Understanding the Epigenetic Landscape of Endothelial Inflammation Due to Diabetes

THESIS

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by

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CERTIFICATE

This is to certify that the thesis titled **Understanding the Epigenetic Landscape of Endothelial Inflammation Due to Diabetes** submitted by **Mr.Sumukh Thakar** ID No **2017PHXF0437P** for award of Ph.D. of the Institute embodies original work done by him under my supervision.

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SUMUKH THAKAR

Abstract

Hyperglycemia has always been shown to lead to vascular dysfunction. As endothelial cells are the first to come in contact with blood glucose, they are generally susceptible to taking in a higher influx of glucose than necessary for metabolism. This causes many issues, oxidative stress and inflammation playing a major role in dysfunction. This persistent damage has the capability to contribute to metabolic memory which was shown as a major drive for epigenetic modulation pathways. Epigenetic mechanisms have emerged as one of the key pathways promoting diabetes associated complications. Herein, we explored the role of enhancer of zeste homolog 2 (EZH2) and its product histone 3 lysine 27 trimethylation (H3K27me3) in high glucose-mediated endothelial inflammation. To examine this, we treated cultured primary endothelial cells (EC) with different treatment conditions—namely, constant or intermittent or transient high glucose. Intermittent high glucose maximally induced endothelial inflammation by upregulating transcript and/or protein level expression of ICAM1 and P-selectin and downregulating eNOS, KLF2, and KLF4 protein levels. We next investigated the underlining epigenetic mechanisms responsible for intermittent hyperglycemia-dependent endothelial inflammation. Compared with other high glucose treatment groups, intermittent high glucose-exposed EC exhibited an increased level of H3K27me3 caused by reduction in EZH2 threonine 367 phosphorylation and nuclear retention of EZH2. Intermittent high glucose also promoted polycomb repressive complex-2 (PRC2) assembly and EZH2's recruitment to histone H3. Abrupt enrichment of H3K27me3 on KLF2 and KLF4 gene promoters caused repression of these genes, further supporting endothelial inflammation. In contrast, reducing H3K27me3 through small molecule and/or siRNA-mediated inhibition of EZH2 rescued KLF2 level and inhibited endothelial inflammation in intermittent high glucose-challenged cultured EC and isolated rat aorta. These findings indicate that abrupt chromatin modifications cause high glucose-dependent inflammatory switch of EC.

Hyperglycemia has been shown to lead to atherosclerosis by mediating vascular inflammation via glucose deregulation and dyslipidemia. However, there is an underlying mechanism which predisposes the cellular systems away from normal homeostasis. Such

pathway involves interplaying with the normal cell cycle of cells as known as cellular senescence. Senescent cells occur transiently at sites of tissue damage where they contribute to wound healing, tissue repair and regeneration, most likely through specific senescence associated secretory proteins (SASP)factors. Endothelial senescence was linked to several cardiovascular diseases. Dysregulation of proteins of the Shelterin complex including TRF2 and TERF2IP causes senescence as it hampers the DNA repair and cell proliferation. Stress in any form, be it metabolic or oxidative can always hamper cell homeostasis and lead to a cell cycle arrest. However, whether hyperglycemia exposure interplays with proteins of the Shelterin complex and further dictating senescent phenotype of the endothelial cells (EC) remains to be explored. Through the present study, we first confirmed increase in the level of two senescence associated biochemical marker, p21 and p53 which associated with endothelial senescence phenotype without causing apoptosis upon intermittent high glucose (IHG) exposure. We next reported an increase in the expression of Shelterin proteins TERF2IP and TRF2 without altering the level of TRF1 and TPP1 upon IHG. Furthermore, a robust induction was detected in p65 level upon IHG challenge. Through ChIP-qPCR experiment, we found enhanced H3K4me3 enrichment in the promoter region of p65, TERF2IP and TRF2. Inhibition of catalysis of H3K4me3 either by pharmacological inhibitor or siRNA mediated knockdown of MLL2 attenuated increase in p65, TERF2IP, and TRF2 level including reversing the level of senescence marker p21 and p53. Interestingly, pharmacological inhibition of NF-κB signaling also diminished the abrupt increase in TERF2-IP and TRF2 levels and further reversed IHG-induced p21 and p53 levels. More importantly, through co-immunoprecipitation and co-localization analysis, we identified that nuclear p65 and MLL2 interact in EC stimulated with IHG. Further, knockdown of either TERF2IP or TRF2 impaired IHG-induced p21 and p53 expression and associated endothelial senescence. Overall, the present study described the interplay of epigenetics in defining NF-κB signaling and shelterin proteins expression which further governs the biochemical and functional state of endothelial senescence in the hyperglycemia milieu.

These findings will help to design better therapeutic approaches to treat diabetes-mediated vascular diseases including improving the cell survival after prognosis of the disease. In conclusion, intermittent hyperglycemia dictates the epigenetic landscape of

endothelial cells further defining cellular health by regulating both inflammation and senescence via parallel epigenetic-dependent pathways, one through EZH2 mediated H3K27me3 enrichment at genes responsible for endothelial homeostasis while the other by elevating H3K4me3 level on p65, shelterin complex genes TRF2 and TERF2IP thereby inducing endothelial senescence.

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T2D – Type 2 diabetes

vSMCs – vascular smooth muscle cells

NF-κB - Nuclear factor kappa B

TGF – Tumour growth factor

ET-1 – endothelin 1

siRNA – silencing RNA

GSK126 – GlaxoSmith Kline 126

cDNA – complementary DNA

eNOS – endothelial nitric oxide synthase

ICAM1 – intercellular adhesion molecule 1

H3K27me3- Trimethylation of histone H3 at lysine 27

H3K4me3- Trimethylation of histone H3 at lysine 4

EZH2- Enhancer of zeste homolog 2

PRC2- Polycomb repressive complex 2

TERF2IP – Telomeric repeat binding factor 2 interacting protein

TRF2 – Telomeric repeat binding factor 2

TRF1 – Telomeric repeat binding factor 2

TPP1 – TIN2(TRF1 TRF2 interacting nuclear protein 2) interacting protein 1

EC – endothelial cells

HUVEC – human umbilical vein endothelial cells

Chapter 1

Introduction and Review

1.1 Introduction

Vascular endothelium maintains homeostasis by acting as a cellular lining of cardiovascular system. Endothelial cells (EC) have anti-coagulative properties and also help in dilation of arteries during stress induced response by secreting mediators to smooth muscle cells. EC synthesizes adhesive cofactors such as von Willebrand factor, fibronectin and thrombospondin, and procoagulant factors such as factor V, and provides a surface on which procoagulant factors can be assembled and activated [1]. These functions of EC represent a mechanism for the localization of clot-promoting activity near the surface of specifically primed blood vessel wall and thus inducing efficient atherosclerotic plug formation. Such priming of endothelial cells is driven by many biophysical or chemical mediators in individuals affected by diabetes. This is particularly important as the metabolic complications leading to diabetes are also a cause for atheroma formation. As we will see in the background below, we will understand the consequences of diabetes arising from hyperglycemic complications. Before that, a brief introduction about diabetes is necessary -

Diabetes is a group of metabolic diseases which occurs due to phenomenon of hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The majority of cases of diabetes fall into three broad etiopathogenetic categories- ***(i) Type 1 diabetes***; It occurs due to deficiency of insulin producing pancreatic beta-cells mainly due to the autoimmune destruction of pancreatic beta cells in islet of pancreas [2] ***(ii) Type 2 diabetes***- This type is referred to as non–insulin dependent diabetes or adult-onset diabetes, and consists of individuals having insulin resistance and usually have relative (rather than absolute) insulin deficiency [1]. In addition to insulin resistance, a demand for greater amounts of insulin by the pancreatic β-cells cannot be compensated in long term due to defects in the function of these cells. ***(iii) Gestational diabetes-*** This type of diabetes primarily occurs in pregnant women wherein there is an increased demand for glucose thereby leading to less insulin secretion. This usually subsides by end of pregnancy, if not it can progress to type 2 diabetes [1].

Approximately 537 million people in the age range of 20 years-79 years have been diagnosed with diabetes in 2021. According to a 2045 projection, it is estimated that the

prevalence of diabetes worldwide will exceed over 12%. The number of people with diabetes is expected to increase by 134% in Africa, 68% in South-East Asia, and 13% in Europe. Half of all people (49.7%) living with diabetes are undiagnosed. Moreover, 541 million are estimated to have impaired glucose tolerance. In line to these statistics, results from a 2020 study conducted by the Indian Council of Medical research (ICMR) found that India now has over 101 million people living with diabetes compared to 70 million people in 2019 [3]. Moreover, 319 million are estimated to have impaired fasting glucose [3]. In patients diagnosed with Type 2 Diabetes, elevated levels of acute-phase proteins (such as C-reactive protein (CRP), cytokines and chemokines) associated with inflammation have been observed. In a study conducted in women suffering from diabetes, elevated levels of interleukin-1β (IL-1β), IL-6 and CRP were found to precede development of Type 2 Diabetes. In animal models, particularly in mice fed on a high fat diet, neovascularization was observed indicating that increase in tissue mass leads to inflammation through hypoxic conditions leading to production of cytokines and homing of macrophages to regions of hypoxia in inflamed tissues. Hyperglycemia in diabetes and elevated levels of free fatty acids have been found to enhance ROS production, leading to decrease in nitric oxide (NO) synthesis in EC via a number of cellular mechanisms. This affects the EC as due to their anatomic location, they are in direct contact with the plasma fluids and also by affecting their vasodilation [4,5]. Such vasodilation is necessary for general development and function of endothelial cells, as given in the next section.

1.2 Function of Endothelial cells in vascular homeostasis

Endothelial cells act as a permeability barrier to the passage of plasma constituents from the lumen to extravascular tissue by selectively controlling passage of several mediators such as clotting factors, cytokines, angiopoietin. The maintenance of vascular tone is accomplished by the release dilator and constrictors like NO, originally identified as endothelium-derived relaxing factor, prostacyclin and bradykinin. The endothelium also produces vasoconstrictor substances, such as endothelin, and angiotensin II. Angiotensin II not only acts as a vasoconstrictor but is also pro-oxidant and stimulates production of endothelin. Unlike dysfunctional endothelial cells, leukocytes do not normally roll over and attach to resting endothelial cells in physiological condition. The main reason for this

would be sequestration of leukocyte-interactive proteins P-selectin and chemokines within specialized secretory vesicles known as Weibel–Palade bodies which in turn suppress the transcription of adhesion molecules, such as E-selectin, vascular cell-adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1). Failure of endothelial cells to adequately perform any of these basal functions constitutes 'endothelial cell dysfunction'. Failure to control coagulation, failure to control permeability, or failure to quiesce leukocytes are also instances of endothelial cell dysfunction [6]. Such dysfunction leads to secretion of cytokines, ultimately leading to inflamed tissue.

1.3 Endothelium role in inflammation

Activation of EC are divided into two types; (i) type I activation; also called stimulation-this is a fast paced one, (ii) Slower responses (type II activation). In both types of activation response, there are three principal components that underlie the four cardinal signs of inflammation: an increase in local blood flow- responsible for rubor and calor of inflamed tissues, localized leakage of plasma-protein-rich fluid (known as an exudate) into the tissue, causing swelling (tumor) of inflamed tissues, and localized recruitment and activation of circulating leukocytes such that they are induced to enter the infected or damaged tissues. Increased blood flow leads to leukocyte delivery to the affected site, the damaged tissues further leak plasma-protein-rich fluid. This creates a matrix to support leukocyte entry into the tissue while increased adhesion helps in leukocyte binding and extravasation [7]. In the next sections, we further study the two major types of inflammatory activation and its mechanisms.

1.4 Activation of type I inflammatory signaling in endothelial cells

Type I activation is mediated by ligands that bind to the extracellular domains of heterotrimeric G-protein-coupled receptor (GPCRs), like histamine H1 receptors, which signal through the intracellular G-protein alpha q subunit. This then leads to activation of phospholipase C, catalyzing the release of inositol-1,4,5-trisphosphate (IP3) from lipid phosphatidylinositol-4,5- bisphosphate. IP3 induces transient intracellular elevations in cytosolic free Ca^{2+} by releasing Ca^{2+} from endoplasmic reticulum stores [8]. In endothelial cells, these changes may take the form of oscillatory, transient Ca^{2+} levels, or produce an

elevated plateau of cytosolic Ca^{2+} levels. Elevated cytosolic Ca^{2+} levels may be sustained by entry of extracellular Ca^{2+} [8]. Activated GPCRs also facilitate the exchange of GDP for GTP on the small G protein RHO (RAS homologue) by activating a RHO guanine-nucleotide exchange factor (RHOGEF) [8]. These two events later combine to mediate the type I activation response. Cytosolic Ca^{2+} ions also form a complex with the adaptor protein calmodulin that activates nitric-oxide synthase 3 (NOS3) to produce NO, which synergizes with PGI. The rise in intracellular Ca^{2+} plays a major role in leukocyte recruitment by activating MLC in endothelial cells, causing plasma protein leakage and the exocytosis of WPBs, bringing P-selectin to the luminal cell surface. The combined display of P-selectin and PAF on the endothelial luminal plasma membrane provides a spatially linked, dual signal (sometimes called a juxtacrine signal) leading to binding of circulating neutrophils (due to P-selectin) followed by integrin activation and cell regulation causing extravasation of neutrophils.

