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MOLECULAR MECHANISMS INVOLVED IN INFLAMMATORY ANGIOGENESIS INDUCED BY MONOCYTE CHEMOTACTIC PROTEIN-INDUCED PROTEIN-1 (MCPIP1)

by

ARPITA ROY
M.Sc. Nagpur University, India, 1999

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida,
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Major Professor: Pappachan E. Kolattukudy

I dedicate this dissertation to my husband, Dr. Avijit Roy, and the two most precious blessings of our lives, daughter Arushi and son Arunit. Their love and unconditional support is one of the reasons I was able to successfully complete my doctoral training. The blessings and moral strength I received from my parents (Maya and Paritosh Kumar Brahma) and in-laws (Santi and Rabindra Chandra Roy) have always helped and guided me to keep my focus and are beyond my expression of gratitude.

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ABSTRACT

Major diseases such as cardiovascular diseases, diabetes, obesity and tumor growth are known to involve inflammatory angiogenesis. MCP-induced protein 1 (MCPIP1) encoded by *ZC3H12A* gene, was reported to promote angiogenesis and is addressed in my dissertation as MCPIP. The mechanism/s involved in the angiogenic differentiation induced by MCPIP was however unknown. The aim of this study was to bridge this gap in our knowledge and delineate the molecular mechanisms and sequential processes involved in angiogenesis mediated via MCPIP. To determine if angiogenesis induced by inflammatory cytokines, TNF-α, IL-1β and IL-8 is mediated via induction of MCPIP, knockdown of MCPIP by its specific siRNA, in human umbilical vein endothelial cells was performed. Oxidative stress, ER stress and autophagy are known to be involved in mediating inflammation. We hypothesized that MCPIP-induced angiogenic differentiation is mediated via induction of oxidative stress, ER stress and autophagy. Chemical inhibitors and specific gene knockdown approach were used to inhibit each process postulated. Oxidative stress was inhibited by apocynin or cerium oxide nanoparticles or knockdown of NADPH oxidase subunit, phox47. Endoplasmic reticulum (ER) stress was blocked by tauroursodeoxycholate or knockdown of ER stress signaling protein IRE-1 and autophagy was inhibited by the use of 3′methyl adenine, or LY 294002 or by specific knockdown of beclin1. Matrigel assay was used as an *in vitro* tool to assay angiogenic differentiation. Inhibition of each step inhibited the subsequent steps postulated. The results reveal that angiogenesis induced by inflammatory agents is mediated via sequential induction of MCPIP that causes

oxidative and nitrosative stress resulting in ER stress leading to autophagy required for angiogenesis.

MCPIP has deubiquitinase and anti-dicer RNase activities. If and how the dual enzymatic activities of MCPIP mediate angiogenesis was unknown. Our results showed that hypoxia-induced angiogenesis is mediated via MCPIP. MCPIP deubiquitinated ubiquitinated hypoxia-inducible factor (HIF-1α) and the stabilized HIF-1α entered the nucleus to promote the transcription of its target genes, cyclooxygenase-2 and vascular endothelial growth factor causing the activation of p38 MAP kinase involved in angiogenesis. MCPIP expression promoted angiogenesis by inhibition of thrombospondin-1 synthesis via induction of silent information regulator (SIRT)-1 and/or via suppression of VEG-inhibitor levels caused by inhibition of NF-κB activation. MCPIP inhibited the production of the anti-angiogenic microRNAs (miR)-20b and miR-34a that repress the translation of HIF-1α and SIRT-1, respectively. Cells expressing the RNase-dead mutant of MCPIP, D141N, that had lost the ability to induce angiogenesis had deubiquitinase activity but did not inhibit the production of miR-20b and miR-34a. Mimetics of miR-20b and miR-34a inhibited MCPIP-induced angiogenesis. These results show for the first time that both deubiquitinase and anti-dicer RNase activities of MCPIP are involved in inflammatory angiogenesis. Results from our study delineate key processes that could be potential targets for therapeutic intervention against inflammatory angiogenesis.

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LIST OF ABBREVIATIONS

ATF	Activating transcription factor
COX2	Cyclooxygenase 2
DHR	Dihydrorhodamine
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
FIH	Factor inhibiting HIF
GRP78	Glucose regulate protein 78
HAT	Histone acetyltransferase
HDAC	Histone deacetylases
HIF-1α	Hypoxia-inducible factor 1, alpha subunit
IL	Interleukin
IRE-1	Inositol-requiring enzyme 1
IKβs	Inhibitor of κb
IKK	κb kinase
3′MA	3-methyladenine
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein-1
MCPIP1	Monocyte chemotactic protein-induced protein 1
MiR	MicroRNA
NADP+	Nicotinamide adenine dinucleotide phosphate
NEMO	NF-κb essential modulator
NF-κB	Nuclear Factor-kappa β

NLS	Nuclear localization signal
PHD	Prolyl hydroxylase domain
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SIRT1	Silent mating type information regulation 2 homolog/sirtuin
TNF	Tumor necrosis factor
TRAF2	Tumor necrosis factor receptor-associated factor 2
TSP	Thrombospondin
TUDC	Tauroursodeoxycholic acid
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VHL	Von hippel–lindau
XBP	Xbox binding protein

CHAPTER1: GENERAL INTRODUCTION

Angiogenesis is a complex physiological processes resulting in the growth of new blood vessels from pre-existing vasculature [1]. It plays a fundamental role in a broad array of physiological processes such as embryonic development [2] and wound healing [3]. Evidence implicates the involvement of angiogenesis in inflammatory pathological disorders such as cardiovascular disease, tumor development, multiple sclerosis, endometriosis and metabolic syndrome-associated disorders, including visceral obesity, atherosclerosis and diabetes that affect millions of people worldwide [4, 5]. A cascade of events regulating angiogenesis involve endothelial cell activation, proliferation, migration and is maintained by a tight balance between pro-angiogenic and anti-angiogenic growth factors and cytokines [6].

Inflammatory Angiogenesis

The inflammatory response is an integral component of the innate immune system. It is a defense mechanism that is triggered by the organism against pathogen attack or physical injury or chemical insult. It is orchestrated by interplay between multiple components that are under stringent controls [7]. Acute inflammation is a short-lived process occurring in response to the attack by the pathogen/s (bacterial, fungal, and viral infections) or tissue injury and is characterized by rapid migration of granulocytes (ie, neutrophils, eosinophils, and basophils) to the inflammatory site. The acute inflammatory phase gets resolved with clearing up of the infection or injury and by cessation of pro-inflammatory signaling that results in removal of the pathogen and cell debris by phagocytosis. Failure in resolving the acute phase of inflammatory response

results in chronic inflammation that is involved several pathologies including, cancer, cardiovascular diseases, obesity-induced type 2 diabetes, rheumatoid arthritis and neurodegenerative diseases, such as Alzheimer's disease [8, 9]. Cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-8 are vital players that contribute to inflammatory response and serve as signaling cues for leukocyte recruitment [10]. Monocyte chemotactic protein, (MCP-1)/CCL2, a well-known potent chemokine has also been implicated in inflammation [11]. It belongs to the CC family of chemokines which on binding to its trimeric G-protein–coupled CCR2 receptor initiates a signaling cascade to induce changes in gene expression and thus help regulate activation, migration and infiltration of the monocytes/macrophages to the site of inflammation caused due to stress, injury or infection [12, 13]. MCP-1, an angiogenic chemokine, plays a crucial role in promoting inflammatory angiogenesis [14].

MCPIP mediates inflammatory angiogenesis

MCP-1 treatment of human peripheral blood monocytes resulted in the transcriptional activation of an array of genes encoding for cytokines and chemokines, extracellular matrix degrading enzymes, cell adhesion proteins, and a set of expression sequence tags (ESTs). The most highly induced EST encoded an unknown protein that was designated as MCP-induced protein1 (MCPIP1) which was mapped to *ZC3H12A* gene, which was later reported also to be induced by interleukin (IL)-1β stimulation of human monocyte-derived macrophages[15].

ZC3H12A belongs to the MCPIP family consisting four members numbered 1 to 4 and is encoded by *ZC3H12A* (at 1p34.3), *ZC3H12B* (at Xq12), *ZC3H12C* (at 11q22.3), *ZC3H12D* (at 6q25.1), respectively. *ZC3H12A* which is mainly deciphered as

a Toll-like receptor (TLR) inducible gene belongs to CCCH zinc finger family. MCPIP1 gene is 8.9 kb in length and contains 5 exons and 4 introns [16]. MCPIP protein contains 599 amino acids and a mass of 65.8 kDa. Protein motif analysis revealed that MCPIP contains two proline-rich activation domains, a putative nuclear localization signal (NLS) and one RNA-binding zinc finger motif containing 3 cysteines and 1 histidine suggesting MCPIP1's potential as a transcription factor[15]. Furthermore, CCCH -type zinc-finger proteins are also known to participate in mRNA metabolism by processes such as mRNA splicing, polyadenylation and decay regulation of the mRNA. Bioinformatic analyses by sequence alignment of the MCPIP1 sequence shows a conserved N-terminal domain (139–297) that shares remote homology to the PilT N-terminus (PIN) domain that is just before the zinc-finger domain (300–324). Structural modeling of MCPIP1 revealed that four acidic residues (D141, E185, D226, D244) in the PIN domain form a conserved, negatively charged pocket that is important for magnesium binding and for its potential enzymatic activity[17]. Studies also report a 80 and 82% sequence identity at the nucleotide and amino acid levels between mouse and human MCPIP1, respectively[15].

MCPIP1 expression induces a variety of genes known to be involved in biological functions ranging from cell death such as in hyperglycemia-induced cardiomyocyte death [18] and in cell differentiation such as adipogenesis [19] and osteoclastogenesis [20]. MCPIP was shown to promote angiogenesis via induction of vital players of angiogenesis, hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF). The enhanced expression of cadherin 12 and 19 in MCPIP1-induced angiogenesis was attributed to the transcription-factor activity of MCPIP1[21]. The

biological processes and the molecular mechanisms by which MCPIP1 expression results in inflammatory angiogenesis was however unknown.

Furthermore, several published data from independent laboratories have established that MCPIP1 is a multifunctional protein and is involved in regulating inflammation[22]. The highlights enlist the documented mechanisms via which MCPIP1 modulates inflammatory signaling:

- MCPIP1 is crucial in inhibiting the development of severe autoimmune responses by targeting the 3′UTR of pro-inflammatory cytokines, IL-6, IL-12p40 and IL-1β [17, 23].
- MCPIP1 acts as a negative regulator of LPS-dependent activation of macrophages. The mechanism of this inhibition involves the deubiquitinase activity of MCPIP1 that blocks LPS- or p65-induced NF-κB activation thereby negatively regulating the expression of inflammatory cytokines. MCPIP1 does not affect the mRNA stability of TNF-α like TTP, a CCCH-zinc finger protein, which promotes the decay of TNF-α mRNA by binding to the ARE element on at the 3′UTR[16].
- MCPIP1 acts as an anti-dicer RNase and suppresses the microRNA biogenesis by cleaving the terminal loops of pre-microRNAs [24].

If and how the enzymatic activities of MCPIP1 may modulate inflammatory angiogenesis is not understood.

HIF-1α

MCPIP expression induces an important angiogenic factor known as hypoxia-inducible factor-1 (HIF-1) [21]. It is an oxygen-sensitive transcription factor and is a

crucial physiological regulator of cell biological processes such as embryonic development and innate immunity. HIF-1 plays an integral role in response to hypoxia leading to the transcriptional induction of a wide spectrum of genes involved in energy homeostasis, cell differentiation, angiogenesis, apoptosis, and other genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia [25]. Since HIF-1 can modulate angiogenesis, it is an important potential target for promoting the vessel proliferation needed for oxygenation in ischemic patients. HIF-1 also facilitates the survival and proliferation of cancerous cells, suggesting the need for the therapeutic inhibition of HIF-1 and its angiogenic properties for the treatment of diseases such as tumor malignancies [26].

HIF-1 domain structure

HIF-1 is a heterodimeric complex and consists of an oxygen-regulated, inducible alpha subunit and a constitutively expressed beta subunit. Both HIF-1α and HIF-1β proteins contain basic helix-loop-helix- Per-ARNT-Sim (bHLH-PAS) motifs that ensure subunit dimerization on binding to the DNA and specific binding to the hypoxia response element (HRE; 5′-RCGTG-3′) in the DNA sequence. HIF-1 α contains an oxygen-dependent degradation domain (ODDD) that regulate its expression levels. The alpha subunit contains two transactivation domains, N-terminal (N-TAD) and C-terminal (C-TAD), both of which help in regulating HIF-1 α expression and binding of coactivators such as CREB binding protein (CBP) and p300 to activate HIF-1 α-target gene transcription [27] and is depicted in **Figure 1**.

Regulation of HIF-1:

The expression levels of HIF-1 are under stringent control through synthesis and degradation and are regulated by multiple mechanisms. Under normoxia, prolyl hydroxylases (PHD1, PHD2 and PHD3) hydroxylate two conserved proline residues (Pro402 and Pro564) located within the oxygen-dependent degradation (ODD) domain of HIF-1 α. Factor inhibiting HIF-1 (FIH-1), an asparaginyl hydroxylase hydroxylates HIF-1α at Asn 803 in the TAD domain. Hydoxylation in the TAD domain abrogates the interaction with coactivator, p300 and thus the HIF-1 α is rendered inept in mediating activation of transcription. Furthermore, hydroxylation either by PHDs or FIH results in binding of von Hippel Lindau (pVHL) E3 ubiquitin ligase complex to the HIF-1 α ODD directing the poly-ubiquitylation followed by proteasomal degradation of HIF-1 α. De-ubiquitination by pVHL-interacting de-ubiquitylating enzyme (VDU2; also called USP20) results in the binding and de-ubiquitylation of HIF-1 α thereby salvaging HIF-1 α from proteasomal degradation. In literature, VDU2 is the only enzyme known to de-ubiquitylate ubiquitinated HIF-1α [28]. If and how MCPIP1 would mediate HIF-1α stabilization has not been elucidated.

HIF-1 target genes: VEGF and COX2

Induction of HIF-1α promotes the transcription of a set of genes, including vascular endothelial growth factor (VEGF) and cyclooxygenase (COX)2 [29]. VEGF is a pro-angiogenic family of proteins and plays an integral role in angiogenesis[30]. Though five members have been reported in the human VEGF family, VEGF-A,-B,-C,-D, and placental growth factor (PlGF), alternative splicing of the pre-mRNAs generates multiple isoforms of VEGF. VEGF has three receptor protein-tyrosine kinases namely,

VEGFR1/Flt-1, VEGFR2/Flk-1/KDR, and VEGFR3/ Flt-4. Binding of VEGF on the tyrosine receptor/s initiates tyrosine phosphorylation that activates downstream signaling enzymes, p38 MAPK, ERK1/2, eNOS and NAPDH oxidase subunits Rac1 and NOX2, resulting in stimulating endothelial cell proliferation and migration[31]. Furthermore, VEGF-induced angiogenesis was reported to induce COX2 expression via activation of p38 MAPK [32].

COX also known as prostaglandin H synthase (PGHS) exists in three isoforms: a constitutively expressed COX1, which regulates “housekeeping’ physiological functions such as intercellular communication ; and an inducible COX2 that controls inflammatory pathways and COX3 that is reported to be a splice variant of COX1. COXs are a family of fatty-acid oxygenases that oxygenates arachidonic acid to prostaglandin G2 by its cyclooxygenase activity followed by reduction of prostaglandin G2 (PGG2) to prostaglandin H2 (PGH2) by its peroxidase activity. PGH2 is a precursor for a group of biologically active molecules called prostanoids known to exert effects on a myriad of physiological and pathophysiological processes such as differentiation and inflammation, respectively[33]. The major difference between the COX1 and COX2 isoenzymes is the substitution of isoleucine at position 434 in COX-1 with a smaller valine residue in COX-2 that prevents stearic hindrance and allows access to a hydrophobic side-pocket in the enzyme and a smaller active site in COX1[34]. Furthermore, presence of hypoxia-responsive element (HRE) on the COX-2 promoter suggests its induction via HIF-1α[29]. While several reports suggest that COX2 induction induces VEGF levels, analysis of several studies pertaining to angiogenesis suggest a significant level of correlations in the expression levels of VEGF and COX2

[35]. Whether and how MCPIP-induced angiogenesis is mediated via induction of HIF-1α target genes has not been elucidated.

Oxidative stress

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) termed as pro-oxidants and their elimination by antioxidant mechanisms. This dysregulation of balance results in significant damage to the cellular organization and important biomolecules, proteins, lipids, and DNA thus leading to a harmful impact as a whole. Oxidative stress has been widely recognized as a contributing factor in various forms of pathophysiology that are mediated by chronic inflammation such as in cancer, diabetes and cardiovascular diseases [36-39].

Endothelial cells (ECs) form a thin layer that line the innermost surface of the entire circulatory system called as the endothelium. ECs reduce the friction caused due to constant blood flow and thus allow smooth flow of blood. Under normal conditions, ECs mediate controlling the volume and the levels of electrolyte content of the intravascular and extravascular spaces in the blood vessels. Any imbalance in these levels is caused due to endothelial dysfunction and is predominant in several pathophysiologies, including cardiovascular diseases such as atherosclerosis. Another form of alteration in the ECs occurs due to an inflammatory response where there is alteration in the permeability or cell-cell adhesion and is called as endothelial activation. This endothelial activation is of significance and occurs during inflammatory angiogenesis.

Superoxide anion ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2) are ROS species generated by ECs that mediate stress and growth responses and are involved in the signaling pathways such as angiogenesis. ROS at physiological concentrations serve as signaling molecules important to mediate biological responses. Though ROS is generated via multiple physiological stimuli such as the mitochondrial electron transport system, xanthine oxidase, cytochrome p450, uncoupled NO synthase (NOS), and myeloperoxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is known to be the major source of ROS production and has been reported to be required for proliferation and migration by ECs [40] . The structure of NADPH oxidase is complex and consists of 5 major components. Two membrane-bound factors ($gp91^{phox}$ or NOX2 and $p22^{phox}$) that form the flavocytochrome b558 and are involved in electron-transporting function of the enzyme and three cytosolic elements ($p67^{phox}$ or NoxA1, $p47^{phox}$ or NoxO1 and $p40^{phox}$) and a low-molecular-weight inactive GTPase (Rac1 or Rac2). The cystosolic components form of a complex containing a NADPH binding site. Upon activation, the cytosolic component $p47^{phox}$ gets phosphorylated by PKC and then is translocated to the cell membrane along with $p67^{phox}$ and $p40^{phox}$ to form an active and complete NADPH oxidase complex [41, 42] . After successful assembly of all its components NADPH oxidase starts the serial production of ROS by converting molecular O_2 to $O_2\cdot^-$ that can be converted to H_2O_2 by another enzyme, superoxide dismutase (SOD), or to peroxinitrite ($OONO.^-$) by reacting with nitric oxidase (NO) or to highly reactive $OH\cdot^-$ [43].

In vitro studies in ECs have reported H_2O_2 to stimulate cell migration and proliferation. *In vivo* studies in pathological conditions such as diabetic retinopathy have also shown that elevated oxidative stress levels directly correlate with the degree of neovascularization [44]. Furthermore, evidences suggest that hypoxia-or VEGF-induced proliferation and migration by endothelial cells is mediated via ROS production [45] and is severely hampered in *in vivo* studies involving knockout of NADPH oxidase subunit, NOX2 [46]. If MCPIP-induced angiogenesis involves oxidative stress production is not known.

ER stress

Endoplasmic reticulum, the first organelle in the secretory pathway, functions to synthesize, modify and deliver the proteins to their proper target sites. In the ER, protein folding occurs along with an array of post-translational modifications coupled with formation of intra- and intermolecular disulfide bonds. Only the correctly folded and modified proteins are exported whereas the proteins that cannot adopt a correctly folded native confirmation are retrotranslocated for proteosomal degradation by a process called as ER-associated protein degradation (ERAD) [47]. An imbalance between the folding capacity and its ability to process the proteins causes ER stress. Multiple factors such as ischemia, hypoglycemia, mutations or viral infections can provoke ER stress as have been reported in inflammatory pathophysiologies, including diabetes and atherosclerosis[48].

