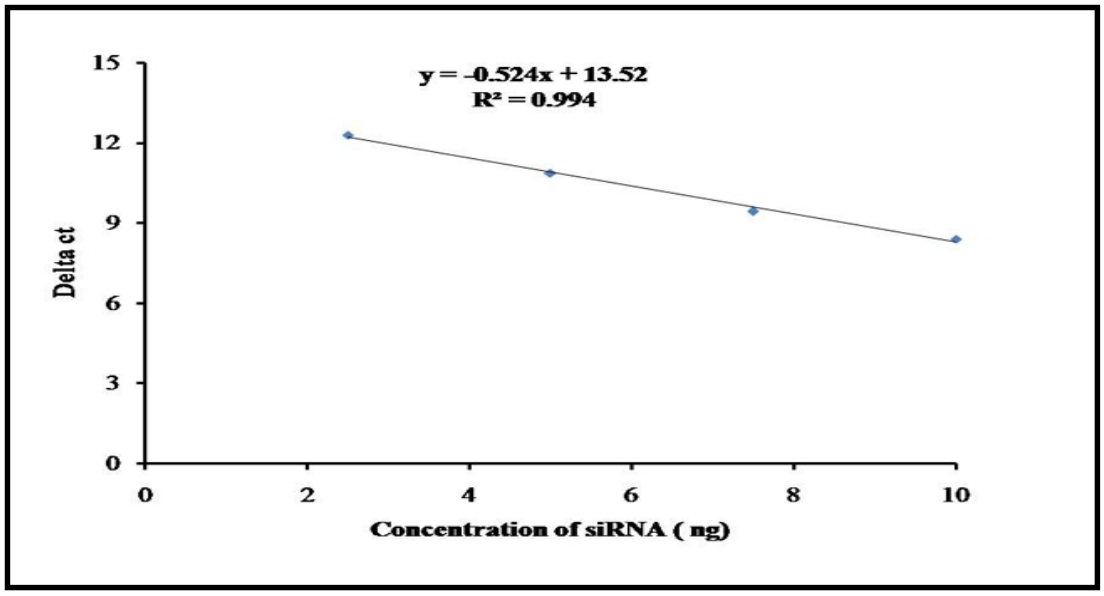


Figure 26: CTR1 mRNA expression at different time points in the presence of Cu. Comparison between control and Cu treatment is represented as # furthermore Cu treatment and siRNA comparison is represented as *.

Results present here indicated that si 1 at 10 nM for 1 h time point to be optimum for mRNA expression studies.

4.4.3 Bioavailability of CTR1 siRNA in HUVEC:

Since it was observed that CTR1 siRNA decreased mRNA expression at 1 h and 4 h time point it needed to be confirmed at the level of siRNA entry. For bioavaibility experiments HUVEC were treated for siRNA 1 h, 2 h and 4 h time points. The pelleted cells were used for small RNA isolation using mirVana kit (Life technologies) and a quantitative real time PCR was performed. PCR showed presence of siRNA within 1 h and levels remained the same till 4 h time point (Figure 27).



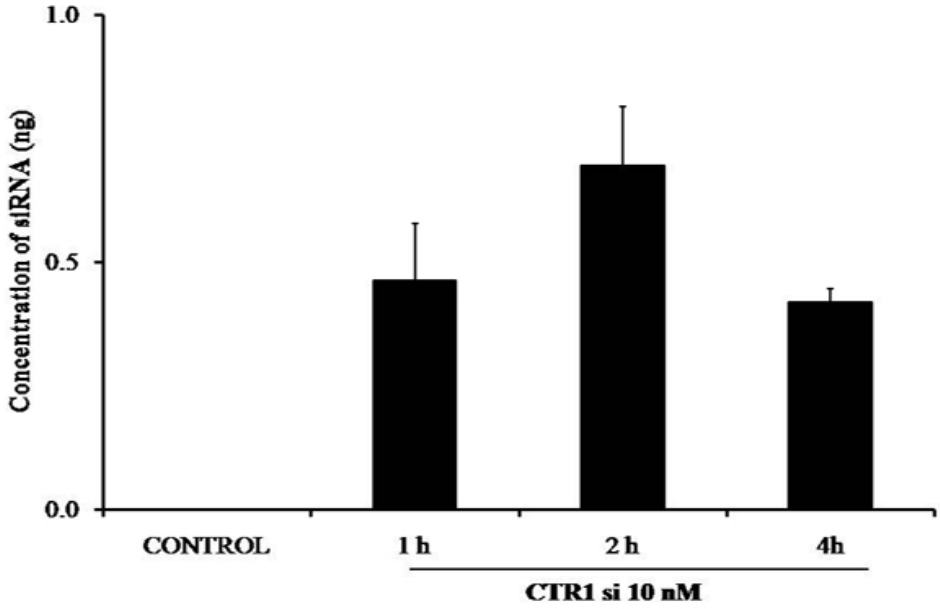


Figure 27: Bioavailability of CTR1 siRNA: a) Standard curve using CTR1 siRNA as standard. b) Intracellular absolute quantification of CTR1 siRNA at different time point of exposure from 1 h to 4 h.

4.4.4 Effect of CTR1 siRNA at the level of protein:

To further confirm siRNA specificity CTR1 ELISA was performed which showed that cells treated with Cu 100 µM had a significant ($p < 0.05$) increase in CTR1 protein (0.12 ± 0.022 ng/mL) compared with control (0.05 ± 0.003 ng/mL). Treatment with si 1 at 10 nM showed a significant decrease ($p = 0.04$) (0.04 ± 0.006 ng/mL) when compared to Cu treated cells. P 800 µM also showed reduction, in CTR1 levels (0.08 ± 0.002 ng/mL) which was not statistically significant when compared to Cu alone treated cells. Whereas, Ssi 10 nM treated did not show any significant change when compared to Cu alone treated cells (Figure. 28A). Western blot for CTR1 was done, and it showed an increase in CTR1 protein level in the presence of Cu, and decreased when treated with si 1 at 10 nM. While, cells treated with 800 µM P treated also showed mild decrease in band intensity when compared to Cu treated (Figure.28B). Similarly, Ssi at 10 nM concentration did not show any significant change. Immunofluorescence of CTR1 showed increase in CTR1 levels in Cu 100 µM and decreased in the presence of si 1 at 10 nM and 800 µM P whereas Ssi at 10 nM did not show any significant change (Figure.28C). Immunocytochemistry showed an increase in CTR1 protein in the presence of 100 µM Cu and si 1 at 10 nM decreased protein expression. Cells treated with 800 µM P showed a mild decrease when compared to Cu treated (Figure.28D). Whereas, Ssi 10 nM treatment did not show any significant change in protein level when compared to Cu alone treated cells.

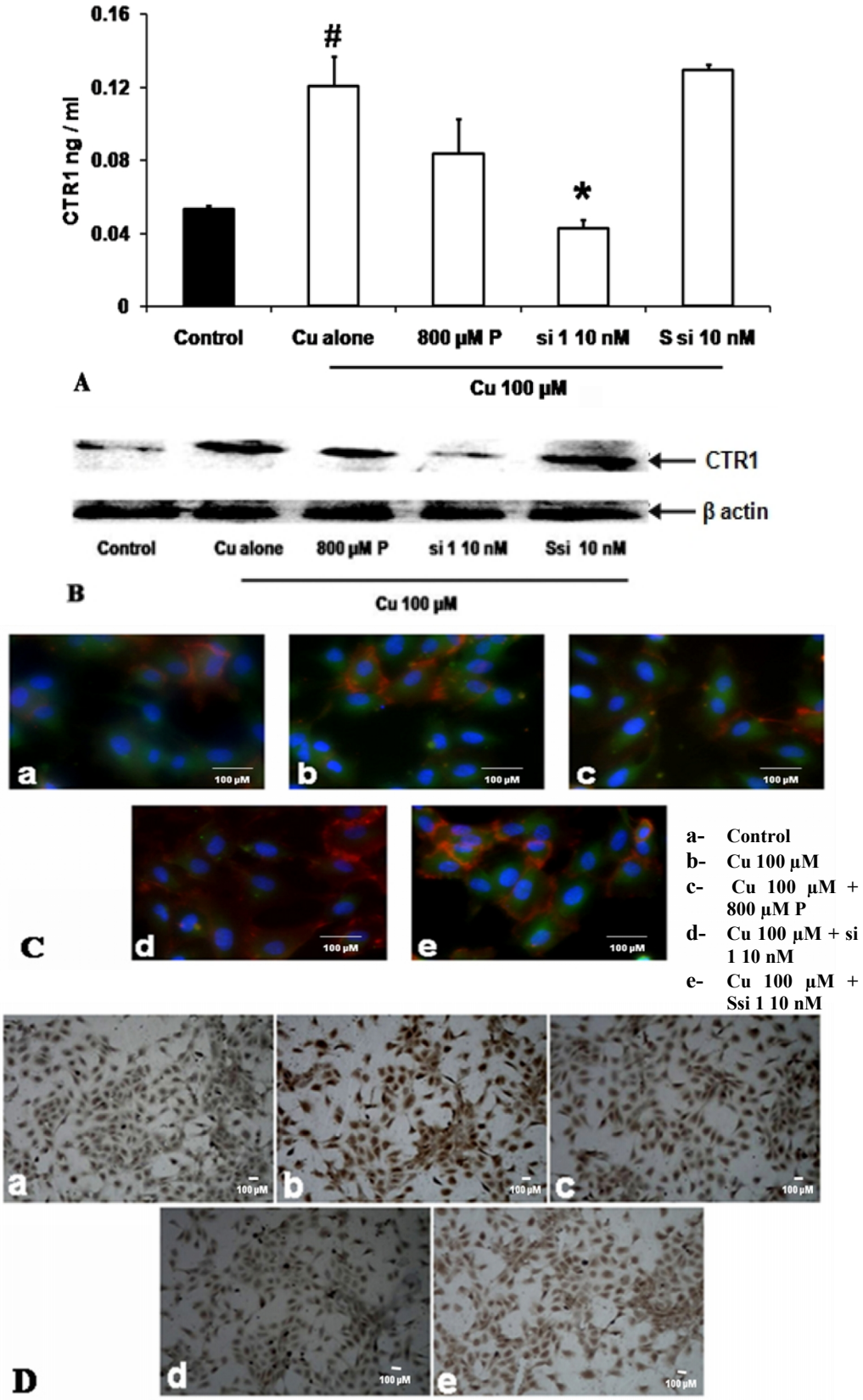


Figure 28: Specificity of CTR1 siRNA: Protein expression pattern of CTR1. A) ELISA for CTR1. B) Western blot panel i) CTR1, ii) β actin. C) IF of CTR1 (green) with CD31 (red), nucleus stained with DAPI. D) ICC for CTR1 showed cells stained brown in colour. Comparison between control and Cu treatment is represented as # furthermore Cu treatment and siRNA comparison is represented as *.

4.4.5 Effect of CTR1 siRNA on other Cu modulators: Further, mRNA expression of SPARC and LOX in the presence of CTR1 siRNA showed no significant change in expression indicating that the selected siRNA did not show any off target effects (Figure. 29).

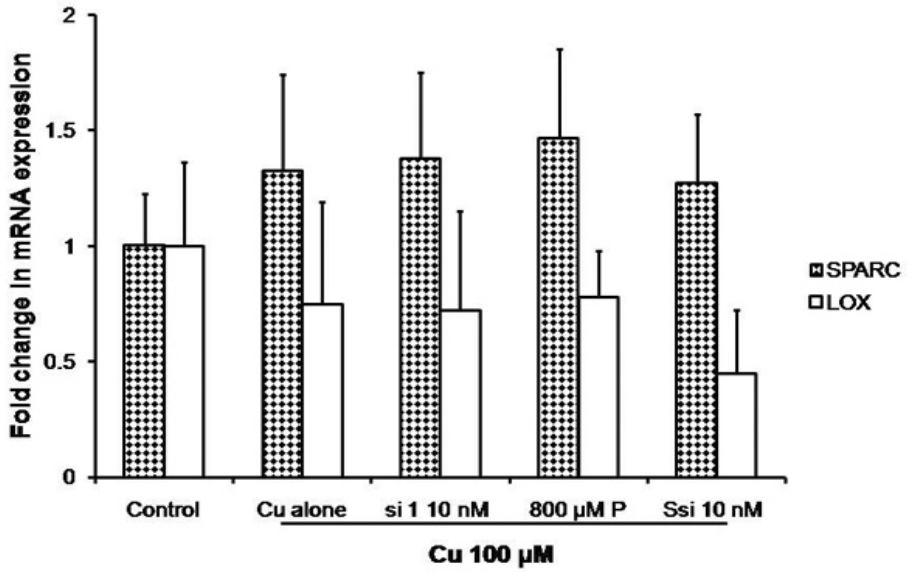


Figure 29: Real time PCR for LOX and SPARC to check the off target effect of CTR1 siRNA in HUVEC.

4.4.6 Effect of CTR1 siRNA on intracellular Cu levels:

Since, Cu increased CTR1 expression, it was imperative to know the intracellular Cu levels. Phen green dye binds to Cu and reduces fluorescence. First a standard graph was plotted using different concentrations of Cu and Phen green dye using a fluorescent plate reader. It was noted that Cu binding to Phen green reduced fluorescence in a dose dependent manner (Figure 30A). Following this, HUVEC were exposed to Cu 100 µM and the three siRNA were screened for CTR1 functional assay by measuring intracellular Cu levels. The results showed that si 1 at 10 nM was effective in regulating intracellular Cu levels, when compared to other siRNAs (Figure 30B).

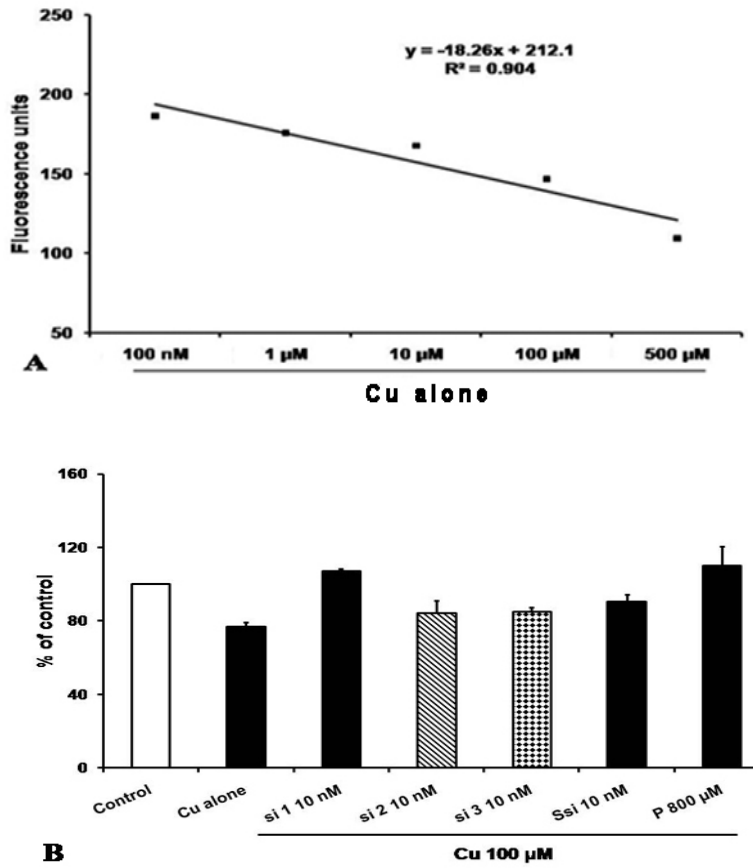


Figure 30: Phen green dye estimation of intracellular Cu level A) Standard graph for Cu in the presence of Phen green from concentration 100 nM to 500 µM showed quenching in fluorescence in the presence of different concentrations of Cu. B) Screening of siRNA for regulation of intracellular Cu levels.

Cu estimation by FACS using Phen Green dye showed that 100 µM Cu reduced the median fluorescence intensity by 50 % which was significant (p = 0.04) when compared with control cells without Cu treatment. Cu 100 µM + si 1 - 10 nM showed an increase in median fluorescence intensity by 60 % which was significant (p = 0.02) when compared to cells treated with Cu. Similarly, 800 µM P treatment also showed 60 % increase which was significant (p = 0.03) in fluorescence intensity when compared to Cu treatment (Figure. 31A). To reaffirm, Cu estimation was done by AAS method in cell lysates. Cells exposed to Cu 100 µM (4.42 µg/mL) showed a 6.4 fold increase in Cu levels when compared with cells without Cu treatment (0.69 µg/mL), (p = 0.0005). Cells treated with si 1 - 10 nM showed a 4 fold decrease in Cu levels (1.08 µg/mL) significantly (p = 0.0008) when compared with Cu. P 800 µM also showed a 2.9 - fold decrease in the Cu level (p = 0.01), which also showed statistical significance. No significant changes in Cu levels were seen in the presence of Ssi (Figure. 31B).