1.5 Activation of type II inflammatory signaling in endothelial cells

Endothelial cells may capture chemokines made by other cells and display them on their luminal surface bound to heparan sulphate proteoglycans. Such stimulation of inflammatory pathways is imparted by binding of cytokines to their respective receptor on endothelial cell membrane. Transient activation of the cytokine receptors primarily causes an intracellular response, however these receptors become desensitized after binding signals by heterotrimeric GPCRs, thereby preventing re-stimulation. Such sensitization of the receptors last for 10–20 minutes, after which the receptors become desensitized, preventing re-stimulation [9]. This ceases the degree of inflammation and neutrophil extravasation that may be caused by type I activation alone. A more sustained inflammatory response requires a more persistent form of endothelial cell activation that is provided by type II activation. Leukocyte recruitment is more effective in type II activation than type I activation. Neutrophil recruitment is triggered by the synthesis and display of chemokines, such as CXC-chemokine ligand 8 (CXCL8 also known as IL-8) and leukocyte adhesion molecules, such as E-selectin [10] which is induced by inflammatory cytokines and P-selectin mobilized by binding of ligands to heterotrimeric GPCRs [10]. In mice, but not in humans, P-selectin synthesis is also increased as a result

of TNFR1 or IL-1R1 signaling [11]. CXCL8, similar to PAF, triggers firm attachment of neutrophils to endothelial cells and induces diapedesis into the tissue. However, it only causes efficient activation on neutrophils that are tethered or rolling on the endothelial cell surface; that is, CXCL8 and E-selectin, similar to PAF and P-selectin, provide a juxtacrine signal. E-selectin synthesis is spontaneously shut off, despite the continued presence of the activating cytokine; this response correlates with the inactivation of AP1 [12]. With somewhat slower induction response than for E-selectin expression, cytokine-activated venular EC increase their expression of VCAM1 and ICAM1 [13]. In combination with the synthesis of other chemokines, such as CC-chemokine ligand 2 (CCL2), such changes in the expression of adhesion molecules favors a transition from neutrophil-rich infiltrates to mononuclear-cell-rich infiltrates, typically occurring between 6 and 24 hours after cytokine-mediated activation. However, for endothelial dysfunction to further subsist and cause damage, a heightened inflammatory mechanism fills the gap as shown in the upcoming section.

1.6 Endothelial cells during Chronic inflammation

If acute inflammatory reactions fail to eradicate the triggering stimulus, and especially if persistent stimulation leads to activation of adaptive immune response, the inflammatory process will evolve to a more chronic form, involving specialized effector cells [6]. EC participate in this process by presenting antigens to circulating effector and memory T cells, endothelial cells will synthesize and express the chemokine CXCL10 [14] which binds to the CXC-chemokine receptor 3 (CXCR3) expressed by effector and effector-memory T cells, and sustain the expression of certain adhesion molecules, such as E-selectin , thus allowing the recruitment of Th1 cells [15]. These changes are induced by IFNγ, creating a positive feedback loop that sustains Th1-cell responses. By contrast, if the inflammatory response is dominated by Th2 cells, characterized by CD4+ T cells that secrete IL-4, IL-5 and/or IL-13, endothelial cells respond to IL-4 or IL-13 by synthesizing chemokines, such as CCL26 (also known as eotaxin-3), and expressing adhesion molecules, such as VCAM1, that favor the local recruitment of Th2 cells, as well as eosinophils [16]. TNF can act as a pro-angiogenic cytokine by binding to TNF receptor 2 (TNFR2) and signaling through cytosolic epithelial/endothelial tyrosine kinase (ETK; also

known as bone-marrow X-linked kinase or BMX) [17]. Activated ETK phosphorylates a subset of tyrosine residues in the cytoplasmic domain of VEGFR2 selectively activating the PI3K–AKT pathway. Angiopoietin-2, which acts through TIE2, enhances the induction of adhesion molecules by TNF especially when TNF is present at low concentrations [18] promoting oxidant-induced injury leading to inflammation [19]; by comparison, angiopoietin-1, which also signals through TIE2, reduces VEGFA induced vascular leakage [18] as well as the expression of tissue factor [20] thus negatively regulating pro-inflammatory signaling.

1.7 How does diabetes mellitus cause endothelial dysfunction?

High glucose and its prolonged exposure has been shown to disrupt the delicate lining of blood vessels. Five major mechanisms for hyperglycemia dependent endothelial dysfunction have been identified so far from clinical reports-

1.7.1 Increased polyol pathway flux

Aldose reductase is a cytosolic, monomeric oxidoreductase that catalyzes the NADPH-dependent reduction of glucose. In hyperglycemia, increased intracellular glucose is enzymatically converted by aldose reductase to the polyalcohol sorbitol, and decreasing level of NADPH (**Fig 1.7.1.1**). Sorbitol is then oxidized to fructose by the enzyme sorbitol

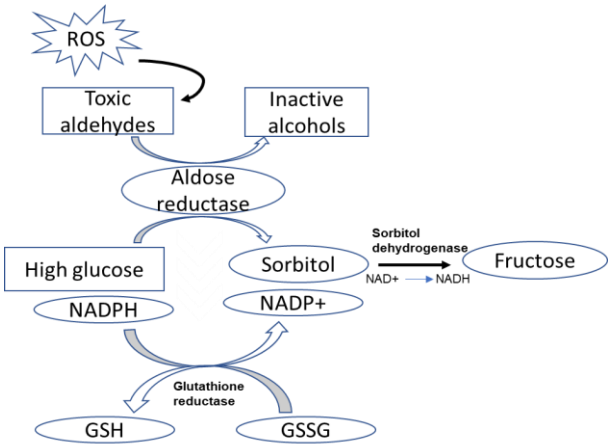


Fig 1.7.1.1. Schematic of the polyol pathway in glucose utilization

dehydrogenase, with NAD+ reduced to NADH. However, Sorbitol does not diffuse easily across cell membranes, and was thought to result in osmotic damage to microvascular

cells. However, sorbitol levels measured in diabetic vessels and nerves were too low to lead to osmotic damage. As NADPH is required for regenerating reduced glutathione (GSH), this could induce or exacerbate intracellular oxidative stress. In a five-year study in dogs, aldose reductase inhibition prevented diabetic neuropathy, but failed to prevent retinopathy or thickening of the capillary basement membrane in the retina, kidney and muscle [21].

1.7.2 Increased intracellular formation of advanced glycation end-products

Advanced glycation end-products (AGE) are found in increased amounts in diabetic retinal vessels [22] and renal glomeruli [23]. AGEs form by intracellular auto-oxidation of glucose to glyoxal [24], by decomposition of the Amadori product (glucose-derived 1-amino- 1-deoxyfructose lysine adducts) to 3-deoxyglucosone and fragmentation of glyceraldehyde- 3-phosphate and dihydroxyacetone phosphate to methylglyoxal [25]. Intracellular AGE precursors led to cell damage by three mechanisms; (i) Altered function of modified intracellular proteins by AGE, (ii) Extracellular matrix components modified by AGE precursors interacting abnormally with the receptors for integrins, (iii) Plasma proteins modified by AGE precursors binding to AGE receptors on endothelial cells, mesangial cells, and macrophages, inducing receptor-mediated production of reactive oxygen species. Ligand binding leads to activation of NF-κB, causing pathological changes in gene expression. AGEs have been known to decrease elasticity in large vessels from diabetic rats, even after degradation of vascular bundle, thereby increasing fluid filtration across the carotid artery [26].

1.7.3 Increased flux through hexosamine pathway

Fructose-6-phosphate is diverted from glycolysis to provide substrates for reactions that require UDP-*N*-acetylglucosamine, for proteoglycan synthesis and the formation of *O*-linked glycoproteins. These further help in transcription of TGF-β and PA-1 genes (**Fig 1.7.3.1**). Inhibition of the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) led to hyperglycemia-induced increase in the transcription of TGF-alpha, TGF-beta [27]. A 2.4-fold increase was seen in hexosamine pathway activity in aortic endothelial cells, resulting in a 1.7-fold increase in Sp1 O-linked GlcNAc and a

70–80% decrease in Sp1 O-linked phosphothreonine and phosphoserine [28]. Concomitantly, a 3.8-fold increase in expression was seen for PAI-1 promoter–luciferase reporter DNA containing two Sp1 sites, but failed to increase expression when the two Sp1 sites were mutated. In addition to transcription factors, many proteins are dynamically modified by O-linked GlcNAc, and show reciprocal modification by phosphorylation in a manner analogous to Sp1. For instance, the inhibition of eNOS activity by hyperglycemia-induced O-acetylglucosaminylation at the Akt site of the eNOS protein is important in diabetic complications. However, many inconsistencies abound, [29] it was reported that participants without diabetes related complications still had metabolic syndrome components, insulin resistance and impaired vasodilatory function which were seen to be proponents for cardiovascular risk.

1.7.4 Selective insulin resistance

Insulin was found to stimulate release of ET-1, a vasoactive peptide from HUVEC produced during stress conditions [30]. In insulin resistance that characterizes T2DM,

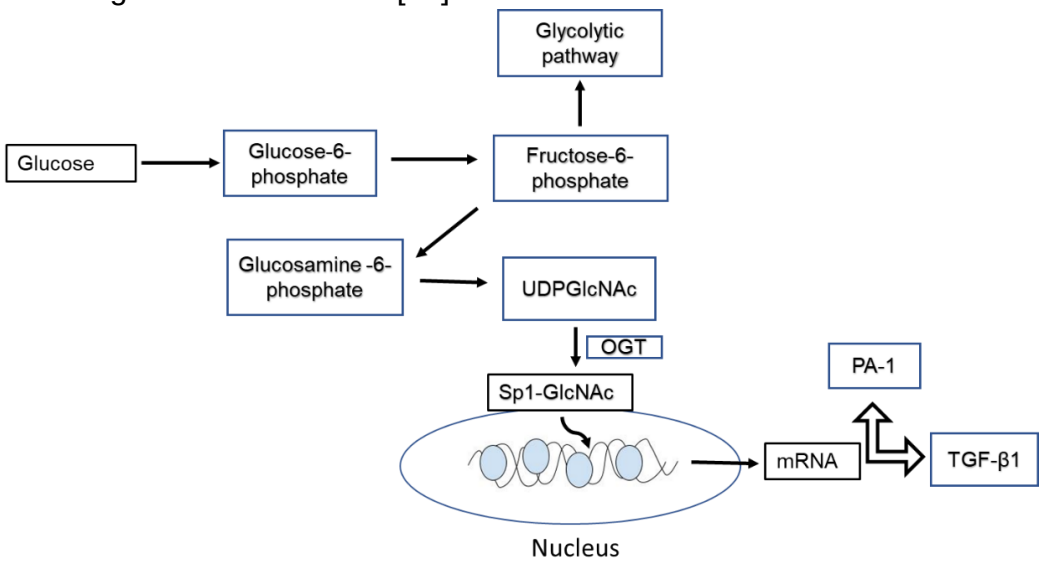


Fig 1.7.3.1. Hexosamine pathway followed after glucose uptake

there is a selective deficiency of the PI3-K pathway, while signaling via the MAPK pathway was found to be unaffected [31]. This resistance can lead to upregulation of either PI3-kinase pathway or the MAP kinase pathway. This also affects bio-metabolism like eNOS

phosphorylation, glycogen uptake and also cause inflammation and disruption in growth signaling (**Fig 1.7.4.1**).

Selective resistance led to hyper-insulinaemia, causing further stimulation of MAPK signaling [32]. This signaling deficiency has been demonstrated experimentally in obese

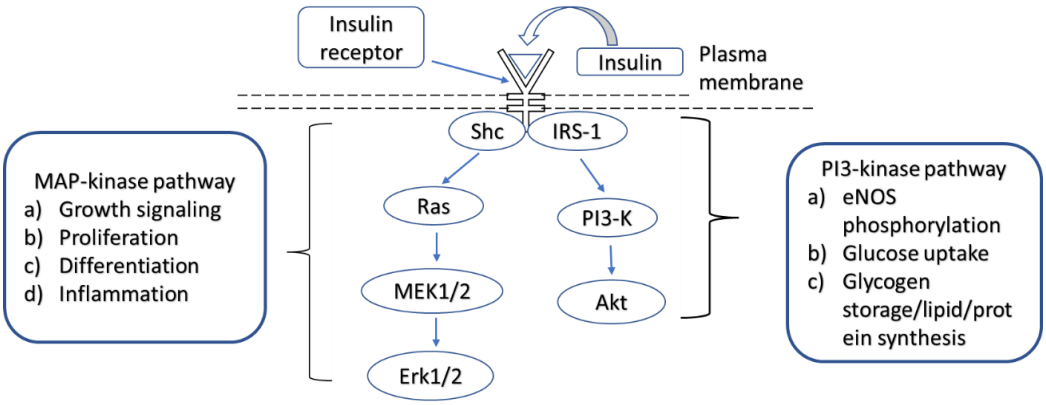


Fig 1.7.4.1. Selective insulin resistance and its outcome

Zucker rats, in which defective PI3-K/Akt signaling consequently resulted in decreased NO bioavailability.

1.7.5 Pro-inflammatory signaling

While acute inflammation as part of innate and adaptive immunity is beneficial, excessive or uncontrolled inflammation shown be detrimental to vascular tissue. Chronic inflammation is thought to be a characteristic feature seen at sites of diabetic complications. Activation of the endothelium in diabetes has been shown to express ICAM-1, VCAM-1, and E-selectins. These adhesion molecules facilitate binding of leukocytes and further extravasation through enabling infiltration into tissues at sites of diabetic complications. Inflammatory cytokine production, in obese animal models showed high levels of TNF-α. [33]. TNF-α activates nuclear factor κB (NF-κB), a transcription factor able to further stimulate expression of inflammatory genes. NF-κB is also activated by free fatty acids (FFA) and the receptor for advanced glycation end-products (RAGE), both of which area recurring feature in diabetic complications. This has been seen in cases of insulin resistance wherein TNF-α has been overexpressed. Obese

mice with null mutations in the TNF-α gene display significantly improved insulin sensitivity [34].

All the above mentioned pathways do not occur singularly, rather effects of hyperglycemia and its complications can occur in tandem with each other. A schematic of the mentioned pathways with a common goal can be used to refer to endothelial dysfunction (**Fig 1.7.5.1**).