In cells not experiencing ER stress, an ER chaperone GRP78/BiP binds to unfolded proteins and luminal domains of ER stress transducers, IRE1, PERK, and ATF6 and thus maintain them in an inactive state [49]. Under ER stress wherein

accumulation of misfolded proteins within the ER results in the activation of a signalling pathway known as the unfolded protein response (UPR), BiP dissociates from the ER stress sensors to help in protein folding. In an effort to adapt and re-establish normal ER functions, activation of the UPR pathway induces transcription of ER stress-responsive genes that potentiate the protein folding capacity of the ER and/or enhance protein degradation to aid in removal of misfolded proteins. ER stress activates the tripartite signaling network via activation of the ER transmembrane proteins-IRE-1, PERK and ATF6 that function in regulating ER stress at both transcriptional and translational levels. PERK activation inhibits protein biosynthesis by phosphorylating the translation initiation factor 2 (eIF2) in an effort to attenuate translation and thus reduce protein folding load although selectively increasing the translation of activating transcription factor 4 (ATF4) that in turn induces the levels of its downstream gene GADD34 that is needed for recovery from ER stress and thus survival. UPR activation causes IRE1-dependent splicing of a 26-nucleotide intron from the XBP-1 mRNA converting it into a potent transcriptional activator that potentiates self-transcription to generate substrate for IRE-1 and thus sustain the UPR response. Activation of the UPR processes ATF6 protein to generate 50–60 kDa cytosolic bZIP which after entering the nucleus acts as a transcription factor to promote the transcription of XBP-1, an IRE-1 substrate [50]. Despite all the efforts by the ER to ameliorate the stress caused due to protein overload, persistent ER stress induces apoptosis by caspase-12 activation [51]. If ER stress is vital in angiogenesis induced by MCPIP expression is unknown.

Autophagy

In an effort to remove the accumulated misfolded protein aggregates that are larger for being degraded by the 26s proteasome or exceeds the capacity of the ERAD system, the autophagy machinery is activated to help the cell undergo the bulk degradation. Furthermore, eIF2 phosphorylation upregulates transcription by ATF4 that promotes the transcription of autophagy gene, ATG12 and induces LC3 conversion thus stimulating autophagy[52].

Autophagy is of physiological importance to the cell and though autophagy is known for the self-digestion mechanism it is mainly activated to protect against cell death [53]. Briefly, autophagy is initiated by sequestering portions of the soluble cytoplasm/organelle/aggregates by a double-membrane bounded autophagic vacuole called the autophagosome which upon maturation fuses with the lysosomal membrane to deliver the contents into the autolysosome, where they are degraded by the lysosomal degradative enzymes and the resulting macromolecules, such as amino acids, carbohydrates are recycled [54], **Figure 3.**

Differentiation

Cell differentiation, an important biological event involves complex signal transduction pathways and requires synthesis of specific macromolecules to continue a differentiated state. Thus cell differentiation is maintained by a delicate balance in synthesis and degradation of these vital macromolecules. Overwhelming evidence suggest the involvement of autophagy in differentiation. Autophagy plays an important role in regulating nutrient supply, renovating the cell cytosol by modifying its protein content and organelle turnover, thus modifying the exposed receptors, transcriptional

factors inside the nucleus, and cytoskeletal dynamics in an effort to help the cell respond better to the extrinsic stimuli/stress. Analysis of *in vitro* studies performed by expression of dominant-negative *atg1* in granule cells of the cerebral cortex from a mouse model resulted in inhibition of neurite outgrowth. Results from studies in neural differentiation suggest that autophagy plays a vital role in retinoic acid-induced differentiation [55]. Knockdown of *atg7* in pre-adipocytes resulted in inhibition of lipid accumulation and differentiation of the cells and adipocyte-targeted Atg7-knockout mice exhibit reduced white adipose tissue [56]. Furthermore, depletion of beclin1 was reported to impair both autophagy and differentiation thus forming a bridge between the two. Moreover, beclin-/- mice exhibited reduced size and developmental delay [57]. Several *in vivo* studies on mice with global knockouts of vital autophagy-related genes, *ambra1, atg5, and atg7*, have revealed that developmental defects occur in the absence of autophagy giving rise to the concept that autophagy is a prosurvival mechanism that is involved in regulating development by modulating critical cellular remodeling functions that are essential for differentiation [58]. Induction of autophagy by overexpression of *atg5* resulted in increased tube formation whereas knockdown of *atg5* was reported to attenuate VEGF-induced angiogenesis in BAECs thus suggesting the involvement of autophagy in angiogenic differentiation [59]. If autophagy is crucial for angiogenic differentiation induced by MCPIP has not been studied.

Sirtuin

Cells possess the ability to detect extracellular stimuli, such as cytokines or stress and execute the appropriate response by the transcriptional induction or repression of distinct sets of genes that is crucial to alter and regulate its cellular

functions [60]. The transcriptional regulation involves the acetylation modifications by the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation of histones on the chromatin releases the suppression and promotes transcription whereas deacetylation results in compacting the chromatin with subsequent inhibition/silencing of the gene transcription. Among the three classes of HDACs, class III HDACs, sirtuins (SIRT1-7)s are unique in their need for nicotinamide adenine dinucleotide (NAD+) as a cofactor. SIRT1 is reported to be involved in regulating cellular differentiation, senescence and to regulate metabolic pathways [61]. SIRT1 mediates its function by deacetylating key transcription factors such as forkhead box o (Foxo) [62] and tumor suppressor, p53, known to mediate cell growth, survival and differentiation. In vitro studies in which 20 residue-long oligopeptide corresponding to residues of p53 protein were acetylated at Lys373 and Lys382 [63]. When the above acetylated oligopeptide was incubated with SIRT1 in presence of NAD+, there was a preferential deacetylation of Lys 382 by SIRT1 suggesting its possible role in repressing p53 and its target genes, such as the well-known angiogenesis inhibitor, thrombospondin (TSP1)[64]. TSP-1 is a matricellular glycoprotein and mediates its effects through interactions with the cell surface receptors CD36. Binding of TSP1 to its CD36 receptor was reported to activate apoptosis and exert the anti-angiogenic activity [65]. It is unknown if and how SIRT-1 induction mediates angiogenesis induced by MCPIP1 expression.

Ubiquitination and deubiquitination

Covalent ligation of ubiquitin (Ub), 76 amino acid polypeptide, to a protein, targets it for degradation. Post-translational modification of the target proteins by Ub

ligation controls multiple cellular processes, including protein degradation, signal transduction pathways, transcriptional regulation, to name a few. Ubiquitination is the formation of an isopeptide bond between the C-terminus glycine of Ub and the ε-amino group Lysine (K) of the target protein catalyzed by sequential action of Ub-activating enzyme E1, Ub-conjugating enzyme E2, and Ub ligase E3 [65]. Furthermore, Ubs can also ligate to additional Ub molecules to form branched poly-Ub chains. Seven potential lysine residues, K6, K11, K27, K29, K33, K48 and K63 in ubiquitin form the polyUb chains. Linear polybiquitination in which the amino and carboxy termini of Ub are linked have also been reported [66]. Length of the poly-ub chain and its position of linkage on the protein determine the fate of the ubiquitinated protein. While K-29 and K-48 linked proteins are targeted for proteosomal degradation [67], K-63 linkage results in mediating signaling pathways such as kinase activation[68]. Evidence suggests that activation of nuclear factor-κB (NF-κB) is regulated by ubiquitination of key signaling molecules[69].

NF-κB is an inducible transcription factor that has been reported to mediate inflammation, stress, and developmental processes, is tightly regulated by the ubiquitination-deubiquitination pathway [70]. NF-κB, a heterodimer, is composed of p50 and p65 (RelA) subunits that reside in a latent form in the cytoplasm of nonstimulated cells. Association of inhibitory family of proteins, IκB, help maintain NF-κB in the cytoplasm by either sterically hindering their nuclear localization signal or by inhibiting their DNA binding and capacity of transactivation. Following stimulation by cytokines, stress or infection, Ser^{32} or Ser^{36} phosphorylation of IκBs by IKKs result in the ubiquitination and thus its degradation by the proteosomal pathway. Briefly, stimulation by TNF-α, IL-1β or LPS results in K-63 linked autoubiquitination of the TRAF2/6 by E3

ligases, RIP1 or IRAK1/4, which causes the recruitment of TAK1 and IKK kinases through binding of ubiquitin-binding adaptors, TAB2/3 and NEMO, leading to NF-κB activation [71].

Given the significance of ubiquitination in NF-κB activation, it is of utmost importance that the process of ubiquitination be under stringent regulation to prevent this well-known inflammatory signaling pathway. Reversal of this protein ubiquitination, by removal of Ub conjugated to the protein, is catalyzed by deubiquitinating enzymes (DUBs). Deubiquitination is a process by which the (a) PolyUb chains are edited to yield Ub monomers or (b) reverses Ub conjugated to proteins so as to prevent the degradation of important cellular proteins or (c) recycle Ub moieties, all mechanisms critical in regulating signaling pathways, including transcriptional regulation, development, and differentiation [72]. DUBs, CYLD and A20 that are induced by NF-κB activation have been reported to modulate cell differentiation by serving as a negative feedback loop to terminate NF-κB activation via multiple mechanisms [73]. A20 functions both as a DUB and E3 ligase. A20 cleaves the K-63 linked polyUb on RIP1 by its DUB activity and then by the E3 ligase activity catalyzes the K-48 linked polyUb on RIP1 to target its degradation thus inhibiting the activation of downstream targets, IKKβ/NEMO and thus downregulating NF-κB activation[74]. Reports on A20 deficient mice show that owing to spontaneous multi-organ inflammation and defects in resolving the inflammatory responses resulted in the premature death of these mice and thus serve as an important insight into the crucial role of A20. A defect in terminating NF-κB activation on TNF-α induction was observed in MEFs cultured from A20 deficient mice thus proving the importance of A20 in negatively regulating NF-κB activation[75, 76]. If

downregulation of NF-κB activation may mediate MCPIP-induced angiogenic differentiation is important but unknown. Furthermore, if the deubiquitinase activity of MCPIP1 may stabilize/destabilize other known players in angiogenesis remains unknown.

MicroRNA

MicroRNAs (miRNAs) have emerged as novel, important regulators of multiple biological functions such as angiogenesis [77]. They are a group of small (20–25 nucleotides in length), non-coding RNAs that form a crucial layer in regulating gene expression at the posttranscriptional level. miRNAs modulate gene expression either by degrading the mRNA or by repressing its translation. miRNAs biogenesis involves two sequential mechanisms: (a) nuclear processing of longer transcripts (termed pri-miRNAs) by ribonuclease drosha to generate smaller (~70) nucleotide pre-miRNAs followed by its export to the cytoplasm by exportin-5 (b) cytoplasmic processing of pre-miRNAs by dicer RNase to generate double stranded mature miRNAs. Of the two strands in the mature miRNAs, the strand with lower stability preferentially associates with RNA-induced silencing complex (RISC) and can degrade the mRNA through direct cleavage or by inhibiting translation thus regulating multiple genes/mechanisms[78, 79].

MiR-20b, was reported to bind the promoters of both HIF-1α and its target gene, VEGF thus downregulating their translation and serving as an anti-angiogenic miR. Inhibitor of mir-20b was also reported to increase protein levels of HIF-1α and VEGF [80]. MiR-34a, an important tumor suppressor, has been found to be deregulated in several forms of cancer. Silent information regulator 1 (Sirt1), novel modulator of

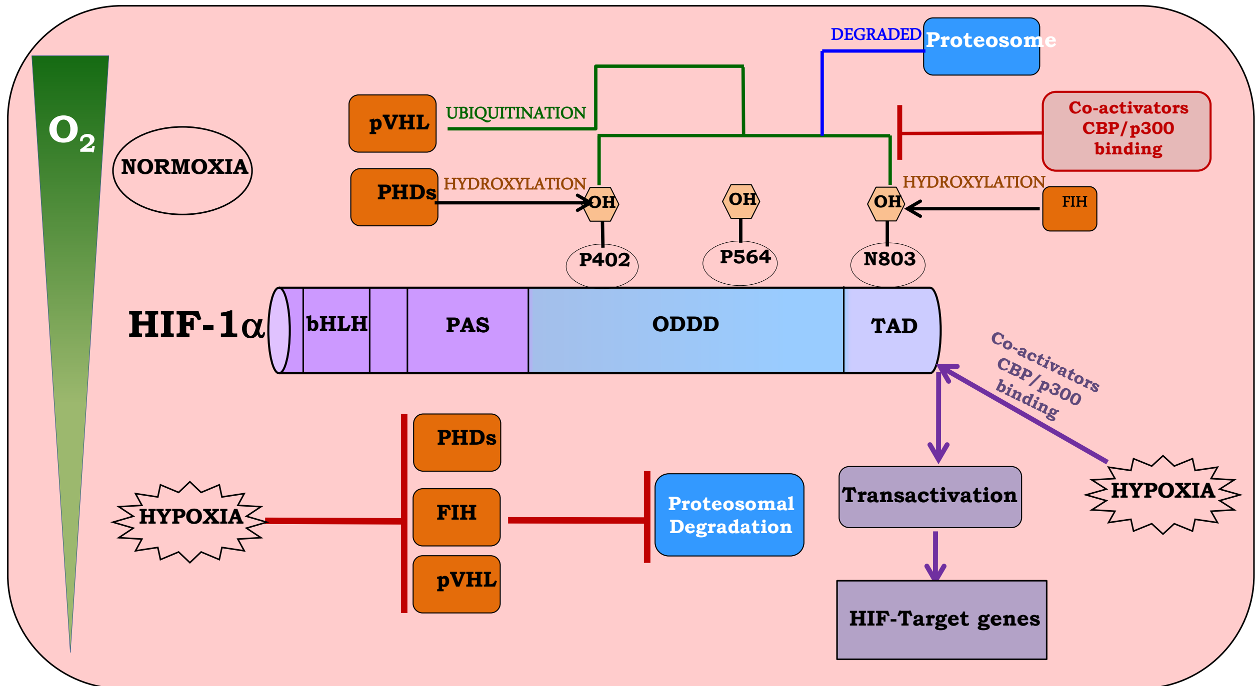
angiogenesis, is one of the potential targets of miR-34a[81]. Additionally, the promoter of miR-34a has NF-κB binding sites suggesting its possible regulation by NF-κB activation [82]. If the anti-dicer activity of MCPIP1 has a role in mediating angiogenic differentiation has not been studied.

Thus, the overall mechanism/s by which MCPIP promotes inflammatory angiogenesis has not been elucidated. We postulated that MCPIP-induced differentiation involves induction of oxidative stress, ER stress and autophagy. Furthermore, we hypothesized that the induction and stabilization of pro-angiogenic factor, HIF-1α involves the deubiquitinase and anti-dicer RNase enzymatic activities of MCPIP. The anti-dicer RNase activity of MCPIP mediates the expression levels of SIRT-1 that down regulates the angiogenic inhibitor, TSP-1. With this current knowledge in the field of inflammatory angiogenesis as presented above, my study aims at understanding the mechanism by which MCPIP1 expression promotes angiogenic differentiation with the following objectives-

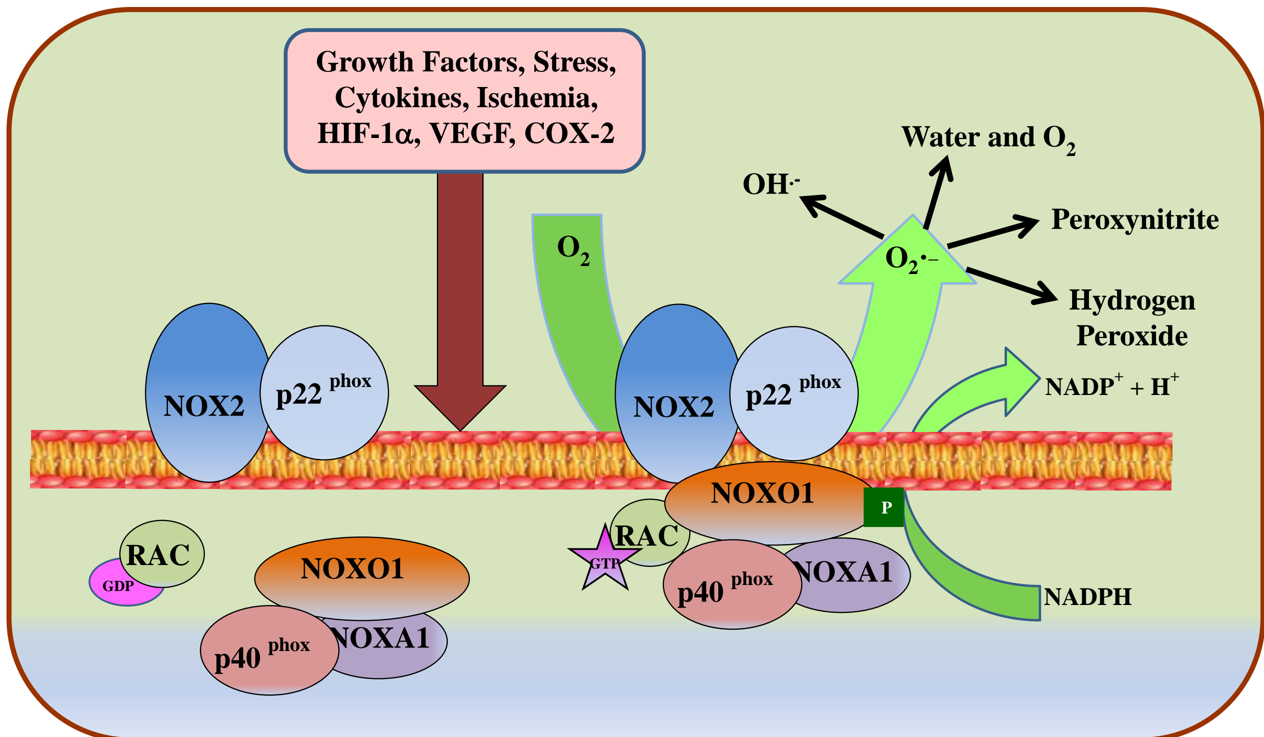
- To determine the biological processes involved in angiogenic differentiation induced by MCPIP expression
- To decipher the mechanism/s by which MCPIP1 expression promotes angiogenesis.

The data presented here suggest for the first time that the sequential induction of oxidative stress, ER stress and autophagy is essential for angiogenic differentiation induced by MCPIP expression. The deubiquitinase activity of MCPIP was shown to be involved both in (a) stabilizing HIF-1α leading to the induction of its pro-angiogenic target genes, VEGF and COX2; and (b) downregulating NF-κB activation resulting in

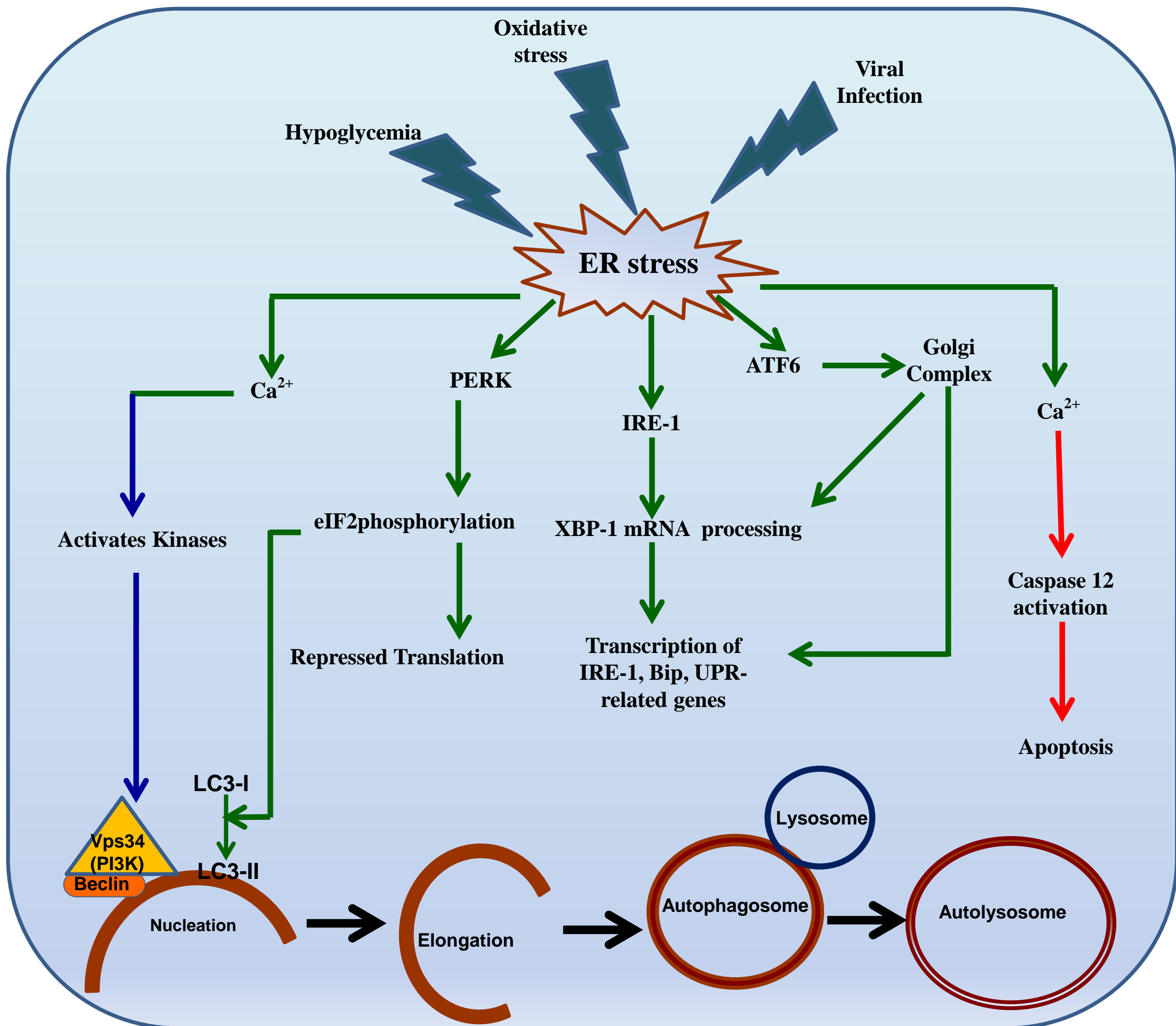
lower levels of anti-angiogenic VEGI. Furthermore, induction of SIRT1 by MCPIP expression resulted in inhibition of anti-angiogenic TSP-1. The biogenesis of anti-angiogenic miRs, mir-20b and mir-34a, was significantly suppressed by MCPIP expression. Reduced levels of mir-20b and mir-34a could no longer inhibit their angiogenic targets HIF-1α and SIRT1, repectively. Thus the overall mechanism of inflammatory angiogenesis mediated by MCPIP expression involves tilting the angiogenic balance by inducing the production of angiogenic molecules and suppressing the production of anti-angiogenic factors.