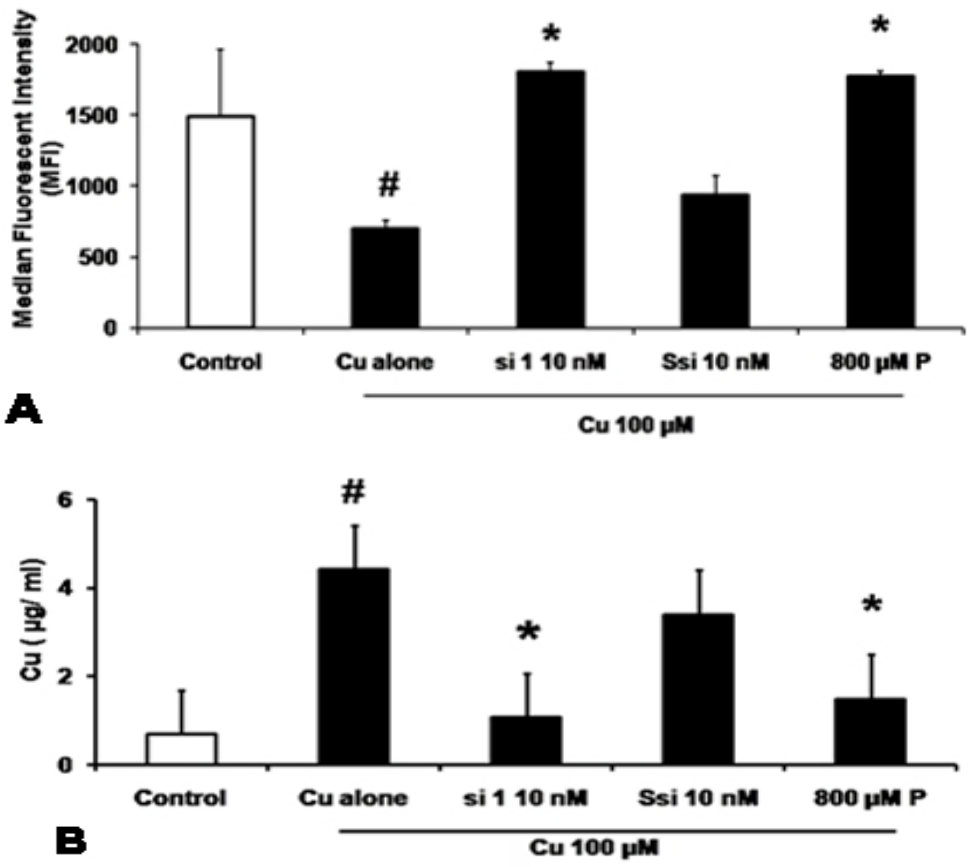


Figure 31: Intracellular Cu estimation A) FACS –.Phen green fluorescent dye. B) AAS – Cu estimation was done by using graphite furnace. Comparison between control and Cu treatment is represented as # furthermore Cu treatment and siRNA comparison is represented as *.

4.4.7 *In - vitro* angiogenesis assays to test the effect of CTR1 silencing on the inhibition of angiogenesis

Cell proliferation assay by tritiated thymidine showed that Cu concentration of 100 µM induced proliferation of the HUVECs by 3 fold (p = 0.0003) compared to cells without Cu treatment, si1 at 10 nM showed 2.5 fold decrease in proliferation was seen in the presence of Cu 100 µM (p = 0.003) and Ssi 10 nM did not show any significant decrease in proliferation. Whereas Cu 100 µM + P 800 µM showed a decrease of 2.8 fold (p = 0.02) when compared with Cu treated cells (Figure 32).

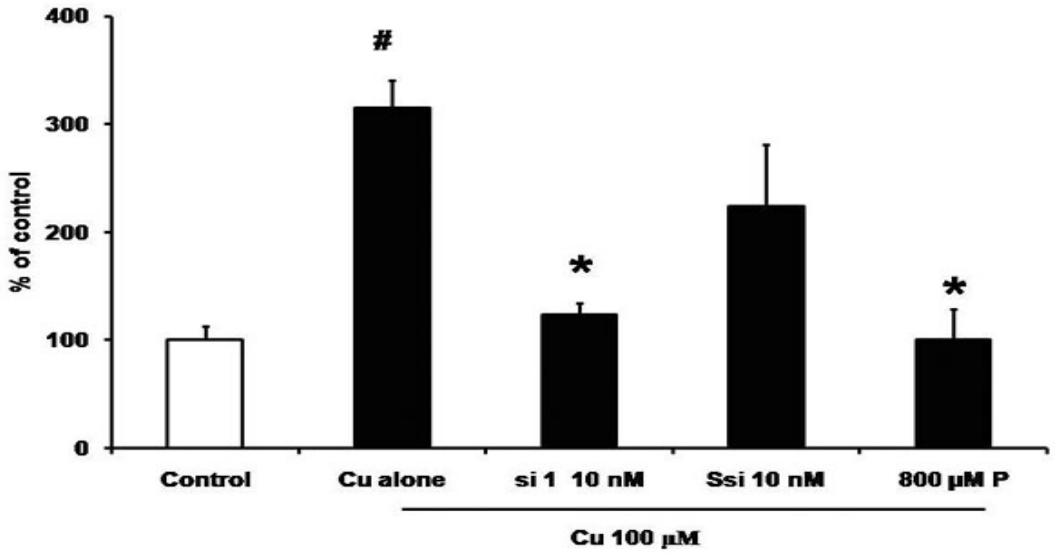


Figure 32: Proliferation assay using tritiated thymidine showed significant increase in proliferation in Cu treatment and CTR1 siRNA and pencillamine decreased proliferation at the end of 48 h of treatment. Comparison between control and Cu treatment is represented as # furthermore Cu treatment and siRNA comparison is represented as *.

Apoptosis assay which measures histone DNA complexes in cytoplasm indicated that there was no cell death induced by siRNA as verified by cell death ELISA method (Figure 33).

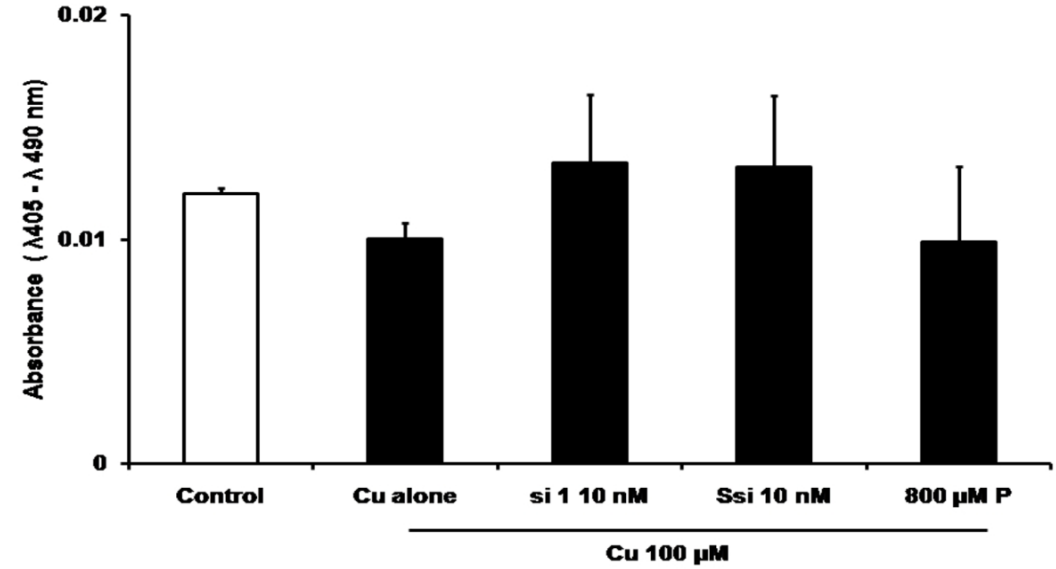


Figure 33: Apoptosis assay – Cell death ELISA method showed no apoptosis of cells in the presence of Cu, siRNA, Ssi and pencillamine when compared to control (untreated).

Attachment assay with Cu concentration of 100 µM showed more attachment of cells to gelatin matrix as compared to control cells with a *p* value of 0.02 and Cu 100 µM + si 1 - 10 nM decreased attachment of HUVEC with a *p* value of 0.00009 respectively. Whereas, Ssi and penicillamine did not show any significant change (Figure 34)

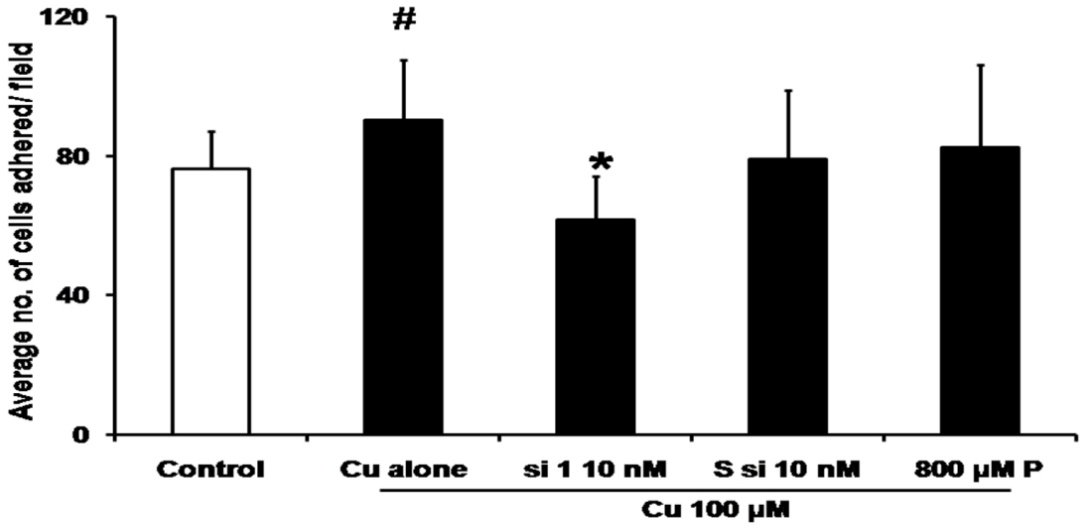


Figure 34: Cell attachment assay. Comparison between control and Cu treatment is represented as # furthermore Cu treatment and siRNA comparison is represented as *.

Tube formation assay showed that Cu concentration of 100 µM induced tube formation. There was a two fold increase in tube length for Cu treated when compared to cells without Cu which was significant (p = 0.008). Treatment with Cu 100 µM + si 1 10 nM concentration showed 3.3 fold decrease in tube formation (p = 0.0015) when compared to Cu treated, which was not observed in Cu 100 µM + Ssi 10 nM. Cu 100 µM + P 800 µM treated condition showed a 1.9 fold reduction in tube formation that was significant (p = 0.03) when compared to Cu alone, but it was not as effective as CTR1 siRNA. Quantification of tubes was done using angioquant software (NIH) in which the number of junctions was measured, and it was found that Cu increased the tube formation and siRNA decreased the same (Figure. 35).

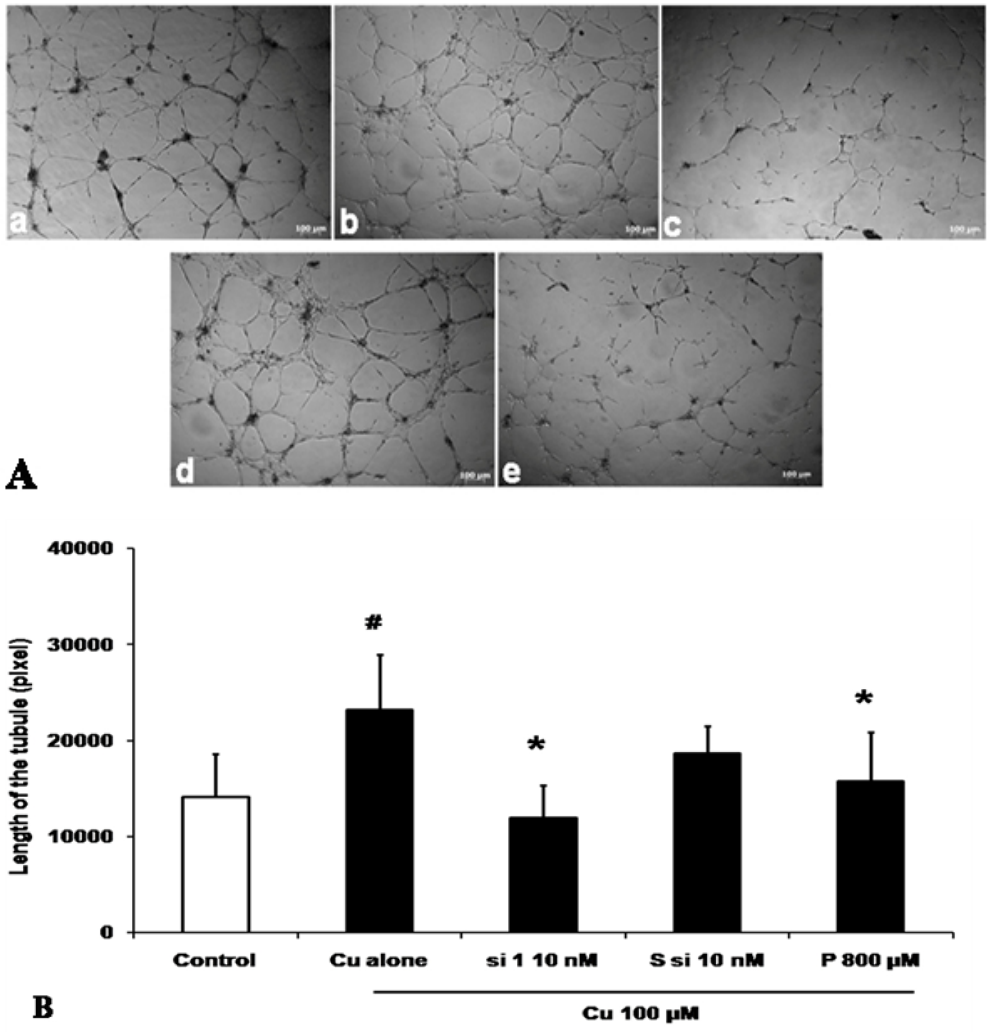


Figure 35: A) Tube formation assay: a) control b) Cu 100 µM c) Cu 100 µM+ si 1- 10 nM d) Cu 100 µM+S si 1- 10 nM e) Cu 100 µM+P 800 µM B) Histogram-Quantification of tubule length using angioquant tool. Comparison between control and Cu treatment is represented as # furthermore Cu treatment and siRNA comparison is represented as *.

Scratch assay showed Cu concentration of 100 µM increased migration of cells and Cu 100 µM + si 1 - 10 nM concentration inhibited the migration. Quantification of migration was done using transwell inserts 8 µm size where Cu showed 88 % increase in migration of cells with a *p* value of 0.00001 when compared to control cells and si 1 - 10 nM concentration reduced migration by 75 % with a *p* value of 0.00001 when compared to Cu concentration of 100 µM treatment. Cells treated with 800 µM P showed significant (p = 0.02) decrease in cell migration whereas Ssi 10 nM and did not inhibit migration (Figure 36).