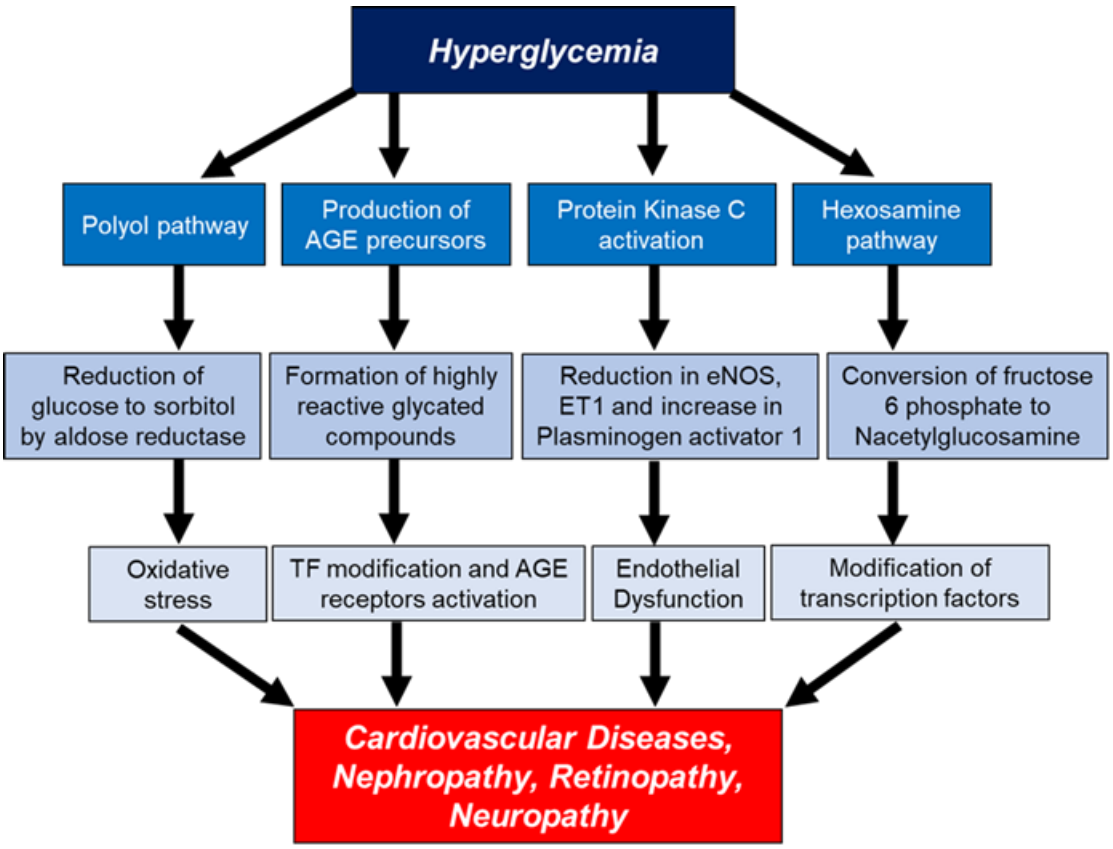


Figure 1.7.5.1 – Schematic showing the versatile pathways of hyperglycemia

1.8 Endothelial Activation during atherosclerosis onset and progression

The first step in atherosclerosis is endothelial dysfunction [35], possibly triggered by oxidized low-density lipoprotein (OxLDL) [36]. Endothelial activation was found to result in the expression of cytokines and chemokines, enhancement in the permeability of the endothelial cell (EC) layer and an increment in the expression of adhesion molecules. Immune and inflammatory cells, such as monocytes and T lymphocytes, are attracted by

chemokines (chemotaxis), following firm adhesion and trans-endothelial migration. Subsequently, infiltration of the sub-endothelium of the vascular wall takes place leading to formation of the ‘fatty streak’ – the first identifiable lesion. Macrophages take up the OxLDL present in the sub-endothelium, however are unable to digest it sufficiently, resulting in the formation of foam cells which show a large amount of lipids in cytoplasm due to defective cholesterol transport [36]. Monocytes and T lymphocytes migrate from the blood and proliferate within the lesion, resulting in the accumulation of inflammatory cells. Eventually focal necrosis is induced leading to the necrotic core found in advanced lesions. In enhanced stages, other cell types like vSMCs become involved. vSMCs start to proliferate as a response to the various signaling molecules present in the lesion, thereby thickening the arterial wall. Eventually the lesion will evolve into a ‘fibrolipid plaque’, consisting of a lipid-rich necrotic core covered by a fibrous cap of vSMCs and a collagen-rich matrix [36], [37]. The resulting thickening of the arterial wall caused by the plaque formation will be partially compensated by gradual dilation (remodeling), however after a certain limit, this cannot compensate enough, leading to decreased lumen size and hampered blood flow.

1.9 Histone methylation as epigenetic regulation of inflammatory gene expression

Overexpression of G9a or inhibition of KDM4D was found to increase the expression of H3K9me2 and reduce adhesion of leukocytes to endothelial cells [38]. This could be due to G9a regulating transcription of adhesion molecules, leading to endothelial activation. In endothelial cells, TNF-α increased the expression of histone demethylases KDM1B and KDM7A which further induced H3K9me2 di-methylation [38]. KDM7A mediated upregulation of ICAM1 was found associated with decreased expression of the transcription factor EB which positively regulates the activity of lysosome formation [39]. In human umbilical vein cell line EA.hy926, elevated di- and tri-methylation of lysine 4 at histone3 for the MCP-1 promotor was found regulated by increased H3K4 histone methyltransferase MLL3, menin and SET7 along with a concomitant decrease in the demethylase LSD1 expression in high glucose conditions [40]. In human aortic endothelial cells (HAECs), Set7 was found to stimulate NF-κB-dependent oxidative and inflammatory signaling by mono-methylation of H3K4 at its promotor. In EC, EZH2 was

reported to be induced by LDL. This was observed to be mediated through the transcription activator, myocyte enhancing factor-2, which decreased the expression of KLF2 [40]. The decreased expression of KLF2 was found to affect the expression of different cellular regulators of atherosclerosis such as thrombomodulin, endothelial NO synthase, and plasminogen activator inhibitor-1, which promote accumulation of platelets on endothelial cells. Since endothelial cells are present in vessels, they are also important in cardiovascular disease. According to Papait et al, the histone methyltransferase G9a was found to have a synergistic effect with the catalytic subunit EZH2 of the PRC2 complex which is responsible for gene silencing [41]. G9a inhibited cardiomyocytes through dimethylation of lysine 9 on histone H3 and interaction with EZH2. Therefore, G9a was essential to maintain correct gene expression in normal cardiomyocytes and to drive changes in the expression of genes associated with cardiac hypertrophy. The results suggest impairment of the G9a function can lead to cardiac dysfunction. G9a may be a potential target for the treatment of early myocardial hypertrophy in the future [41]. Kurozumi et al. showed induction of p-STAT3 activation and recruitment of jumonji domain-containing protein (JMJD)2B recruitment on stimulation of interleukin (IL)-6/SIL-6R [42]. Runx2 gene expression is increased in human VSMCs (hVSMCs) by bivalent histone modification of the transcription enhancer trimethylation of lysine 4 of histone H3 (H3K4me3) and the transcription suppressor trimethylated histone 3 lysine 9 (H3K9me3). In another study activation of IL-6/SIL-6R resulted in the recruitment of JMJD2B to targeted sites in the Runx2 promoter region, which demethylated H3K9me3. This caused an increase in the osteoblast differentiation markers (ALP and OPN mRNA) expression, while enhancing osteoblast differentiation and vascular calcification [42]. SM22a encoded by Transgelin (TAGLN) is expressed smooth muscle cells. Maleszewska et al. reported a role for polycomb methyltransferase EZH2 with respect to regulation of TAGLN/SM22a expression [43]. Downregulated IL-1β and transforming growth factor-β (TGFβ)-2 inhibited the TAGLN expression, due to a repressive mark H3K27me3 level at the proximal promoter region of TAGLN. SM22a is essential for the maintenance of smooth muscle cell phenotype and function, dysregulation of which was found to cause calcification [43]. In conclusion, these studies suggest that histone methylation is strongly associated with progression of vascular disease and its pathologic mechanism.

1.10 Epigenetics behind glucose induced endothelial dysfunction

High glucose has been shown to induce increased histone H3 lysine 9 dimethylation in THP1 monocytes. Miao et al. showed that high glucose exposure caused increased H3K4me2 and H3K9me2. They reported increased H3K4me2 was associated with increased methylation of nine genes, including ICAM3, FOS, GSTA-4, IL-8, and BCL-9, showed decreased methylation following HG exposure [44]. Similarly, H3K9me2 methylation resulted in increased methylation of 39 genes and decreased methylation of 11 genes. They further observed increased H3K9me2 at the coding and promoter regions of two candidate genes (IL-1A and PTEN) in blood monocytes of diabetic patients, indicating that diabetic milieu induced aberrant histone methylation is an important contributor to diabetes-associated complications. High glucose exposure altered ratio of cytoplasmic/nuclear ratio of Set7 protein without changing overall level of Set7 in vascular endothelial cells, indicating a role of Set7 and its role in hyperglycemia-induced gene activation of vascular endothelial cells. H3K9-specific demethylase JHDM2A (also known as JHMJD1A and KDM3A) has also been shown to be involved in regulating the expression of metabolic genes, strengthening the role of epigenetic regulation of metabolic genes in microvascular complications of diabetes [45]. These authors observed that JHDM2A regulates the expression of PPARα and β-adrenergic signaling pathway genes and suggested that JHDM2A might regulate energy mediated β-adrenergic signaling pathway [46].

In a recent study, Li et al. [47] showed that increased p21 expression seen in high glucose-treated mesangial cells was mediated by reduced histone H3-lysine9-dimethylation (H3K9me2), increased histone H3-lysine4 methylation (H3K4me1/3) and increased translocation of SET7/9 at the p21 promoter region. El-Osta [48] reported that short-term exposure of aortic endothelial cells to high glucose-induced promoter DNA methylation of NF-kB p65 subunit, an important mediator of cardiac fibrosis. TNF-α-mediated increased promoter methylation of sarcoplasmic reticulum Ca-ATPases (SERCA2a) resulting in decreased SERCA2a expression has been observed in high glucose-treated cardiomyocytes [49]. These results suggest that diabetic milieu can cause increased or decreased histone methylation in promoters of different genes,

resulting in their aberrant expression in cardiovascular system. Thus, there is substantial evidence to suggest that hyperglycemia causes aberrant methylation of regulatory regions of many genes resulting in their dysregulated expression.

In diabetic rat mesangial cells, increase in expression of profibrotic genes COL11A1, CTGF, and SERPINE1 was found due to a decrease in the H3 lysine 9 trimethylation 3 (H3K9me3) mark was found at promoter. These are important as they contribute to the development of diabetic nephropathy [50]. The commonly occurring repressive mark H3K27me3was also identified at the promoter region of profibrotic and inflammatory genes in diabetic rat renal mesangial cells [51]. Increased levels of the activating epigenetic mark H3K4 demethylation were also observed in kidneys of OVE6 mice and STZ-induced diabetic rats [52]. In STZ-induced diabetic rats treated with curcumin, an Increase in acetylation of histone H3, with increased dephosphorylation of histone H3, was reported in kidneys [53]. High glucose levels were important for enhancing H2A and H2B mono-ubiquitination levels in cultured glomerular mesangial cells [54]. However, it cannot be overlooked that H2AK119 and H2BK120 ubiquitination were increased in the whole kidney of diabetic animals, whereas they were reduced in the glomeruli. This could be due to initial damage induction in the glomeruli and then progressively damaging the kidney. [54]. In vascular smooth muscle cells from diabetic db/db mice, decreased levels of H3K9me3 were identified at promoter regions of IL-6, MCSF, and MCP-1 even after cells were cultured in vitro, indicating metabolic memory [55]. Even the activating H3K4me1 mark persisted at the promotor site of the NF-κB subunit p65 in aortic endothelial cells even when the cells were removed from the hyperglycemic environment [34,41]. A genome wide histone H3K9/K14 hyper-acetylation analysis in primary human aortic endothelial cells exposed to high or low glucose showed different levels of acetylation patterns. Hyperacetylation was an occurrence in regions of genes responsible for diabetes, coronary artery disease, and other cardiovascular diseases following hyperglycemia [58]. Chronically high glucose levels caused changes in histone H3K4 and K9 demethylation in human monocytes [56]. In DCCT/EDIC participants, H3K9 acetylation (H3K9Ac) in monocytes were found to regulate HbA1c levels. Participants with a history of higher HbA1c levels had greater number of promotor regions enriched with H3K9Ac in their isolated monocytes. These genes were related to various diabetes

complication related pathways, including the TNFR2 signaling and the NF-κB pathway [57]. Miao et al observed similar findings [44,45]. An *in vitro* study on human monocytes exposed to high glucose levels showed suppressed pro-inflammatory gene expression after the treatment with curcumin by decreasing histone acetylation [60].

Contradictory findings were observed in retina for diabetics. Histone hyperacetylation was observed in the retinas of diabetic rats, together with increased expression of inflammatory proteins [61]. But for STZ-induced rats exposed to high glucose levels, the global acetylation of histone H3 was decreased, which persisted even after the reversal, a clear case of metabolic memory [62]. High glucose levels caused aberrant expression of SET7/9, a methyltransferase, stimulating the expression of extracellular matrix genes in renal mesangial cells [33,49], inflammatory genes in monocytes [64], and cultured vascular smooth muscle cells [65]. In renal mesangial cells, the downregulation of EZH2, responsible for H3K27me3 mark and upregulation of its demethylases KDM6B and KDM6A were identified [51]. Levels of SUV39H1 methyltransferase were lowered in diabetic conditions in vascular smooth muscle cells and mesangial cells [51, 52]. Single nucleotide polymorphism rs17353856 in the SUV39H2 gene, a methyltransferase, was associated with diabetic retinopathy in individuals with T1D [67]. In cultured human monocytes, high glucose caused upregulation of p300, which is a major histone acetyltransferase and coactivator of NF-kB along with downregulation of histone deacetylase activity. These were reversed after treatment with curcumin. Atherosclerosis can also ultimately lead to a phenomenon of cell cycle arrest leading to senescence in which the cell would be unable to proliferate. However, it can still respond to environmental receptor signaling and mechanisms. How hyperglycemia can lead to senescence is another debate, however here we will describe senescence, its types and its influence on epigenetics occurring in hyperglycemia in endothelial cells.

1.11 Senescence- a brief introduction

Senescence is mainly a non-proliferative nature of cells seen normally or in case of chronic disease. Senescent ECs are detected in animal models and human tissues in many different patho-physiological conditions. Single and double balloon denudations of rabbit carotid arteries showed EC senescence as revealed by SA-β-gal staining [68], a

commonly used marker of cellular senescence. The authors postulated that vascular cell senescence may contribute to atherogenesis and postangioplasty re-stenosis. Hyperglycemia has been shown to lead to atherosclerosis via vascular inflammation via glucose deregulation and dyslipidemia. However, there is an underlying mechanism here which predisposes the cellular systems away from normal homeostasis. This is the cell cycle arrest pathway, known as senescence. The senescence response can be beneficial or deleterious, depending on the physiological context. Senescent cells occur transiently at sites of tissue damage where they contribute to wound healing, tissue repair and regeneration, most likely through specific SASP factors. Senescent cells increase with age in most mammalian tissues, where they appear to persist. Whether this increase is due to increased production or decreased clearance, for example by the immune system, is unclear. High glucose has also been shown to promote senescence in other cell types, namely fibroblasts and retinal endothelial cells. Cell cycle arrest in senescence is largely mediated via activation of either one or both p53/p21WAF1/CIP1 and p16INK4A/pRB tumor suppressor pathways. Prolonged overexpression of any of these four critical components (p53, pRB, p16INK4A, p21WAF1/CIP1) is sufficient to induce senescence.