- **Figure 1: Regulation of HIF-1α**
- **Under normoxia, HIF-1α is degraded. Hydroxylation of proline residues by PHDs result in the ubiquitination of HIF-1α followed by proteosomal degradation. FIH, an aspargine hydroxylase hydroxylates the aspargine residue and prevents the binding of co-activators, CBP and p300 that are known to help transactivation of HIF-1α to the nucleus. During hypoxia, PHDs and FIH can no longer hydroxylate the proline or aspargine residues thus preventing the ubiquitination of HIF-1α resulting in stabilized HIF-1α that can enter the nucleus to function as a transcription factor.**



- **Figure 2: NADPH oxidase in endothelial cells**
- **In stimulated endothelial cells, the cytoplasmic subunits of NADPH, NOXO1, NOXOA1 (p47 phox, p40 phox, RAC) translocate to the membrane to form an active NADPH complex on binding to the membrane subunits, NOX2, p22phox. NADPH oxidase generates ROS by converting molecular molecular oxygen O2 to O2·_ that is converted to H2O2 by superoxide dismutase (SOD), or to peroxinitrite (OONO._) by nitric oxidase (NO) or to highly reactive OH·_**



- **Figure 3: ER stress and Autophagy**

- **Oxidative stress induces ER stress resulting in processing of ER proteins, PERK, IRE-1 and ATF6. PERK activation results in eIF2 phosphorylation that results in repressing protein synthesis and inducing autophagic vacuole formation. IRE-1 activation processes the XBP-1 mRNA thus activating XBP1 as a transcription factor that promotes the transcription of IRE-1 and UPR-related genes. ATF6 after cleavage in the Golgi complex also promotes the synthesis of XBP-1 thus acting as a positive feedback loop. Uncontrolled ER stress could result in autophagy by the excessive**

calcium release or it may also result in the activation of Caspase12 and promote apoptosis.

CHAPTER 2: MONOCYTE CHEMOTACTIC PROTEIN-INDUCED PROTEIN (MCPIP) PROMOTES INFLAMMATORY ANGIOGENESIS VIA SEQUENTIAL INDUCTION OF OXIDATIVE STRESS, ENDOPLASMIC RETICULUM STRESS AND AUTOPHAGY

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is involved in important physiological processes such as embryogenesis and wound repair as well as in the pathophysiology of many inflammatory diseases such as atherosclerosis, ischemic heart disease, rheumatoid arthritis, diabetes and obesity [83, 84]. Vessel growth also contributes to tumor growth and metastasis [1, 85, 86]. The multi-step complex process of angiogenesis is controlled by a wide range of activating and inhibitory chemokines involving degradation of extracellular matrix, disruption of cell-cell contacts, proliferation, migration and capillary-like tube formation of endothelial cells. There is clear evidence for the role of pro-inflammatory cytokines monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin- (IL)-1β and IL-8 in angiogenesis and several angiogenesis-related disorders [87-89] including the development of cardiovascular diseases in humans [90-92].

MCPIP (MCP-1-induced protein), originally discovered as a novel zinc finger protein, ZC3H12A, induced by treatment of monocytes with MCP-1 is also induced by other inflammatory agents [15, 17, 21, 93]. MCP-1 treatment of human umbilical vein endothelial cells (HUVECs) induces angiogenic differentiation resulting in capillary-like

tube formation via induction of MCPIP [21]. MCPIP can induce hypoxia inducible factor (HIF)-1α and vascular endothelial growth factor (VEGF), that are known to be involved in angiogenesis [21]. VEGF can stimulate reactive oxygen species (ROS) production via activation of NADPH oxidase and nitric oxide production [94, 95] that are involved in angiogenic differentiation [96]. ROS generated from NADPH oxidase activation serves as secondary messengers for stimulating multiple signaling pathways leading to angiogenesis [46, 97]. Oxidative and nitrosative stress has been reported to induce endoplasmic reticulum (ER) stress [98-100]. Furthermore, ER stress can lead to autophagy [101] that can be involved in cell differentiation [102, 103]. Based on such scattered observations we postulate that angiogenesis induced by inflammatory agents is mediated via MCPIP that leads to induction of oxidative and nitrosative stress causing ER stress resulting in autophagy involved in angiogenic differentiation. The experimental data presented here show that angiogenesis induced by inflammatory agents, TNF-α, IL-1β, MCP-1 and IL-8, is mediated via MCPIP and that the inflammatory angiogenesis is mediated via oxidative stress, ER stress and autophagy. The results provide the first molecular insight into the probable mechanism involved in inflammatory angiogenesis and thus contribute to the pathological conditions related to angiogenic differentiation such as cancer, cardiovascular diseases and obesity.

Material and methods

Cell culture conditions

The human umbilical vein endothelial cells ([HUVECs]; CC-2519, LONZA) were grown in endothelial cell basal medium (CC-3124) as recommended by the

manufacturer. HUVECs were used between passages 4 and 8. All cells were maintained at 37°C in presence of 5% CO_2.

Transfection procedure

The human MCPIP cDNA encoding the full-length MCPIP [Accession number: AY920403] was cloned in pEGFP/N1 vector to generate MCPIP-GFP fusion protein as previously described [15]. HUVECs were transfected with vectors expressing MCPIP fused to GFP or GFP alone using Lipofectamine and PLUS Reagents (Invitrogen) according to manufacturer's protocol. The transfection efficiency was about 60-70% and was determined by the green fluorescence.

Knockdown with small interfering RNA (siRNA)

HUVECs were transfected for 6 hours with 100 nmol/l of a chemically synthesized siRNA targeted for the MCPIP or IRE-1 or beclin geneor non-specific siRNA (Santacruz) using Lipofectamine and PLUS Reagents (Invitrogen) prior to transfection with MCPIP–GFP or GFP expression vectors.

HUVEC treatments

MCP-1 (100ng/ml), TNF-α (10ng/ml), IL-1β (10ng/ml) and IL-8 (100ng/ml) were used to treat HUVECs for 24 hours. HUVECs were treated with following chemical inhibitors 3 hours before transfection with expression constructs for MCPIP-GFP or GFP alone: ROS inhibitors, apocynin (50μM) and cerium oxide nanoparticles (CeO_2) nanoparticles (100nM); RNS inhibitor, L-Name (50 μM); ER stress inhibitor,

tauroursodeoxycholate (TUDC; 50μM); and autophagy inhibitor, 3′Methyl Adenine (50μM); LY 294002 (10μM).

In vitro capillary-like tube formation assays

After treatment under various conditions, HUVECs were trypsinized and then seeded onto the surface of the polymerized matrigel (1 $\times 10^4$ cells / per well, BD Matrigel™ Basement Membrane Matrix (Cat#354234) in 96-well plates according to the manufacturer's protocol followed by incubation in EBM medium for 24 hours at 37ºC in 5% CO_2. Tube formation was photographed under a phase-contrast microscope and quantified as previously described[21]. The experiments were repeated at least three times. The results were expressed as the mean percentage of branches and expressed as a ratio to the untreated or empty vector (control).

ROS measurements

HUVECs were treated with inhibitors or transfected with specific siRNAs for 3-6 hours before being transfected with MCPIP–GFP or GFP expression plasmids. After 24 hours the cells were incubated with 1 μmol/l DHR123 (dihydrorhodamine 123) for 30 min at 37°C and 5% CO_2. ROS was measured with a fluorimetric plate reader (excitation wavelength 550 nm and emission wavelength 590 nm) as per manufacturer's recommendations.

Real-time PCR

Total RNA was isolated from HUVECs using Trizol reagent (Invitrogen). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied

Biosystems) utilizing 1 μg of total RNA (DNase-treated). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) served as an internal control. The sequences of primers designed for RT–PCR are as mentioned in earlier study [21]. The sequences of other primers used are:

IRE-1-Forward-5′ GCGAACAGAATACACCATCAC-3′, IRE-1-Reverse - 5′ACCAGCCCATCACCATTG-3′; Beclin- Forward-5′AAGAGGTTGAGAAAGGCGAG-3′, Beclin-Reverse - 5′ TGGGTTTTGATGGAATAGGAGC-3′

phox47 antisense – CCAGCAGGGCGATGTGACGGATGAA ;phox47 sense-GGAGTAGTGCGTAGTGAGCCTTGAC.

Immunoblot analysis

HUVECs were lysed with CellLytic lysis Buffer (Sigma) and the cell lysate was collected from different experimental conditions. An equal amount of protein sample from each condition was subjected to immunoblot analysis using the primary antibodies namely, anti-mouse polyclonal GAPDH (1:1000); anti-mouse GRP 78 (1:500); anti-rabbit beclin (1:1000) and HRP-conjugated secondary antibodies (1:5000). Immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) kit (Amersham, UK). Immunoblots were quantified as a ratio over endogenous GAPDH.

Statistical analysis

Each experiment was repeated three times. The error bars represent ± S.E.M. An asterisk (*) indicates a significant difference as compared to the control as indicated in each experiment. P value of < 0.05 was considered significant as determined by student's t-test.

Results

Inflammatory cytokines induce angiogenesis via MCPIP:

HUVECs were treated with inflammatory agents, TNF-α, IL-1β, IL-8 and MCP-1 with or without siRNA specific for MCPIP. Knockdown of MCPIP significantly inhibited the angiogenic differentiation induced by inflammatory cytokines Figure 4 a and b. Efficiency of MCPIP knockdown on MCPIP protein levels was determined by western blot analysis Figure 4c.

MCPIP overexpression induces oxidative stress, endoplasmic reticulum stress, autophagy and angiogenesis:

Since it is known that ROS signaling plays a major role in angiogenesis [104] we tested whether forced expression of MCPIP induces ROS production. Flourometric analysis with the redox sensitive dye, dihydrorhodamine 123, showed that overexpression of MCPIP induced oxidative stress (Figure 5 a and b). iNOS transcript levels were measured to determine nitrosative stress in MCPIP expressing HUVECs. Nitrosative stress was also measured by the diazotization of Griess reagent by nitrite produced as a result of MCPIP overexpression. Our data show that forced expression of MCPIP induced oxidative and nitrosative stress (Figure 5 c and d). It is known that NADPH oxidase is a major source of ROS production in endothelial cells and membrane association of phox47 subunit is essential for the formation of an active NADPH oxidase complex. Forced expression of MCPIP caused increase in the protein level of phox47 in HUVECs (Figure 5e) and induced translocation of phox47 to the membrane (Figure 5f).

Experimental data suggest that endoplasmic reticulum is sensitive to oxidative stress resulting in an ER stress response [105]. We determined whether forced expression of MCPIP would result in ER stress. Our findings show that forced expression of MCPIP resulted in significant increase in transcript and protein levels of the ER stress markers GRP78, and IRE-1 (Figure 6a and b).

Since it is known that ER stress can trigger autophagy in some cases [52, 101, 106, 107] we tested whether forced expression of MCPIP would also induce autophagy in HUVECs. Forced expression of MCPIP caused significant increase in protein levels of autophagy marker, beclin, compared to the cells expressing the vector alone (Figure 7a). Furthermore, HUVECs expressing MCPIP showed increased appearance of autophagosomes as detected by LC3 immunostaining thus demonstrating that autophagy is induced by MCPIP in HUVECs (Figure 7b).

Since autophagy can play a role in development and differentiation [58], we tested whether forced expression of MCPIP induced tube formation in HUVECs. Data shown in Figure 8 indicate that forced expression of MCPIP-GFP induced tube formation as compared to cells expressing GFP alone.

Inhibition of ROS production reduces MCPIP-induced ER stress and autophagy and attenuates angiogenesis

We tested whether MCPIP-induced oxidative stress is essential for the induction of ER stress response in HUVECs. Inhibitors of oxidative stress, apocynin and CeO_2 nanoparticles reduced oxidative stress as shown in Figure 9 and knockdown by antisense phox47 nucleotides reduced phox 47 levels (Figure 10a), and also

decreased transcript levels of a major signaling component of ER stress response, IRE1, and GRP78 in MCPIP-GFP expressing cells (Figure 10b and c). We next tested whether MCPIP-induced autophagy is mediated via oxidative stress. Inhibition of oxidative stress with apocynin and CeO_2 nanoparticles resulted in marked decrease in the transcript and protein levels of autophagy marker, beclin, in MCPIP-GFP expressing cells (Figure 10d). That oxidative and nitrosative stress is essential for angiogenesis mediated by MCPIP, was demonstrated by the observation that inhibition of oxidative stress by treatment with apocynin, CeO_2 nanoparticles, L-NAME or phox47 antisense oligonucleotides before transfection with MCPIP expression vector attenuated MCPIP-induced tube formation in HUVECs (Figure 10e).

Inhibition of ER stress reduces MCPIP-induced autophagy and attenuates tube formation:

To determine whether ER stress is critical for MCPIP-induced autophagic response and angiogenesis in HUVECs, the effect of inhibition of ER stress by chemical inhibitor, TUDC or by knockdown of IRE-1, an ER stress marker, with its specific siRNA, on autophagy marker expression and tube formation were determined. Inhibitors of ER stress showed reduced level of ER stress marker, GRP 78 (as shown in Figure 11). Efficiency of si-IRE-1 knockdown on IRE-1 protein levels was determined by western blot analysis (Figure 12a). Inhibition of ER stress by levels lowered both the transcript and protein levels of autophagy marker, beclin (Figure 12b-d) and also attenuated MCPIP-induced tube formation in HUVECs (Figure 12e). These findings suggest that ER stress is involved in MCPIP-induced autophagy and angiogenesis.

Inhibition of autophagy attenuates MCPIP-induced tube formation:

Since it is known that autophagy can be involved in differentiation in diverse cellular contexts [103, 108], we tested if MCPIP-induced tube formation is mediated via autophagy. Efficiency of beclin knockdown by its specific siRNA on beclin protein levels was determined by western blot analysis, (Figure 13a) and inhibition of beclin by autophagy chemical inhibitors, 3′MA and LY 294002 was determined as shown in Figure 13b. We found that inhibition with 3′MA (3-methyladenine) and LY 294002 and knockdown of beclin-1 with specific siRNA significantly attenuated MCPIP-induced tube formation (Figure 13c).

Discussion

Pro-angiogenic factors, TNF-α, IL-8, IL-1β, and MCP-1 play a key role in inducing inflammatory angiogenesis [21, 89, 99, 109-113]. It has been reported that MCP-1 mediates angiogenesis in HUVECs by inducing a novel zinc-finger protein, MCPIP [21, 114]. However, it is unknown if other pro-inflammatory cytokines induce angiogenesis via MCPIP. Furthermore, the molecular and cellular processes by which MCPIP mediates its angiogenic effects has not been elucidated. Our findings show that inflammatory angiogenesis induced by TNF-α, IL-8, IL-1β is mediated via MCPIP. Experimental data presented here provide a molecular insight into the mechanism by which MCPIP induces angiogenesis. In this study, we sought to delineate the sequential processes induced by MCPIP that lead to inflammatory angiogenesis. Our study tested the postulate that angiogenic differentiation induced by inflammatory cytokines is mediated via MCPIP that leads to production of oxidative stress resulting in ER stress

causing autophagy. Specific inhibition of each postulated process with chemical inhibitors or gene knockdown inhibited all subsequent steps postulated.

Inflammatory angiogenesis has been reported to be mediated via HIF-1α and VEGF [115]. Experimental results from our previous studies show that MCPIP induces HIF-1α and VEGF in HUVECs. In the present study we found that MCPIP-induced HIF-1α results in VEGF production (data not shown). Studies also confirm that VEGF expression directly correlates with elevated levels of oxidative stress in pathological conditions, diabetic retinopathies and atherosclerosis [30, 116]. Evidence also suggests that VEGF induction results in excessive ROS production via NADPH oxidase complex in a Rac-1 dependent manner [117]. Our finding that inhibiting MCPIP over expression induces oxidative stress and angiogenic differentiation are in line with the previous observations.

In the current study, we show that MCPIP -induced angiogenesis is mediated via ROS and RNS production. Oxidative stress refers to the imbalance between the ROS production and the ability of the cell to have an anti-oxidant response. Though ROS is generated from a multitude of sources including mitochondrial electron chain system, xanthine oxidase and NO synthase, NADPH oxidase family is a major source of ROS production in endothelial cells [118]. Our results indicate that MCPIP mediates the induction and membrane translocation of NADPH oxidase subunit, phox47 that is required for activation of NADPH oxidases. Inhibition of oxidative and nitrosative stress attenuated tube-formation induced by MCPIP in HUVECs. Previous studies suggest that

vascular inflammation in the pathogenesis of atherosclerosis is mediated by elevated levels of oxidative stress and also plays a significant role in the development and progression of Type II diabetes and heart failure [119] . Elevated levels of ROS play a vital role in tumorigenesis and are thus the target for therapeutic intervention [120]. Many studies also report that NADPH oxidase complex as an important component in mediating the angiogenic signaling cascades and is vital in the regulation of angiogenesis [37, 119, 121]. Our results are consistent with a variety of previous observations concerning the potential role of oxidative stress in angiogenesis and places oxidative stress in the context of the sequential events involved in angiogenesis. Experimental evidence suggests that oxidative stress plays a vital role in inducing ER stress [105, 108, 114, 122, 123]. Recent findings in different *in vitro* studies also suggest that ER plays an important role in response to damage induced by oxidative stress[105].

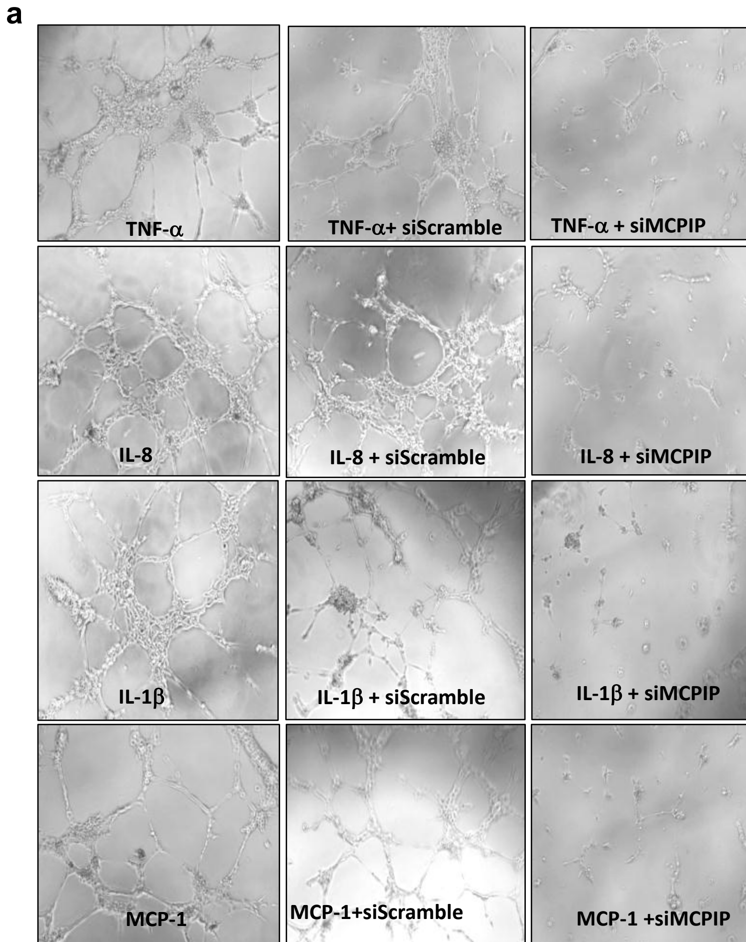
Furthermore, in transgenic MCP-mice (cardiomyocyte-targeted expression of MCP-1), in which MCPIP expression was elevated [114], also exhibited ROS and RNS production along with elevated ER stress levels [124, 125]. Our results from the present study demonstrate that MCP-1 treatment of HUVECs induces ER stress response and this response is mediated via MCPIP. Increased accumulation of ROS results in induction of unfolded protein response (UPR) by complex intracellular signal transduction pathways. GRP78 (Bip) and inositol-requiring protein 1 (IRE1) are important members of UPR signaling pathway[52]. Our results show that forced expression of MCPIP in HUVECs results in elevated transcript and protein levels of ER

stress-related proteins, GRP 78 and IRE-1. Furthermore, inhibition of oxidative stress reduced the levels of MCPIP-induced ER stress. In addition, ER stress inhibition attenuated the MCPIP-induced angiogenic differentiation thereby suggesting that MCPIP-induced oxidative stress is involved in elevating the ER stress response that mediates angiogenic differentiation in HUVECs. Our findings are consistent with the accumulating evidence that persistent oxidative stress induces ER stress. Studies show that ER stress mediates the differentiation of human embryonic stem cells under retinoic acid treatment by upregulating GRP78/Bip and XBP-1[126]. Inhibition studies of IRE-1 show that reduced levels of IRE-1 result in decreased growth rate, reduced angiogenesis and lower blood perfusion in tumours [127], consistent with our findings that ER stress inhibition attenuates MCPIP-induced angiogenic differentiation.