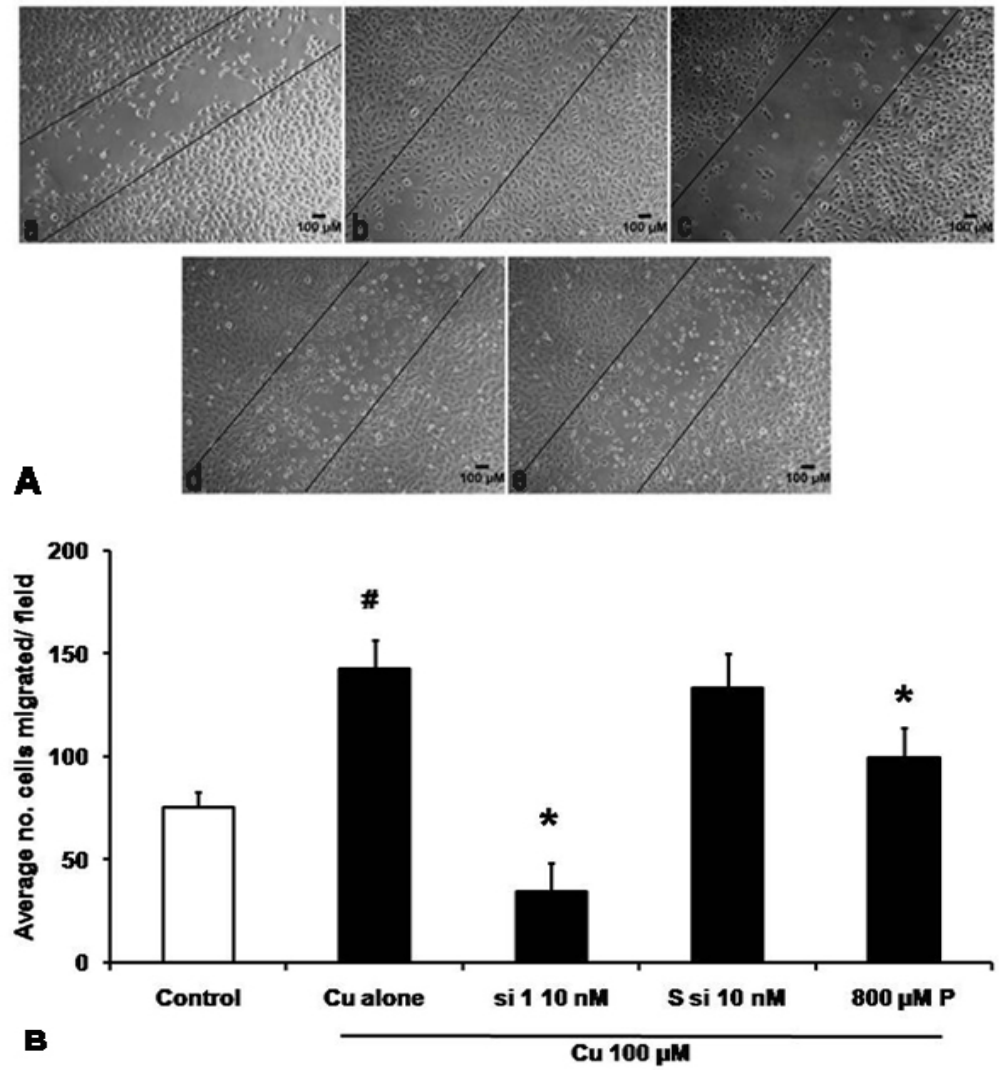


Figure 36:Migration assay A) Scratch assay : a) control, b) Cu 100 µM, c) Cu 100 µM+ si 1- 10 nM, d) Cu 100 µM+S si 1- 10 nM, e) Cu 100 µM+P 800 µM. B) Histogram - Transwell migration assay. Comparison between control and Cu treatment is represented as # furthermore Cu treatment and siRNA comparison is represented as *.

4.4.8 *In vivo* assay to test the efficacy of the developed antisense si RNA:

To test the efficacy of the siRNA it was tested on closed CAM model and it showed regression of blood vessels in the presence of siRNA treatment. To various condition such as control, Cu concentration of 25 µM, Cu with si RNA and Cu with Pencillamine at 0^{th} h time point and at the end of 18 h. Photographs were taken near the implant which showed increased vessel formation, in Cu treated and regressed vessel formation indicated in si 10 nM treated CAM model pencillamine treatment did not show much changes (Figure 37).

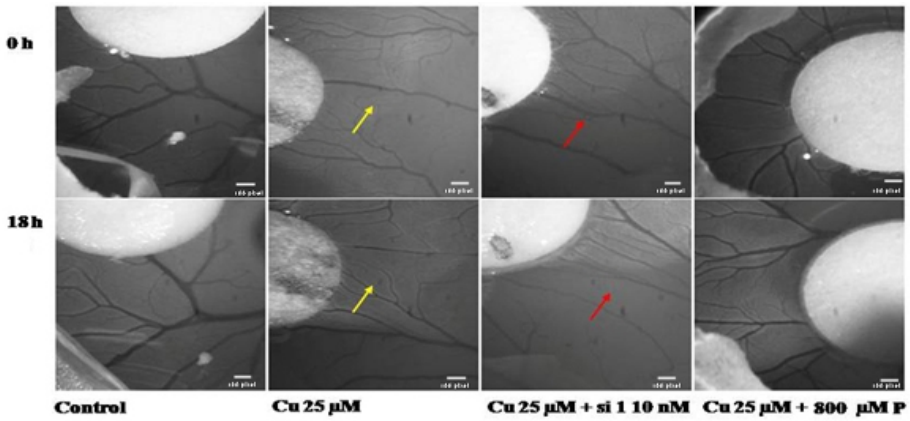


Figure 37: CAM assay after 18 h time point showed increased in blood vessel formation in the presence of Cu 25 µM (indicated in yellow arrows) and siRNA 10 nM showed decreased blood vessel formation (indicated in red arrows).

CTR1 siRNA was modified to (si 1 100 ng) 2'– O – methyl for better stability. This was tested in New Zealand male rabbit *in vivo* by performing corneal packet assay. Matrigel implanted cornea showed progression in new blood vessel formation which was regressed in siRNA treated group when compared to matrigel treated animal. The regression of blood vessels was seen from day 3, but by day 11 there was a significant reduction in blood vessel formation. PBS given as a vehicle control did not show regression in blood vessel formation when compared with matrigel implant (Figure.38).

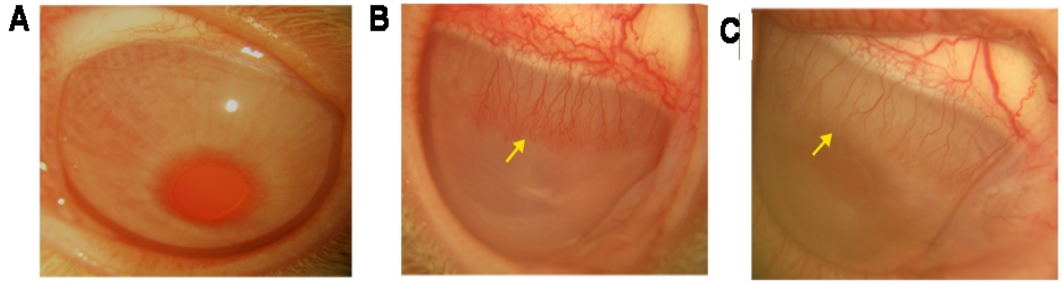


Figure 38: Rabbit corneal packet assay representative microphotographs A) Control- Normal cornea without matrigel. B) Vehicle control – matrigel implant PBS treated showed increased blood vessel formation (indicated yellow arrow). C) CTR1 siRNA 100 ng treated- matrigel implant CTR1 siRNA treated (indicated in yellow arrow).

4.4.9 Mechanism of anti-angiogenic effect of CTR1 siRNA:

Cu has been shown to induce VEGF expression through a similar pathway to HIF 1α pathway, and it has also been showed that it was inhibited by Cu chelating agents (Sen et al. 2002). In this study we observed that Cu concentration of 100 µM increased VEGF mRNA levels by 1.73 fold (p = 0.015) when compared to control. Cells treated with CTR1 si 1 at 10 nM mRNA expression reduced in the presence of by 45% (p = 0.013) when compared to cells treated with Cu. Treatment with P 800 µM showed decreased mRNA levels by 1.12 fold whereas Ssi showed no significant change (Figure 39A). Protein expression showed increased VEGF protein in the presence of Cu and inhibiting Cu entry inside the cell by silencing the CTR1 decreased VEGF expression (Figure 39B).

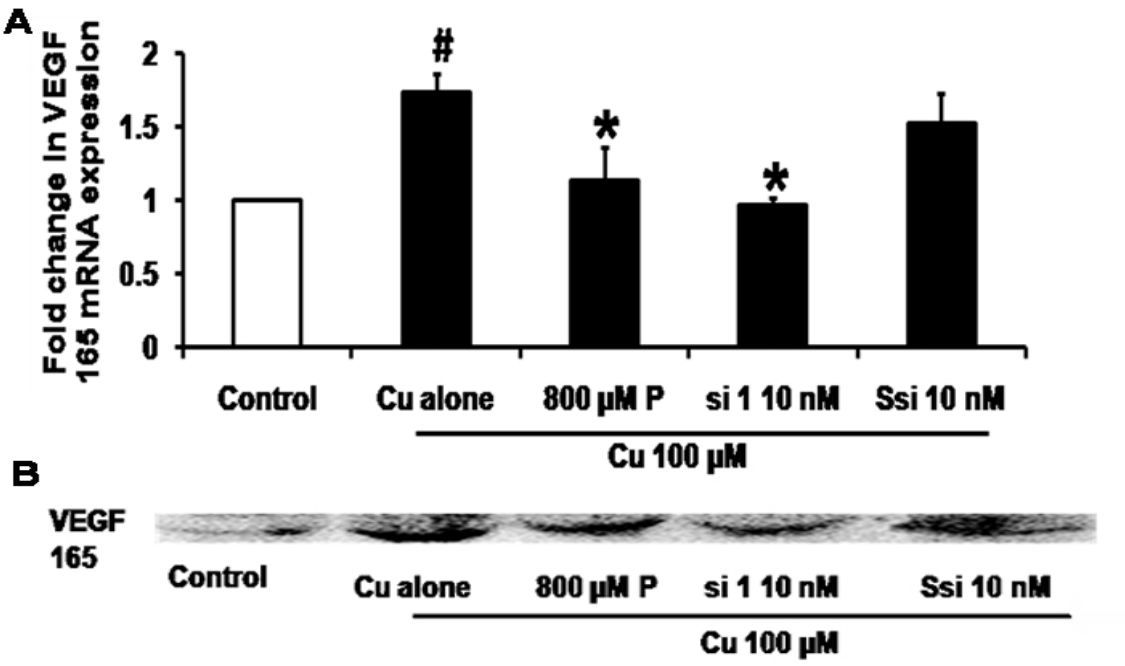
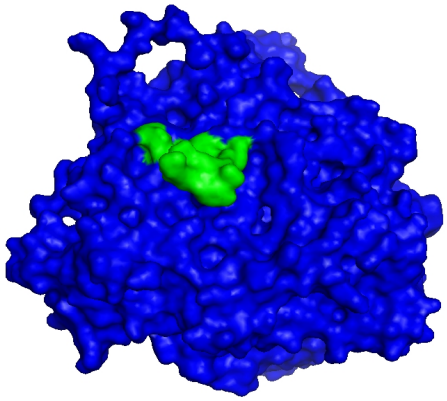


Figure 39: Mechanism of CTR1 silencing A) VEGF mRNA expression, B) VEGF western blot. Comparison between control and Cu alone treatment is represented as # furthermore Cu treatment and siRNA comparison is represented as *.

The developed CTR1 siRNA inhibited angiogenesis in HUVEC model by decreasing VEGF levels.

4.5 DESIGN OF PEPTIDE FRAGMENTS FROM CTR1 TARGETING ANGIOGENESIS

Using bioinformatics software Hex, CTR1 interactions with CP was studied. Three motifs from CTR1 which were already known to interact with CP were taken and docking studies were performed (Figure 39-41). These studies were also performed by Zatulovskiy *et al*., to show CTR1 "histidine rich" motifs interacts with CP (Zatulovskiy et al. 2007).



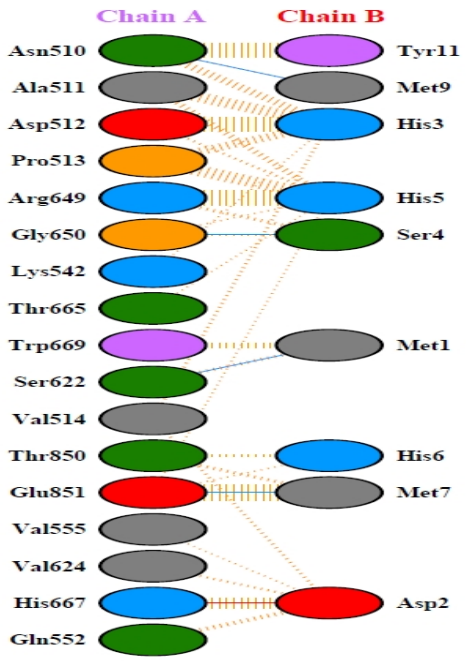
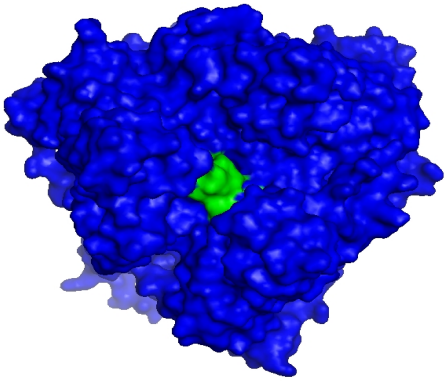


Figure 40: Docking of CTR1 motif 1 with whole CP protein. Results shows interactions at methionine (1,7&9), histidine residues mainly (3,5,&6).CP (blue) interaction with motif 1 of CTR 1(green) PDB sum result: Chain A (CP) –Chain B (Motif 1)



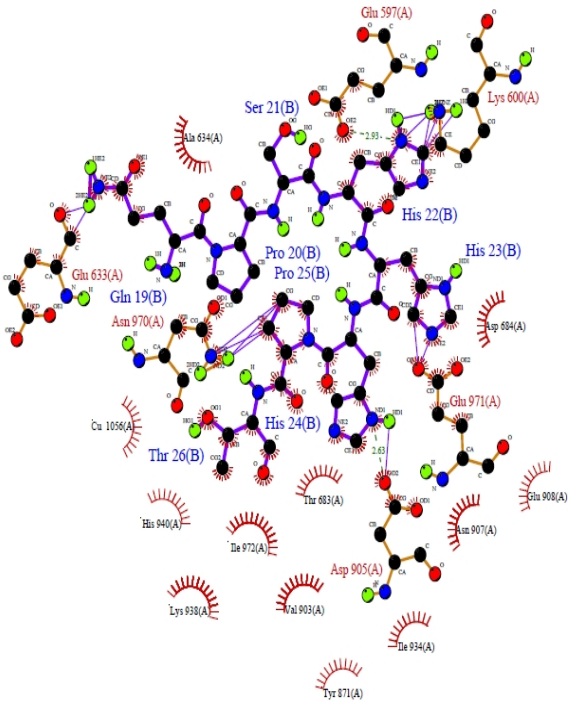
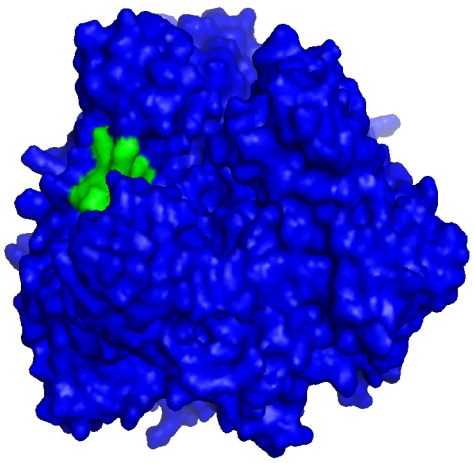


Figure 41: Docking of CTR1 motif 2 with whole CP protein. Results show interactions at histidine (22, 23 &24), proline (20&25) and serine (21) residues. CP (blue) interactions with motif 2 of CTR1 (green). PDB sum result: Chain A (CP) –Chain B (Motif 2).



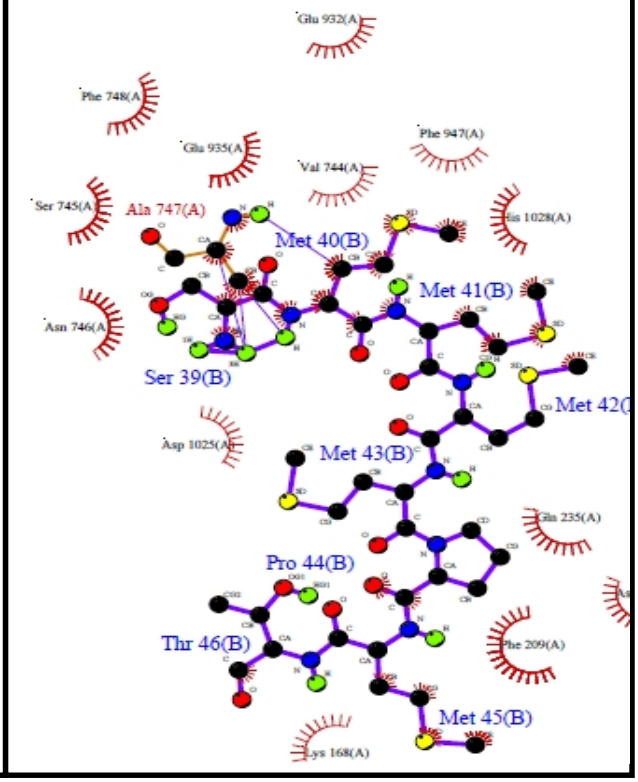


Figure 42: Docking of CTR1 motif 3 with whole CP protein. Results shows interactions at methionine (40-43 &45), proline (44) and serine (39) residues. CP (blue) interactions with motif 3 of CTR 1(green) PDB sum result: Chain A (CP) –Chain B (Motif 3).