1.11.1 DNA Damage

Three phosphoinositide 3-kinase-related kinases control the DNA damage occurring due to genotoxic and oxidative stress : Ataxia-telangiectasia mutated (ATM), ATM- and Rad3-related, and DNA-dependent protein kinase [69,70]. The DNA damage response is responsible for repair of DNA damage coordinating with other cell mediated responses like proliferation, cell survival, apoptosis, and senescence. p53-mediated signaling downstream of ATM is one of the key branches of the DNA damage response [71], which plays important roles in cellular senescence. In response to DNA damage, cGAS-STING signaling is also important in promoting cellular senescence [72]. Human primary ECs are more sensitive to genotoxic substances such as benzopyrene, a component in cigarette smoke and a common mutagen in the environment than human primary smooth muscle cells, and pericytes, because nucleotide excision repair proteins Excision repair cross complementing-group 1 (ERCC1), XPF, and ligase I are expressed at lower levels in

human primary ECs in culture. However Unrepaired DNA damage contributes importantly to vascular aging and the development of cardiovascular disease [73–76]. For example, ERCC1 is a mammalian endonuclease required for both nucleotide excision repair and Fanconi anemia inter-strand crosslink repair. ERCC1 is also important in preventing endogenous DNA damage [77]. ERCC1-deficiency in mice accounts for accelerated aging in mouse model. Its deficiency leads to cellular senescence in cells and mice [78] and is dependent upon ATM kinase [79]. ATM is activated in senescent cells in culture and tissues from ERCC1-deficient mice and naturally aged mice. Genetic and pharmacologic inhibition of ATM attenuates senescent phenotypes in ERCC1-deficient cells and mice. In a study by Bautista-Nino et al, mice with EC-specific loss of ERCC1 died at the age of 5.5–6 months [80]. These mice developed an increased permeability of the renal microvasculature. Aorta and iliac arteries of ERCC1 EC-specific knockout mice exhibited decreased endothelium-dependent relaxations in response to acetylcholine associated with reduced NO availability and increased oxidative stress. ERCC1 EC-specific knockout mice also showed a hypertrophic aorta wall with outward remodeling. These data show that endothelial genomic instability can lead to vascular aging and macrovascular and microvascular dysfunction.

1.11.2 Mitochondrial Dysfunction

Mitochondria are dynamic organelles and their function is maintained through proper coordination of mitochondrial biogenesis, dynamics (fission and fusion), and turnover (mitophagy). Alterations in mitochondrial morphology and function in ECs have been observed in obesity and diabetes [80,81]. In venous ECs freshly isolated from patients with diabetes mellitus, increased mitochondrial fission was observed [81]. Defects in mitochondrial function play a key role in the regulation of cellular senescence through mitochondria-borne reactive oxygen species [60–63]. In comparison to young HUVECs, old senescent HUVECs have extended and interconnected mitochondria, which is associated with the reduced expression of Fis1 and Drp1, responsible for mitochondrial fission [64,65]. Silencing of Drp1 induces senescence in young HUVECs with increased SA-b-gal staining, elongated mitochondria, impaired autophagy and an increase in p21 and p16 expression. Drp1 expression is also reduced in the endothelium of aorta in old

rats associated with impaired autophagic processing. Drp1 knockdown is able to disrupt autophagic flux in the vascular endothelium of common carotid arteries in rats [88]. Loss of protein disulfide isomerase A1 causes EC senescence through inducing mitochondrial fragmentation and mitochondrial reactive oxygen species production. Knockdown of protein disulfide isomerase A1 results in mitochondrial fragmentation by increasing Drp1 sulfenylation at cysteine 644 and in turn Drp1 activity [89].

1.11.3 Oxidative Stress

Hydrogen peroxide is routinely used for induction of cellular senescence in cell culture. Being largely a signaling mediator under physiological conditions, it is an endogenous reactive oxygen species that can induce oxidative stress and cellular senescence in vivo. The main sources of hydrogen peroxide are NAPDH oxidase, xanthine-oxido-reductase, endothelial nitric oxide synthase, and mitochondrial respiration complexes in vascular endothelium [89,90]. The levels of hydrogen peroxide also increase in vascular endothelium resulting from impaired antioxidant species such as loss of thioredoxin reductase 2 [92]. When hydrogen peroxide levels exceed the antioxidant capacity of cells, it induces cellular senescence through the following - The levels of intracellular reactive oxygen species increase in late passage EC which contribute to cellular senescence by accelerating telomere shortening [92,93]. This is mediated by inducing the nuclear export of telomerase reverse transcriptase into the cytosol [93]. Early studies indicated that oxidative stress rather than telomere shortening plays a dominant role in inducing cellular senescence of ECs isolated from atherosclerotic chronic smokers [95]. Oxidative stress causes oxidative DNA, lipid, and protein damage, all can promote cellular senescence of vascular endothelium. However, the *in vitro* experiments would find themselves at crossroads, as the exogenously added hydrogen peroxide does not mimic the more selective spatiotemporal pattern of endogenous hydrogen peroxide flux, its concentration being much higher than endogenous hydrogen peroxide, and having different functional consequences [96].

1.12 Pathways of cellular senescence

1.12.1 p53/p21WAF1/CIP1 Pathway

p53/p21WAF1/CIP1 is activated in response to DNA damage caused by telomere attrition, oxidative or oncogenic stress. Chronic activation of p53 due to DNA damage response induces cellular senescence through Interaction of p53 with FOXO4 [97]. p21WAF1/CIP1, is also regulated by the p53 protein transcriptionally. It is capable of inactivating all CDKs, thereby inhibiting cell cycle progression [98]. By inhibiting the the kinase activity of cyclin-CDK complexes, it leads to inhibition of phosphorylation of the RB family of proteins and subsequent association with E2Fs and formation of the DREAM complex thereby leading to a cell cycle arrest. High levels of p21 have been shown to inhibit the kinase activity of cyclinD/CDK4,6 complex leading to arrest of cell cycle whereas low levels of p21WAF1/CIP1 act as an assembly factor for cyclinD/CDK4,6 complex and promote its activation resulting in cell cycle progression. Members of the Krüppellike factor (KLF) transcription factor (TF) family can activate the CDKN1A gene by cooperating with p300-CREBBP [99],[100]. Transient stress leads to induction of p53 which activates DNA repair and leads to quiescence [101],[102].

1.12.2 p16INK4A/pRB Pathway

An important target of the CDK complex is the RB family of pocket proteins. These bind to and inactivating E2F complexes leading to repression of E2F target gene transcription. There are three members of the RB pocket protein family: RB1 (pRB), RBL1 (p107), and RBL2 (p130). These proteins share a common bipartite pocket region (LXCXE motif), which allows them to interact directly with other proteins [103].

pRB when dephosphorylated binds to E2Fs, forming RB-E2F complex, binding to the promoter regions of E2F target genes thereby inhibiting the transcription of genes required for cell cycle progression [104]. At the restriction point it is antagonized by hyperphosphorylation of RB by cyclinE-CDK2 which leads to release of E2Fs, thereby promoting transcription of S phase genes and hence progression of the cell cycle [105]. INK4/ARF locus encodes p16INK4A and p14ARF encoded by CDKN2A gene and p15INK4B by CDKN2B gene. The polycomb protein complexes PRC1/PRC2 can bind to and repress the INK4/ARF locus bt H3K27me3 deposition at promoter regions [106]. Hence, senescence induced by p16INK4A can be disrupted by silencing PRC1/PRC2

complex components such as CBX7, BMI1 or EZH2 followed by decrease in levels of H3K27me3.

1.12.3 DREAM Complex Mediated Cell Cycle Arrest

In mammalian cells, phosphorylation of p130 led to dissociation of the DREAM complex resulting in the MuvB core complex recruiting B-MYB to activate late S-phase genes, and FOXM1, in G2 phase, to activate mitotic gene expression [107],[108],[109]. However, a working mechanism by which p53 mediates transcriptional repression of genes was not understood, however this changed after the availability of genome-wide ChIP data on p53 binding sites and the discovery of the mammalian DREAM complex along with its target genes [84,85]. The p53-DREAM pathway is defined by the upregulation of p21WAF1/CIP1 via direct binding of p53 to promoter regions present in the p21WAF1/CIP1 promoter [110]. p21 also blocks phosphorylation of the pRB related pocket proteins, p107 and p130 as well as RB. In the unphosphorylated state p107 and p130 proteins bind to the MuvB core complex promoting the assembly of the repressive DREAM complex. In such a case, p53 would activate and shift the equilibrium from the activating MMB-FOXM1 complex to the repressive DREAM complex in a p21WAF1/CIP1 dependent manner [110].

1.13 Epigenetics of senescence mediated signaling

Replicative potential of human cells is due to an epigenetic setting which depends on the progressive accumulation of double-strand breaks (DSBs) at the chromosome ends This can be observed in pre-senescent cells, distal from telomeric junctions. This leads to initiation of an epigenetic biology that dictates the replicative potential of human cells [111]. Late passage IMR90 and WI38 human fibroblasts were found to be characterized by a reduced expression of core histone H3 and H4, linker histone H1 and the histone chaperons ASF1A/B and CAF1-p150/p60 [112]. However, the issue here is H1 is post translationally regulated rather than decreased neo-synthesis for H3 and H4. This means that after effects are experienced by late fibroblasts [113]. Moreover, alternative spliced histone mRNAs belonging to the HIST1 cluster were also found to be accumulated in quiescent and RS-arrested human fibroblasts [114]. Replicative senescent cells are

characterized by the deposition of the histone variants H3.3, H2A at genomic loci involving genes responsible for proliferation. These would be promoting the transcription of (i) tumor suppressors [115] (ii) inflammatory genes marking the SASP and iii) the cleavage of H3.3. This is important for also mediating the repression of E2F/RB target genes [115]. While in senescence, the HIRA-mediated deposition of H3.3 is responsible for cell-cycle arrest [115], and in embryonic stem cells ATRX and DAXX recruit H3.3 to repress the transcription of endogenous retroviruses (ERVs) [116]. The redistribution of H3.3 between the proliferating and senescent cells, which depends on the detachment from ATRX/DAXX and the complexing to HIRA, is at the basis of the activation of ERVs observed in senescence and aging [117]. The regulated deposition of all these histone variants is necessary [114] and sufficient to sustain cell-cycle arrest [31]. Interestingly, genes encoding these histone variants are frequently mutated in cancer, as a confirmation of their tumor-suppressive properties [112,116].

Replicative senescence in human fibroblasts is however governed by a global decrease in H3K9me2/3 and H4K20me and an increase in H3K9me1 levels [117,118]. But in proliferating cells, the heterochromatin marker H3K27me3 and the euchromatin marker H3K4me3 are mostly redistributed. This redistribution correlates well with the expression profile of senescent cells [119,120]. The repressive mark H4K20me3 and the activating mark H4K16ac follow a different pattern. Although their enrichment is observed in the regulative elements of genes modulated during replicative senescence, they do not correlate with gene expression changes observed in senescent cells [114]. Such a paradox would be comprehended the senescence-specific activation of super-enhancers (marked by H3K27ac/H3K4me1) and the activation of neighbor genes involved in SASP and metabolism [123]. A detailed comparison of H3K4me3 and H3K27me3 levels in proliferating and senescent human fibroblasts evidenced large-scale chromatin modifications during replicative senescence [124]. The augmented levels of H3K4me3 and H3K27me3 have been found to frequently co-localize in defined areas called as “mesas” which extend for many kilobases. Larger domains of replicative senescence genome (up to 10Mb) defined as “canyons” were characterized by decreased levels of H3K27me3 [124]. H3K4me3 and H3K27me3 mesas colocalize in LMNB1-associated domains and overlap DNA hypomethylation. Canyons on the other hand are enriched in

gene bodies and enhancers and H3K27me3 loss correlates with the up-regulation of senescent transcriptional programs [124].

On exposing human cells to oxidative stress in vitro, they undergo stochastic transcriptional changes leading to an aged tissue phenotype. Recently, it has been demonstrated that oxidative stress contributes not only to aging but also to age-related diseases [125]. H_2O_2 particularly is an important metabolic cause for aging. Global histone methylations for H3K4, K27 and K9 were increased on exposing BEAS-2B cells to H_2O_2, while preincubation with ascorbate reversed these changes [126]. The general heterochromatinization observed after H_2O_2 treatment is achieved in two steps. 1.Reducing acetylation (H3K9ac, H4K8ac, H4K16ac) in a HDAC-dependent manner [127]. 2. Recruiting histone methyltransferases (HMTs) and inducing increase in levels of H3K27me3, H3K9me3 and H4K20me3. Cell cycle arrest in cells can lead to the up-regulation of CDKi, the DDR response and SASP production, similarly to cells undergoing replicative senescence [128]. Also, mitochondrial dysfunctions in IMR90 human fibroblasts were found to be a cause for ROS–JNK retrograde signaling pathways, thereby promoting SASP, majorly through cytoplasmic DNA fragments [128]. In addition, the epigenetic homeostasis perturbation achieved through HMTs or HDACs inhibition [129], was reported after exposing cells to endogenous ROS production [134] In particular, ROS generating compounds abrogate action of PRC2 methyltransferases to allow the focused demethylation and transcriptional activation of CDKi [128]. Similarly, the senescence entry of PAK2 knocked-down MEFs cultured in normoxia is delayed because of the decreased deposition of H3.3 on CDKi loci [130]. At the DNA level, SIPS is characterized by a global DNA hypomethylation that only partially overlaps with the one observed during RS [131].

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are the most widely studied histone-modifying enzymes in vascular aging. HATs transfer acetyl group onto histone proteins, which in turn neutralizes the charge of histone, and weakens its interaction with DNA. Acetylation of H3K122 and H3K64 breaks the interaction between histone tails on adjacent nucleosomes and loosens the connection between nucleosomes

[132]. HDACs have opposite effects to that of HATs and a dynamic balance between the activities of HATs and HDACs is responsible for gene expression.