It is known that UPR is initiated by ER stress to ameliorate the protein overload on the ER [52]. However, if these measures fail to reestablish proper ER homeostasis, prolonged ER stress upregulate the autophagy machinery [128, 129]. Consistent with these findings our results show that forced expression of MCPIP in HUVECs increased levels of autophagy marker, beclin-1 and autophagosome formation. Inhibition of oxidative stress and ER stress resulted in decreased levels of MCPIP-induced beclin expression. However, inhibition of autophagy did not result in reduction of oxidative stress or ER stress levels (data not shown). Our findings thereby suggest that MCPIP induced oxidative and ER stress is upstream of autophagy. Several reports suggest that autophagy sustains the cell survival mechanism, although the final outcome of autophagy induction is dependent on the cell type and intensity of the stimulus. There is accumulating evidence to suggest that autophagy is vital for development and

differentiation. Autophagy plays an important role in recycling cellular proteins and organelles thereby maintaining ATP levels during situations of cellular stress so that the cell can maintain important cellular processes such as replication, transcription, protein synthesis and growth [103, 130-132]. MCPIP has been reported to mediate both death and differentiation in cells depending on the cell type [93] . MCPIP can mediate death in post-mitotic cardiomyocyte resulting in cardiomyopathies [108] or MCPIP can mediate differentiation in pre-adipocytes, monocytic cells and endothelial cells resulting in adipogenesis, osteoclastogensis and angiogenesis, respectively[93]. The results from the present study demonstrate that inhibition of autophagy attenuates MCPIP-induced cell differentiation thereby suggesting that autophagy is involved in MCPIP-induced angiogenic differentiation in HUVECs. Autophagy probably recycles some of the cellular proteins to provide the aminoacids needed for the synthesis of the new proteins needed for angiogenic differentiation.

The results reveal for the first time the sequence of events involved in angiogenesis mediated by pro-inflammatory cytokines and consequent MCPIP induction **Figure 14**. The experimental data presented suggest that MCPIP-induces differentiation via induction of oxidative stress that leads to ER stress that causes autophagy involved in tube formation in HUVECs. In future studies, it will be important to ascertain whether or not MCPIP actually accelerates vasculogenesis *in vivo* and also to determine its therapeutic potential to treat multiple diseases associated with inflammatory angiogenesis.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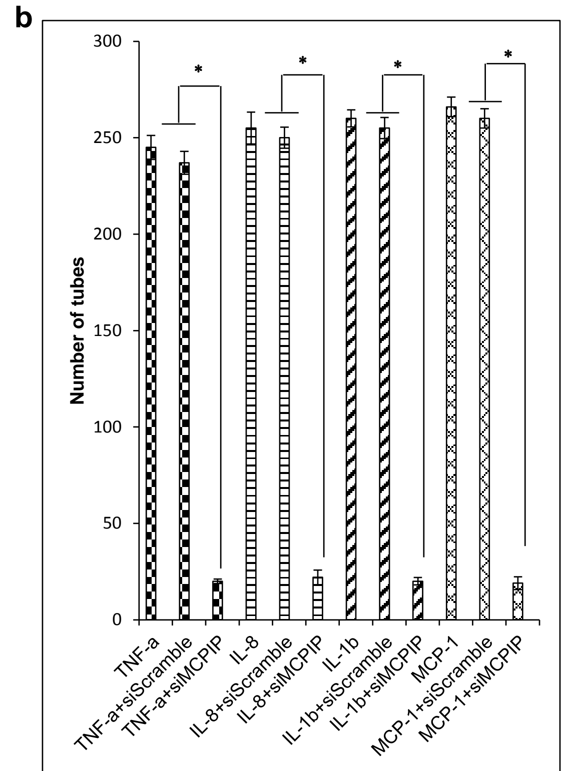
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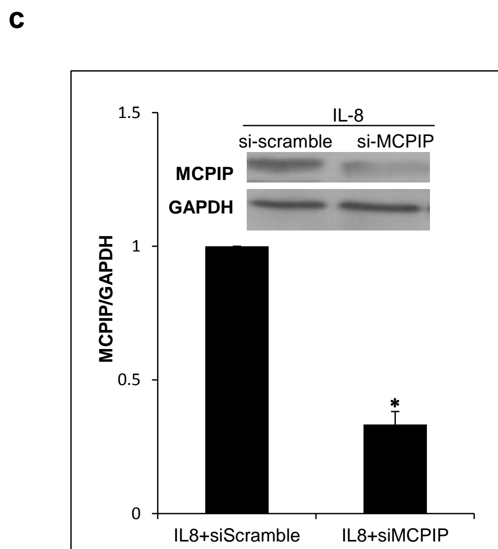
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1.5
1
0.5
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IL8+siScramble
IL8+siMCPIP

- **Figure 4: Effect of MCPIP knockdown on TNF-α, IL-1β, IL-8 and MCP-1 treatments on HUVECs**
- **HUVECs were transfected with siMCPIP and siScramble for 6 hours before treating with MCP-1 (100ng/ml), TNF-α (10ng/ml), IL-1β (10ng/ml) or IL-8 (100ng/ml). (a) After 24 hours cells were trypsinized and placed on matrigel. (b)Quantification of phase-contrast photomicrographs of the tube formation is represented; ∗=p <0.001. (c) Efficiency of MCPIP knockdown on MCPIP protein levels induced by IL-8 was determined by western blot analysis. Quantification of the immunoblot was performed and normalized to GAPDH; ∗=P<0.01.**

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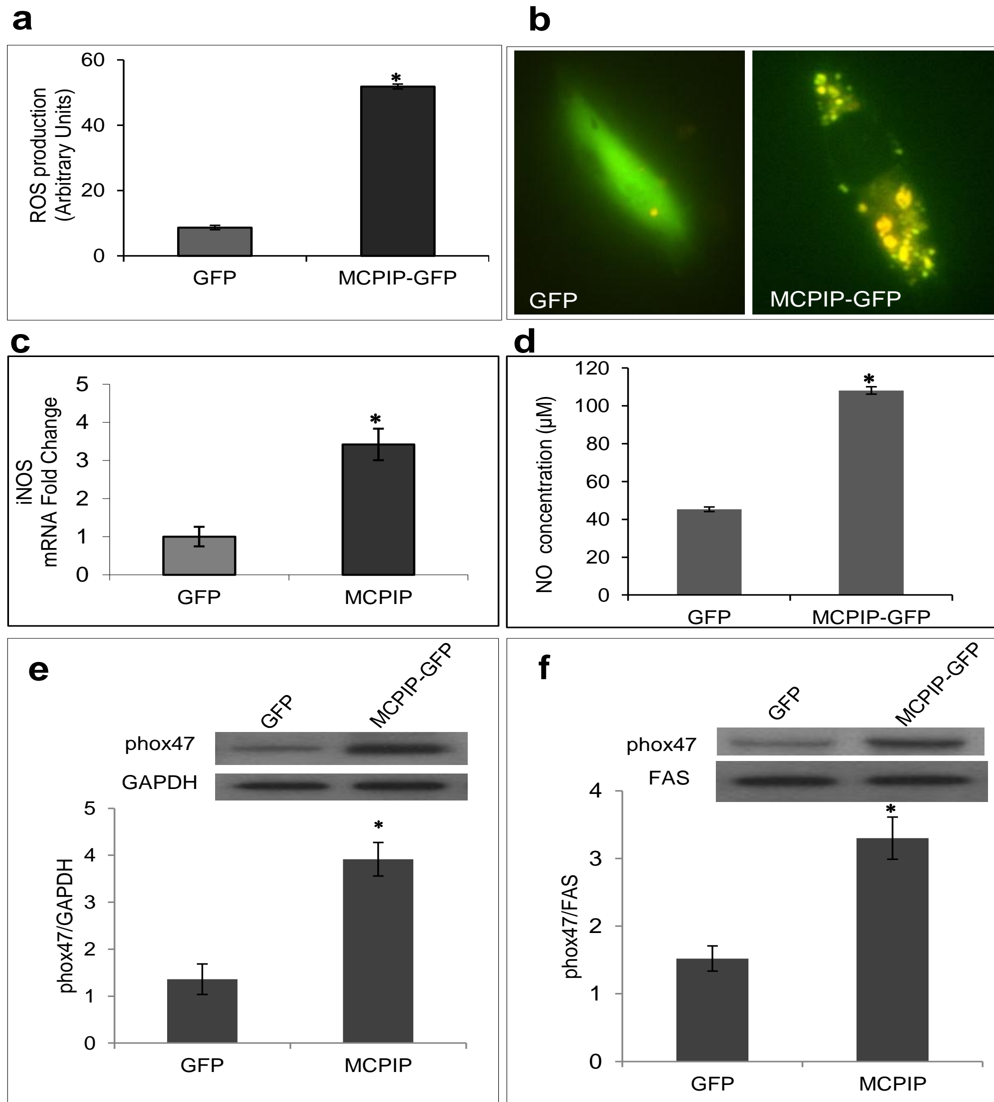
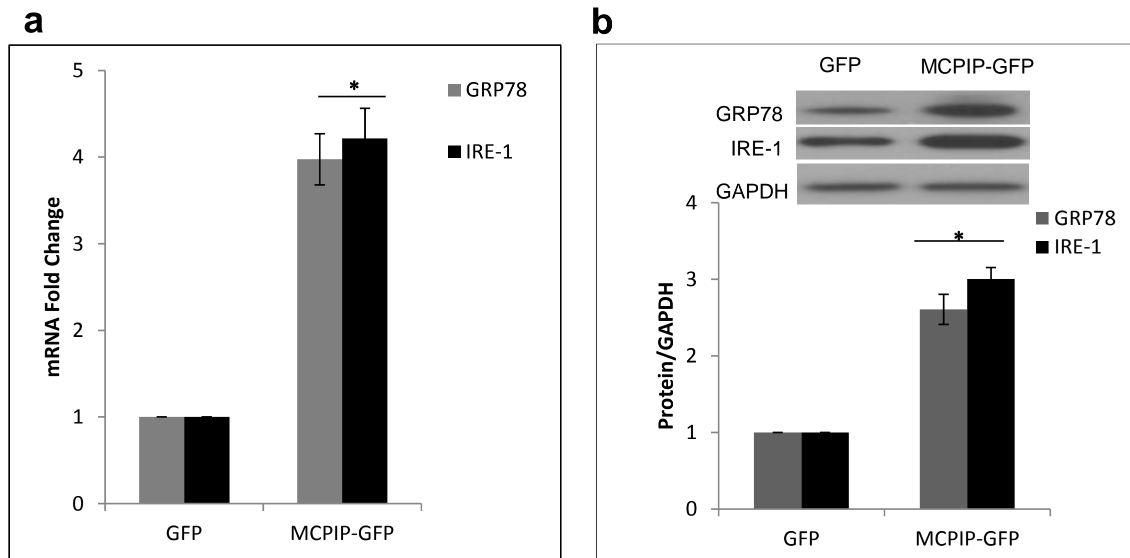
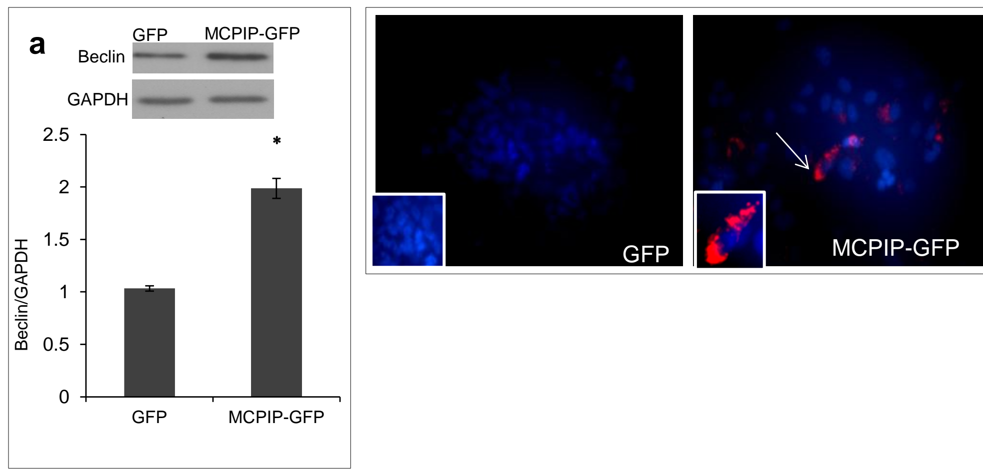


- **Figure 5 : MCPIP overexpression induces ROS/RNS in HUVECs**
- **(a) Total ROS/RNS was measured at 24 hours by treating these cells with 1µmol/L DHR123 for 30 min and (b) examined flurometrically (excitation wavelength 550nm and emission wavelength 590nm); ∗=*P*<0.01; (c) Real time RT-PCR was used to assess iNOS transcript levels. ∗=*P*<0.01. (d) Cell culture media was used to determine nitrite levels on MCPIP-GFP and GFP transfection of HUVECs. Griess diazotization reaction was used to**

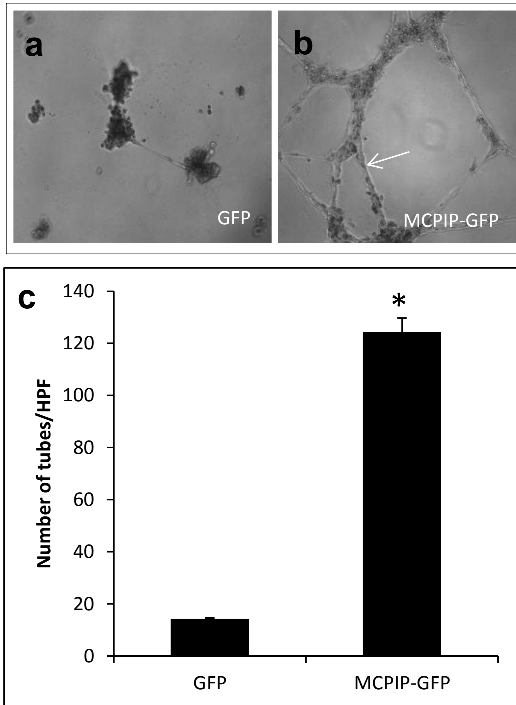
spectrophotometrically (548nm) detect NO present in the media. (e) Densitometric analysis of total phox47 was performed and normalized against GAPDH; ∗=*P*<0.01. (f) Plasma membrane was isolated from total cell lysate and was subjected to immunoblot analysis using antibodies specific for phox47 or the plasma-membrane-specific protein FAS. Quantification of the immunoblot was carried out and normalized to FAS; ∗=*P*<0.01.



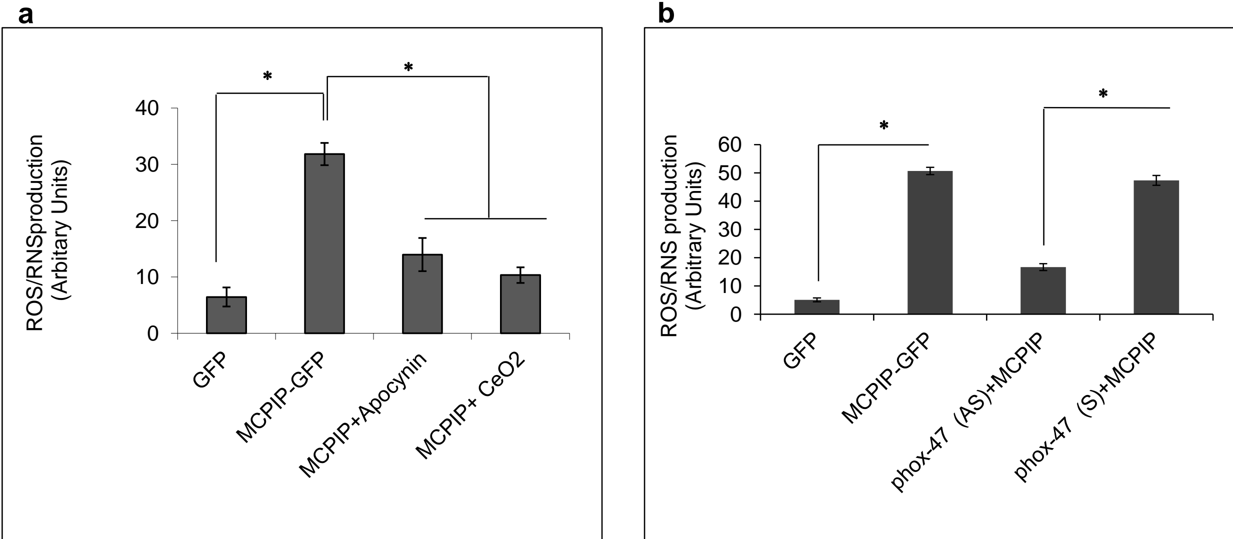
- **Figure 6: Forced expression of MCPIP induces ER stress response in HUVECs**
- **HUVECs were transfected with MCPIP-GFP or GFP expression vector. After 24 h RNA and total cell lysate were isolated for transcript (a) and protein analysis (b) of ER stress markers GRP78 and IRE-1; ∗=P< 0.01.**



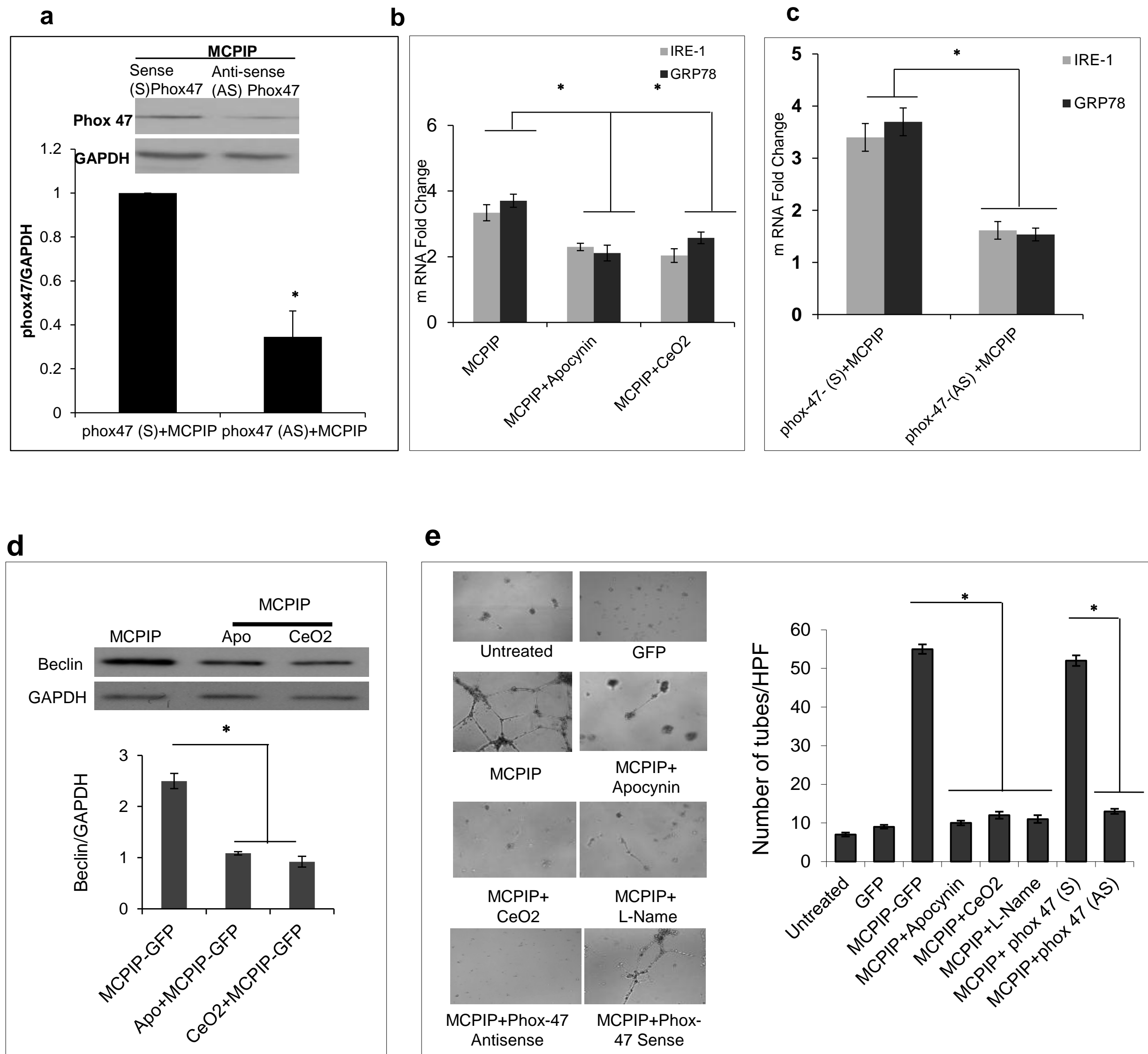
- **Figure 7: MCPIP over expression induces autophagy in HUVECs**
- **(a) HUVECs were transfected with MCPIP–GFP or GFP expression vector. After 24h, cell lysates were subjected to immunoblot analysis with antibody against beclin. Quantification of immunoblot data is shown after normalizing against GAPDH (*=*P* <0.02). (b) LC3 immunostaining of autophagosomes (arrows) after 24hours of HUVECs transfected with GFP and MCPIP-GFP. DAPI staining was used to detect cell number.**