CTR1 derived peptides were synthetically produced (USV peptides, India) and were tested for purity using Agilent HPLC system. All the peptides of 10 µg concentrations were injected in HPLC showed a single peak in VWD detector indicating the purity of the synthesized peptides (Figure 43).

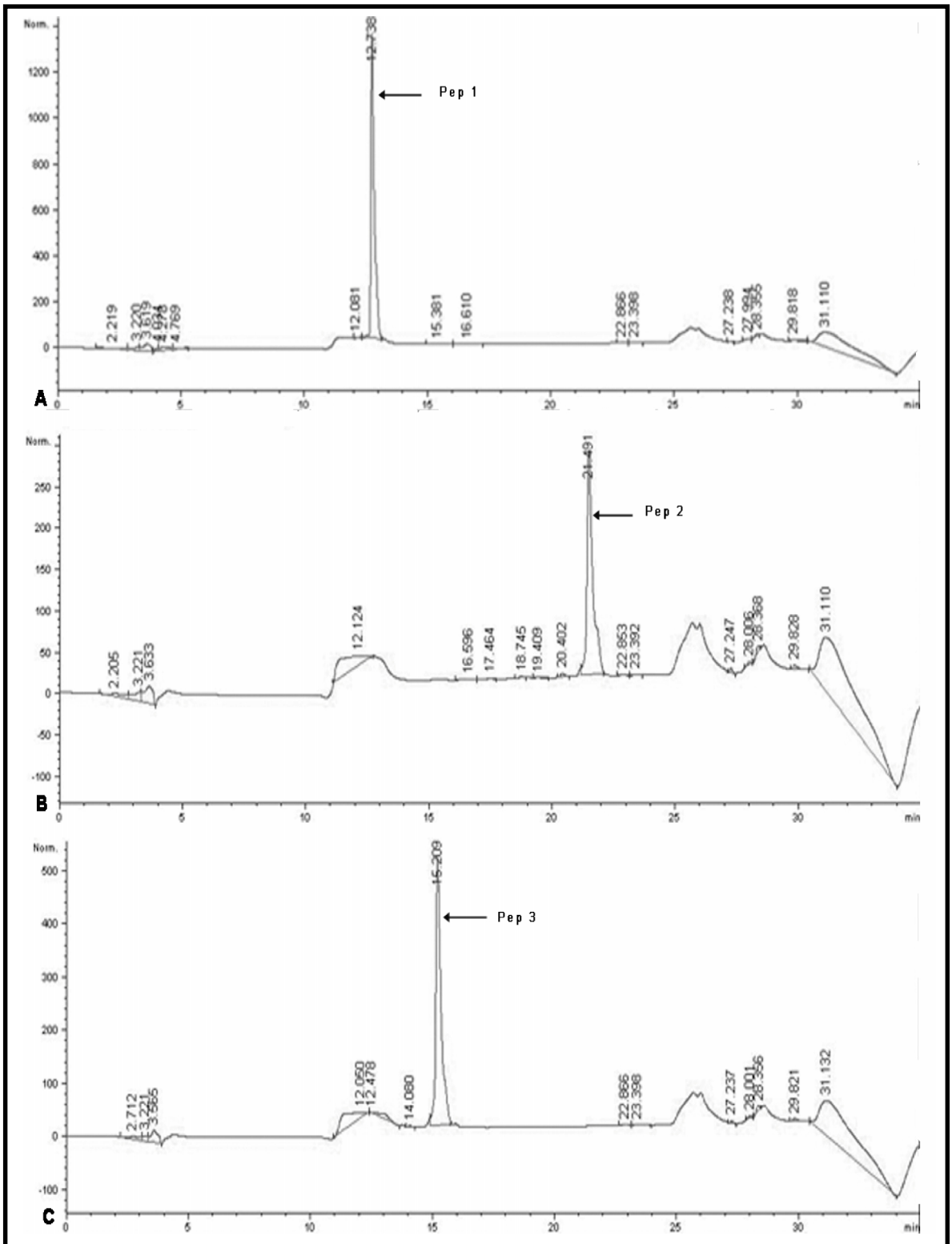


Figure 43: HPLC chromatogram of CTR1 derived peptides. A) Pep1 showed a retention time of 12.73 min, B) Pep 2 showed a retention time of 21.49 min and C) Pep 3 showed a retention time of 16.02 min respectively at 215 nm.

4.5.1 Cytotoxicity assay of CTR1 derived peptides:

Peptides derived from histidine rich 27 amino acids peptide of endostatin which bound to zinc was reported to have anti-angiogenic effect (Kolozsi et al. 2009). Similarly, in this work CTR1 derived peptide rich in histidine and methionine motifs were tested for their role in Cu binding and anti-angiogenic activity.

MTT assay was performed to check the cytotoxicity of the designed peptides from CTR1 on HUVEC. Concentration ranges from 1- 10 µM for 1 h time point of Pep 1, 2, 3 and Scp did not show any cytotoxic effect tested (Figure 44).

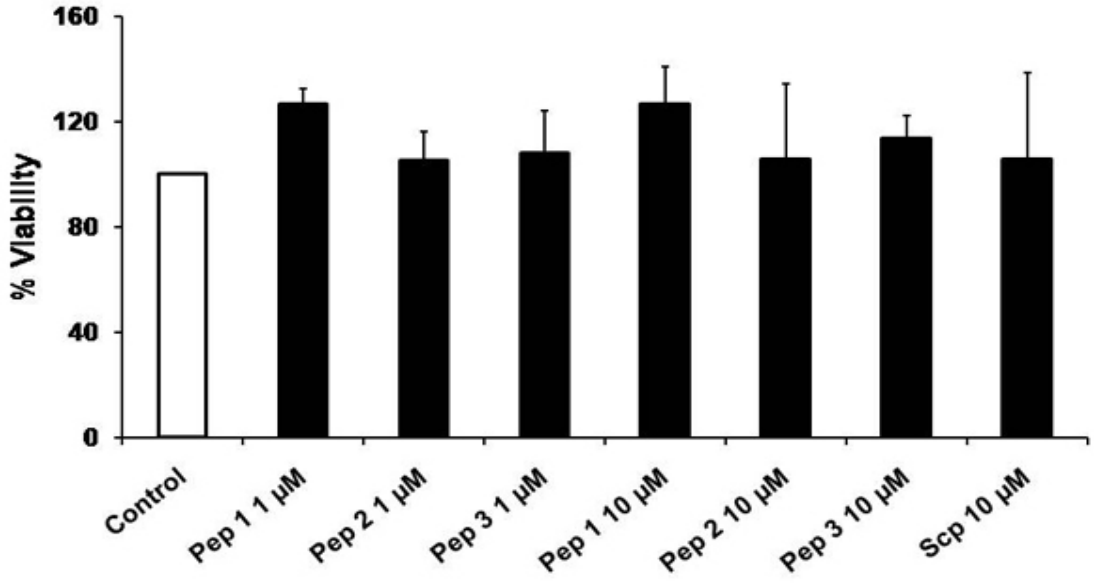


Figure 44: Cytotoxicity assay on HUVEC for CTR1 derived peptides.

4.5.1.1 Standardisation of peptide concentration for angiogenesis assay:

Migration assay:

Peptide concentrations from 1- 100 µM were tested for migration assay in HUVEC. All the three peptides screened showed inhibition in migration in tested concentration indicating that 1 µM of the peptides could be used for further studies (Figure 45).

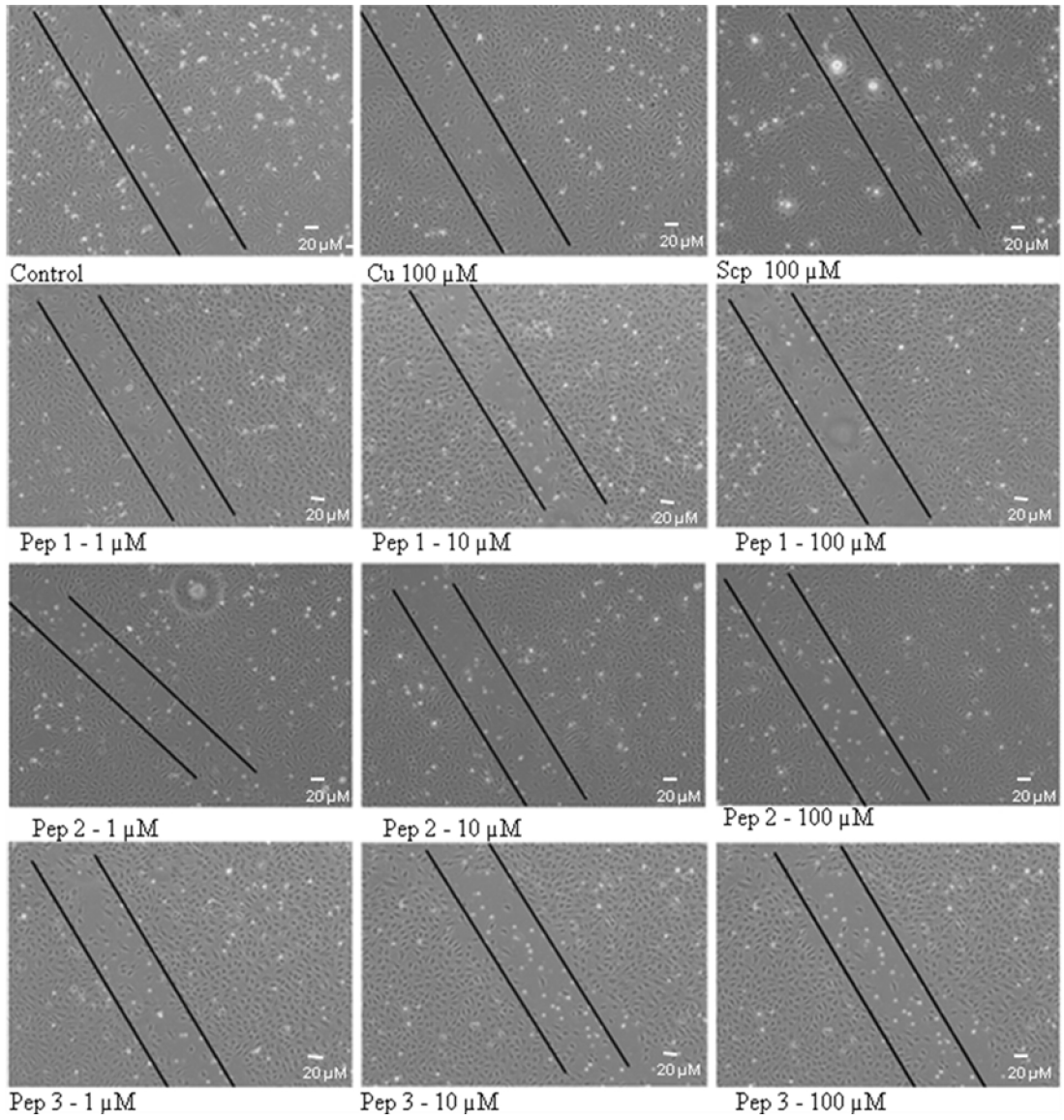
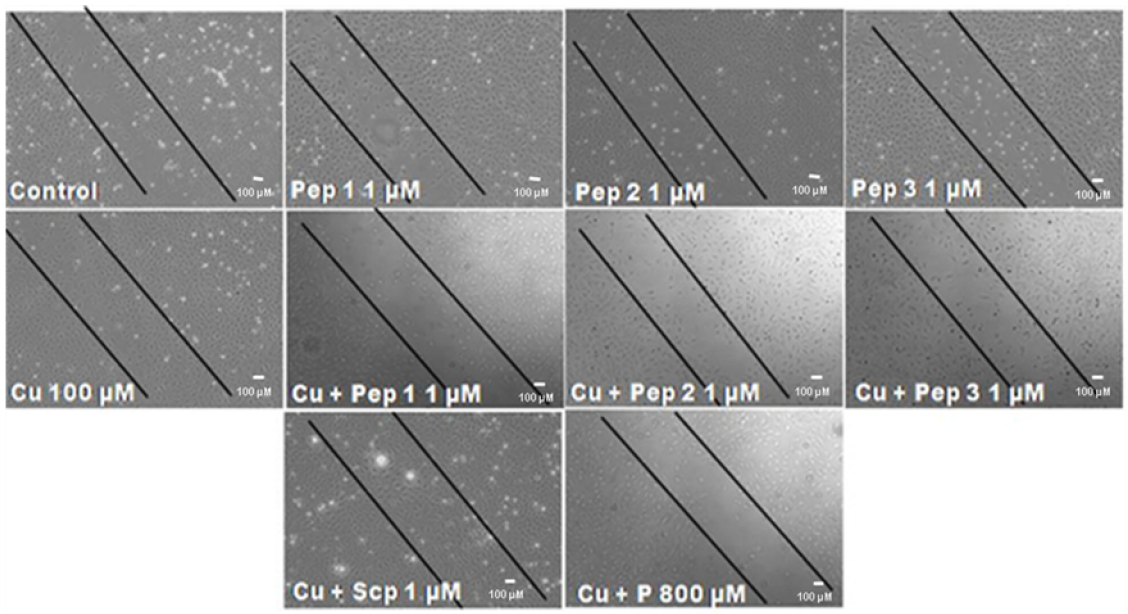


Figure 45: Standardisation of peptide concentration for angiogenesis assay in HUVECs in 1% EBM medium.

4.5.1.2 *In vitro* angiogenesis assay:

Scratch assay showed CTR1 derived peptides (Pep 1, 2 and 3) at 1 µM concentration inhibited migration when compared to control whereas Scp 1 µM did not show any effect. This inhibition of peptide was also seen in presence of Cu 100 µM dependent migration. At the end of 8 h, migration was quantified using 8 µM transwell inserts which showed significant decrease in migration by 52 % for Pep 1 - 1 µM (p= 0.001), 64 % for Pep 2 - 1 µM (p= 0.01) and 65 % for Pep 3 - 1 µM (p= 0.02) when compared to control. Whereas Scp did not show any significant change when compared to control. Further CTR1 derived

peptides were tested in presence of Cu concentration of 100 µM. Cu showed 74 % increase in migration of cells with a *p* value of 0.00003 when compared to control. Decreased migration was seen in cells treated with Cu 100 µM + 1 µM of Pep 1 by 60% (*p*= 0.000002), Cu 100 µM + 1 µM of Pep 2 by 53 % (*p*= 0.0000003), Cu 100 µM + 1 µM of Pep 3 by 38 % (*p*= 0.00018) when compared to Cu 100 µM treatment. P 800 µM treated cells inhibited migration by 63% (*p*= 0.000028) when compared to Cu treated (Figure 46).



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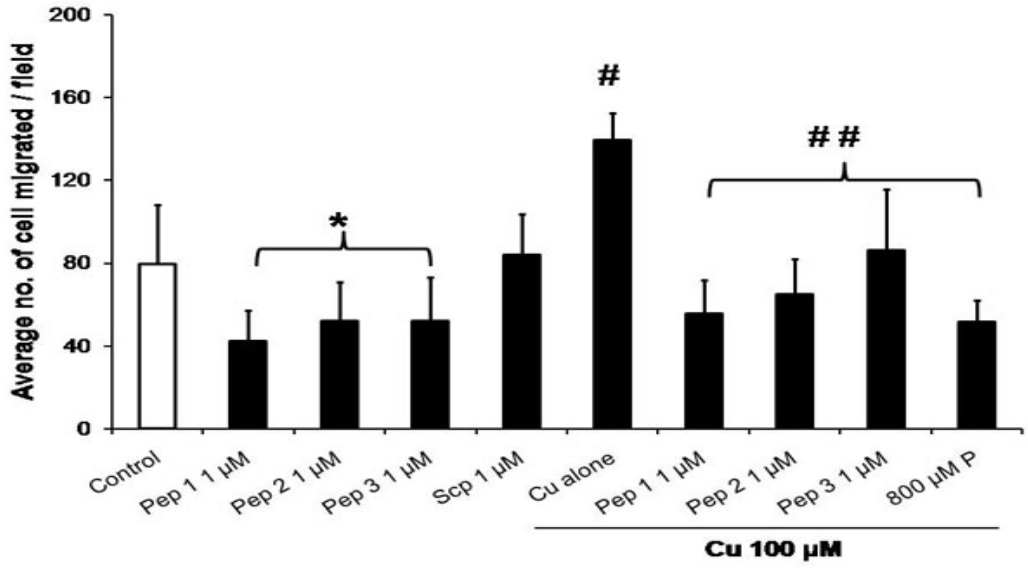


Figure 46: Effect of CTR1 derived peptide on migration assay A) Scratch assay B) Transwell migration assay. Comparison between control and peptide alone treatment is represented as *. Cu treatment condition comparison was done with control and peptide treatment that are represented as #.