HDACs have a role in vessel homeostasis also. Indeed, HDAC4 has been found to abrogate vascular inflammation via activation of autophagy in endothelial cells [133] while the deletion of HDAC9 reduced inflammation and the reverse cholesterol transport mechanism in atherosclerosis and coronary heart diseases [134]. In addition, aging has been found to be accelerated by elevation of acetyltransferase p300 [135]. Histone H3 lysine 4 (H3K4) methyltransferase Smyd3 (SET and MYND domain containing proteins) directly binds to the promoter region of Cdkn1a. This interaction led to an increase in H3K4me3 causing activation of p21 giving rise to senescence associated secretory phenotype. Reversal by the Smyd3-specific inhibitor EPZ031686 decreased this phenotype [136], however it was enhanced on binding of Hsp90α to Smyd3 [137]. The SIRT family proteins are positively associated with longevity. The action of SIRT1 in reduction of vascular senescence, inflammation, DNA damage, and atherosclerosis are widely reported [138]. SIRT3 was found to enhance the expression of the blood pressure regulator GATA5 (GATA-binding protein 5). The endothelial specific loss of GATA5 causes vascular endothelial dysfunction via the inhibition of transcriptional repressor Nkx3 mediated by deacetylation. An important anti-atherosclerotic function handled by SIRT6 is in the maintenance of endothelial function via its ability to deacetylate H3K9 in the promoter region of the pro-atherogenic gene TNFSF4 [139]. Other components associated with vascular function include the p66shc, an epigenetic factor associated with diabetes-induced vascular senescence via its ability to inactivation of miR-34a and SIRT3. [138,139].

On basis of all points mentioned, we further narrowed the research down to endothelial dysfunction through endothelial inflammation and senescence due to hyperglycemia and resulting complications. Following are the gaps in our research and the objectives required to fulfil them -

1.14 Gaps and Objectives

Endothelial cells form the lining of blood vessels, and form an integral part of vascular physiology. They help in maintaining the blood pressure and are senescent. They release many mediators essential for maintaining phenotype and homeostasis like eNOS, VEGF-A. As they are the first to come in contact with blood, they are easily inflamed by bacterial infection, high cholesterol, high glucose. The activated cells will show expression of Nf-κB which is a proinflammatory marker and also expression of selectins and integrins on the cell surface leading to leukocyte recruitment at the affected area. However, in a diabetic individual, only chronic hyperglycemic shocks are seldom seen. Rather they show alternating normoglycemic and hyperglycemic periods. Most of the research has so far been focused on chronic hyperglycemia, rather than pulsatile hyperglycemic shocks. By this, we also want to uncover the gray area here and hence we decided on an intermittent model. We decided only on time kinetics, and kept only two glucose concentrations- 5.5 and 25mM.

In clinical studies and also in individuals affected with diabetes, recurrent vascular deregulatory effects were still found to occur even after medication. In some cases, these were found even after a period of 10 years. This led to the concept of metabolic memory, wherein the inflamed cells have an imprint of the damage caused by high glucose even after decrease in inflammation. As important genes in functioning pathways are affected both on a transcriptional as well as on a translational level, this is also known as epigenetic memory. In many studies, epigenetic alterations have been involved in vascular inflammation. For diabetes and associated vascular disease, long non coding RNAs and DNA methylation were found, however histone methylation was lacking extensive study. Hence we decided to focus on this particular area hoping to recognize leads regarding intermittent hyperglycemia.

Many papers show that repetitive insult on the cells leaves a genetic imprint on the cells, even after many years of prognosis. This has been called as metabolic memory. For these genes to be distinctly expressive in featuring cellular characteristics, there was bound to be an epigenetic control. Hence this was also called as epigenetic memory. This involves DNA methylation, histone tail modifications and long non coding RNAs. We decided to focus on histone tail modifications, particularly on methylation. Our main objective was to

find a singular mode which would be strong enough to prevail over others and lead to a potent phenotypic control in intermittent hyperglycemia.

Further also we were interested to decipher role of epigenetic mechanisms in regulating hyperglycemia driven endothelial inflammation, we singled out the PRC2 complex which is responsible for EZH2 mediated trimethylation in promoter regions of genes. The complex is formed of SUZ12 and EED which recruits EZH2. Hyperglycemia leading to senescence is a common concept, mostly occurring due to cell damage by ROS production and metabolic dysfunction. The telomere complex of proteins was found to be dysregulated in many cases of senescence. It was found that disruption of the telomere prevented cell damage and tissue growth in diabetic condition. Increase in expression of these proteins in a healthy person led to age related senescence. These have also been known to act as transcriptional factors in angiogenesis and during stem cell development. In vascular inflammation, shelterin knockouts have been found to progress to senescence, whereas if these were induced for a gain in expression, it was found to reduce inflammation. However, in a clinical setting, the difference could only be seen in high grade inflammation, particularly in lymphocytes. In order for senescence to proceed, it would involve expression of the cell cycle control proteins – CDK 40, p16, p21, p27, particularly the tumour suppressor gene p53. A normally dividing cell would stop proliferation under two conditions- the first being replicative senescence and the second being induced due to stress, in this case being inflammation. MLL1 particularly has been shown to be implicated in disruption of the cell cycle genes and promotion of DNA damage response leading to senescence. MLL1 inhibition was to show prevention of cell cycle escape, especially in case of p53 and p16 and prevented activation of ATM-NF-κB signaling axis. p53 has been shown to link with p65 in an attempt to prevent proliferation via phosphorylation of p65 via DNA damaging agents. This tells us that NF-κB can also have a role in senescence via interaction with antiproliferative proteins. However, this has been shown in cancer cells and fibroblasts. In such studies even though cell cycle deficit has been observed, there has shown to be no changes in telomere length. The shelterin complex proteins instead have been shown to have transcription factor properties in activating gene expression by binding to promoter regions. This suggests that even though there may be increase in expression of these proteins, it gives no

exclusivity for binding to telomeric regions and aiding in replication induced senescence. A telomeric independent role has never been seen in endothelial cells, particularly in hyperglycemia. We therefore formulated an action plan based on available data to get a

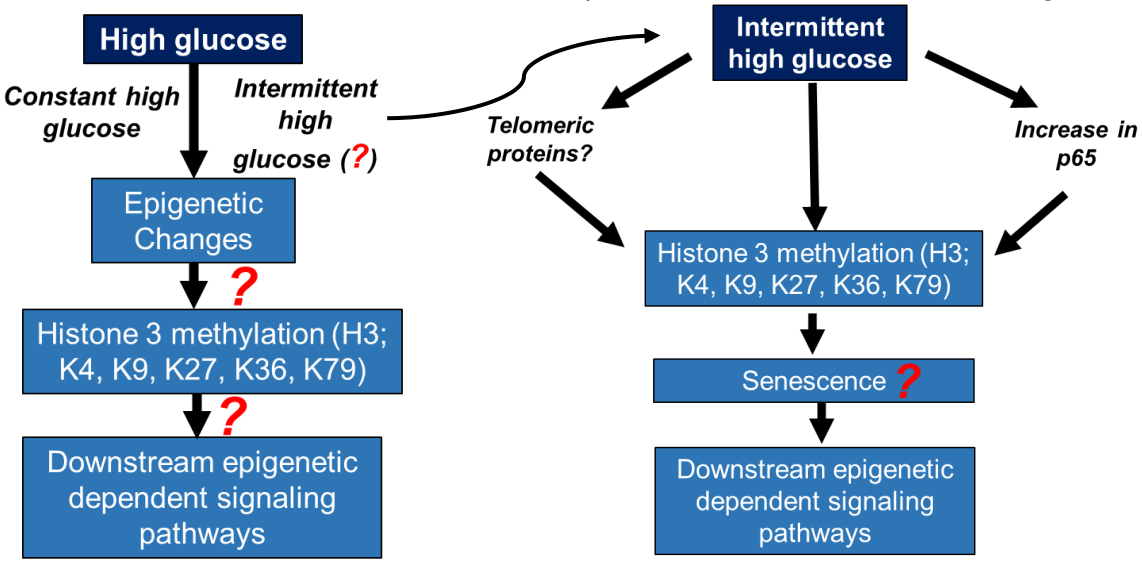


Fig 1.14.1. Flow diagram showing the progressive gaps in deciphering the signaling pathways

regressive signaling cascade. (**Fig 1.14.1**)

Keeping all such issues in mind, we formulated the following objectives-

Objective 1: Studying the effect of differential high glucose treatment on endothelial inflammation

Objective 2: Identifying hyperglycemia dependent epigenetic mechanism(s) associated with endothelial inflammation.

Objective 3: Evaluating the role of the epigenetic mechanism(s) in regulating hyperglycemia driven endothelial inflammation.

Objective 4: Dissecting the epigenetics dependent underlining signaling mechanism(s) associated with diabetes induced vascular dysfunction.

Chapter 2

Materials and Methods

2.1 Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Hi Media (CL002-T25, Mumbai, India) and cultured using HiEndoXL^{TM} Endothelial Cell Expansion medium (AL517, Hi Media) supplemented with 2% endothelial growth supplement, 5% fetal bovine serum (FBS), and 1% pen/strep. HUVEC were grown in HiEndoXL cell medium containing 5.5 mM of basal glucose. EA.hy926, an immortalized human umbilical vein cell was purchased from ATCC (#CRL-2922) and was used for many parts of the study. The cells were cultured and passaged every 2 or 3 days in Dulbecco's modified Eagle's medium (DMEM) (Hi Media Laboratories) supplemented with 10% FBS (Hi Media Laboratories) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Both cells were cultured at 37ºC and in a humidified incubator with a 5% CO2 atmosphere. They were then trypsinized and seeded in six-well plates at a confluency of $1*10^5$. After achieving a 70% confluency, they were then subjected to different time kinetics of hyperglycemia, alternating between normal glucose level (5.5 mM) and high glucose level (25 mM); a constant high glucose (referred to as CHG) treatment condition was imparted on EC by incubating the cells for 72 h in high glucose (25 mM)-containing media; an intermittent high glucose (referred to as IHG) treatment condition was established through 12 h of high glucose (25 mM) and 12 h of normal glucose (5.5 mM) cycle for 3 cycles, totaling 72 h of treatment time; a transient high glucose (referred to as THG) treatment condition was achieved through 24 h of high glucose (25 mM) followed by 48 h of normal glucose (5.5 mM) exposure. A control treatment condition (cells constantly exposed to normal glucose (5.5 mM)) is represented as "Ct" in multiple figures. EA.hy 926 cells were made tolerant to 15mM glucose through multiple passages by lowering down glucose concentration from 25mM. The resulting cell line was then given 30mM as intermittent high glucose treatment condition.

2.2 Animal Dissection and Treatment Conditions

All experimental procedures for the rodent studies were approved by the Institutional Animal Ethics Committee of BITS Pilani, Pilani Campus. Male Wistar rats aged 12 to 16 weeks were selected for the *ex vivo* experiment. The rats had been fed on normal chow diet. They were anaesthetized, and dissection was then performed by a cut from the

ventral end. Hearts and the aortas were perfused with PBS to remove any blood cells from the vessels. The primary aortas were then collected and the fatty tissue layers were removed, followed by cutting into cylindrical pieces measuring 2 mm lengthwise to obtain aortic rings. These rings were washed with PBS and further cultured in HiEndo endothelial cell medium in 24-well plates for the experiment. Prior to initiating any treatment condition, these aortic rings were allowed a 12 h incubation period in the culture medium containing 5.5 mM glucose. The aortic rings were subjected to an intermittent glucose treatment, as explained earlier (12 h alternating periods of normal (5.5 mM) and high glucose (25 mM) for three cycles), along with a combination of GSK126 (10 µM). After 3 days, the tissue fractions were homogenized, suspended in RIPA lysis buffer, and sonicated. Protein estimation was done by Bradford assay, followed by SDS-PAGE and immunoblot. Antibodies used for probing are summarized in the given table.

2.3 Inhibitor Treatment Condition and RNA Silencing in Cultured Endothelial Cells

Prevalidated human-specific EZH2 small-interfering RNA (SignalSilence® Ezh2 siRNA I #6509) and control siRNA (SignalSilence® Control siRNA#6568) were from Cell Signaling Technology, TERF2IP siRNA and TRF2 siRNA were designed and ordered from GeneCrust. MLL siRNA was ordered from Thermo Fisher. All were used at a concentration of 40 nM. OptiMEM containing lipofectamine 2000 (#11668030, Thermo Fisher Scientific, Waltham, MA, USA) and siRNA were subjected to HUVEC for 4 h. OptiMEM was later replaced with HiEndo endothelial culture medium and subjected to the intermittent high glucose (25 mM) treatment condition. For EA.hy926, DMEM culture medium was used and 30mM was used for intermittent high glucose treatment condition. Cells were finally harvested upon completion of the intermittent high glucose treatment condition, as specified earlier. For comparative analysis, all nonsiRNA - transfected cells were transfected with scrambled siRNA. For all EZH2 inhibition studies, a competitive SAM inhibitor GSK126 was used at a final concentration of 10uM.For all MLL2 siRNA inhibition studies, an MLL-WDR5 inhibitor OICR was used at a final concentration of 10uM. DMSO was used as vehicle control.

2.4 Cell Viability Assay

Viability of cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) assay. HUVEC were cultured in 96-well plates overnight, followed by exposing them to differential high glucose treatment conditions: a constant high glucose (25 mM) treatment condition for 72 h; two different conditions of intermittent high glucose treatment — (i) 8 h of high glucose (25 mM) and 16 h of normal glucose (5.5 mM) cycle for 3 cycles and (ii) 12 h of high glucose (25 mM) and 12 h of normal glucose (5.5 mM) cycle for 3 cycles; and two different conditions of transient high glucose treatment — (i) 4h of high glucose (25 mM) followed by 68 h of normal glucose (5.5 mM) exposure and (ii) 24 h of high glucose (25 mM) followed by 48 h of normal glucose (5.5 mM) challenge. For GSK126 treatment groups, HUVEC were treated with GSK126 (10 mM) for 72 h. Thereafter, MTT was added to all treated and control cells, and cells were incubated for 4 h. Formazan crystals were solubilized in DMSO, and readings were obtained at 495 nm with a differential filter of 630 nm using a micro-plate reader (Start-fax 2100). Percentage of viable cells was calculated as % viability = (mean absorbance value of drug-treated cells)/(mean absorbance value of control) * 100.