- **Figure 8: MCPIP expression induces angiogenic differentiation in HUVECs**
- **(a,b) Huvecs were tranfected with MCPIP or MCPIP-GFP expression vector. After 24 hours of treatment, HUVECs were trypsinized and placed on matrigel for 24 hours. Quantification of phase-contrast photomicrographs of the tube formation is represented; ∗=*P* <0.01.**



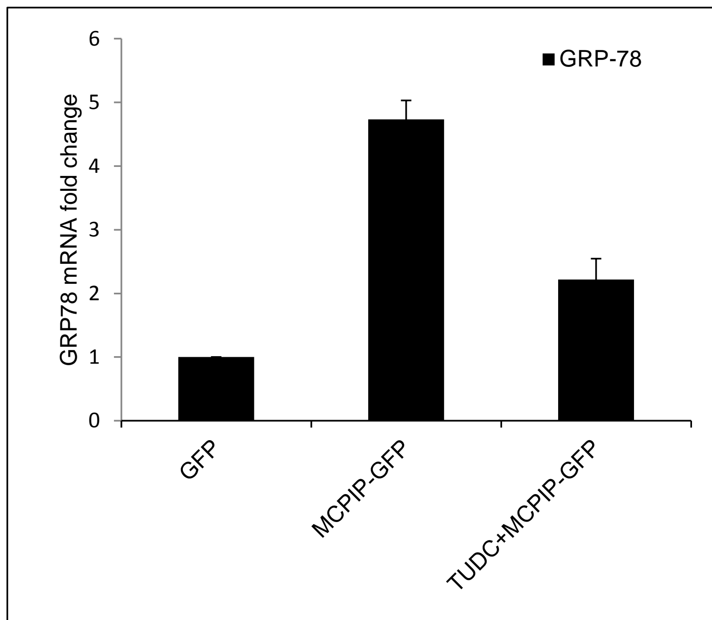
- **Figure 9: Inhibition of ROS/RNS induced by MCPIP**
- **(a) HUVECs were treated with apocynin or cerium oxide nanoparticles b) Antisense and sense oligonucleotides specific for phox47 before transfecting with MCPIP-GFP expression vector. Total ROS was detected at 24 hours with 1μmol/L DHR123 for 30 min and then examined flurometrically (excitation wavelength 550nm and emission wavelength 590nm; ∗=P<0.01).**



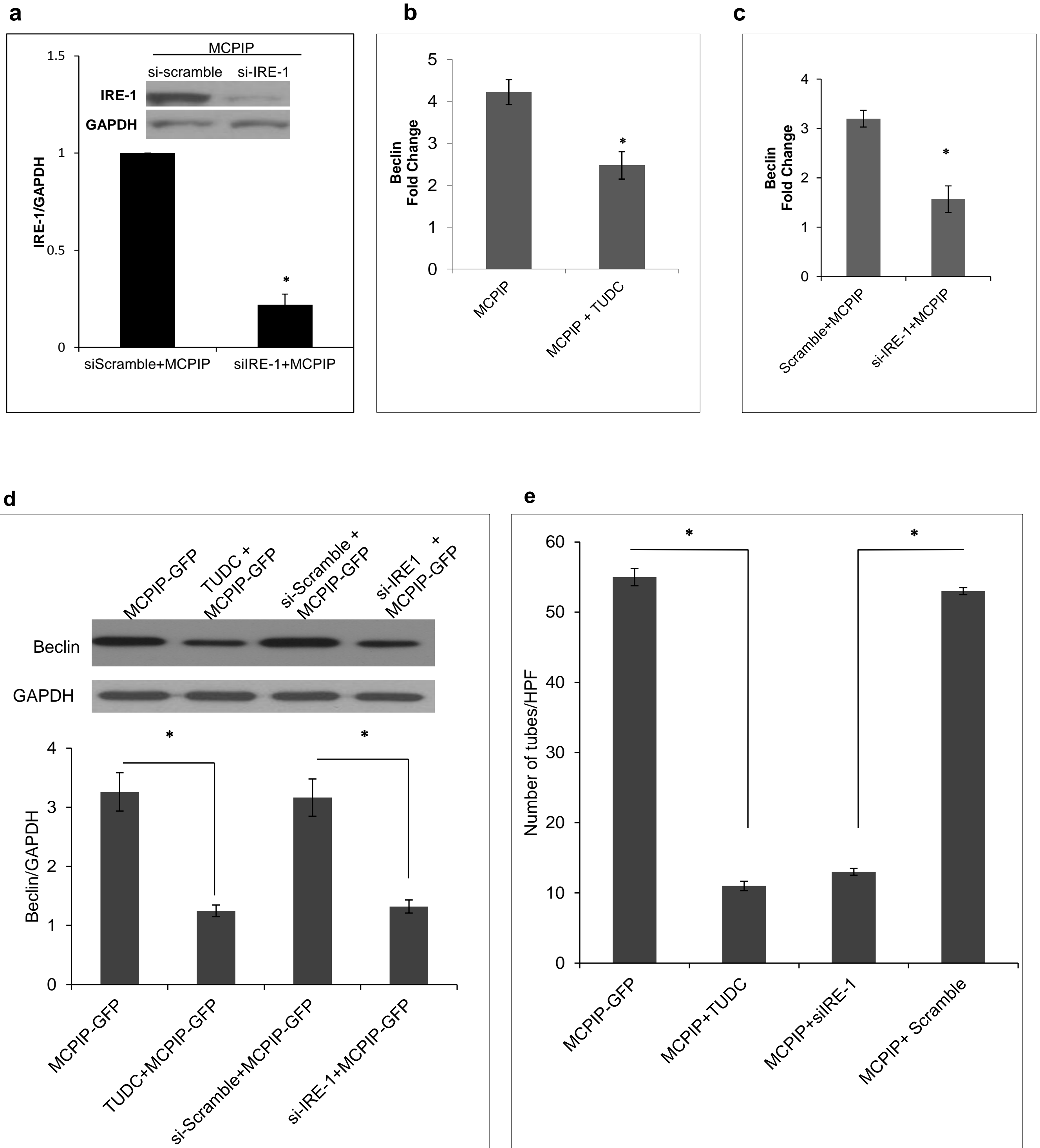
- **Figure 10: Inhibition of ROS production reduces MCPIP-induced ER stress and autophagy and attenuates tube formation**

- **HUVECs were treated with or without apocynin, or free radical scavenger CeO2 nanoparticles or phox47 antisense oligonucleotides prior to transfection with MCPIP-GFP or GFP expression vectors. (a) Efficiency of phox47 knockdown by antisense phox47 nucleotides on phox47 protein levels was determined by western blot analysis. Quantification of the immunoblot was performed and normalized to GAPDH; ∗=P<0.02. After 24**

h, transcript levels were evaluated for the ER stress proteins IRE1 and GRP78 (b and c) and protein levels (d) were evaluated for the autophagy marker, beclin. After 24 hours of treatments, HUVECs were trypsinized and placed on matrigel for 24 hours. (e) Phase-contrast photomicrographs of the tube formation were quantified at 24 hours, ∗=P <0.01.



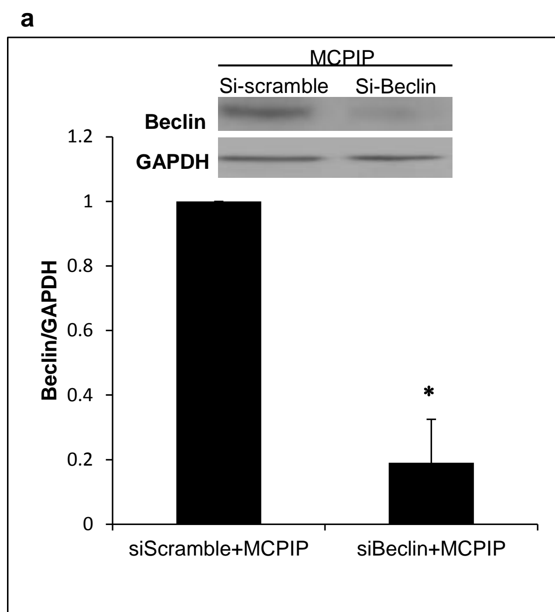
- **Figure 11: Inhibition of ER stress induced by MCPIP by TUDC treatment**
- **Huvecs were treated with TUDC before transfection with MCPIP-GFP expression vector. After 24 hours, GRP78 transcript analysis was performed by real time PCR; ∗=*P* <0.01**



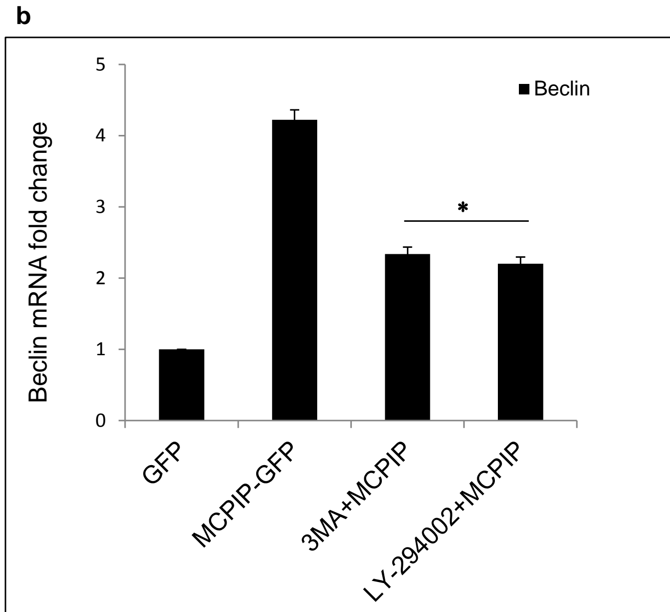
- **Figure 12: Inhibition of ER stress reduces MCPIP- induced autophagy and attenuates tube formation**

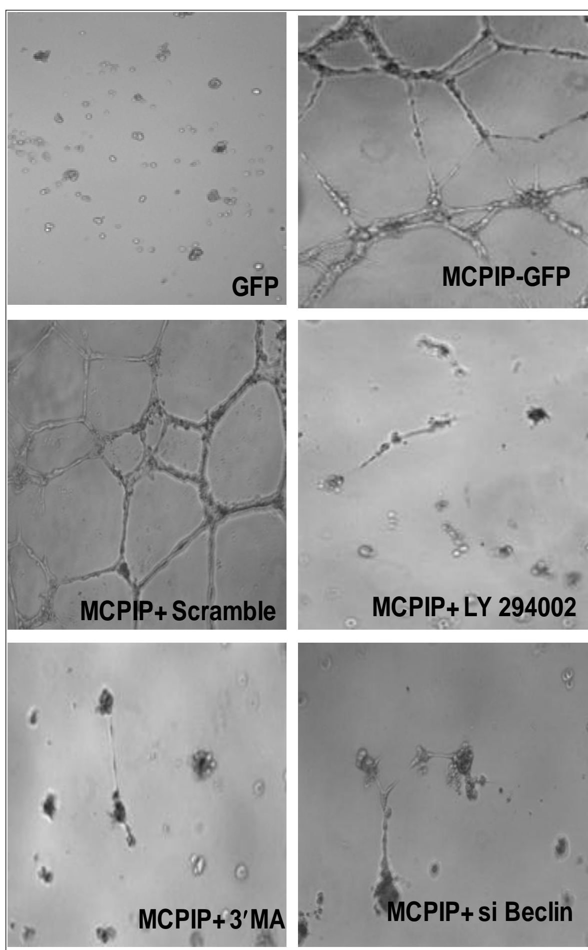
- **HUVECs were treated with or without ER-stress-specific inhibitor TUDC (tauroursodeoxycholate), or siIRE1 or si-scrambled before transfection with MCPIP-GFP and GFP expression vectors. (a) Efficiency of si-IRE-1 knockdown on IRE-1 protein levels was determined by western blot analysis. Quantification of the immunoblot was performed and normalized to GAPDH; ∗=P<0.01. After 24 hours of treatments, cells were evaluated for the autophagy marker, beclin (b and c) using real time RT–PCR ∗=P <0.01; and immunoblot analysis and quantification (d); after normalizing against GAPDH (*P <0.01) is shown. (e) After 24 hours of treatments, HUVECs were trypsinized and placed on matrigel for 24 hours. Quantification of phase-contrast photomicrographs of the tube formation is represented; ∗=P <0.02.**

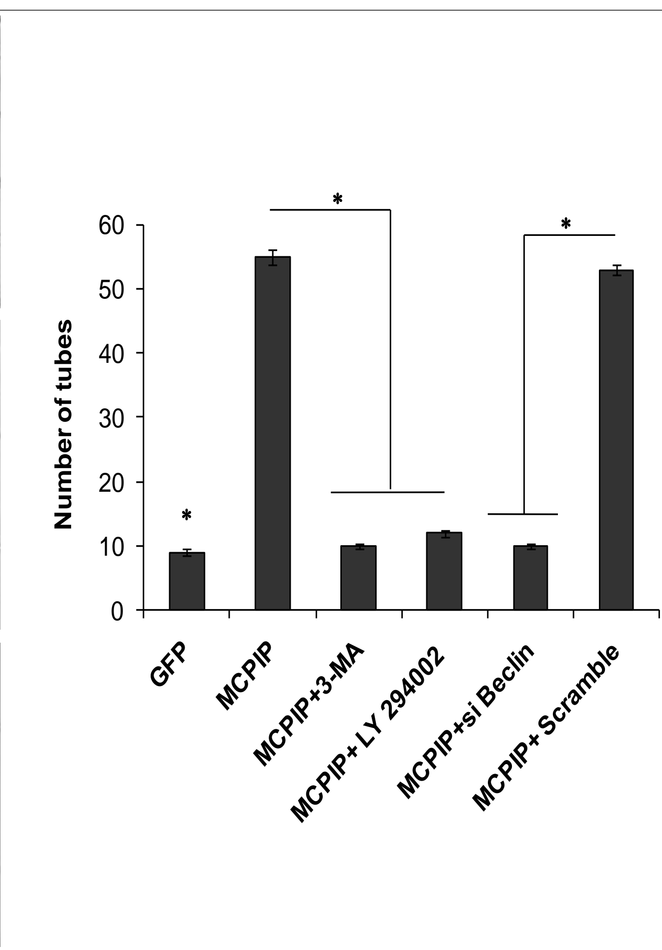
a



b

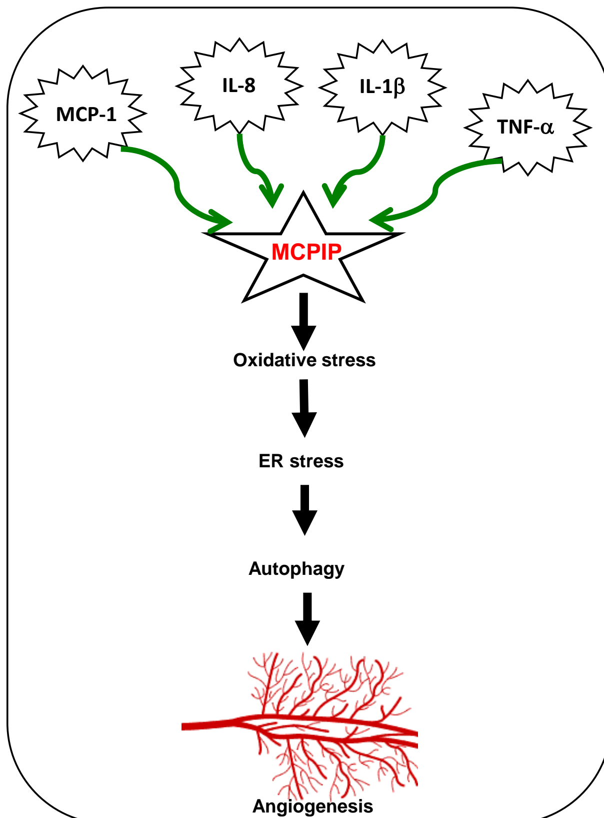






- **Figure 13: Inhibition of autophagy attenuates MCPIP-induced tube formation**

- **HUVECs were treated with and without autophagy inhibitors, 3′MA (3-methyladenine) and LY 294002 three hours prior to transfection and knockdown of beclin by siRNA, 6 hours before transfection with MCPIP-GFP and GFP expression vectors. Scrambled siRNA served as a control. . (a and b) Efficiency of beclin knockdown by its specific siRNA on beclin protein and transcript levels were determined by western blot analysis and real time PCR. Quantification of the immunoblot was performed and normalized to GAPDH; ∗=P<0.01. (c) Phase-contrast photomicrographs of the tube formation in the matrigel assay were quantified at 24 hours; ∗=*P* <0.01.**



- **Figure 14: Summary of events - Sequential processes that mediate inflammatory angiogenesis via MCPIP in human endothelial cells**

CHAPTER 3: INVOLVEMENT OF BOTH THE DEUBIQUITINASE AND ANTI-DICER RNASE ACTIVITIES OF MONOCYTE CHEMOTACTIC PROTEIN-INDUCED PROTEIN-1 (MCPIP1) IN INFLAMMATORY ANGIOGENESIS

Introduction

Angiogenesis is a tightly controlled process involving proper balance between the levels of pro-and anti-angiogenic factors [85]. Dysregulation of these processes is involved in inflammatory diseases such as, psoriasis, cardiovascular diseases, obesity and cancer [114]. A recent study suggests that inflammatory cytokines TNFα, IL-1β, IL-8, and MCP-1 mediate angiogenesis via the induction of *ZC3H12A* gene encoding MCP-1-induced protein-1 (MCPIP1) [133], originally identified as a protein induced by MCP-1 treatment of human monocytes[13]. MCPIP1 is the first member of a novel CCCH-type zinc finger protein family [16] and we refer to it as MCPIP in this paper. MCPIP has been shown to mediate several biological functions such as angiogenesis[133, 134], adipogenesis[135], osteoclastogenesis[20] and hyperglycemia-induced death of cardiomyocytes [18]. MCPIP was reported to have deubiquitinase activity [93, 136] and RNase activity [17, 22, 23]. If and how the dual enzymatic activities of MCPIP are involved in mediating any of its biological functions remains unknown.

HIF-1α that is known to be involved in angiogenesis is a key transcription factor that is activated under hypoxic conditions. It plays important roles in many biological processes such as embryonic development and in pathophysiological processes involving ischemia[26]. HIF-1 is a crucial regulator that induces genes assisting in

cellular processes such as oxygen transport, glucose metabolism, angiogenesis and cell survival[25]. HIF-1 is a heterodimeric protein complex consisting of hypoxia-inducible subunit, HIF-1 α and constitutively expressed HIF-1 β subunit. Under normoxic conditions, HIF-1 α is an unstable protein with a half-life of ~5 minutes and is under stringent negative regulation by multiple mechanisms. HIF-1α is hydroxylated in an oxygen-dependant manner by prolyl hydroxylase domain (PHD) enzymes at proline residues in its oxygen-dependent degradation domain [137]. Upon HIF-1α hydroxylation, von Hippel–Lindau protein, an E3 ligase, binds to it resulting in the ubiquitination of HIF-1 α and its degradation by the ubiquitin-proteosome pathway [28, 138]. Under hypoxic conditions PHD can no longer hydroxylate HIF-1α resulting in its stabilization and consequent entry into the nucleus to form a complex with HIF-1β subunit. In the nucleus the dimer can bind to the hypoxia response element (HRE; RCGTG) on the promoters of its target genes. COX2 and VEGF are HIF-1α target genes. (COX)-2 is an inducible isoform of the cyclooxygenases (COX) family of enzymes that are involved in the production of biological mediators of inflammation, prostanoids generated from arachidonic acid [33, 139]. Induction of COX-2 is influenced by pro-inflammatory stimuli and has been implicated in pathologies involving inflammatory angiogenesis, such as cancer [140, 141]. VEGF is a well-established pro-angiogenic factor[31]. MCPIP is known to cause elevation of HIF-1α levels during MCPIP-induced angiogenesis[134]. The molecular mechanism by which MCPIP causes stabilization of HIF-1α is unknown. It is unknown whether MCPIP-induced angiogenesis could be mediated via stabilization of HIF-1α by removal of the ubiquitin moieties linked to HIF-1α by the MCPIP's deubiquitinase activity, that was reported to negatively

regulate NF-κB activation [136]. Moreover, it is unknown whether inhibition of NF-κB activation by MCPIP is involved in mediating angiogenesis. Our study aims to decipher whether MCPIP deubiquitinates ubiquitinated HIF-1α and promotes its nuclear entry to mediate transcription of pro-angiogenic genes, VEGF and COX2, and if inhibition of NF-κB, a key pro-inflammatory transcription factor, promotes angiogenesis.