CTR1 derived peptides were tested for **tube formation assay**. Cells treated with 1 µM of Pep 1 showed 0.33 fold reduction in tube length (p=0.006), Pep 2 (p=0.03) and Pep 3 (p=0.02) showed 0.2 fold reduction in tube length significantly, when compared to control, whereas Scp 1 µM did not show any significant change. There was a two fold increase in tube length in Cu treated when compared with cells without Cu which was significant (p = 0.006). Treatment of CTR1 derived peptides Pep 1, 2 and 3 at 1 µM with Cu 100 µM showed 1.5 fold significant decrease in tube formation (p = 0.0003, p = 0.002 and p = 0.01) when compared to Cu treated. Cu 100 µM + P 800 µM treated condition also showed a 1.6 fold reduction in tube that was significant (p = 0.006) when compared with Cu (Figure 47).

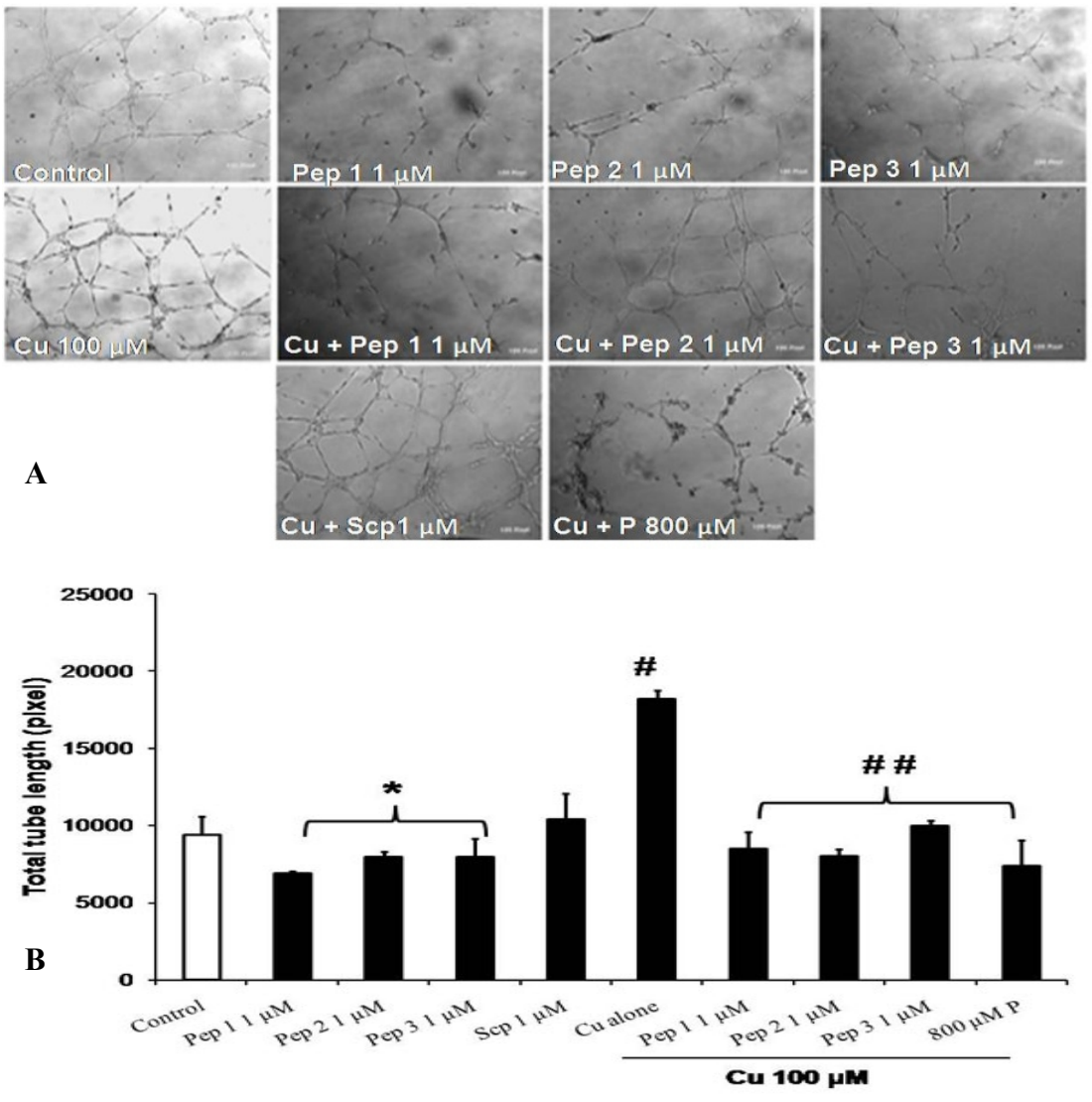


Figure 47: Effect of CTR1 derived peptide on tube formation assay. A) Tube formation assay. B) Quantification of tubes using angioquant software. Comparison between control and peptide alone treatment is represented as *. Cu treatment condition comparison was done with control and peptide treatment that are represented as #.

Cell proliferation assay showed that 1 µM of Pep 1 significantly (p = 0.04) decreased proliferation by 0.25 fold when compared to control. Whereas, 1 µM of Pep 2, 1 µM of Pep 3 showed 0.12 and 0.17 fold reduction and Scp 1 µM did not show any significant change in proliferation when compared to control cells Cu concentration of 100 µM induced proliferation of the HUVECs by 2.2 fold (p = 0.003) compared to cells without Cu treatment.CTR1 derived peptides Pep 1, Pep 2 and Pep 3 of 1 µM concentration showed significant (p = 0.003, p = 0.02, and p = 0.003) inhibition in proliferation in the presence of Cu 100 µM by 1.5 fold. Similarly, Cu 100 µM + P 800 µM also showed a 1.5 fold decrease (p = 0.003) when compared with Cu (Figure 48).

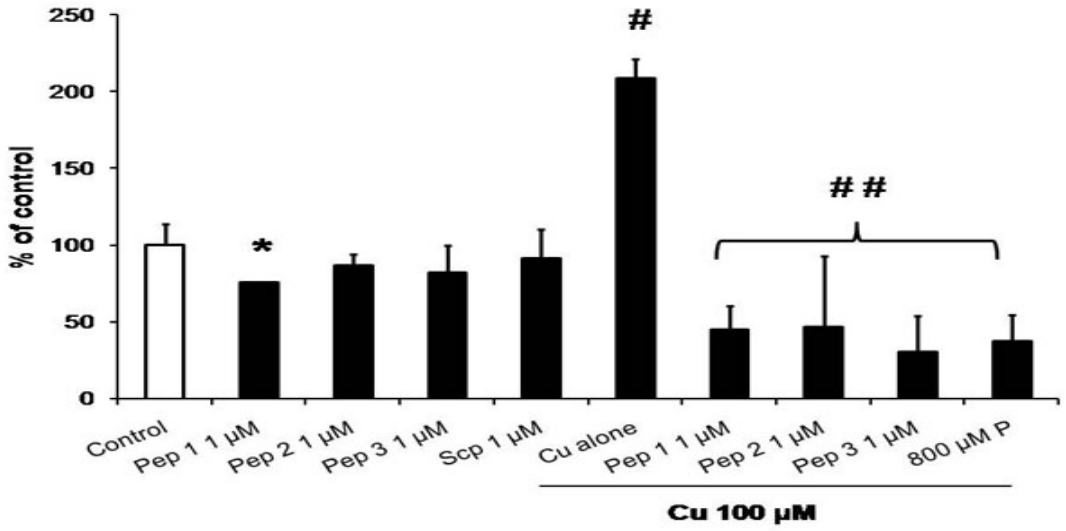


Figure 48: Proliferation assay using tritiated thymidine for CTR1 derived peptide. Comparison between control and peptide alone treatment is represented as *. Cu treatment condition comparison was done with control and peptide treatment that are represented as #.

Further, since these regions were designed from Cu binding sites of CTR1, it was interesting to estimate intracellular Cu level. **Cu estimation was done by AAS** showed cells exposed to 1 µM concentration of CTR1 derived peptides. Pep 1 showed 50 % reduction in intracellular Cu levels (1.56 µg/mL), Pep 2 showed a 48 % reduction in intracellular Cu levels (1.61 µg/mL), and Pep 3 showed a 37 % reduction in intracellular Cu level (1.24 µg/mL) whereas Scp 1 µM did not show any significant change. Cells exposed to Cu concentration of 100 µM (15.85 µg/mL) showed a 4.73 fold increase in Cu levels when compared with cells without Cu treatment (3.35 µg/mL), (p = 0.02). Cells treated with Cu 100 µM and 1 µM concentration of CTR1 derived peptides. Pep 1, Pep 2, Pep 3 µM showed a 2.7, 2.5 and 2.8 fold reduction in Cu levels (5.88 µg/mL, 6.216

µg/mL, 5.5 µg/mL) significantly (p = 0.01, p = 0.01, p = 0.04), when compared with Cu treatment (Figure 49).

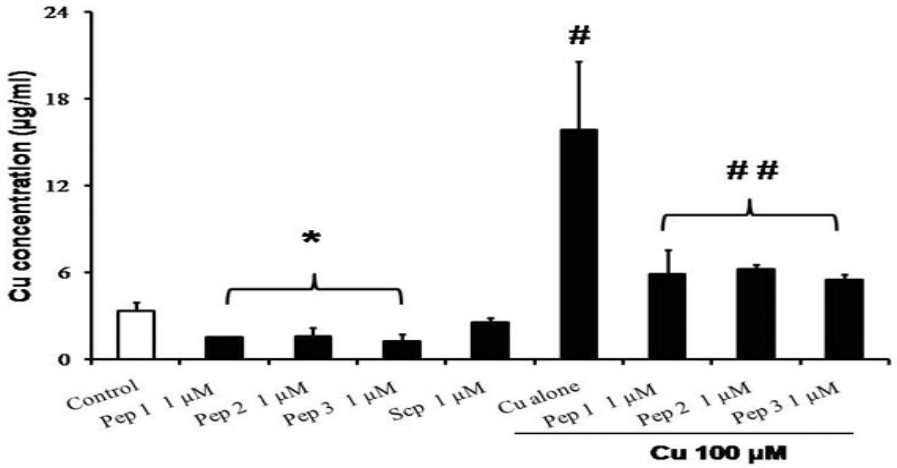
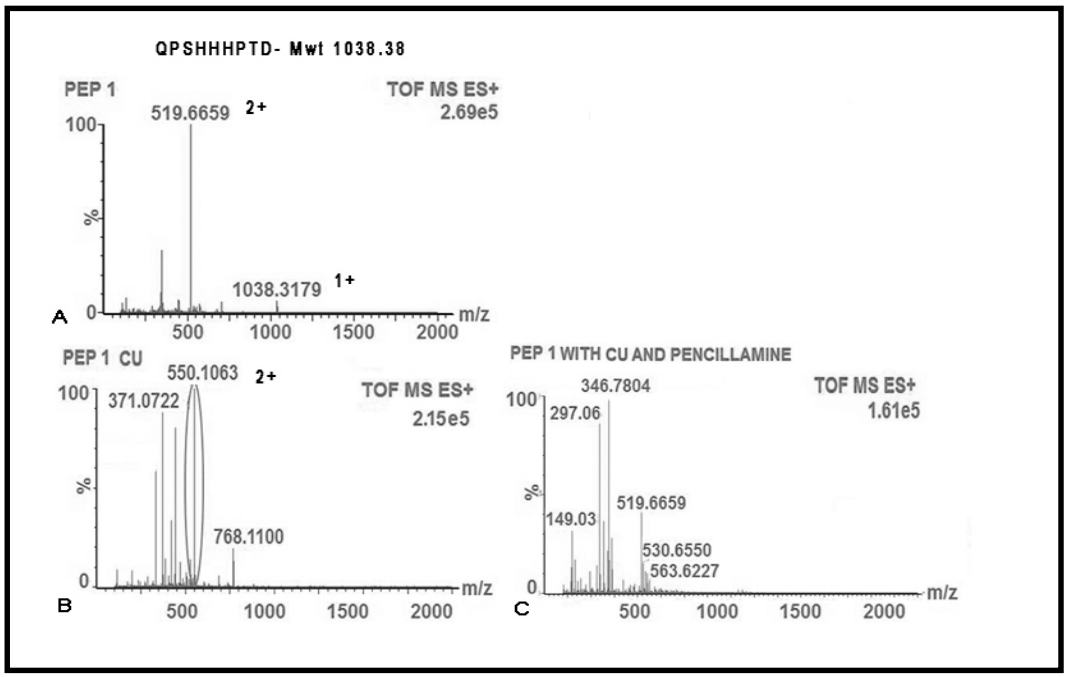


Figure 49: Intracellular Cu estimation by AAS for CTR1 derived peptides. Comparison between control and peptide alone treatment is represented as *. Cu treatment condition comparison was done with control and peptide treatment that are represented as #.

4.5.1.3 Copper binding function of CTR1 derived peptides

CTR1 derived peptides were analysed for their Cu binding function. CTR1 derived peptides of 1 µM concentration (Pep 1, 2 and 3) were incubated with equimolar Cu concentration. MS (XevoG2SQTof) data revealed all peptides were bound to Cu whereas Scp peptide did not show any Cu binding function. Further when Cu chelator penicillamine was added to the mixture it showed that Cu binding to peptide was lost (Figure 50).



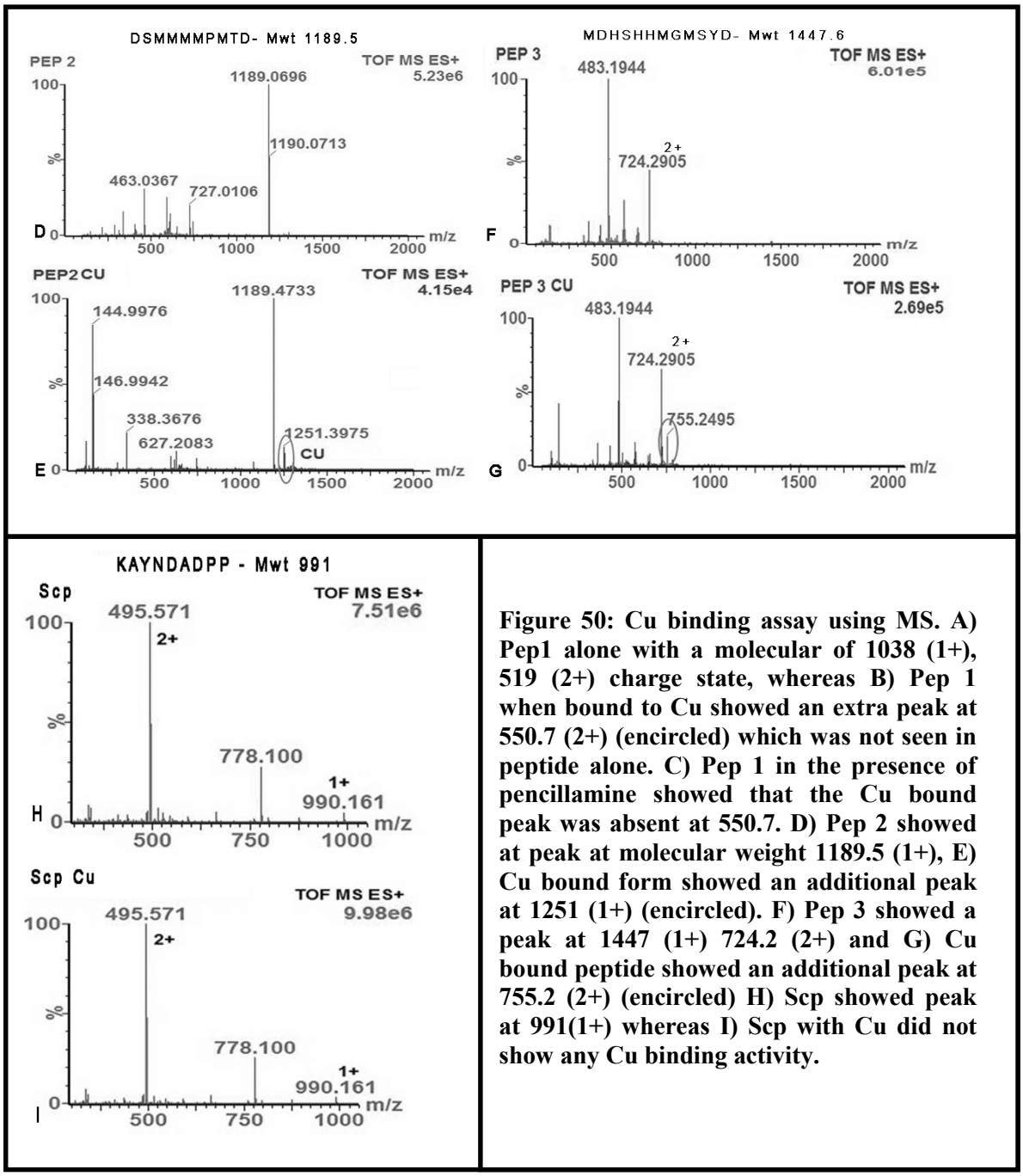


Figure 50: Cu binding assay using MS. A) Pep1 alone with a molecular of 1038 (1+), 519 (2+) charge state, whereas B) Pep 1 when bound to Cu showed an extra peak at 550.7 (2+) (encircled) which was not seen in peptide alone. C) Pep 1 in the presence of pencillamine showed that the Cu bound peak was absent at 550.7. D) Pep 2 showed at peak at molecular weight 1189.5 (1+), E) Cu bound form showed an additional peak at 1251 (1+) (encircled). F) Pep 3 showed a peak at 1447 (1+) 724.2 (2+) and G) Cu bound peptide showed an additional peak at 755.2 (2+) (encircled) H) Scp showed peak at 991(1+) whereas I) Scp with Cu did not show any Cu binding activity.