2.5 RNA Isolation

RNA isolation was performed following the manufacturer's protocol (#15596026, TRIzol™ Reagent, Life Technologies, Thermo Fisher Scientific). In brief, HUVEC were grown in six-well plates up to 70% confluency and subjected to the earlier-mentioned treatment regimes. After 72 h, treated cells were incubated in Trizol reagent. Upon collecting the cells in Trizol reagent, organic layer separation was carried out using chloroform, and further RNA in the aqueous layer was precipitated using isopropanol. Next, precipitated RNA was washed with 75% ethanol, and RNA pellets were air dried. Finally, RNA pellets were dissolved in sterile nuclease-free water, and quantity and quality were analyzed through NanoDrop measurement.

2.6 cDNA Synthesis and Quantitative Analysis through Reverse Transcriptase-Quantitative Polymerase Chain Reaction

To measure the transcript level of different genes, we carried out reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). In brief, total RNA (1 µg) was taken

from the cDNA preparation using iScript™ cDNA Synthesis Kit (#1708891, Bio-Rad Laboratories, Hercules, CA, USA). Prior to cDNA synthesis, isolated RNAs were pre-incubated with DNAse to remove any DNA contamination. Real-time PCR was then performed using iTaq™ Universal SYBR® Green Supermix (#1725124, Bio-Rad Laboratories), with a total master mix volume of 10 µL. Analysis was carried out by calculating delta-delta Ct. GAPDH was used as the housekeeping gene. Primer sequences for specific genes used in Real Time PCR (**Table 2.6.1**) are given below.

Gene	Forward sequence	Reverse sequence
eNOS	GCCGGAACAGCACAAGAGTTAT	AGCCCGAACACACAGAACC
KLF2	CGGCAAGACCTACACCAAGA	TGGTAGGGCTTCTCACCTGT
GAPDH	TCGGAGTCAACGGATTTGGT	TTCCCGTTCTCAGCCTTGAC
ICAM1	TTCGTGTCCTGTATGGCCC	CACATTGGAGTCTGCTGGGA
VCAM1	TGGATAATGTTTGCAGCTTCTCA	CGTCACCTTCCCATTCAGTG
P-Selectin	CCAACCTGCAAAGGCATAGC	GCGTTGCAGCCAAAGTAACA
KLF4	CCACCTTCTTCACCCCTAGA	AAGGTTTCTCACCTGTGTGG
TERF2IP	CCTTGTGGAAAGCGATGGAG	TTATTCTGTGGTTCCCCGCT
TERF2	TTCTCAACCAACCCCTCCCT	CATCTGGTGCTGCCTGAACT
TPP1	ATTCGGGAGCTGATTCTGGG	GGCGTCCTGTAGTACCTCAA
TRF1	ACAGCGCAGAGGCTATTATT	GGGCTGATTCCAAGGGTGTA

Table 2.6.1 – Primer sequences for Real Time PCR

2.7 Subcellular Fractionation

HUVEC challenged with or without intermittent hyperglycemia, were harvested and washed with PBS. Upon centrifugation, the cell pellet was re-suspended in ice cold PBS containing 0.1% Nonidet P-40 (NP-40) and 1% protease inhibitors (#P8340, Sigma-

Aldrich, MI, USA). The nuclear fraction was pelleted down by centrifugation for 10 min at 10,000 rpm, while the supernatant was collected as the membrane and cytoplasmic fraction. Furthermore, the nuclear lysate was obtained by resuspending the nuclear pellet in PBS containing 0.1% Nonidet P-40 (NP-40) and 1% protease inhibitor (#P8340, Sigma). Both the nuclear and cellular fractions were sonicated for 10 s (2) and then separated by SDS-PAGE and immunoblotted for TRF2, TERF2IP, EZH2. GAPDH and H3 were used as loading controls for cytoplasmic and nuclear localization.

2.8 Co-Immunoprecipitation

Proteins were extracted from HUVEC using RIPA lysis buffer (#R0278, Sigma-Aldrich) containing protease inhibitor (#P8340, Sigma-Aldrich). A total of 1000 ug of protein was used for pulldown with the antibody for EZH2 (1 g/uL, #5246, Cell Signaling Technology), followed by separating in SDS-PAGE. Antibodies directed to SUZ12 (1:1000, #3737), EED (1:1000, #51673), and histone H3 (1:1000, #4499) (Cell Signaling Technology) were used to confirm co-immunoprecipitation with EZH2. Similar was done for pulldown with antibody for TRF2 and p65. Antibodies specific to MLL2, MLL1-N, MLL-C. Resulting bands were visualized by Chemi-Doc using the Clarity™ (#1705061, Bio-Rad Laboratories) or Clarity™ Max (#1705062, Bio-Rad Laboratories) Western Blotting ECL Substrates and analyzed by expression levels relative to 5% input obtained using ImageJ software.

2.9 Immunofluorescence Imaging and Analysis

HUVEC were grown on gelatin (#TC041, HiMedia) coated coverslips up to 70% confluency. After completion of treatment, they were washed with PBS and subjected to fixation with 4% ice-cold paraformaldehyde. Cells were incubated with 0.1% Triton X for permeabilization. Cells were then incubated with BSA (1%) for 1 h, followed by overnight incubation with EZH2 antibody (1:1000, # 5246, Cell Signaling Technology). Cells were washed with PBS to remove unbound primary antibody, and were subsequently incubated with Alexa fluor 555 secondary antibody (1:4000; # A32732, Thermo Fisher Scientific) for 2 h. To stain F-actin, cells were incubated with rhodamine-tagged phalloidin (1:5000; # R415, Thermo Fisher Scientific) for 30 min, followed by incubation with DAPI

(# D9542, Sigma-Aldrich) for nuclear staining. For EA.hy926 cells, similar procedure was followed, overnight incubation for primary antibody was TERF2IP or TRF2 in different treated sets at 30mM IHG. Fluorescence images were captured using a Zeiss ApoTome.2 microscope (Carl Zeiss, Jena, Germany), and intensities were measured using ImageJ software. For Co- immunofluorescence, similar protocol was followed. After fixation with 4%PFA for 5min and Triton X-100 treatment, cells were incubated with MLL2 antibody overnight. The next day, cell were washed with PBS to remove unbound primary antibody and incubated with F'ab secondary antibody 488 for 2hours(1:2000, 4412S). Cells were then again incubated with p65 primary antibody (1:1000) overnight and successively incubated with secondary Alexa Fluor IgG 555 as per abovementioned protocol. After staining with DAPI and mounting with 70% glycerol, they were imaged to find co-localization through confocal microscopy. Analysis was performed using Image J software.

2.10 Chromatin Immunoprecipitation (ChIP) and Subsequent Quantitative PCR

A ChIP assay was performed using an Imprint® Chromatin Immunoprecipitation Kit (#CHP1, Sigma-Aldrich, # 86652 CUT & RUN assay kit, Cell signaling Technology). In brief, exponentially growing HUVEC (80% cell density) were subjected to intermittent high glucose treatment. Treated HUVEC were harvested (1 * 10^6 cells), washed, and cross-linked with 1% formaldehyde in HiEndoXL Endothelial Cell Expansion medium (10 min at room temperature). After washing in PBS, the cell pellet was re-suspended in Nuclei Preparation Buffer (200 L per 106 cells) and kept on ice for 10 min. The nuclear pellet thus obtained was re-suspended in shearing buffer (100 L per 10^6 cells) supplemented with protease inhibitor cocktail (1 L per ml of shearing buffer) and further sheared by sonication for 30 s (40). The sheared chromatin (containing 100 to 500 bp long sheared genomic DNA) was immunoprecipitated with antibodies directed against H3K27me3 at a concentration of 1:50 or H3K4me3 at a concentration of 1:50. The samples were then washed, reverse cross-linked, and treated with proteinase K to obtain purified DNA fragments. qPCR was performed using primers targeted to amplify regions of human gene promoters. The promoter primers used for amplification of genes are provided below. (**Table 2.10.1**)

Gene	Forward primer	Reverse primer
TERF2IP	CTCCAGGGATTTGGGAGTGG	TCTGGGCCTCACTAAGGTCA
TERF2	GTGGGGCTGGTAGGACAATC	AGGACCACCCATAGTCCCAT
p65	TTCTGTCACCTGAAGTCGGC	GTGGCTGGCCCTGATTAGAA
KLF2	AGTAAAGGAACCCAGCCTGC	CCCAACCTCAATAAGCCCGT
KLF4	TGAGCCGAAGGAACGAGTTG	TGACTTTTCAAGCCACGCAC

Table 2.10.1 – Promoter primer sequences used for Chromatin Immunoprecipitation

2.11 Flow cytometry analysis of cell cycle

EA.hy 926 cells seeded at 60% density were subjected to a five day treatment regime of intermittent high glucose and a combination of intermittent high glucose with TERF2IP siRNA and TRF2 siRNA. All were used at a concentration of 40 nM. OptiMEM containing lipofectamine 2000 (#11668030, Thermo Fisher Scientific, Waltham, MA, USA) and siRNA were subjected to HUVEC for 4 h. OptiMEM was later replaced with DMEM culture medium and subjected to the intermittent high glucose (30 mM) treatment condition. Cells were finally harvested upon completion of the intermittent high glucose treatment condition, as specified earlier. For comparative analysis, all nonsiRNA-transfected cells were transfected with scrambled siRNA. Cells were harvested by trypsinization and washed with PBS followed by 5000 rpm for 10 min at 4°C, the pellet was re-suspended in a fixative (100 µL of PBS and 900 µL of ice cold 70% ethanol) and incubated at room temperature for 45 minutes. Cells were centrifuged at 5000rpm and the pellet was re-suspended in 400 µL PBS with 50 µL of propidium iodide staining solution (PI; 2 mg/ml). The samples were then incubated in dark for 30 min followed by acquisition using flow cytometer (Cytoflex, Beckmann Coulter) and analysis using CytExpert software.

2.12 Immunoblotting

HUVEC were grown up to 70% confluency for high glucose treatment conditions. Medium was removed and cells were briefly washed with sterile 1X phosphate-buffered saline (PBS). RIPA buffer containing protease inhibitor was then used for protein extraction.

Cells were incubated in RIPA buffer for 1 h, followed by sonication. Cell lysates were centrifuged (10,000 rpm for 8 min), followed by collecting the supernatant and measuring protein concentration using Bradford reagent. Equal amounts of protein for different treatment groups were then applied in SDS-PAGE, followed by transferring the proteins onto a nitrocellulose membrane. Membranes were then blocked in 5% nonfat milk or 5% bovine serum albumin for 1 h. Membranes were then probed overnight at 4ºC with different primary antibodies. The blots were then incubated with a peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (1:2000) (# 7074 or #7076, respectively; Cell Signaling Technology). The antibody–antigen reactions were detected using the Clarity™ or Clarity™ MaxWestern Blotting ECL Substrates (Bio-Rad). Densitometry analysis was performed using ImageJ software. The primary antibodies used for probing of respective proteins and their dilution may be referred in **Table 2.12.1**.

2.13 Flow cytometric analysis of apoptotic cells using propidium iodide (PI) uptake

Propidium Iodide is a cell impermeable dye. Dead cells are able to retain the dye, while exclusively labeling only apoptotic cells. For the determination of apoptotis, cells were seeded in 24 well dishes at a density of $1 * 10^5$ cells/dish. The cells were treated with glucose with the desired IHG regime and a combination of IHG+GSK126 for a required period of time(72hrs). Thereafter, the cells were harvested, washed with PBS, and re-suspended in 500 µl of fresh PBS. To detect the percentage of dead cells, PI was added and incubated for 20 min in dark. Thereafter flow cytometric (Cytoflex, Beckmann Coulter) analysis was performed and the acquired data were analyzed using CytExpert software

2.14 Statistical analysis

All the values are expressed as the mean ± SD. All analysis data in bar graphs are presented as relative to control treatment condition. Statistical significance was determined by one-way ANOVA followed by Fisher least significant difference post hoc test for multiple groups comparison or by two-tailed Student's *t* test for comparison between two groups, unless otherwise stated. Statistical analyses were performed using GraphPad Prism software. A p value of less than 0.05 was considered statistically significant.

Antibody	**Catalogue number**
eNOS Rabbit mAb (1:500)	32027, CST
ICAM1 pAb (1:500)	4915, CST
EZH2 Rabbit mAb(1:1000)	5246, CST
H3K27me3 Rabbit mAb (1:1000)	9733, CST
p53 Mouse Ab(1:1000)	2524, CST
p21 Rabbit mAb (1:1000)	2947, CST
TRF2 Rabbit mAb (1:1000)	13136, CST
TERF2IP Rabbit mAb (1:1000)	5433, CST
TPP1 Rabbit mAb (1:1000)	14667, CST
p16 Rabbit mAb (1:1000)	92803, CST
Lamin B1 Rabbit mAb (1:1000)	13435, CST
TNFα Rabbit mAb (1:1000)	6945, CST
MMP3 Rabbit mAb (1:1000)	14351, CST
MLL1 Rabbit mAb (amino-terminal,1:1000)	14689, CST
MLL1 Rabbit mAb (Carboxy-terminal antigen,1:1000	14197, CST
MLL2 Rabbit mAb (Carboxy-terminal,1:1000)	63735, CST
IKKα Mouse Ab (1:1000)	11930, CST
NF-κB p65 Rabbit mAb (1:1000)	8242, CST
IκBα (1:1000)	4814, CST
Trimethyl H3K4 (1:1000)	9751, CST
KLF4 (1:1000)	4038, CST

KLF2 Rabbit mAb (1:500)	PA5-40591, Invitrogen
TRF1 Rabbit mAb (1:1000)	04-638-25 UG, Merck
PhosphoEZH2 (Thr367) Rabbit pAb	PA5-106225, CST

Table 2.12.1 – Antibodies used in immunoblotting

Chapter 3

Intermittent High Glucose Elevates Nuclear Localization of EZH2 to Cause H3K27me3-Dependent Repression of KLF2 Leading to Endothelial Inflammation

3.1 INTRODUCTION

The vascular endothelium maintains homeostasis by acting as a cellular lining of the circulatory system, and dysfunction of these cells leads to many pathophysiologies, including vasoconstriction and atherosclerosis [7]. Endothelial dysfunction is a common finding in patients affected by diabetes and hyperglycemic conditions that damage the endothelial lining of blood vessels, causing vascular complications [142]. Such dysfunction of the endothelium upon hyperglycemia is primarily manifested by a reduction in protective genes and expression of inflammation-associated genes in endothelial cells (EC) [143]. Hyperglycemic conditions in type 2 diabetes have been shown to cause epigenetic disturbances in many tissue types, including pancreatic islets, adipose tissue, skeletal muscle, and vascular tissues [144–148]. EC exposed to hyperglycemia exhibited heightened enrichment of di- and tri-methylated histone H3 lysine4 (H3K4me2/3) on the MCP-1 promoter [48]. Such an increase in H3K4me2/3 was due to an increase in the expression level of histone methyltransferase MLL3, menin, and SET7, along with a concomitant reduction in the demethylase LSD1. In parallel, Set7 was found to stimulate nuclear factor kappa-light chain- enhancer of activated B cells (NF-κB) dependent oxidative and inflammatory signaling by mono-methylation of H3K4 at its promoter in human aortic endothelial cells [149]. In an independent study, H3K9-specific demethylase JHDM2A (also known as KDM3A) was shown to be involved in regulating the expression of metabolic genes, indicating epigenetic regulation as key to diabetes-dependent microvascular complications [150]. The work of El-Osta et al.,2008 demonstrated a dynamic and coordinated alteration in H3K9/K14 acetylation and methylation of histones H3K4 that was responsible for p65-dependent inflammatory switch of EC [48]. Interestingly, the hyperglycemia-driven induction of gene expression that is responsible for endothelial dysfunction occurred through inversely modulating the acetylation of H3K9/K14 and methyl-CpG content of DNA [44]. Histone methyltransferase EZH2 is the enzymatic subunit of PRC2 and is primarily responsible for catalysis of H3K27me3, which finally executes PRC2-dependent gene repression through heterochromatinization [151]. EZH2 and its product H3K27me3 are critical in regulating signaling pathways responsible for development and diseases. In a recent study, human umbilical vein EC isolated from gestational diabetes mellitus-affected individuals (also referred as GDM-HUVECs)

displayed impaired endothelial function via miR-101 upregulation and a concurrent reduction in EZH2 and H3K27me3 levels [152]. Although, said study described the role of EZH2 in maintaining endothelial homeostasis and function, it failed to narrate the exact underlying mechanisms through which EZH2 engendered an inflammatory state of EC upon high glucose challenge.