Silent information regulator (SIRT) -1 enhances the angiogenic potential of endothelial cells by deacetylating forkhead box O (FoxO), a negative regulator of angiogenesis [142]. It is a member of the sirtuins family of nicotinamide adenine dinucleotide (NAD+) dependent histone deacetylases that regulate several biological processes including cell survival, metabolism, longevity, inflammation, and tumorigenesis [60, 143]. SIRT1 SIRT1 regulates cellular differentiation by deacetylating p53, a tumor suppressor resulting in the inhibition of p53 transcription. A target gene of p53 is thrombospondin (TSP) -1, an inhibitor of angiogenesis [81, 144-146]. Whether MCPIP mediated angiogenesis involves SIRT1 or TSP-1 is unknown.

MicroRNA (miR)s play a vital role in regulating inflammation [147]and in modulating the levels of HIF-1 α and SIRT-1. MiR-20b binds to the 3′UTR of HIF-1 α and thus inhibit translation of HIF-1α. Inhibition of miR 20b production increased the levels of HIF-1α, thus suggesting its anti-angiogenic role [80]. miR 34a is anti-angiogenic and SIRT-1 is one of its targets [148]. It was reported that miR-34a inhibits SIRT-1 translation by binding to the 3′UTR of SIRT-1 mRNA [81]. MCPIP can cleave the terminal loops of precursor miRNAs and this anti-dicer activity can suppress miRNA

biogenesis [24]. Whether the anti-dicer RNase activity of MCPIP plays a role in angiogenesis-induced by MCPIP is not known.

We tested whether angiogenic differentiation in human umbilical vein endothelial cells (HUVECs) induced by MCPIP is mediated via the deubiquitinase and/or anti-dicer RNase activities of MCPIP that might regulate the stability and levels of angiogenic players, HIF-1α, VEGF, COX2 and SIRT-1. Results from the present study suggest that MCPIP would stabilize HIF-1 α by its deubiquitinase activity and thus promote angiogenesis via induction of pro-angiogenic factors, VEGF and COX2. Our findings suggest that MCPIP-induced angiogenic differentiation is also mediated via suppression of anti-angiogenic factors. Thus, MCPIP induces of SIRT-1 that downregulates anti-angiogenic TSP-1. Furthermore, we show that the anti-dicer activity of MCPIP causes the inhibition of production of anti-angiogenic miRNAs, miR-20b and miR-34a, thus promoting angiogenic differentiation of HUVECs. MCPIP suppresses the production of anti-angiogenic VEG-inhibitor (VEGI) via inhibition of NF-κB activation. In conclusion, our findings suggest that both the deubiquitinase and anti-dicer RNase activities of MCPIP are involved in promoting angiogenesis thus elucidating, for the first time, role of the two enzymatic activities in one of the biological functions of MCPIP.

Materials and Methods

Cell culture conditions

The human umbilical vein endothelial cells (HUVECs; CC-2519, LONZA, NJ, USA) were cultured in endothelial cell basal medium (EBM, CC-3124, LONZA) according to manufacturer’s protocol. HUVECs used were between passages 4-8. All

cells were maintained at 37°C in 5% CO_2. Experiments under hypoxic conditions [149] were performed in the chamber with 1% O_2 at 37°C and 5% CO_2.

Plasmid construction

The human wildtype MCPIP (Accession No: AY920403) was sub-cloned into the pCMV-MAT-FLAG vector (Sigma-Aldrich). MAT-FLAG sequence and HISx8 tag were added at the 5' and the 3' end, respectively. The D141N mutation was produced using the QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's directions using pCMV-MAT-FLAG wildtype-MCPIP as template DNA and D141N mutagenic primers (Sense: 5'-AGA-CCA-GTG-GTC-ATC-AAC-GGG-AGC-AAC-GTG-GCC-3'; Antisense: 5'-GGC-CAC-GTT-GCT-CCC-GTT-GAT-GAC-CAC-TGG-TCT-3'). The Qiagen Plasmid Maxi Kit was used to prepare the pCMV-MAT-FLAG MCPIP-WT and D141N expression vectors.

Transfection procedure

HUVECs were transfected with vectors expressing MCPIP or empty vector using Lipofectamine and PLUS Reagents (11668; 11514, Life Technologies, NY, USA). The transfection efficiency was 60-70% and was determined by the immunoblotting with antibody against FLAG (1:500; Sigma).

Treatment/Transfection of HUVECs

HUVECs were treated with following chemical inhibitors: p38 MAPK inhibitor, SB 203580 (20μM), three hours prior to transfection with expression construct for MCPIP or empty vector. HUVECs were transfected for 6 hours with 100 nmol/l of a chemically

synthesized siRNA targeted for the HIF-1α or SIRT-1 or COX2 with 100 nmol/l non-specific siRNA (Santa Cruz Biotechnology, Inc.) using Lipofectamine and PLUS Reagents (Life Technologies) according to the manufacturer's protocol prior to transfection with MCPIP–MAT or empty vector.

Real-time PCR

Total RNA was isolated from HUVECs by using Trizol reagent (Invitrogen). cDNA was synthesized utilizing 1 μg of total RNA (DNase-treated) as previously described[134]. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) served as internal controls for transcript analysis. Results presented are of three independent experiments, each measured in triplicate. The sequences of the primers used for real time analysis are as stated below:

HIF-1α-Forward-5 CTTTTACCATGCCCCAGAT-3′;

HIF-1α-Reverse - 5′ CATTGACCATATCACTATCCACA-3′;

VEGF-Forward -5 CGAGGCAGCTTGAGTTAA -3′,

VEGF -Reverse - 5′ GCGTGGTTTCTGTATCGATC -3′;

SIRT-1- Forward -5′CCCTCAAAGTAAGACCAGTAGC -3′,

SIRT-1- Reverse - 5′ CACAGTCTCCAAGAAGCTCTAC -3′

TSP-1 - Forward –5′ CTCCCCTATGCTATCACAACG -3′

TSP-1 - Reverse –5′ AGGAACTGTGGCATTGGAG--3′

COX-2-Forward-5′CTATGGCTACAAAAGCTGGG-3′;

COX-2-Reverse - 5′CCACAATCTCATTTGAATCAGG-3′

MicroRNA analysis

MicroRNAs were isolated from HUVECs after treatments using Trizol method. Primers (5S rRNA: 203906; U6 snRNA: 203907; hsa-miR-20b: 204755; hsa-miR-34a: 204318) for microRNA analysis were purchased from Exiqon and were used as per manufacturer's recommendations. Mimics of miR-20b (MC10975) and miR-34a (MC11030) and negative control (4464058) were ordered from LifeTechnologies, CA, USA. All real time PCR reactions were performed using the 7500 real-time PCR system (Applied Biosystems). The amplification steps consisted of denaturation for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 10 s and then annealing at 60°C for 1 min. using the SYBR green master Mix (203450, Exiqon, USA). U6 and 5s were used as endogenous controls and fold changes were calculated for each gene. Each RNA sample assay was run in triplicate and the assay repeated for three times.

In vitro capillary-like tube formation assays

Matrigel assay was performed as described [134] . Briefly, HUVECs after treatment for 3 hours were transfected with expression vector for MCPIP or empty vector for 24 hours before being trypsinized and seeded onto the surface of the matrigel according to the manufacturer's protocol, followed by incubation in at 37ºC in 5% CO_2 for 24 hours. Tube formation was quantified using photographs captured by phase-contrast microscope.

Purification of MCPIP

HEK293 cells were transfected with pCMV-MAT-FLAG -MCPIP expression vector for 48 hrs. Transfected cells were lysed at $4^{o}C$ in Cell-Lytic M lysis buffer (C2978; Sigma-Aldrich, MO, USA) supplemented with a protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Upon centrifugation, the cleared lysate was loaded onto a column containing Ni-NTA Agarose beads (Qiagen) that was previously prepared as per manufacturer's protocol. The column was washed several times with wash buffer (50mM Tris-HCl [pH7.5] and 150mM NaCl) along with increasing concentrations of Imidizole (10mM, 50mM). MCPIP protein was eluted using 500mM Imidizole.

Preparation of Ubiquitinated HIF-1α substrate

Human embryonic kidney (HEK) cells were transfected with HA-HIF1α-pcDNA3 expression vector (cat # 18949, Addgene, MA, USA). Hypoxic conditions were induced by $CoCl_2$ (200μM) and proteasome inhibition by MG132 (10μM) for 24 hours. The lysates were subjected to immunoprecipitation with HA-coated beads to yield HIF-1α-HA as the substrate. *In vitro* ubiquitination of the substrate was performed by Ubiquitin-Protein Conjugation Kit, (Cat. # K-960, Boston Biochem, MA, USA) to yield ubiquitinated-HIF-1α substrate.Ub-HIF-1α was incubated with purified MCPIP enzyme with or without ubiquitin aldehyde, a deubiquitinase inhibitor or MCPIP mutant, D141N. Hydrolysis of the Ub-HIF-1α substrate was observed by immunoblotting with ubiquitin antibody.

Deubiquitinase Assay:

Experiments to determine ubiquitin hydrolysis were performed by a) Ub-AFC assay: Purified MCPIP or D141N protein (1μg) was incubated with 1μM Ub-AFC (Cat# U-551, Boston Biochem) in buffer containing 50 mM Tris-HCl, pH 7.0, 10 mM DTT and 150mM NaCl in a final volume of 200μl. Assays were performed at 37°C for 4 hours. The fluorescence signals were detected at Ex400nm and Em505nm in a time-dependent manner. b) High Molecular Weight K-63 linked PolyUbiquitin (Cat# UC 316, Boston Biochem) (1μg) was incubated in buffer containing 50 mM Tris-HCl, pH 7.0, 10 mM DTT and 150mM NaCl in a final volume of 200μl with purified MCPIP or D141N protein (1μg) at 37°C for an hour. The reaction mixture was used for immunoblot analysis with anti-ubiquitin antibodies.

p38 MAPK activity assay:

Assay to determine activity was performed by p38 MAPK Activity Assay Kit (Cat# CS0250, Sigma, Missouri, USA) as per manufacturer's recommendations. Briefly HUVECs were transfected with MCPIP expression vector with or without prior treatment of p38 inhibitor SB203580. The whole cell lysate was immune precipitated with p38 antibody and elute was incubated with ATF2 substrate at 30°C. After 30 min the reaction mixture was run on SDS-PAGE and immunoblotted against phospho –ATF2 antibody.

Immunoblot analysis

HUVECs from different experimental conditions were lysed with Cell Lytic lysis Buffer (Sigma). Protein samples (50μg) were subjected to SDS-PAGE using 10% polyacrylamide or 4–20% (NB10-420, NuSep, GA, USA) Tris-HCl gels and transferred using standard protocols. Immunoblot analysis was performed using the primary antibodies from SantaCruz Biotechnology, USA, anti-mouse GAPDH (1:1000; Cat. No 47724,); anti-rabbit HIF-1α (1:500; Cat. No 10790); anti-mouse SIRT-1 (1:500; Cat. No 74504), anti-mouse TSP-1(1:500; Cat. No 74538) and HRP-conjugated Mouse (1:5000; Cat. No 2005), and rabbit antibodies (1:5000; Cat. No 2317) and ubiquitin (1:1000; Cat. No VU 101; Life sensors). Immunoreactive proteins were analysed using enhanced chemiluminescence (ECL) kit.

Statistical analysis

All experiments were repeated three times. The error bars are represented as ± S.E.M. An asterisk (*) indicates a significant difference when compared to the control as indicated in each experiment. *P* value of < 0.05 was considered significant and was determined by student's *t*-test.

Results

Hypoxia-induced angiogenesis is mediated via MCPIP:

Hypoxia-induced angiogenesis is augmented by cytokine production [150]. MCPIP was reported to mediate angiogenesis induced by inflammatory cytokines [133].

To determine if MCPIP mediates hypoxia-induced angiogenesis, HUVECs were transfected with siRNA specific for MCPIP or non-specific scrambled control before hypoxic (1% oxygen) or normoxic (21% oxygen) incubation for 6 hours. siRNA specific for MCPIP, but not scrambled siRNA, significantly inhibited hypoxia-induced production of HIF-1α and angiogenesis thus strongly suggesting that hypoxia-induced angiogenesis is mediated via MCPIP **(Figure 15 A-D)**.

MCPIP expression results in the HIF-1α localization in the nucleus and induction of its target genes, COX2 and VEGF:

To determine whether MCPIP expression resulted in HIF-1α localization in the nuclei, HUVECs were transfected with MCPIP expression vector or empty vector for 24 hours. Immunocytochemistry using antibody against HIF-1α was performed. DAPI was used for counterstaining the nuclei. Fluorescence microscopic images showed that MCPIP expression resulted in nuclear localization of HIF-1α when compared to the empty vector control **(Figure 16A)**. Expression levels of HIF-target genes, COX2 and VEGF, were also higher in cells expressing MCPIP. Furthermore, specific knockdown of HIF-1α inhibited MCPIP-induction of VEGF and COX2 **(Figure 16B)**. These results suggest that MCPIP expression promotes entry of HIF-1α into the nucleus resulting in induction of VEGF and COX2 production. Since p38MAPK activation was reported to mediate VEGF-induced angiogenesis [151, 152], we sought to determine if MCPIP induces the activation of p38 MAPK by transfecting HUVECs with MCPIP expression vector. After immunoprecipitation of total p38 from the whole cell lysates, the kinase activity of p38 was checked on its model substrate, ATF2. The reaction mixture was immunoblotted with phospho-ATF2 antibody. Results showed that cells expressing

MCPIP had increased ATF2 phosphorylation as compared to the cells transfected with empty vector. SB 203580, a p38 MAPK inhibitor blocked phosphorylation of ATF2 **(Figure 17A)**. Our results suggest that MCPIP expression induces p38 MAPK activation. Furthermore, to determine if MCPIP- induced angiogenic differentiation involves the induction of p38 MAPK, HUVECs were treated with p38 MAPK inhibitor, SB 203580 prior to transfection with MCPIP expression vector. Our results indicate that MCPIP-induced tube formation was drastically reduced by p38 MAPK inhibition **(Figure 17B)**. Our data thus suggest that angiogenesis MCPIP-induced angiogenesis is mediated via induction of p38 MAPK activation.

SIRT-1 mediates MCPIP-induced angiogenic differentiation:

Studies have shown that loss of SIRT-1 function blocks angiogenesis [143] thus suggesting its pro-angiogenic role. To determine if SIRT-1 is induced by expression of MCPIP, real time PCR and immunoblot analysis were performed to examine the effect of MCPIP expression on the levels of SIRT-1 **(Figure 18A and B)**. Our results show that SIRT-1 levels were significantly elevated in cells expressing MCPIP thus suggesting that MCPIP induces SIRT-1.To determine if MCPIP-induced endothelial differentiation is mediated via SIRT-1, HUVECs were transfected with siRNA specific for SIRT-1 or siScramble before transfection with MCPIP expression vector. Knockdown of SIRT-1 inhibited MCPIP-induced tube formation **(Figure 18C and D)**. Our results suggest that angiogenesis induced by MCPIP is mediated via SIRT-1 induction.

MCPIP expression reduces the levels of anti-angiogenic factors, TSP-1 and VEGI:

TSP-1 is a well-known inhibitor of angiogenesis [153]. To determine if MCPIP expression has an effect on TSP-1 levels, transcript and protein analysis were performed. Expression of MCPIP resulted in lower levels of angiogenesis inhibitor, TSP-1 as compared to the empty vector **(Figure 19A and B).** Furthermore, knockdown of SIRT-1 gene resulted in higher levels of TSP-1 in cells expressing MCPIP **(Figure 19C and D).** This result suggests that reduction in TSP-1 level caused by MCPIP expression is mediated via SIRT-1. Furthermore, since it was reported that MCPIP negatively regulated NF-κB activation [136], we sought to determine if this mechanism would be of importance in promoting MCPIP-induced angiogenesis. Our data show that expression of MCPIP resulted in a reduction in nuclear levels of p65 as compared to cells transfected with empty vector **(Figure 19E)**. Our results also show that MCPIP expression caused significant decrease in the levels of anti-angiogenic VEGI **(Figure 19F)** whose production is known to require NF-κB activation[154]. Thus, our data suggest that inhibition of NF-κB activation by MCPIP would contribute to the angiogenic activity of MCPIP by reducing the level of anti-angiogenic VEGI.

Enzymatic activities of MCPIP involved in its induction of angiogenesis:

To explore the potential involvement of the enzymatic activities of MCPIP in its promotion of angiogenesis, a MCPIP mutant, D141N, that is known to have lost the RNase activity [17, 22, 23], was used for the *in vitro* matrigel assay. Results suggest that cells expressing D141N mutant showed significantly reduced tube formation when

compared to the cells expressing wild-type MCPIP **(Figure 20A)** suggesting the importance of RNase activity of MCPIP in angiogenic differentiation.

MCPIP and its RNase-dead mutant, D141N, deubiquitinates ubiquitinated HIF-1α : To promote the transcription of angiogenesis-related genes, HIF-1α has to be stable and thus non-ubiquitinated. We tested whether MCPIP can deubiquitinate ubiquitinated HIF-1α *in vitro*, HEK cells were transfected with HA-HIF-1α expression vector under conditions described in Methods section. HA-HIF-1α isolated by immunoprecipitation was ubiquitinated i*n vitro*. After incubation of the ubiquitinated HIF-1α substrate with purified MCPIP in the presence or absence of ubiquitin aldehyde, a deubiquitinase inhibitor, or purified MCPIP mutant, D141N that was found to have lost the ability to induce angiogenesis. The mixture was immunoblotted with ubiquitin antibody. Results demonstrate that MCPIP deubiquitinates the ubiquitinated HIF-1α substrate and the deubiquitinase inhibitor, ubiquitin aldehyde, prevented the hydrolysis. Immunoblot analysis also showed that MCPIP mutant, D141N, that was reported to have no deubiquitinase activity against octa-ubiquitin [136] hydrolysed the ubiquitinated HIF-1α substrate **(Figure 20B)**. Purified D141N also showed deubiquitinase activity similar to the wild type MCPIP when assayed with a model substrate, Ub-AFC **(Figure 20C)**. Since D141N was reported to be incapable of hydrolyzing octa-ubiquitin [136], we tested whether the mutant could hydrolyze high molecular weight polyubiquitin (Poly Ub). Our results showed that both MCPIP and its mutant D141N were able to hydrolyze poly Ub **(Figure 20D)** suggesting that MCPIP mutant, D141N has deubiquitinase activity on substrates relevant to the biological functions of MCPIP.

Anti-dicer RNase activity of MCPIP suppresses the levels of miRs modulating HIF-1α and SIRT-1 expression:

Since angiogenic activity of MCPIP was severely compromised by D141N mutation, inspite of having intact deubiquitinase activity, we suspected that its anti-dicer RNase activity might be involved in promoting angiogenesis. To test this possibility, HUVECs were transfected with expression vector for MCPIP or its D141N mutant, that has been reported to have lost RNase activities by three different laboratories [17, 22, 23], and production of miRNA that could be involved in the regulation of angiogenesis was examined. MiR-20b has been reported to reduce HIF-1α protein levels. To determine if the RNase activity of MCPIP suppresses the levels of miR-20b, thus resulting in induction of HIF-1α, RT-PCR of the miRNAs isolated from cells transfected with expression vectors for MCPIP or D141N mutant or empty vector was performed. Expression of MCPIP resulted in reduced levels of miR-20b. This reduction was however not seen with the RNase-dead mutant D141N **(Figure 21A)**. Furthermore, transcript level of miR-34a, a microRNA known to suppress SIRT-1 levels [155], was measured. Results showed that expression of MCPIP significantly reduced the levels of miR-34a but the RNase-dead mutant, D141N, failed to inhibit miR-34a production **(Figure 21B)**. If MCPIP-induced angiogenesis involves inhibition of biogenesis of these anti-angiogenic microRNAs, mimetics of their miRs should inhibit MCPIP-induced angiogenesis. To test this possibility HUVECs were transfected with mimetics of mir-20b or mir-34a or negative control prior to transfection with MCPIP expression construct. Our results showed **(Figure 21C)** that mimetics for miR-20b or miR-34a inhibited MCPIP-induced tube formation. These results strongly suggest that anti-dicer RNase

activity of MCPIP represses the levels of anti-angiogenic miRs and thus promotes angiogenesis. The lack of anti-dicer RNase activity of MCPIP mutant, D141N, is probably the reason for its inability to induce angiogenesis.