4.5.1.4 *In vitro* screening of rhodamine labelled CTR1 derived peptides for cellular entry

The challenge was to understand how these peptides were anti-angiogenic in nature when they could bind to Cu. Thus amino terminal labelling of CTR1 derived peptides with rhodamine showed that at different time points peptides were unable to penetrate the cell membrane and thus bound with extracellular Cu (Figure 51).

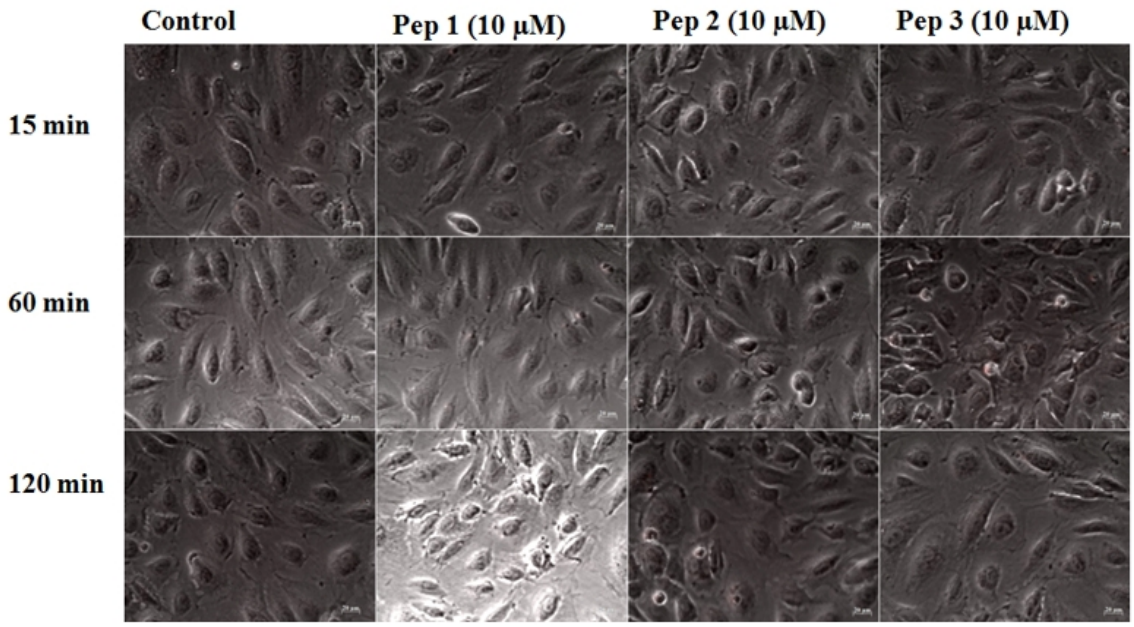


Figure 51: Intracellular identification of rhodamine labelled CTR1 derived peptide.

4.5.1.5 *In vivo* CAM open model to screen CTR1 derived peptides for angiogenesis

Open CAM model was performed for CTR1 derived peptides to screen for their anti-angiogenic function and 100 nM concentration of Pep 1, Pep 2 and Scp were tested. Data showed Pep 1, Pep 2 showed decreased vessel formation when compared to Scp (Figure 52).

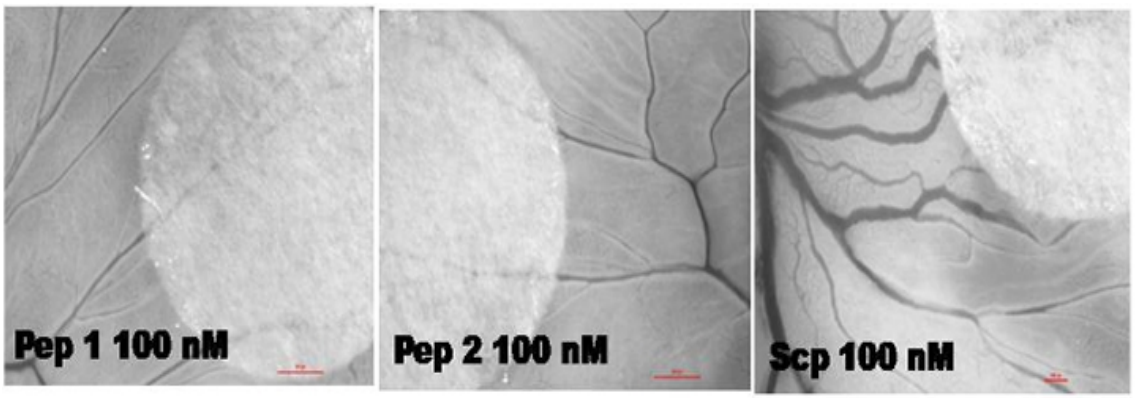
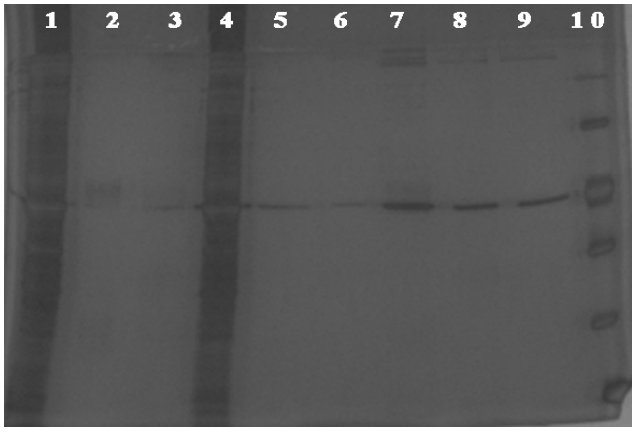


Figure 52: Open CAM assay to screen anti angiogenic property of CTR1 derived peptides.

4.6 INTRACELLULAR INTERACTING PARTNERS OF CTR1

IP for CTR1 was done in PBMC collected from three healthy volunteers. The eluted proteins were run in a gel. Gel showed the presence of eluted protein at 50 kDa and some high molecular proteins were also seen (Figure 53). The samples were subjected to insol digestion and submitted to 2D Nano LC Xevo G2S Qtof MS and analysed. The triplicate samples were analysed and the proteins hits were further narrowed down based on the same protein hits obtained in all the samples.



Lane 1- Crude PBMC cell lysate
Lane 2 - Ab cross linked bead
Lane 3 – Ab flow through
Lane 4 – unbound protein
Lane 5- Wash 3
Lane 6- Wash 7
Lane 7- elute 1
Lane 8- elute 2
Lane 9- elute 3
Lane 10 – Mwt ladder

Figure 53: CTR1 IP performed in PBMC using direct IP kit

List of protein obtained from MS control IP samples were found to be 22 proteins. These hits were subjected to bioinformatics online tool PANTHER and pathways were identified. Majority of the proteins belonged to angiogenic related pathways (41%) and inflammatory pathway (23%). Others included Alzheimer's disease (9%), Huntington (13%) and nicotinic acid receptor signalling pathway (14%) which are shown below in a pie chart (Figure 53).

Three control samples were subjected to MS in duplicate. Proteins were short listed from the above pathway based on their repeatability across samples and more no. of matched peptide residues. The proteins from the samples which were found in all the samples and proteins that had more matched peptide coverage were selected from which 5 proteins that were involved in angiogenic pathway are listed below (Table 13):

Table 13: List of protein hits obtained from MS for CTR1 IP.

S.No	Uniprot Accession ID	Protein name	Peptide match	Biological function
1.	P06396	Gelsolin	14	Plays a role in ciliogenesis
2.	P02787	Serotransferrin	15	Iron transport, also has a role in cell proliferation
3.	P07996	Thrombospondin 1	8	Anti-angiogenic molecule
4.	Q05682	Caldesmon	24	Plays an important role in cellular mitosis
5.	P36955	PEDF	35	Anti-angiogenic molecule and over expression is involved in inhibition of retinal inflammation.

MS data showed out the 5 hits, 3 proteins were involved in proangiogenic pathway whereas 2 proteins were involved in anti-angiogenic pathway. So far in literature reports are present for COX, ATOX and CCS. Apart from that CTR1 has been found to activate FGF, PDGF through MAPK pathway (Tsai et al. 2012). In a recent study Tsai *et al*., reported the interaction of CTR1 with IRS4 (Tsai et al. 2014). In this study we identified Gelsolin and Caldesmon to be interacting with CTR1. Gelsolin, which acts as a proangiogenic molecule by activating HIF1-α, is also known to interact with ceruloplasmin and Caldesmon (Nishio and Matsumori 2009). Thus, from the above experimental data it can be concluded that angiogenesis could be mediated through interactions of CTR1, gelsolin and caldesmon.

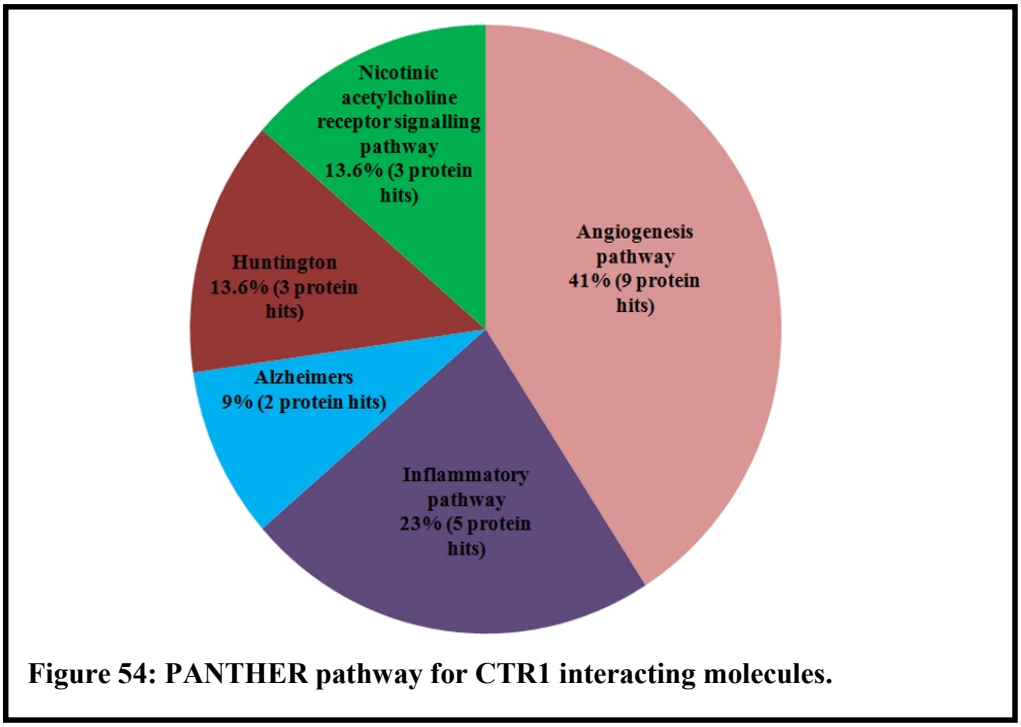


Figure 54: PANTHER pathway for CTR1 interacting molecules.

CHAPTER 5: DISCUSSION

Angiogenesis is a process of development of new blood vessel from a pre-existing vasculature (Folkman 1971). Excessive angiogenesis supports tumour growth and survival. Angiogenesis plays major role in ocular disease like Eales' disease, proliferative diabetic retinopathy, age related macular degeneration and retinopathy of premature (Biswas et al. 2002; Spranger et al. 2001; Witmer et al. 2003). Till date, a lot of therapeutic strategies to target angiogenesis have been developed. Drugs targeting VEGF (Bevacizumab) and its receptors (Sunitinib) are used to treat angiogenesis. Still, there are many challenges in using these drugs, like re-occurrences in residual tumour and its influences on other cell types such as fibroblast and hematopoietic cells (Riely and Miller 2007). In treated patients, side effects such as resistance to anti-VEGF treatment, neutropenia, bleeding problems, anemia, hypertension and thrombus have also been reported (Elice and Rodeghiero 2012). Thus, it is very important to develop new strategies to target angiogenesis which are easy to use and have fewer complications.

This thesis work is aimed to inhibit angiogenesis by targeting Cu entry into endothelium. Cu is an essential micronutrient required for various metabolic processes as cofactor like respiration, detoxification, iron absorption, and elastin cross linking (Goodman et al. 2004). Dietary Cu from liver is transported by ceruloplasmin (Lowndes and Harris 2005). Cu (I) across the plasma membrane is transported by CTR1 and delivered to intracellular chaperones like CCS, ATOX and COX 17 for the synthesis of SOD, GSH and MT (Barry et al. 2010; Lee et al. 2002b; Lutsenko et al. 2007). Cu is exported by ATP7A and ATP7B from intracellular to extracellular space to maintain homeostasis which is affected in Menke's disease (Ashino et al. 2010; Tumer and Moller 2010). In various types of tumor such as breast cancer, carcinoma of bladder, chronic lymphoid leukemia, prostate, colon, lung, and brain increased Cu levels in serum and tissue are reported (Margalioth et al. 1983). Increased Cu levels in vitreous are also shown in ocular diseases with neovascularisation such as eales' disease and proliferative diabetic retinopathy (Konerirajapuram et al. 2004).

Since, Cu has role in angiogenesis and is involved in disease pathology targeting CTR1 could be a new strategy in disease management. Therefore, present work will have translational importance for further developing drugs, which can be used topically otherwise as intravitreal/intratumoral injections. Cu is involved in modulating angiogenesis by altering the growth factors and also the extracellular matrix (Harris 2004). Goodman *et*

al., suggested that Cu deficiency can act as an anti cancer strategy (Goodman et al. 2004). Li *et al*., had showed Cu 5 µM – 100 µM induced angiogenesis by increasing VEGF level and Cu chelation with tetrathiomolybdate reduced VEGF levels in rat arotic ring culture (Li et al. 2014).