Previous studies established that intermittent or oscillating glucose levels, such as those detected in diabetic individuals, have more deleterious effects than constant high glucose has on endothelial function and oxidative stress [153,154]. Intermittent high glucose exposure to HUVEC caused activation of protein kinase C and NAD(P)H-oxidase, leading to oxidative stress and further promotion of endothelial apoptosis [153,154]. Furthermore, individuals with type 2 diabetes showed heightened oxidative stress and impairment of endothelial function when challenged with an oscillating glucose level [16,17]. Therefore, altogether, these findings suggest a stronger association between endothelial oxidative stress and dysfunction and intermittent/oscillating glucose level. Transient hyperglycemia induces long-lasting activation of epigenetic changes in the promoter of the nuclear factor p65 in aortic endothelial cells, both in vitro and in nondiabetic mice, which cause increased p65 gene expression [48]. A recent study by Flynn et al. described transient intermittent hyperglycemia as causing proliferation of monocytes by promoting myelopoiesis through the RAGE pathway in atherosclerosis [155]. Although inflammation-dependent epigenetic alteration upon hyperglycemic exposure is well studied in many cell types, the epigenetic mechanism that is responsible for high glucose-dependent endothelial inflammation is yet to be investigated. In this paper, we aimed to study the role of epigenetic regulation through EZH2–PRC2 pathways in intermittent high glucose-exposed EC and to further unravel the underlining mechanisms that facilitate inflammatory signaling in EC.

3.2 RESULTS

3.2.1 Intermittent High Glucose Treatment Maximally Induced Endothelial Inflammation In Vitro and Ex Vivo

Intermittent or oscillating glucose causes more deleterious effects on endothelial function than constant high glucose, especially on its oxidative balance [153,154]. Therefore, we first undertook experiments to evaluate the effectivity of intermittent high glucose in inducing endothelial inflammatory signaling compared with constant or transient high glucose treatment conditions. Initially, to determine the treatment conditions, we assessed the viability of HUVEC that were exposed to differential high glucose treatment conditions. Although subtle changes in cell viability were detected in some of the high glucose treatment conditions, such differences were statistically insignificant (**Fig 3.2.1 A**). To analyze endothelial inflammation, we then measured the transcript level of endothelial inflammatory cell adhesion molecule ICAM1 in cells exposed to the differential high glucose challenge. Cells in the intermittent high glucose treatment condition, where HUVEC were incubated in 12 h high and normal glucose cycles, exhibited the highest transcript-level expression of ICAM1 (**Fig 3.2.1 B**). Based on these observations, we next shortlisted three unique high glucose treatment regimens — constant high glucose (72 h of constant high glucose), intermittent high glucose (12 h high and normal glucose cycles for three cycles), and transient high glucose (24 h high glucose followed by 48 h of normal glucose)-or comprehensive analysis of endothelial inflammatory markers. Transcript-level expression of ICAM1, VCAM1, and P-selectin were carried out. A significant increase in the transcript level of ICAM1 and P-selectin were detected in cells exposed to intermittent high glucose, while no changes in ICAM1 were detected in constant or transient high glucose-treated cells (**Fig 3.2.1 A,B**). We did not detect any changes in VCAM1 transcript level in any of the treatment conditions (data not shown). We then measured the protein-level expression of ICAM1 and eNOS and further observed a significant increase in ICAM1 expression and reduction in eNOS level in cells treated with intermittent high glucose, while no changes in these proteins were detected in EC exposed to constant or transient high glucose relative to normal glucose-incubated HUVEC (**Fig 3.2.1 C**).To exclude the possibility that the intermittent high glucose effect on endothelial inflammation

was due to osmolality changes, we simultaneously analyzed these markers in HUVEC exposed to intermittent mannitol, which generates a comparable osmolality that is similar to intermittent high glucose. We did not observe any alteration in eNOS (**Fig 3.2.1.1 A**) and ICAM1 (**Fig 3.2.1.1 B**) protein level in EC challenged with intermittent mannitol. which generates a comparable osmolality that is similar to intermittent high glucose. We did not observe any alteration in eNOS (**Fig 3.2.1.2 A**) and ICAM1 (**Fig 3.2.1.2 B**) protein level in EC challenged with intermittent mannitol. To confirm such in vitro findings in an animal model, we isolated rat aortas and exposed them to the intermittent high glucose treatment condition ex vivo. Through such experiments, we observed a reduction in eNOS level and a concurrent increase in ICAM1 expression in aortas exposed to intermittent high glucose (**Fig 3.2.1 D**). We also confirmed the presence of EC in the rat aortic tissue lysate by detecting the presence of CD144, an endothelial-specific cell-surface marker.

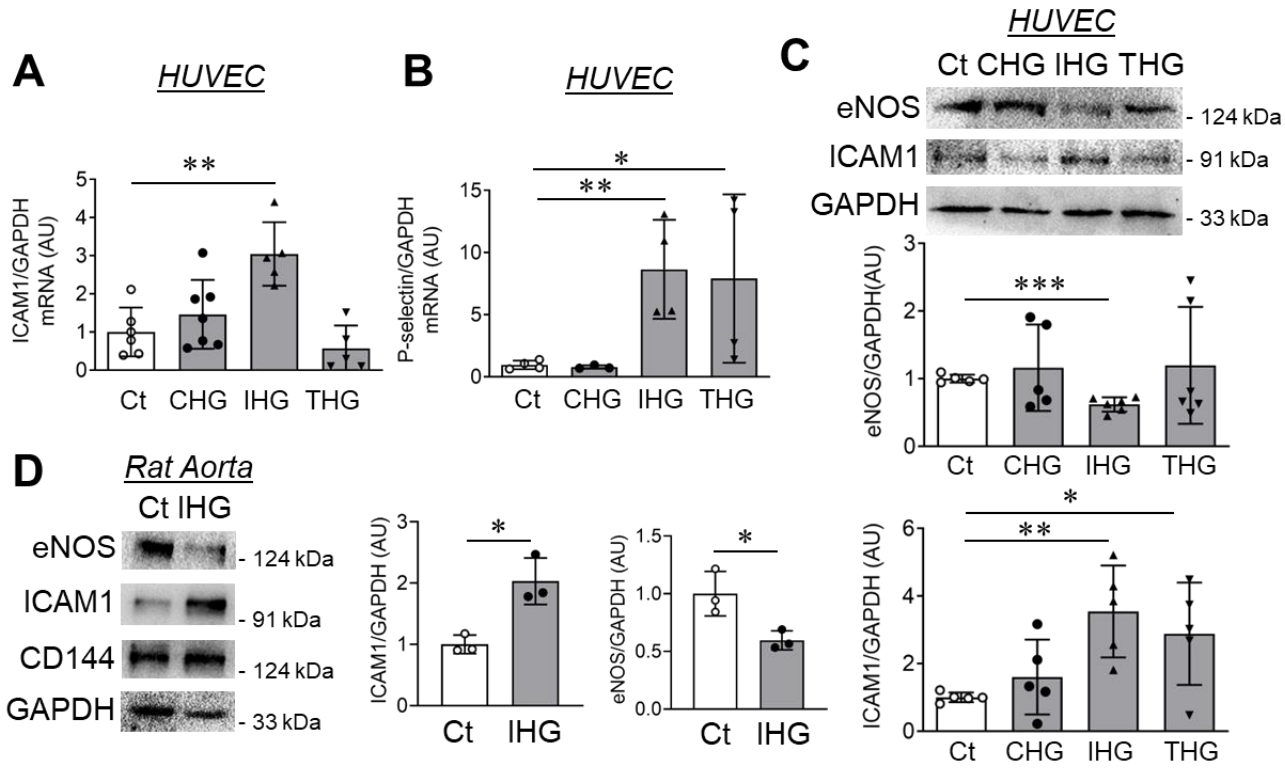


Fig 3.2.1. Intermittent hyperglycemia triggers an inflammatory state of endothelial cells. *(**A**, **B**) Transcriptomic analysis of ICAM1 ((**A**), n = 5) and P-selectin (**B**), n = 4–6) in HUVEC exposed to differential high glucose treatment conditions; control, constant (72 h high glucose, 25 mM), intermittent (alternating 5.5 mM and 25 mM cycle of 12 h for 3 transient (24 h high glucose, 25 mM, followed by 48 h of normal glucose, 5.5 mM). (**C**)*

*Immunoblotting and quantitation for eNOS and ICAM1 in HUVEC challenged with previously mentioned high glucose treatment conditions (n = 5–6). (**D**) Immunoblotting and quantitation of eNOS, ICAM1, and CD144 in tissue lysates of rat aortic rings (2 mm length) that were exposed to intermittent high glucose (n = 3). Control treatment condition (cells constantly exposed to normal glucose (5.5 mM)) represented as "Ct" in the figure. All analyzed data were normalized to the control treatment condition. All analyzed data were normalized to the control treatment condition. Values represent the mean ± SD. * p < 0.05, ** p < 0.01 and *** p < 0.001 by one-way ANOVA analysis or by unpaired t test.*

Ct- Normal glucose (5.5mM)

72- 72 hours high glucose (25mM)

8/16- Three cycles of 8 hours high glucose (25mM) 16 hours normal glucose (5.5mM)

12/12- Three cycles of 12 hours high glucose (25mM) 12 hours normal glucose (5.5mM) 4/68 - 4 hour high glucose (25mM) 68 hours normal glucose (5.5mM)

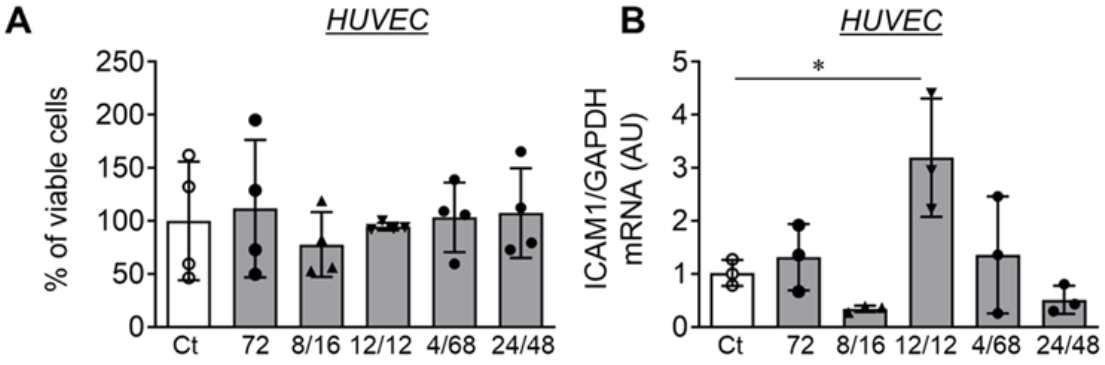


Fig 3.2.1.1. Differential high glucose treatment condition did not alter the viability of cells while specific intermittent high glucose treatment induced ICAM1 expression. *(**A**) Cell viability of HUVEC was detected using MTT that are exposed to differential high glucose treatment condition as mentioned in the figure. (**B**) ICAM1 transcript level was detected in HUVEC challenged with high glucose treatment condition as mentioned in the figure. All analyzed data were normalized to control treatment condition. Values represent the mean ± SD. *P < 0.05 by unpaired t test.*

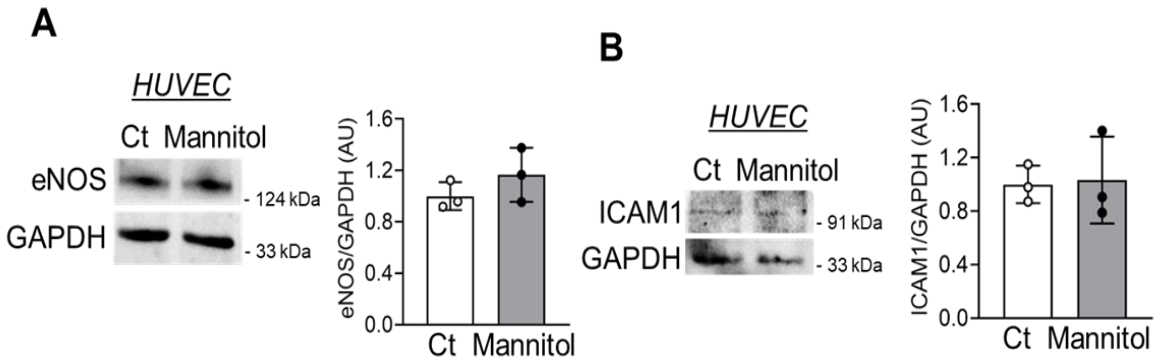


Figure 3.2.1.2. Intermittent mannitol treatment that imparts similar level of osmolality as like intermittent high glucose did not alter inflammation associated genes in EC. ***(A-B)*** *Immunoblotting and quantification of eNOS (**A**), ICAM1 (**B**) in HUVEC under exposure to intermittent high glucose (12 hours normal (5.5mM) and 12 hours high glucose (25mM) in three cycles) n=3.*