Discussion

Chronic inflammation plays a major role in several diseases such as cancer, cardiovascular diseases, obesity and is marked by elevated levels of proinflammatory cytokines, including TNFα, IL-1β, IL-8 and MCP-1. Inflammatory cytokines are known to induce a novel zinc-finger protein, MCPIP encoded by the *ZC3H12A* gene [13, 17, 147]. A recent study has shown that MCPIP mediates angiogenesis induced by inflammatory cytokines [133]. Results from the present study provide new insights into the possible underlying mechanisms that mediate MCPIP-induced angiogenesis. We show that MCPIP promotes the expression of pro-angiogenic molecules and inhibits the synthesis of anti-angiogenic molecules, thus tilting the balance towards promotion of angiogenesis. Deubiquitination of ubiquitinated HIF-1α by MCPIP demonstrated here indicates that MCPIP would stabilize HIF-1α thus allowing HIF-1α to enter the nucleus and promote the transcription of its angiogenic target genes, COX2 and VEGF. Induction of VEGF levels would result in p38 MAPK activation thus mediating MCPIP-induced angiogenesis. Experimental data also suggest that inhibition of NF-κB activation contributes to the angiogenic activity of MCPIP by reducing the levels of anti-angiogenic VEGI. Furthermore, the anti-dicer RNase activity of MCPIP inhibits the synthesis of miR-20b that is known to reduce the levels of HIF-1α. MCPIP expression also induces SIRT-1 expression, which results in the inhibition of anti-angiogenic TSP-1 production. Furthermore, our results also show that the anti-dicer RNase activity of

MCPIP results in the inhibition of synthesis of miR-34a that is known to suppress SIRT-1 levels. Moreover, mir-34a production is also known to be suppressed by inhibition of NF-κB activation. Thus experimental results presented here reveal how the deubiquitinase and anti-dicer RNase activities of MCPIP would mediate MCPIP-induced angiogenesis.

An imbalance between the levels of oxygen supply and its demand is critical in the development of inflammatory diseases such as diabetic retinopathy, psoriasis and tumorigenesis[156]. Cellular adaptations under hypoxia are modulated by the induction of pro-inflammatory cytokines and HIF-1α[157]. HIF-1α is a key regulator and a transcription factor that mediates an array of cellular pathways such as angiogenesis by promoting the transcription of several target genes including COX2 and VEGF [29, 158, 159]. Under normoxia, HIF-1α is tightly regulated by O2-dependent prolyl hydroxylation that aids in polyubiquitination by E3 ubiquitin ligase, pVHL, leading to the degradation of HIF-1α by the proteosomal pathway [160]. Under hypoxic conditions, however, the hydroxylation of prolyl is inhibited thus resulting in accumulation and increased activity of HIF-1α. State of ubiquitination is an important biochemical modification, which regulate a wide range of cell biological processes[73]. Deubiquitination, a mechanism of reversing ubiquitination adds another important modulatory modification in regulating cellular functions. VDU2, a pVHL-interacting deubiquitinating enzyme 2, has been known to deubiquitinate and stabilize HIF-1α, thus preventing HIF-1α from proteosomal degradation [28]. MCPIP is known to have deubiquitinase activity[136]. Furthermore, angiogenic differentiation induced by MCPIP was reported to be mediated via HIF-1α induction [134]. However, the mechanism/s underlying HIF-1α induction by MCPIP was

unknown. Our demonstration that MCPIP can deubiquitinate ubiquitinated HIF-1α suggests that MCPIP would stabilize HIF-1α via its deubiquitinase activity. The stabilized HIF-1α would enter the nuclei and promote the transcription of its target genes VEGF and COX2, important players in angiogenesis. Thus MCPIP-induced deubiquitination of ubiquitinated HIF-1α is a probable mechanism by which MCPIP promotes angiogenesis. Moreover, it was reported that the deubiquitinase activity of MCPIP negatively regulates NF-κB activation [136]. Our results showing reduced nuclear levels of p65 subunit of NF-κB support that finding and also suggest a probable mechanism by which MCPIP may promote angiogenesis.

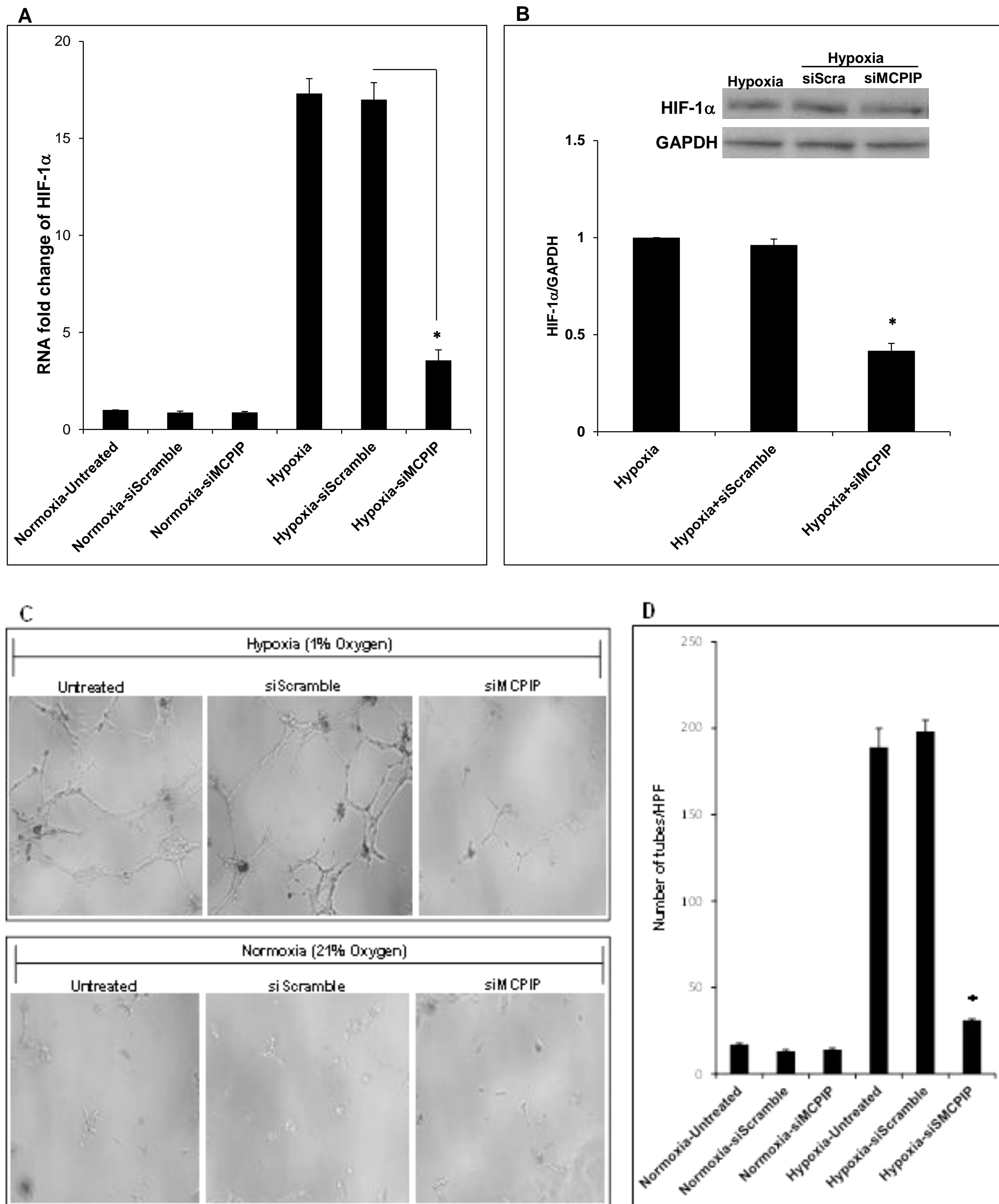
Sirtuins (SIRTs) are a family of nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylases (HDAC) that regulate gene expression [161, 162]. SIRT1 plays a vital role in regulating cellular differentiation by transcriptional repression of several transcriptional regulators including, forkhead box type O transcription factors (FOXO), and tumor suppressor protein, p53 [142, 143]. It was reported that inhibition or knockdown of SIRT-1 expression in both zebrafish and mice resulted in impairment of vasculature development suggesting that SIRT1 mediates angiogenic signaling [163]. Studies have shown that hyperacetylation of p53 results in its stabilization and results in onset of apoptosis. Conversely, p53 deacetylation by induction or overexpression of SIRT1 would reduce p53 activity and promote cell survival [164].SIRT-1 down-regulates the stability of p53 [165]. p53 promotes the transcription of TSP-1, an inhibitor of angiogenesis [64]. Our findings suggest that SIRT1 mediates MCPIP-induced angiogenic differentiation. This induction of SIRT1 resulted in the repression of the inhibitor of angiogenesis, TSP-1. Furthermore, it was reported that SIRT-1 deacetylates

lysine 310 on RelA/p65 protein in the NF-κB complex thus inhibiting the transactivation capacity of the NF-κB complex [166] and thus preventing NF-κB activation in HUVEC as demonstrated by our data. Also, since there are multiple NF-κB binding sites on the promoter of TSP-1, inhibiton of NF-κB activation may also reduce TSP-1 levels thus adding a new dimension to the mechanism by which SIRT1 regulates MCPIP-induced angiogenesis.

We found that D141N mutant of MCPIP could not induce angiogenesis even though we demonstrate that it has deubiquitinase activity. Therefore, the anti-dicer RNase activity, that had been shown to be lost in this mutant [24], appeared likely to be involved in promoting angiogenesis. A recent study suggested that MCPIP cleaves the terminal loops of precursor microRNAs thus antagonizing dicer activity to inhibit miRNA biogenesis [24]. Since miRNAs are known to regulate many aspects of angiogenesis, it was essential to determine if the RNase activity of MCPIP would modulate MCPIP-induced angiogenic differentiation. The present findings suggest that MCPIP expression results in the suppression of the levels of miRNAs, miR-20b and miR-34a that are known to bind to the 3′UTR of HIF-1α [80]and SIRT-1[81, 167], respectively. Interestingly, mir-34a transcription was reported to be induced by NF-κB activation as the promoter of mir-34a has NF-κB binding sites [82]. Thus, the reduction in mir-34a levels caused by MCPIP expression could be due to inhibition of NF-κB activation possibly by induction of SIRT1 and/or via its own deubiquitinase activity or by the anti-dicer RNase activity of MCPIP repressing the microRNA levels. That the anti-dicer RNase activity of MCPIP is involved in the induction of angiogenesis by its ability to inhibit biogenesis of miR-20b and miR-34a was supported by the finding that MCPIP

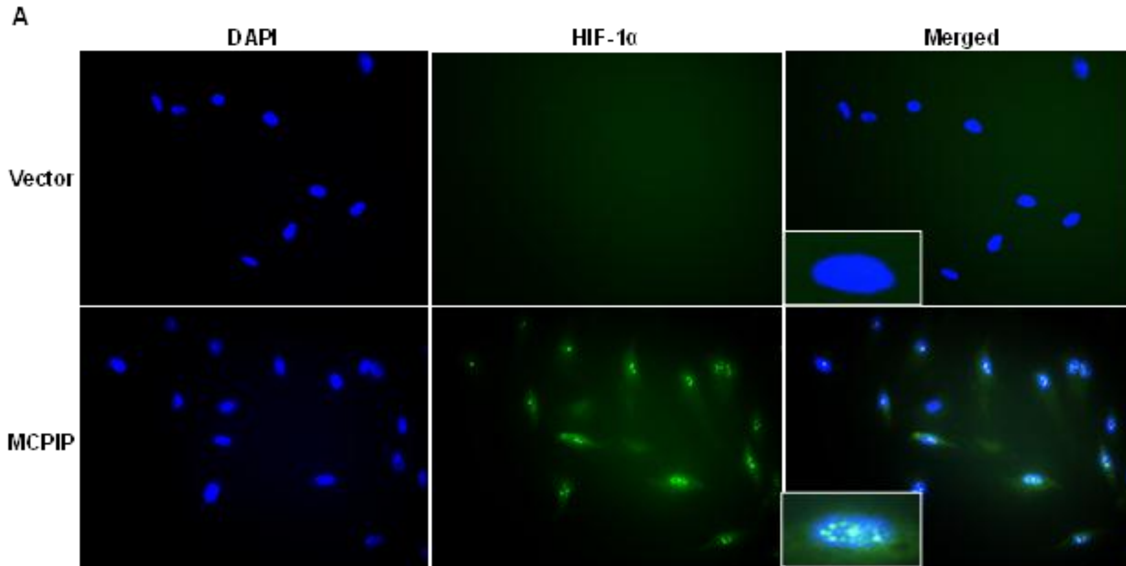
expression inhibited the production of the miRNAs and their mimetics inhibited MCPIP-induced angiogenesis. This conclusion is further strengthened by our finding that transfection with expression vector for RNase-dead mutant D141N [24], caused increased levels of miR-20b and miR-34a and also showed reduced angiogenic differentiation as compared to the cells expressing MCPIP.

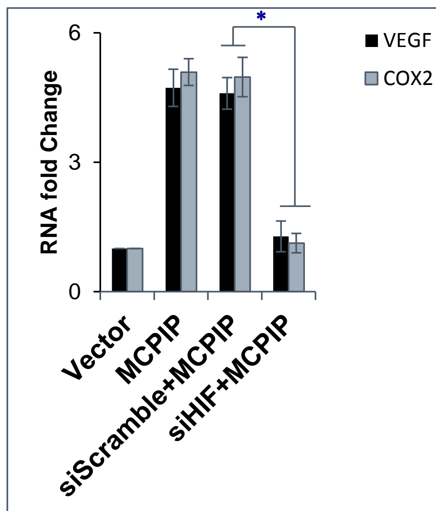
Taken together, the findings of the present study delineate the molecular mechanisms by which MCPIP mediates inflammatory angiogenesis (**Figure 22**). In summary, MCPIP mediates angiogenic differentiation by promoting the synthesis of pro-angiogenic VEGF and COX2, and by suppressing the production of anti-angiogenic microRNAs, mir-20b and mir-34a, TSP-1 and VEGI, thus tilting the balance towards angiogenesis. Both the deubiquitinase and the anti-dicer RNase activities are involved in the mediation of inflammatory angiogenesis by MCPIP. This is the first demonstration of the involvement of the two enzymatic activities of MCPIP in any of its biological functions. Deubiquitination [76] and microRNA biogenesis[168] have been shown to be involved in regulating several human pathologies such as impaired wound healing, cancer and heart disease[77]. Findings from our study thus reveal potential targets that may contribute to the development of novel therapeutic strategies.



- **Figure 15: Hypoxia-induced angiogenic differentiation is mediated via MCPIP**
- **HUVECs were transfected with siRNA specific for MCPIP or non-specific scramble siRNA for 4 hours and then incubated under 1% oxygen (hypoxia)**

for 6 hours. The controls were kept at 21% oxygen (normoxia) after transfection. After 6 hours, (A) transcript levels and (B) protein levels were evaluated for HIF-1α ; ∗=*p*<0.05. Under normoxia, HIF-1α protein levels were undetectable (data not shown). After 4 hours of transfection as in (a and b) cells were trypsinized and placed on matrigel before induction with 1% oxygen (hypoxia) or 21% oxygen (normoxia) for 6 hours. (C) Phase-contrast photomicrographs of the tube formation is represented (D) Quantification of phase-contrast photomicrographs of the tube formation; ∗=*p* <0.005.

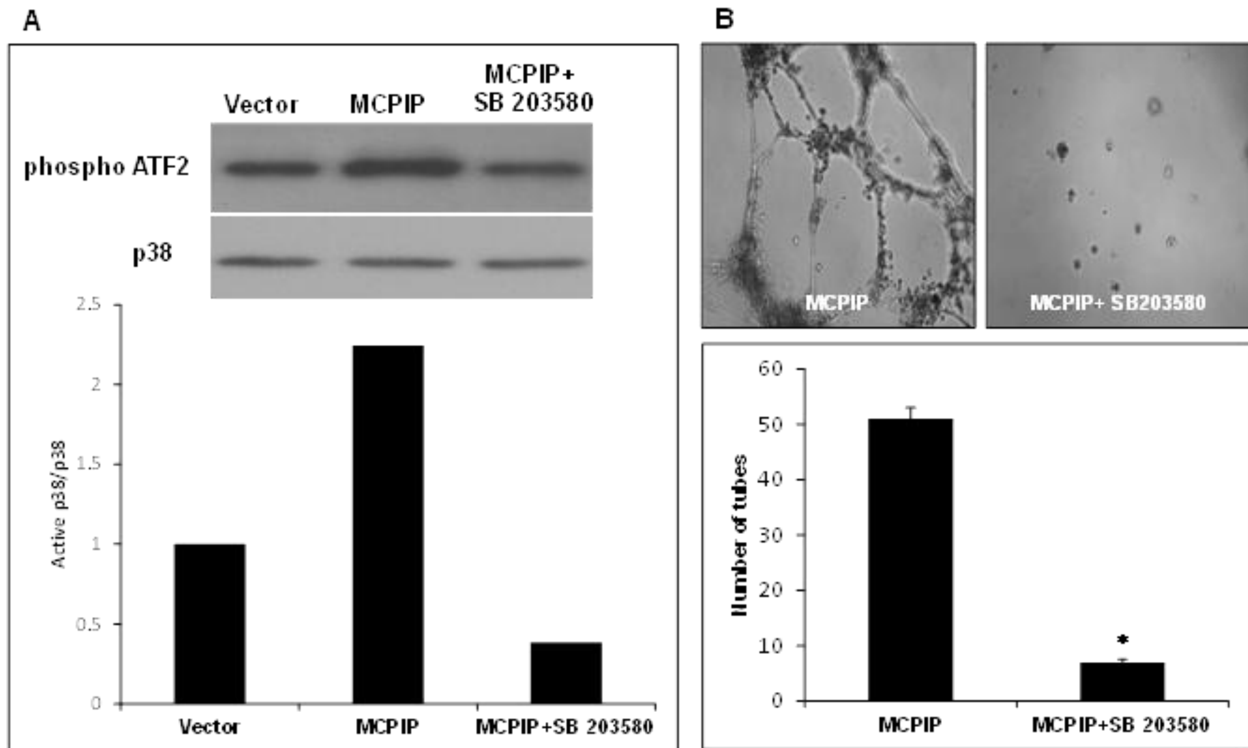




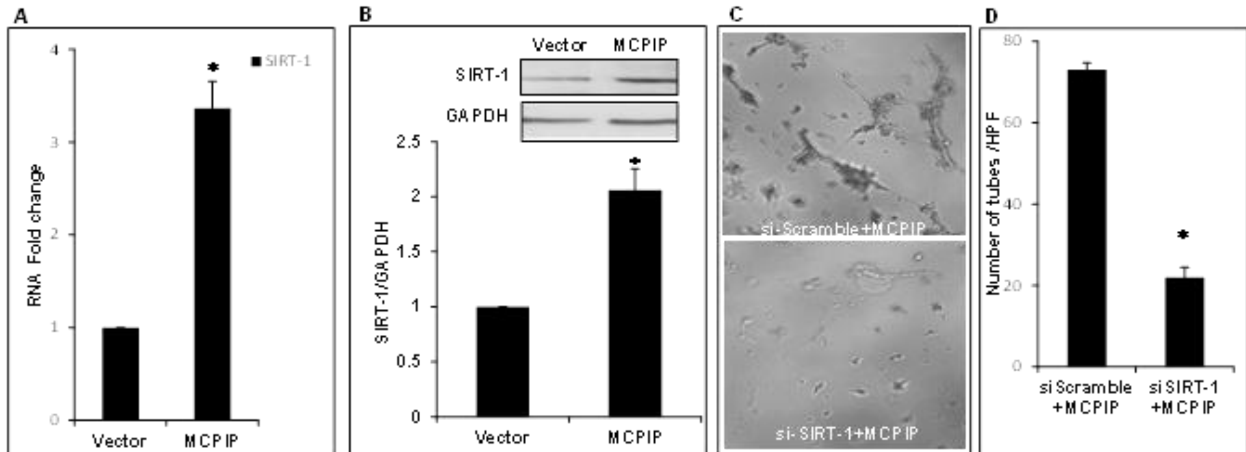
- **Figure 16: Forced expression of MCPIP resulted in the nuclear entry of HIF-1 and induction of COX2 and VEGF**

- **HUVECs were transfected with MCPIP expression vector or empty vector for 24 hours. (A) Cells were fixed and immunocytochemistry was performed using antibody against HIF-1α. Nuclei were counterstained with DAPI. The images were merged. Inset (40X) shows the nuclei. (B and C) HUVECs were treated with siRNA specific for HIF-1α or scrambled siRNA as a control 3 hours before being transfected with MCPIP. After 24 h, RNA was isolated**

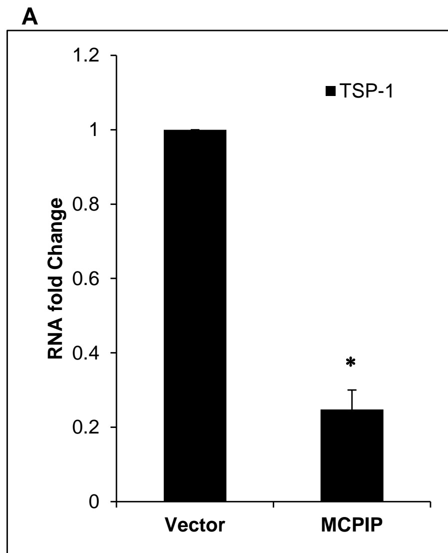
for transcript analysis by real time PCR to detect COX2 and VEGF expression, ∗=p<0.05.