However, drugs like penicillamine and tetrathiomolybdate help in removing Cu through urinary excretion; it has several disadvantages such as liver toxicity, non-specificity and therefore inhibit Cu dependent molecules such as SOD and CP (Hopkins and Failla 1997). There are several molecules which function through Cu and are involved in angiogenesis. Among these related proteins CP, CTR1, LOX and SPARC were selected for this study (Listed in Table1). Literature evidences have showed that all of these molecules are increased in serum of patients with tumour thereby regulating tumour growth and metastasis. Osawa *et al*., suggested that LOX expression increased in melanoma cell line and renal carcinoma model and was also associated with tumour metastasis (Osawa et al. 2013). Holzer *et al*., demonstrated that CTR1 protein expression was increased in melanoma, adenocarcinomas and renal cell carcinoma when compared to normal tissues (Holzer et al. 2006). Ros-Bullon *et al*., reported that serum ceruloplasmin levels were significantly increased in melanoma when compared, to healthy control, (Ros-Bullon et al. 2001) and also can be as used as a serum biomarker in solid tumours (Senra Varela et al. 1997). Koukourakis *et al*., reported that SPARC levels are increased in tissues of non small lung carcinoma patients when compared to healthy control (Koukourakis et al. 2003). SPARC is also involved tumour progression, aggressive phenotypes and increased SPARC levels have also been attributed to resistance to therapy (Tai and Tang 2008). Currently it is known from these literature studies that, Cu can work through these molecules and thus targeting these Cu responsive molecules could be beneficial in pathological angiogenesis.

The hypothesis was increased Cu leads to angiogenesis; if CTR1 plays a vital role in regulating Cu level, silencing CTR1 will help in suppressing angiogenesis. For testing this hypothesis HUVECs were used as a *in vitro* model to screen molecules involved in angiogenesis. These cells exhibit all three functions like migration, tube formation and proliferation which are involved in angiogenesis. Established HUVEC expressed PECAM (CD31) protein an EC specific marker (Figure13). Initial mRNA expression of Cu responsive molecule showed Cu influenced expression of CTR1 in a dose dependent manner whereas those changes were not observed in SPARC and LOX (Figure 16). CP mRNA was found to be absent in HUVEC and expression was not influenced in the presence of LPS treated condition (Figure 14&15). However, other cell types (ARPE19,

HeLa, HEpG2, A549 and retina) showed CP mRNA expression. Mazumder *et al*., had also demonstrated that mRNA of CP was absent in endothelial and smooth muscle cells even after exposure to IFN-γ for 24 h (Mazumder et al. 1997). When tested by MTT assay, IC_{50} for Cu was found to be 500 µM (Figure 18) and Cu concentration of 100 µM induced migration and tube formation of HUVEC (Figure 19&20). Comparably, Li *et al*., also showed that Cu concentration of more than 200 µM was found to be cytotoxic to HUVEC (Li et al. 2012). Whereas, Hu *et al*., reported 500 µM was proangiogenic in HUVEC (Hu 1998). In this study, penicillamine concentration of 800 µM showed inhibition of CTR1 mRNA expression and was thus used as a postive control in the study (Figure 17). Gupte *et al*., had shown that pencillamine 400 µM was effective for Cu concentration of 10 µM in MCF cell lines (Gupte and Mumper 2007) which was comparable to the concentration used in this study.

Until now, no studies have been published on the presence of CTR1 in human ocular tissues and one report even showed that it was absent in ocular tissues (Holzer et al. 2006). Holzer *et al*., reported the absence of CTR1 based on only one sample using immuno histochemistry which is less sensitive when compared to immunofluorescence. Interestingly, in this report ocular tissues demonstrated CTR1 at the level of mRNA and protein ranged from 0.29 – 0.08 ng/µg of tissue. Confocal microscopy revealed presence of CTR1 in the inner and outer nuclear cell layer (Figure 21&22). Apart from human tissue Kuo et al., have also reported the presence of CTR1 in mouse RPE, outer limiting membrane and outer plexiform layer of the retina (Kuo et al. 2006). Other than ocular tissues CTR1 has been identified in brain tissues (Davies et al. 2013). CTR1 protein expression was found to be the highest in retina. Thus, to understrand the role of CTR1 in retinal diseases with retinal neovascularisation and Cu excess condition were selected. Sulochana *et al*., showed increased Cu in vitreous of ED patients (0.128 ± 0.04 µg/ml) when compared to control (0.082 ± 0.02 µg/ml) (Konerirajapuram et al. 2004). Since no ocular tissues are available during their treatment in ED, alternatively PBMC were used for the study. PBMC from ED showed 2.4 fold increase in CTR1 mRNA and 3 fold increase in protein when compared to control. Role of Cu in Alzheimer's disease (AD) a neurodegenerative disease in mouse model studied by Zheng *et al*., showed increased Cu and increased CTR1 mRNA expression which could lead to aggregation of β amyloid protein. They were unable to perform CTR1 protein studies because of non availability of specific antibodies (Zheng et al. 2010). Increased Cu and CTR1 protein were observed in North Ronaldsay sheep. These sheep demonstrate enhanced Cu absorption due to their Cu

deficient habitat. Their liver Cu levels correlate with brain Cu levels since they depict unusual features with Cu import mechanism. Haywood *et al*., also showed that increased Cu and CTR1 protein expression in blood brain barrier implicating its role in neurodegenrative diseases of brain (Haywood and Vaillant 2014).

Since CTR1 played a role in ED and angiogenesis, siRNA targeting CTR1 was designed using Ambion web tool. Treatment with siRNAs has been used in various disorders due to their sensitivity and specificity. Pachyonychia congenital, a skin disorder (autosomal dominant), was targeted by TD 101 siRNA concentrations (1 - 8.5 mg/ml) for keratin 6a mutated mRNA which is in Phase Ib clinical trial. It was found to be effective in decreasing the callus in the foot (Leachman et al. 2010). Similarly, Phase I clinical trial for liver cancer (primary and secondary) was targeted using BI 2536 siRNA for serine/threonine- Polo like protein kinase 1 of concentration (10 – 100 nM) and was found to induce apoptosis in malignant cells (Steegmaier et al. 2007). Systemic administration of nanoparticle conjugated siRNA was delivered to target M2 subunit of ribonucleotide reductase protein for treating malignant melanoma showed 5 fold reduction in mRNA and protein expression of the target gene (Davis et al. 2010). In ocular diseases like neovascular AMD, Kaiser *et al*., in their phase 1 clinical trial demonstrated that a single intra vitreal dose of siRNA (1600 μg/eye) targeting VEGF R1 improved visual acuity (Kaiser et al. 2010) indicating RNAi technology is effective *in vivo*. Delivery of siRNA with antisense strand, 5' phosphorylation of antisense, double stranded have been developed and tested in cell culture models (Martinez et al. 2002). Inspite of these variations, in their structure design it is understood from literature that all these siRNA bind to sense strand of mRNA, thereby, inhibiting the target gene (Holen 2005).

So far, CTR1 siRNA have been used to study its role of CTR1 in embryonic development (Lee et al. 2001), Cu transport (Kim et al. 2009) and cisplatin uptake (More et al. 2010). However, till date to our knowledge, there were no studies on the role of CTR1 in angiogenesis. In this study, CTR1 mRNA was targeted by antisense siRNA to understand its role in angiogenesis. Out of the three CTR1 siRNAs, si 1 was found to inhibit CTR1 mRNA by 71% whereas si 2 and si 3 did not show any significant change (Figure 25). In addition, intracellular Cu estimation by Phen green dye (quenches fluorescence when bound to Cu) showed, si 1 to be effective when compared to si 2 & 3 (Figure 26). Thus, si 1 at 10 nM concentration was chosen as it was found to be effective in decreasing CTR1 mRNA and intracellular Cu levels.

The half life of CTR1 protein varied on the amount of Cu present in the extracellular region. Peña *et al*., had shown in yeast the half life of CTR1 protein in the presence of Cu concentration of 10 μM was found to be only 15 min in yeast (Peña et al. 2000). In this study, exposure to Cu concentration of 100 μM showed increased CTR1 mRNA expression at 1 h and 4 h significantly. At the same time interval, si 1 at 10 nM concentration decreased mRNA expression by 50 %, whereas Ssi did not show any change. Penicillamine concentration of 800 μM also showed decrease in CTR1 mRNA expression (Figure 26). Since, 1 h exposure was sufficient to bring optimal, reproducible changes in mRNA levels in HUVEC, this time point was fixed. From the above experiments it was seen that 1 h showed CTR1 silencing but siRNA experiments are usually done for 24-72 h time point. Therefore to check these bioavailability studies of siRNA was done using Taqman real time PCR which confirmed the presence intracellular siRNA at 1- 4 h time point (Figure 27). Stalder *et al*., had shown single stranded siRNA targeting Sjogren syndrome antigen B was found to be functionally effective at 25 nM concentration in Hela cells within 1 h time point and was found to be to conjugated with Ago2 (Stalder et al. 2013).

CTR1 protein levels increased by 2 fold in the presence of Cu. The inhibition of CTR1 by si 1 at 10 nM reduced the Cu levels and CTR1 protein by 50 % indicating the specificity of the designed siRNA (Figure. 28). Caco-2 cells during hypoxia when exposed to Cu concentration of 50 μM showed 25% increase in CTR1 protein (Pourvali et al. 2012). Addition of Cu concentration of 100 μM increased intracellular Cu levels and si 1 at 10 nM concentration decreased Cu levels which were confirmed by Phen meth dye and AAS experiments (Figure 31). Cu increased proliferation, migration and tube formation of HUVEC, while siRNA treatment inhibited Cu induced effect (Figure 32-36). Ashino *et al*., had shown that the PDGF stimulated vascular smooth muscle cells demonstrated inhibition in migration in the presence of CTR1 siRNA (Ashino et al. 2010). Cai *et al*., reported that shRNA targeting CTR1 significantly lowered intracellular ^{64}Cu thereby decreasing proliferation and growth of prostate cancer xenograft model (Cai et al. 2014). Cu influx activates Erk1/2 through Cu – MEK 1 interaction. Brady *et al*., studied the role of BRAF 1 an oncogene which was overexpressed in tumours and also activated MEK1. Decreasing the levels of CTR1 (Cu transporter 1), or mutations in MEK1 that disrupt Cu binding, decreased BRAF-driven signalling and tumorigenesis (Brady et al. 2014). Thus, intracellular Cu increased angiogenesis and si 1 at 10 nM showed inhibition. Studies have reported that Cu chelators like penicillamine are used in concentrations of 1.6 - 19

mmol/day to treat glioblastoma (Brem et al. 2005). Jain *et al*., in phase II clinical trial study on breast cancer patients showed that tetrathiomolybdate of 100 mg concentration administration was effective. Cu depletion reduced circulating progenitor endothelial cells, CP and was thus beneficial in maintaining the tumor at dormancy (Jain et al. 2013). CTR1 siRNA showed inhibition at 10 nM concentration whereas, penicillamine showed similar effects at 800 μM indicating lower dosage of CTR1 siRNA itself is sufficient for inhibiting angiogenesis. Since specificity of siRNA is a problem because it can lead to off target effects (Fedorov et al. 2006). The specificity of CTR1 siRNA was also indicated in real time PCR of other Cu binding pro angiogenic molecules such as LOX, SPARC (Figure 29) and additionally even scrambled siRNA did not show any significant change in HUVEC. These results indicate the specificity of the selected siRNA in silencing CTR1. To further authenticate the data i*n vivo* study in CAM model was performed. Cu concentration of 25 μM induced angiogenesis in CAM assay which was inhibited in the presence of si 1 at 10 nM at the end of 18 h (Figure 37). Similarly, corneal packet assay in rabbits showed that the O- methyl CTR1 siRNA (100 ng) showed a regression in blood vessel formation when compared to matrigel treated control after 11 days of treatment (Figure. 38). This O methyl chemical modification at position 2 of the guide strand has been reported to reduce off target effect (Jackson et al. 2006). It also provides more stability against endogenous nuclease activity, improved pharmacokinetic and toxicological properties (Prakash et al. 2005). Certain restrictions in corneal packet assay are it is an invasive technique, expensive and cornea is exposed to atmospheric oxygen therefore cannot completely mimic the tumour environment.

The angiogenic role of Cu has been attributed to increasing VEGF expression. Sen *et al*., reported Cu concentration of 25 μM for 6 h showed increased VEGF expression in HaCaT cell. Further, they also reported in excision wound model in Balb C mice showed improved wound closure from day 1 to 9 in the presence of Cu (0.625 nmol), when compared to control (Sen et al. 2002). Qui *et al*., demonstrated that Cu concentration of 25 μM for 2 h in HUVEC induced transcriptional activation of HIF1-α through CCS which further, induced VEGF level (Qiu et al. 2012). Zimnicka *et al*., also had shown that silencing CTR1 decreases migration, proliferation and tube formation by decreasing intracellular Cu levels in pulmonary smooth muscle cells both during normoxia and hypoxia (Zimnicka et al. 2014). Therefore, in this work it was necessary to check whether CTR1 siRNA had any effect on VEGF expression at the protein level. VEGF expression was measured in the presence of Cu with and without siRNA. Results showed that Cu

concentration of 100 μM increased VEGF mRNA expression by 1.5 fold by real time PCR and increase in the protein by western blot (Figure 39). The designed CTR1 siRNA not only reduced the Cu levels but also inhibited Cu induced angiogenesis. Other Cu chaperones and Cu enzymes have not been studied in the presence of si 1 in this study. Site specific delivery can be achieved by conjugation with tumour specific proteins thereby allowing normal tissues to remain unaffected (Schiffelers et al. 2004). Similar to prostrate specific target, CTR1 specific siRNAs can be conjugated with cell/ tumour specific molecules for targeted delivery (Kim et al. 2009). Further, this molecule can be studied for its stability and bioavailability to be developed as a therapeutic molecule for treating angiogenesis related diseases. In this study, different permeable agents were not used or *in vivo* pharmacokinetic assay was not done to test the efficiency of the developed CTR1 siRNA. Although CTR1 siRNA showed inhibition angiogenesis understanding the structure function of proteins are also essential.

Proteins perform their functions through domains or motif which are interactive. Various peptides have been designed from endogenous proteins which are anti-angiogenic in nature (Sulochana and Ge 2007). TGF β binding region of decorin derived peptides LRR5, have proved to be anti-angiogenic in HUVEC model by inhibiting VEGF mediated angiogenesis and activating the caspase mediated pathway (Sulochana et al. 2005). Similarly, biomimetic peptides (10 mg/Kg body weight) derived from α5 fibril collagen IV also showed by targeting integrins inhibit angiogenesis of breast tumour cells *in vivo* (Rosca et al. 2011). CP interacts with CTR1 during Cu delivery into the EC. Similar to Zatulovskiy *et al*., in this study Hex online bioinformatics tool enabled to dock CTR1 with CP for understanding the interacting residues (Figure 40-42) which were further designed as peptides (Zatulovskiy et al. 2007). Crystal structure of CP is available whereas CTR1 crystal structure is still unknown. Thus, motifs which bind to Cu and function in Cu transport would help in understanding the structure function relationship.

CTR1 derived peptides (10 ng) were checked for purity which showed single peak when injected in HPLC (Figure 43). Cytotoxicity assay showed that peptides did not decrease viability in HUVEC in the concentrations tested (1- 10 μM). CTR1 derived peptides were found to inhibit migration, tube formation and proliferation (Figure 31 -41). Intracellular Cu levels were found to be reduced by CTR1 derived peptides by 0.3 to 0.5 fold and Scp did not show any change when compared to control. Whereas, in the presence of Cu 100 μM concentration CTR1 derived peptides reduced intracellular Cu levels by 2.5- 2.8 fold respectively.

CTR1 peptide regions, rich in histidine and methionine residues have Cu binding property which is pH dependent. At neutral pH, Cu binding was found to be more in histidine when compared to methionine whereas at pH 4.5 methionine was found to have more Cu binding activity than histidine (Rubino et al. 2011). Mass spectroscopy results indicated that CTR1 derived peptides specifically bound to Cu, whereas Scp did not show any binding (Figure 49). Similarly, Cu binding region of LOX derived peptides were found to be anti-angiogenic in HUVEC (Mohankumar et al. 2014). Histidine rich peptides derived from the N terminal of endostatin binds to Cu and could contribute to anti-angiogenic activity of the peptides (Kolozsi et al. 2009). Whittal *et al*., using ESI MS reported Cu binding properties of prion proteins (Whittal et al. 2000). Cu binding region of GHK tripeptide is also anti-inflammatory in function (Hostynek et al. 2011). Microplusin an antimicrobial peptide functions by binding and sequestering Cu (Silva et al. 2009) which can be used in ointments and sprayed to cure infection.

The question unanswered, was how these peptides which were binding to Cu are anti-angiogenic in nature? To answer this end, peptides were rhodamine labelled at the N terminal and tested for their internalisation. Peptides were tested in different time points (15- 120 min) showed no entry inside the cell. These results indicated, that they bound to extracellular Cu and thereby, decreasing Cu availability to cells (Figure 44). Reports of intracellular distribution of Tat derived peptides (5 μM), labelled with Texas Red after 30 min at 37°C or 4°C in HeLa cells when observed under confocal laser microscopy have been reported (Takeshima et al. 2003). Octa arginine conjugated with anti-miRNA penetrated the cell, and thus reduced target miR-21 expression by decreasing migration in glioblastoma cell line (Zhang et al. 2014). However, peptides derived from CTR1 did not show entry at the different time point examined. These peptides derived from CTR1 can be developed further and tested in xenograft models and in various tumours for their antitumor properties.

To check the efficacy of CTR1 derived peptides *in vivo* open CAM model were performed on day 4. This technique is less expensive when compared to other animal model and large number of samples can be screened (Staton et al. 2004). CAM assay was performed for pep 1 and 2 (100 nM) for 6 h showed decrease in blood vessel formation when compared to Scp indicating that they are effective *in vivo* also (Figure 52). However, there are certain limitations of CAM assay such as highly established vasculature after day 8 hence early CAM has to be considered for experiments to overcome this problem. Some species – species variations may arise of the molecules tested (Ribatti 2014). Prodrugs that require

metabolic activation to become active (e.g. Tafenoquine an antimalarial drug requires cytochrome P450) cannot be tested on CAM model (Marcsisin et al. 2014).

The role of CTR1 in angiogenesis is still poorly understood. Reports up till now indicate that CTR1 interacts with FGF signalling with ERK pathway in Xenopus (Haremaki et al. 2007). CTR1 also plays a role in activation of FGF, PDGF through MAPK and receptor tyrosine kinase pathway (Tsai et al. 2012). Cu induced internalisation of CTR1 showed interaction with insulin receptor 4 (IRS4), which has role in modulating IGF1 induced PI3K pathway and thus indicating its role in angiogenesis (Tsai et al. 2014). In order to arrive at the molecular interactive partners, an IP pull down followed by mass spectrometry was performed. This revealed that, gelsolin was found to interact with CTR1. Gelsolin is an actin binding protein reported to bind with ceruloplasmin to activate NFκB mediated inflammatory pathway (Lokamani et al. 2014). Gelsolin plays a vital role in activating HIF-1α by translocating to the nucleus (Nishio and Matsumori 2009). Recent study by Zheng *et al.,* showed the role of CTR1 in AD, in which they demonstrated IFN-γ a proinflammatory cytokine regulated Cu homeostasis and thereby inducing CTR1 mRNA expression (Zheng et al. 2010). Caldesmon is also an actin binding protein which was also a hit obtained in MS result for CTR1 IP. Investigation of caldesmon interaction with gelsolin showed that they both are regulated by cGMP- dependent protein kinase 1 β. Thereby, leading pro invasive and promigratory pathway in human breast cancers (Nishio and Matsumori 2009). In summary CTR1 interacts with gelsolin and caldesmon thereby, leading to proangiogenic pathway. Validation of IP results can be done, by fluorescence resonance energy transfer (FRET) or knockdown experiments. The IP has been performed to identify transient interacting partner, cross linkers such as disuccinimidyl suberate or bis (sulfosuccinimidyl) suberate can also be used to stabilise these transient interactions. These attempts may throw more information on gelsolin-caldesmon interactions with respect to their angiogenic activity (Figure 55).

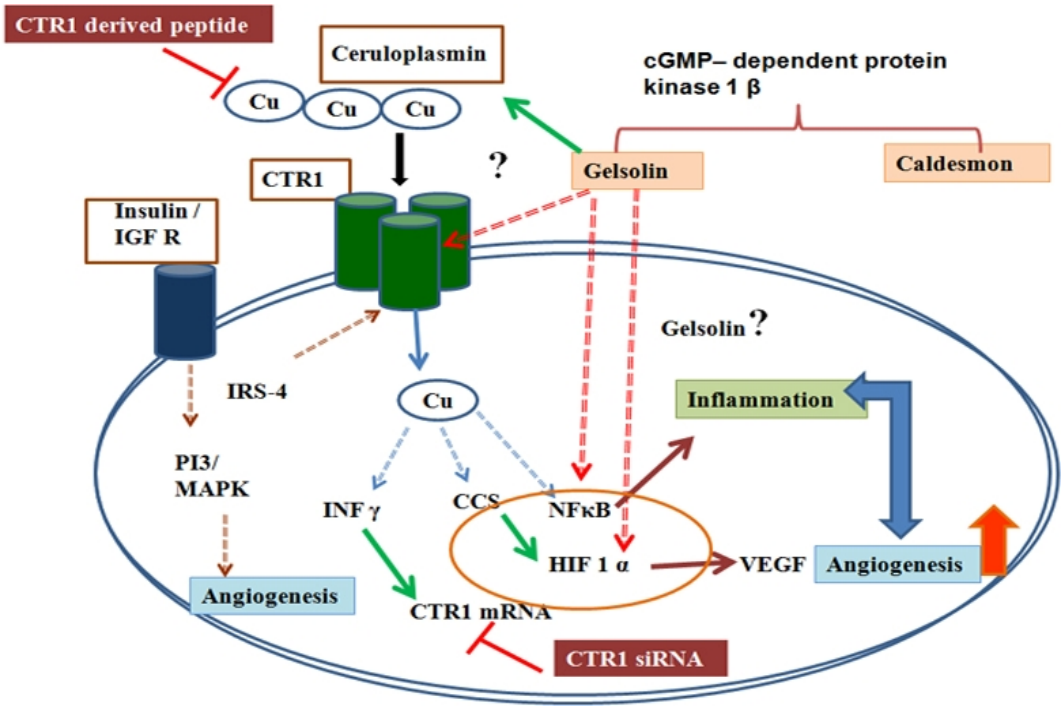


Figure 55: Possible mechanism of CTR1 mediated angiogenesis. Dotted brown lines and blues lines indicate existing interactions; new interactions are indicated in red dotted lines.

SPECIFIC CONTRIBUTIONS:

Limited literature studies are available on the role of Cu as an angiogenic molecule. CTR1 protein as a target was identified by screening ED which also has increased vitreous Cu and angiogenesis.

CTR1 silencing suppressed angiogenesis not only *in vitro* but also *in vivo*, particularly when it was delivered topically. The CTR1 siRNA, reduced CTR1 expression specifically whereas, the other molecules tested such as SPARC and LOX remained unaltered. When compared to pencillamine a Cu chelator was effective at 800 μM concentrations whereas siRNA was effective even at 10 nM.

Peptides rich in histidine methionine motifs of CTR1 protein show anti-angiogenic effect by binding to Cu and thus can be used for chelating extracellular Cu.

IP for CTR1 interacting partner showed gelsolin and caldesmon interaction could play role in CTR1 mediated angiogenesis.

In this study *in vitro* and *in vivo* models have been used to identify novel drug target. These targets will be of translational use which has to be checked for bio availability and pharmacokinetic study. These experiments were performed using sophisticated instruments such as FACS, HPLC, AAS and Mass spectrometry.

CHAPTER 6: CONCLUSION

Cu induced angiogenesis is reported in literature. The role of CTR1 in ocular angiogenesis was studied in this work and the approach of inhibiting Cu induced angiogenesis by CTR1siRNA and peptide.

1. CTR1 was localised in human ocular tissues
2. CTR1 was found to be involved in ocular angiogenesis like PBMC from Eales' disease wherein CTR1 protein was found to be increased.
3. Designed CTR1 siRNA, moreover validated for its specificity and sensitivity. CTR1 siRNA also did not show any off target effects.
4. Silencing CTR1 in human umbilical vein endothelial cell model using si 1 showed a decrease in Cu mediated migration, tube formation and proliferation.
5. Further, the designed siRNA was also validated *in vivo* by CAM and rabbit corneal packet assay.
6. *In-silico* approach for designing peptides using HEX bioinformatics software helped in identifying three peptides from CTR1 protein interacting with ceruloplasmin.
7. CTR1 derived peptides decreased intracellular Cu levels thereby inhibiting angiogenesis. These results were also validated by *in vivo* CAM experiments.
8. Thus both siRNA and peptide designed in the study can be developed as a drug to treat Cu mediated angiogenesis.
9. Gelsolin and Caldesmon interactions with CTR1 also indicate the role of CTR1in angiogenesis. Identification of interactive partners of CTR1 through IP revealed its role beyond angiogenesis including inflammation.
10. Thus, CTR1 is a novel target to treat Cu mediated angiogenesis as it regulates intracellular Cu level.

FUTURE SCOPE AND LIMITATIONS OF THE STUDY:

- The developed CTR1 siRNA can be tested with other cell permeable agents to increase its bioavailability. Detailed bioavailability, pharmacokinetics and pharmacodynamics are not part of this thesis.
- CTR1 targeting siRNA and CTR1 derived peptides can be tested in xenograft model in various tumours for their efficacy. Studies involving the specific amino acid residue important in binding to Cu in these peptides can be identified.
- IP for CTR1 using linker such as DSS and BS3 can be performed. Gelsolin and Caldesmon role in Cu induced angiogenesis can be validated by FRET and knockdown model.
- Tandem affinity purification using over expression system can be done to understand the molecular mechanism of CTR1 clearly.

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APPENDIX 1

S.No	Product	Company
1.	Vented flask and culture dishes	Nunc
2.	Fibronectin	Sigma
3.	Collagenase	Sigma
4.	Antibiotic solution	Gibco
5.	Endothelial growth medium 2	Lonza
6.	Gelatin	Sigma
7.	Dimethyl sulfoxide	Merck
8.	MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)	Sigma
9.	Paraformaldehyde	Merck
10.	Transwell inserts	Millipore
11.	ECM matrix	Millipore
12.	Phen green FL	Invitrogen
13.	Cell death ELISA kit	Roche
14.	TRIzol	Sigma
15.	Chloroform	Merck
16.	Iso propanol	Merck
17.	Ethanol	Merck
18.	iScript RT-PCR Kit	Biorad
19.	SYBR Green	Eurogentech
20.	Bradford	Thermoscientific
21.	Bicinchoninic acid assay (BCA)	Thermoscientific
22.	Nitrocellulose membrane	Millipore
23.	Horse radish peroxidase tagged antibody	Santacruz
24.	CTR1 ELISA kit	USCN
25.	Co immunoprecipitation-kit	Pierce
26.	Oasis column	Waters
27.	Dithiothreitol (DTT)	Biorad
28.	Iodaoacetic acid (IAA)	Biorad

29.	Sodium dodecyl sulphate	Sigma
30.	Acrylamide	Sigma
31.	Bisacrylamide	Sigma
32.	TEMED	Biorad
33.	Ammonium per sulphate	Biorad
34.	Copper chloride	Sigma
35.	CTR1 derived Peptides	USV peptides
36.	siRNA	Merck
37.	ICA-fectine	Eurogentech
38.	EDTA	Merck
39.	Tris	Sigma
40.	Glycine	Sigma
41.	Trypsin for MS	Proteogen
42.	Trypsin for cell culture	Hi media
43.	Foetal bovine serum	Hi media
44.	mirVana – miRNA isolation kit	Life technologies
45.	Primers	Eurogentech
46.	siRNA quantification- Taq man probes	Life technologies

PUBLICATIONS

1. Narayanan Gomathy, SR.Bartahidevi et al. (2013), 'CTR1 silencing inhibits angiogenesis by limiting copper entry into endothelial cells', *PLoS One,* 8 (9), e71982.
2. Karunakaran Coral, Narayanasamy Angayarkanni, Narayanan Gomathy**,** Muthuvel Bharathselvi, Rishi Pukhraj, and Roy Rupak. Homocysteine Levels in the Vitreous of ProliferativeDiabetic Retinopathy and Rhegmatogenous Retinal Detachment: Its Modulating Role on Lysyl Oxidase. Invest Ophthalmol Vis Sci, 2009.50(8):3607-12
3. N. Gomathy, V.N. Sumantran, A. Shabna, K.N. Sulochana, Tolerance of ARPE 19 cells to organophosphorus pesticide chlorpyrifos is limited to concentration and time of exposure, Pesticide Biochemistry and Physiology.

MANUSCRIPT UNDER PREPARATION:

1. Role of Gelsolin and Caldesmon in CTR1 mediated angiogenesis
2. Copper binding regions of CTR1 derived peptides are anti-angiogenic in nature.
3. Role of CTR1 in ocular tissue and in inflammation mediated angiogenesis

PATENT FILED:

1. Patency no: 3221/che'2012- Anti-angiogenic property of CTR1 siRNA
   Inventor: Dr. K.N.Sulochana,
   Co- inventor, Iyer Gomathy Narayanan & Dr. SR. Barathidevi

POSTER PRESENTED:

1. Participated in international conference- Asia ARVO- 2013- Identification of Copper Transporter 1 as a Target for Ocular Angiogenesis.
2. Best poster award- Runner up – Contempary trends in biological and pharmaceutical research (BITS Pilani 2010) - Role of Copper Transporter-1 in Angiogenesis.
3. Poster presented in SBCI 2009 – NCCS Pune – Effect pesticide cholorpyrifos on ARPE 19 cells.

WORKSHOP ATTENDED:

International workshop on Wound Healing and angiogenesis

Workshop on Scientific writing 2014- Elite school of optometry.

AWARD:

The poster on "Role of copper transporter-1 in angiogenesis" presented by Ms. Iyer Gomathy Narayanan won a runner up award in the category of best post at BITS- CTBR March, 2011.

BRIEF BIODATA OF CANDIDATE

Ms. Iyer Gomathy Narayanan obtained her B.Sc. Biochemistry degree from Valliammal College for women in 2002. She did her MS in medical laboratory technology in 2005 from Birla institute of Technology & Science – Pilani in collaboration with Medical research foundation. She did MSMLT internship in biochemistry dept on the topic of standardising homocysteine estimation in HPLC and in ocular fluids which was published in IOVS. She later joined the department of Biochemistry and cell biology as a junior scientist in 2008. She worked under Dr.Venil. N. Sumantran in intramural funded project focusing on exposure of pesticide chlorpyrifos in ARPE 19 cells. Later she registered for PhD in 2009 under the guidance of Dr. K.N. Sulochana. She was recruited as a senior research fellow in DBT funded grant. During which she made 3 poster presentations, 1 oral presentation and attended 2 workshops. She has published 1 paper in her PhD topic and other 3 manuscripts are under preparation. She has one patent filed and one more patent application to be sent for filing in India. She established HUVEC cell culture in this department and also handled several cell lines like ARPE 19, Y79 and cells isolated from peripheral blood mononuclear cells. Her keen interest is on angiogenesis related diseases. She is also involved in patient care and teaching classes for optometry students.

BRIEF BIODATA OF SUPERVISOR

Prof. K.N. Sulochana is currently Sr. Professor & Director, R.S. Mehta Jain Department of Biochemistry and Cell Biology, Vision Research Foundation, Chennai. She has been Reader in Biochemistry Research Dept, Sankara Nethralaya for nearly 10 yrs, Sr. Research Fellow, National University Singapore, for 2 yrs and as a Sr. Lecturer at Bradford University Singapore campus and Sr. Research Scientist, in pharma industry for 3 yrs. She did her B.Sc.(Chemistry); M.Sc. (Biochemistry)–University of Madras and Ph.D. (Biochemistry- Enzymology) SV. University, India.

She has keen interest in research, teaching and patient care. Her teaching area includes Basic Biochemistry, Biomolecules, Instrumentation, Clinical Biochemistry and Ocular biochemistry. Patient care includes optimization and Development of clinical investigations for oxidative stress, analysis of vitamin, amino acids, and enzymes.

Her current interests are Ocular Angiogenesis, Protein- Protein interactions, Molecular mechanisms of metabolic diseases, Drug targets, Design and development of inhibitors of angiogenesis, Pharmacokinetics and Preclinical studies. She has more than 50 Research article published, 5 reviews and 4 Book Chapters.

She has participated in many National and International conferences. She has received Dr. BC. Roy Award, Silver Jubilee Research Award of Medical Council of India, 1996, SwarnLata Punshi award as "Best Research Worker" Medical Research Foundation, 1997, Women of the Year American Biographical Institute, Inc (2000), The biographical sketch inclusion, 19th edn of Who's Who in the World by Marquis (2001) and Outstanding Woman of the 21st Century", American Biographical Institute, NC, USA, 2001.

She's also a life member of professional bodies in Society of Biological Chemistry (I) (Life Member), Association for the promotion DNA fingerprint DNA technology (Life Member), Active participant in Annual Meeting of IERG for the past 7 yrs, Member in ARVO, Member in Association for clinical biochemist Singapore since 2002 (SAC), Member of the Management development institute of Singapore (MDIS) since Jan-2008.