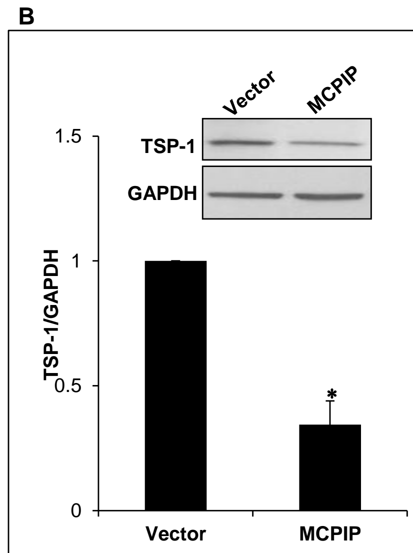


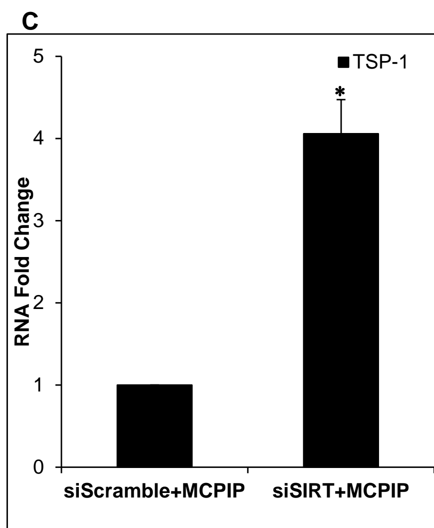
- **Figure 17: MCPIP induced angiogenesis via p38 MAPK activation**
- **HUVECs were treated with or without SB203580 (20 μM) before transfection with MCPIP expression vector. After 24 hours of transfection, cells were trypsinized and (A) the whole cell lysate was immunoprecipitated with beads coated with p38 antibody. After elution, p38 MAPK was incubated with substrate, ATF2. Phosphorylation of ATF2 was used as a measure to determine p38MAPK activity, (B) placed on matrigel for 24 hours. Phase-contrast photomicrographs of the tube formation are represented. Quantification of phase-contrast photomicrographs of the tube formation; ∗=*p* <0.002.**

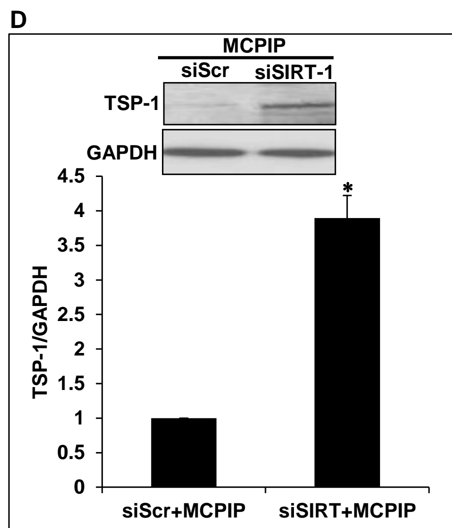


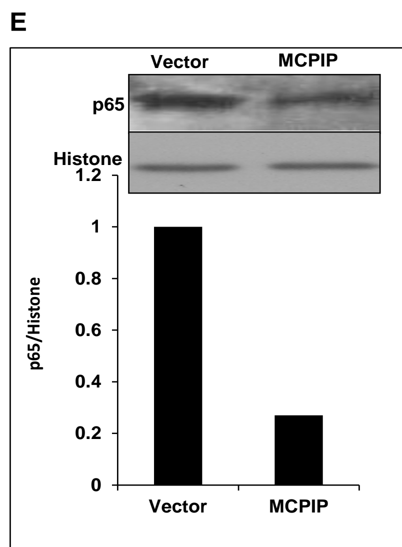
- **Figure 18: MCPIP-induced angiogenesis is mediated via SIRT-1 induction**
- **HUVECs were transfected with empty vector (MAT) or MCPIP (MAT-MCPIP). After 24 h, (A) transcript levels and (B) protein levels were evaluated for SIRT-1;∗=*p* <0.05.(C) cells were trypsinized and placed on matrigel. (d) Quantification of phase-contrast photomicrographs of the tube formation is represented; ∗=*p* <0.005.**

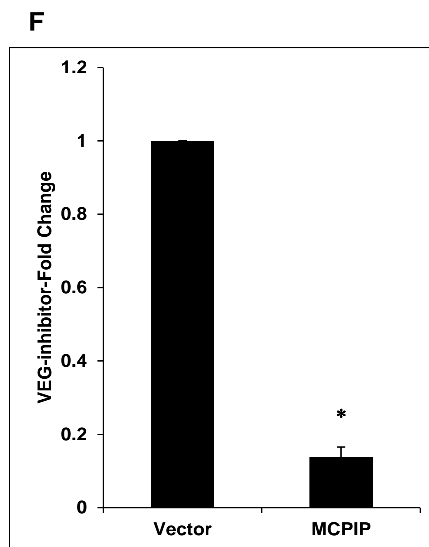

A
TSP-1
RNA fold Change
1.2
1
0.8
0.6
0.4
0.2
0
*
Vector
MCPIP


B
Vector
MCPIP
TSP-1
GAPDH
TSP-1/GAPDH
1.5
1
0.5
0
*
Vector
MCPIP

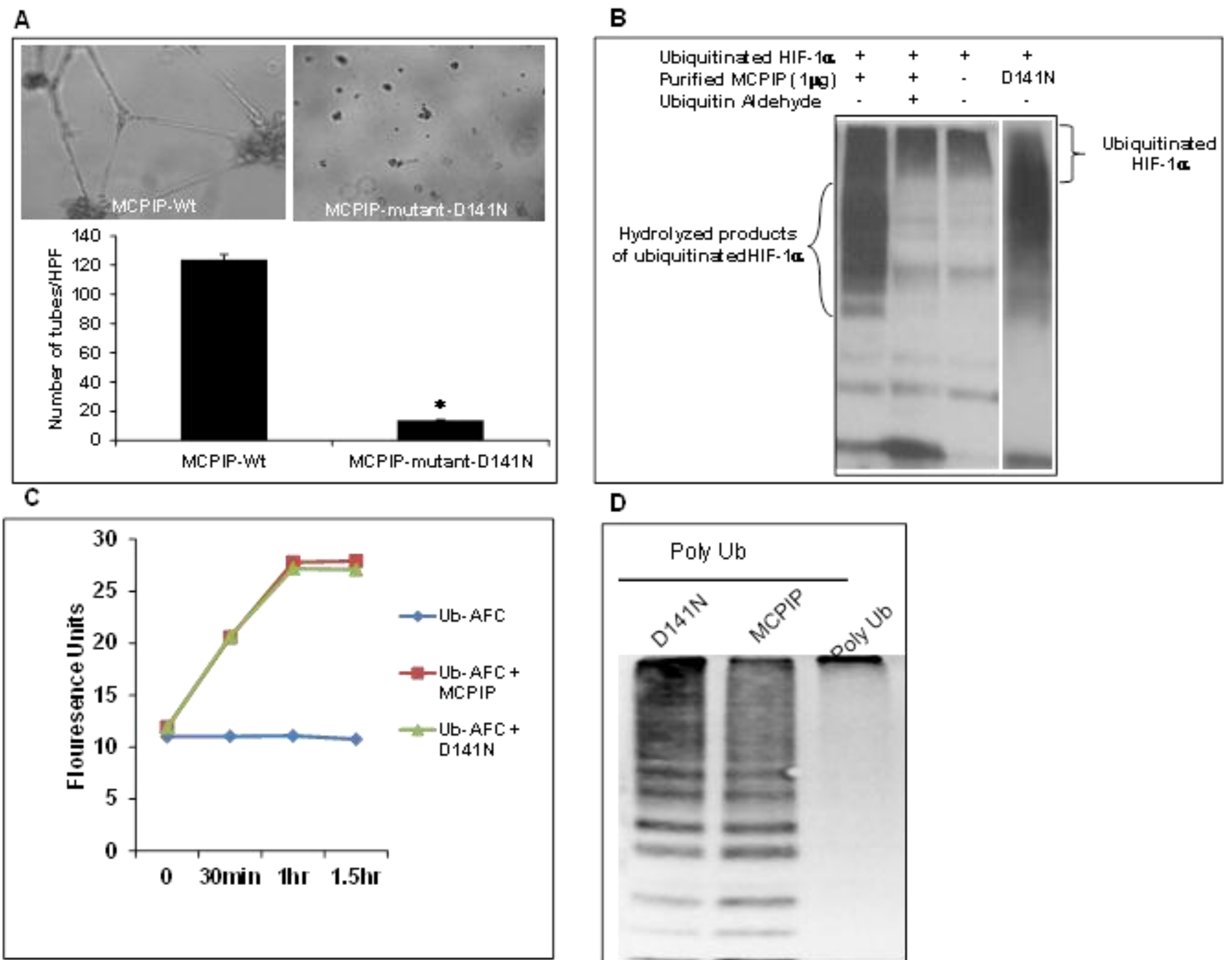

C
TSP-1
RNA Fold Change
5
4
3
2
1
0
*
siScramble+MCPIP
siSIRT+MCPIP


D
MCPIP
siScr
siSIRT-1
TSP-1
GAPDH
TSP-1/GAPDH
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
siScr+MCPIP
siSIRT+MCPIP


E
Vector
MCPIP
p65
Histone
p65/Histone
1.2
1
0.8
0.6
0.4
0.2
0
Vector
MCPIP


F
VEG-inhibitor-Fold Change
1.2
1
0.8
0.6
0.4
0.2
0
*
Vector
MCPIP

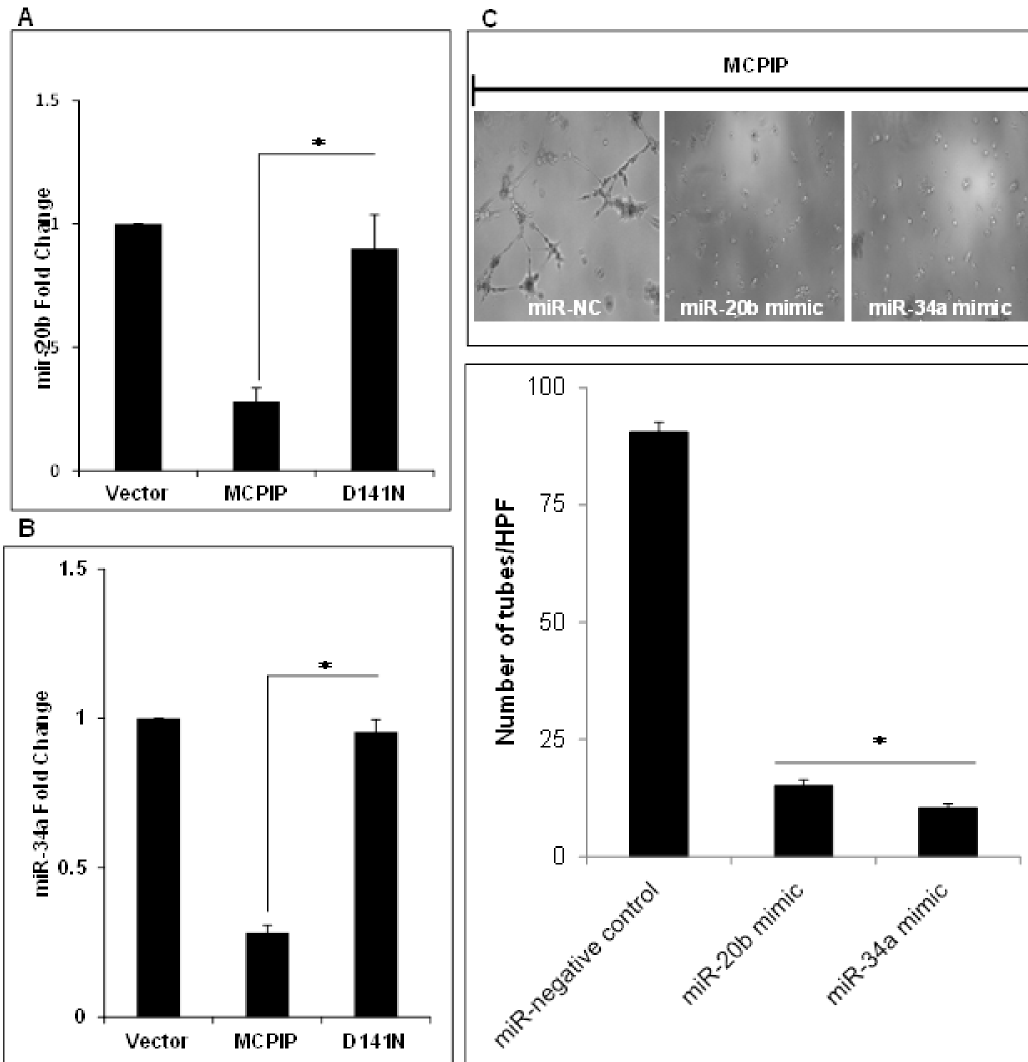
- **Figure 19: MCPIP overexpression reduced levels of anti-angiogenic factors, TSP-1 and VEGI**
- **HUVECs were transfected with MCPIP expression vector or empty vector for 24 hours (A) After 24 h, transcript levels were evaluated for TSP-1; ∗=p<0.02 (B) protein levels were assayed by immunoblot analysis with antibody against TSP-1 with GAPDH as a control. HUVECs were transfected with siRNA specific for SIRT-1 or scrambled (Scr) siRNA for 3 hours before transfecting with MCPIP expression vector After 24 h, (C) transcript levels were evaluated for TSP-1; ∗=p<0.02 (D) protein levels were assayed by immunoblot analysis with antibody against TSP-1 with GAPDH as a control∗=p<0.05. (E) HUVECs were transfected with for MCPIP expression vector or empty vector was used as a control. p65 nuclear protein levels were measured by immunoblot analysis. Histone was used to check for purity of the nuclear extract and as a control for densitometric analysis, (F) Transcript levels of VEG-inhibitor were analysed by RT-PCR, ∗=p <0.05.**



- **Figure 20: Deubiquitinase activity of MCPIP critical for angiogenic differentiation**

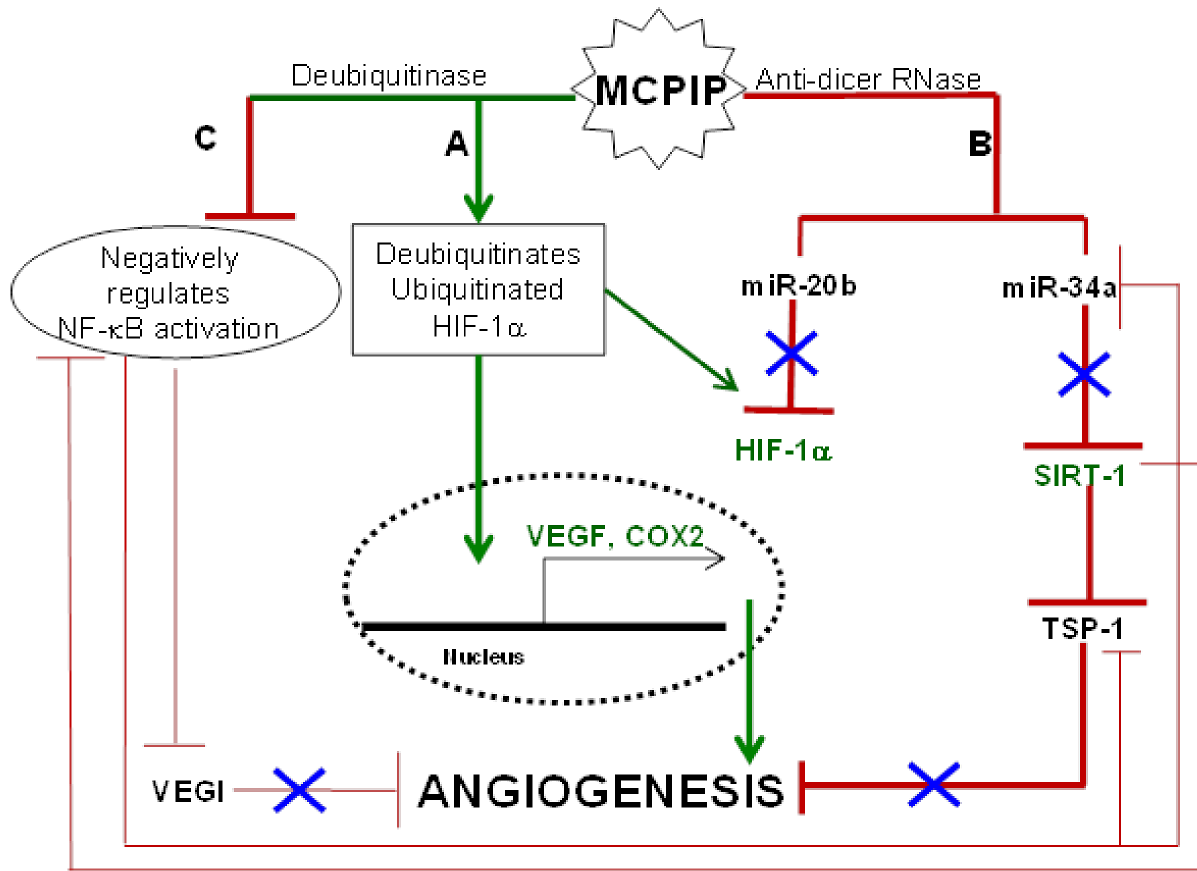
- **HUVECs were transfected with MCPIP-wild type and MCPIP-mutant-D141N expression vectors. (A) After 24 hours cells were trypsinized and placed on matrigel. Quantification of phase-contrast photomicrographs of the tube formation is represented; $*=p<0.005$. (B) Ubiquitinated HIF-1α substrate of MCPIP was incubated with purified MCPIP (1μg) or purified D141N mutant (1μg) at 37°C. After 1 hour the mixture was immunoblotted against ubiquitin antibody. (C) Ub-AFC (1μM) was incubated with MCPIP1 or its mutant, D141N (1μg) at 37°C. Ub-AFC without any protein was used as a negative control. Fluorescence was measured at Ex400nm and Em505nm in a time-dependent manner. (D) High Molecular weight polyubiquitin (1μg) was**

incubated with purified MCPIP (1μg) or MCPIP mutant D141N (1μg) at 37°C for 1 hour. The reaction mixture was immunoblotted with ubiquitin antibody.



- **Figure 21: MCPIP inhibits the production of anti-angiogenic miR-20b and miR-34a and their mimetics inhibit MCPIP-induced angiogenesis**

- **HUVECs were transfected with empty vector or MCPIP or RNase dead mutant, D141N. After 24 h, levels of (A) miR-20b or (B) miR-34a were measured by RT-PCR. (C) HUVECs were transfected with miR-negative control (NC) miR-20b mimic or miR-34a mimic for 3 hours before transfecting with MCPIP expression vectors. After 24 hours cells were trypsinized and placed on matrigel. Quantification of phase-contrast photomicrographs of the tube formation is represented; ∗=*p* <0.005.**



- **Figure 22: Schematic representation of the mechanisms involved in MCPIP-induced differentiation**

- **MCPIP mediates angiogenic differentiation by inducing HIF-1α and SIRT-1 levels and by inhibiting NF-κB activation via dual mechanisms: (A) deubiquitination of ubiquitinated HIF-1α thus stabilizing HIF-1α and (B) suppressing the levels of mir-20b and mir-34a thus augmenting the levels of HIF-1α and SIRT-1. Stabilized HIF-1α enters the nucleus for promoting the transcription of VEGF and COX2. Increased levels of SIRT-1 repress levels of the angiogenesis inhibitor, TSP-1, thus promoting MCPIP-induced angiogenesis. SIRT-1 is also reported to inhibit NF-κB that in turn results in reduced levels of TSP-1 and mir-34a levels.**

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