3.2.2 Intermittent High Glucose Causes Nuclear Localization of EZH2 through Its Threonine 367 Phosphorylation, Thereby Elevating H3K27me3 Level

Once we established that intermittent high glucose was most potent in causing the inflammatory switch of EC, we next undertook experiments to evaluate the underlining epigenetic mechanisms. Alterations in epigenetic mechanisms are prevalent in different cells exposed to hyperglycemic conditions. In a recent study, hyperglycemia was shown to promote SIRT6 and TETs, in turn causing dynamic changes in 5 methyl cytosine and 5 hydroxy methyl cytosine in WBCs collected from diabetes mellitus type 2 (T2DM) patients [156]. In addition, EC exposed to constant hyperglycemia exhibited heightened enrichment of di- and tri-methylated histone H3 lysine4 (H3K4me2/3) on MCP-1 promotor [157] through histone methyltransferase MLL3, menin and SET7. We therefore questioned whether intermittent high glucose alters the epigenetic landscape of EC through regulation of EZH2 and its product, H3K27me3. Immunoblot analysis of H3K27me3 and EZH2 in HUVEC treated with different high glucose treatment conditions revealed increased H3K27me3 in cells exposed to intermittent high glucose (**Fig 3.2.2 A**). Surprisingly, we did not detect any changes in the methyltransferase EZH2 (**Fig 3.2.2 B**) or H3K27me3-associated demethylase UTX (**Fig 3.2.2.2 A,B**) and JMJD3 (**Fig 3.2.2.2 C,D**) in EC exposed to intermittent high glucose. Unlike intermittent high glucose,

intermittent mannitol treatment (considered as osmolality control) in EC did not alter cellular H3K27me3 levels (**Fig 3.2.2.1**). Considering that EZH2 having a dispersive localization within cells and that its cytosolic localization was shown to execute certain cytosolic functions [158], we further examined the localization of EZH2 protein in EC exposed to intermittent high glucose. Intermittent high glucose treatment enhanced the nuclear localization of EZH2 in EC (**Fig 3.2.2 D**). As the studies up to now describe that p38-dependent phosphorylation of EZH2 protein at threonine 367 (pThr367EZH2) causes its cytosolic localization [159], we determined the level of pThr367EZH2 in EC exposed to intermittent high glucose. Through such analysis, we detected a significant reduction in endothelial pThr367EZH2 upon intermittent high glucose treatment (**Fig 3.2.2 E**), thereby allowing EZH2 nuclear translocation in EC upon such treatment condition. Furthermore, we next confirmed these in vitro observations in an ex vivo model of rat aortic rings, which indicated a robust increase in H3K27me3 (**Fig 3.2.2 F**) level without altering the expression level of EZH2 (**Fig 3.2.2 G**) upon intermittent high glucose exposure.

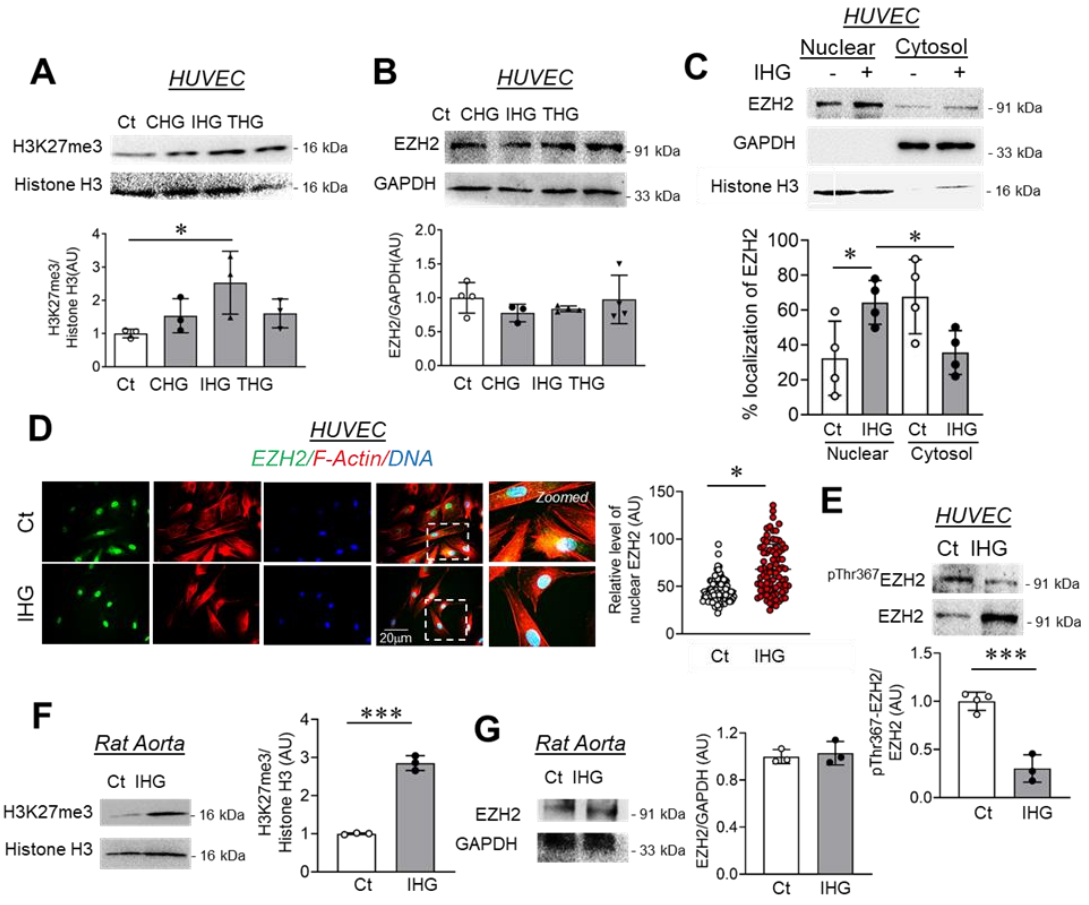


Fig 3.2.2. Intermittent high glucose induces nuclear localization of EZH2 through de-phosphorylation of threonine 367. *(**A**,**B**) Immunoblotting and quantitation of differential hyperglycemia-treated HUVEC lysates for H3K27me3 ((**A**), n = 3) and EZH2 ((**B**), n = 4). (**C**) Subcellular fractionation, immunoblotting, and quantitation of nuclear and cytosolic level of EZH2 in intermittent high glucose-challenged HUVEC (n = 4). (**D**) Immunofluorescence and quantitation of HUVEC for EZH2 (green) after intermittent high glucose treatment. F-actin staining through phalloidin shown in red and DAPI staining shown in blue. Fluorescence intensity values taken for individual cells are each indicated by dots, and at least n > 100 cells were analyzed per group from three independent experiments. (**E**) Immunoblotting and quantitation of threonine 367 phosphorylated EZH2 in intermittent high glucose-exposed HUVEC (n = 4). (**F**,**G**) Tissue lysates of intermittent high glucose-treated rat aortic rings were immunoblotted and quantified for H3K27me3 (**F**) and EZH2 (**G**) (n = 3). Control treatment condition (cells constantly exposed to normal glucose (5.5 mM)) represented as "Ct" in the figure. All analyzed data were normalized to the control treatment condition. Values represent the mean ± SD. * p < 0.05 and *** p < 0.001 by unpaired t test.*

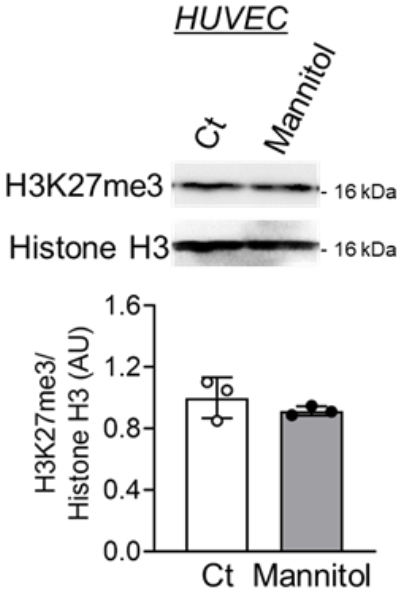


Figure 3.2.2.1. Immunoblotting and quantification of H3K27me3 (C) in HUVEC under exposure to intermittent high mannitol *(12 hours normal (5mM) and 12 hours high glucose (25mM) in three cycles) (n=3). Values represent the mean ± SD.*

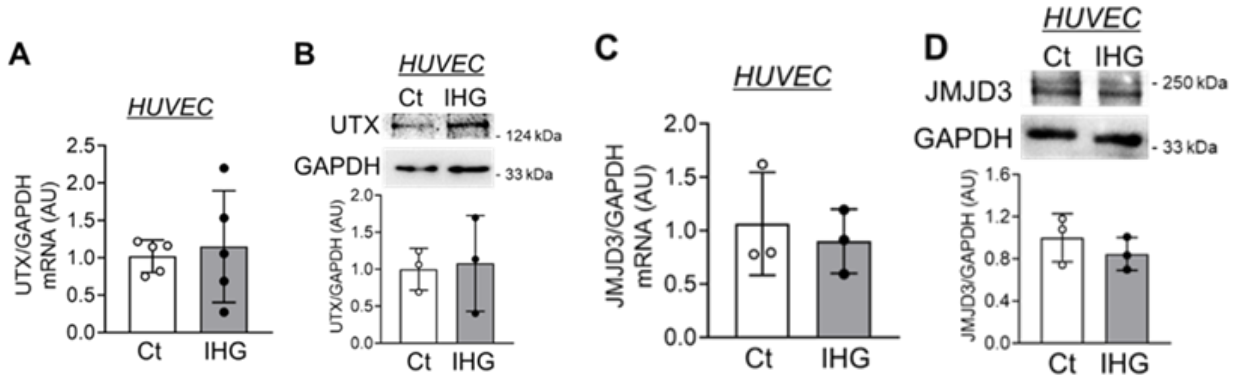


Figure 3.2.2.2. Intermittent hyperglycemia does not alter the expression of Jumonji C domain containing H3K27me3 demethylase UTX and JMJD3. *(**A-D**) Real time PCR for UTX transcript (**A**, n=5) and JMJD3 transcript (**C**, n=3) and subsequent immunoblot and quantification of UTX protein (**B**, n=3) and JMJD3 protein (**D**, n=3) in HUVEC treated with intermittent high glucose (alternating 5.5mM and 25mM cycle of 12 hours for three cycles). Values represented as mean ± SD.*

3.2.3 In Endothelial Cells Treated with Intermittent High Glucose, Nuclear EZH2 Assembles PRC2 That Endorses H3K27me3 Enrichment on KLF2 and KLF4 Promoters and Further Suppresses Their Expression

EZH2, along with other proteins including EED and Suz12, assembles PRC2 that catalyzes the H3K27me3 [160]. As an increase in the level of nuclear EZH2 was detected in EC exposed to intermittent high glucose, we wondered whether such elevated nuclear EZH2 assembles PRC2 to cause gene-specific H3K27me3 enrichment. To address this, we performed a co-immunoprecipitation experiment to evaluate the assembly of the PRC2 complex. By doing so, we detected a greater association of EED and Suz12 with EZH2 protein upon intermittent high glucose exposure (**Fig 3.2.3 A, IP panel**). Furthermore, in intermittent high glucose-treated EC, EZH2 was also found to be robustly associated with histone H3 (**Fig 3.2.3 A**). Immunoblotting of total cell lysate revealed unaltered protein expression of EED and Suz12 upon intermittent high glucose exposure (**Fig 3.2.3 A, input panel**). Transcription factors KLF2 [24,25] and KLF4 [161] are essential for maintaining endothelial homeostasis [162] and coordinate a gene expression pattern that inhibits the inflammatory switch of EC. Because we observed an elevated

level of endothelial inflammation and concurrent increase in H3K27me3 level, we therefore questioned whether such increase in H3K27me3 may affect KLF2 and KLF4 genes through regulation of their corresponding gene promoter regions. To address this, we then performed a ChIP-qPCR assay to confirm the enrichment of H3K27me3 in KLF2 and KLF4 gene promoters in intermittent high glucose-exposed EC. We detected a more than 8-fold enrichment (although such increase in enrichment was found to be statistically insignificant due to large variation between the replicates, $p = 0.058$) of H3K27me3 at the KLF2 gene promoter in intermittent high glucose-treated EC (**Fig 3.2.3 B**), while the same treatment condition caused a nearly 5-fold increase in H3K27me3 enrichment at the KLF4 gene promoter region (**Fig 3.2.3 C**). Since we observed elevated promoter-level enrichment of H3K27me3 on KLF2 and KLF4 gene promoters in EC challenged with intermittent high glucose, we next detected the transcript and protein-level expression of KLF2 and KLF4. We detected significant depletion in KLF2 (**Fig 3.2.3 D**) and KLF4 (**Fig 3.2.3 E**) mRNA in EC exposed to intermittent high glucose. Further, we observed that intermittent high glucose exposure led to a significant reduction in KLF2 (**Fig 3.2.3 F**) and KLF4 (**Fig 3.2.3 G**) protein levels. We did not detect any changes in KLF2 (**Fig 3.2.3.1 A**) and KLF4 (**Fig 3.2.3.1 B**) proteins in EC exposed to intermittent mannitol treatment. We also determined the expression level of KLF2 and KLF4 in tissue lysates collected from intermittent high glucose-challenged rat aortic rings and detected a significant reduction in KLF2 protein level (**Fig 3.2.3 H**) and an unaltered KLF4 protein level (**Fig 3.2.3 I**). Such observation of unaltered KLF4 protein level in intermittent high glucose-exposed rat aortic rings could likely be due to the complexity of the tissue and with the presence of other cells types, including smooth muscle cells that predominantly express KLF4 [163].

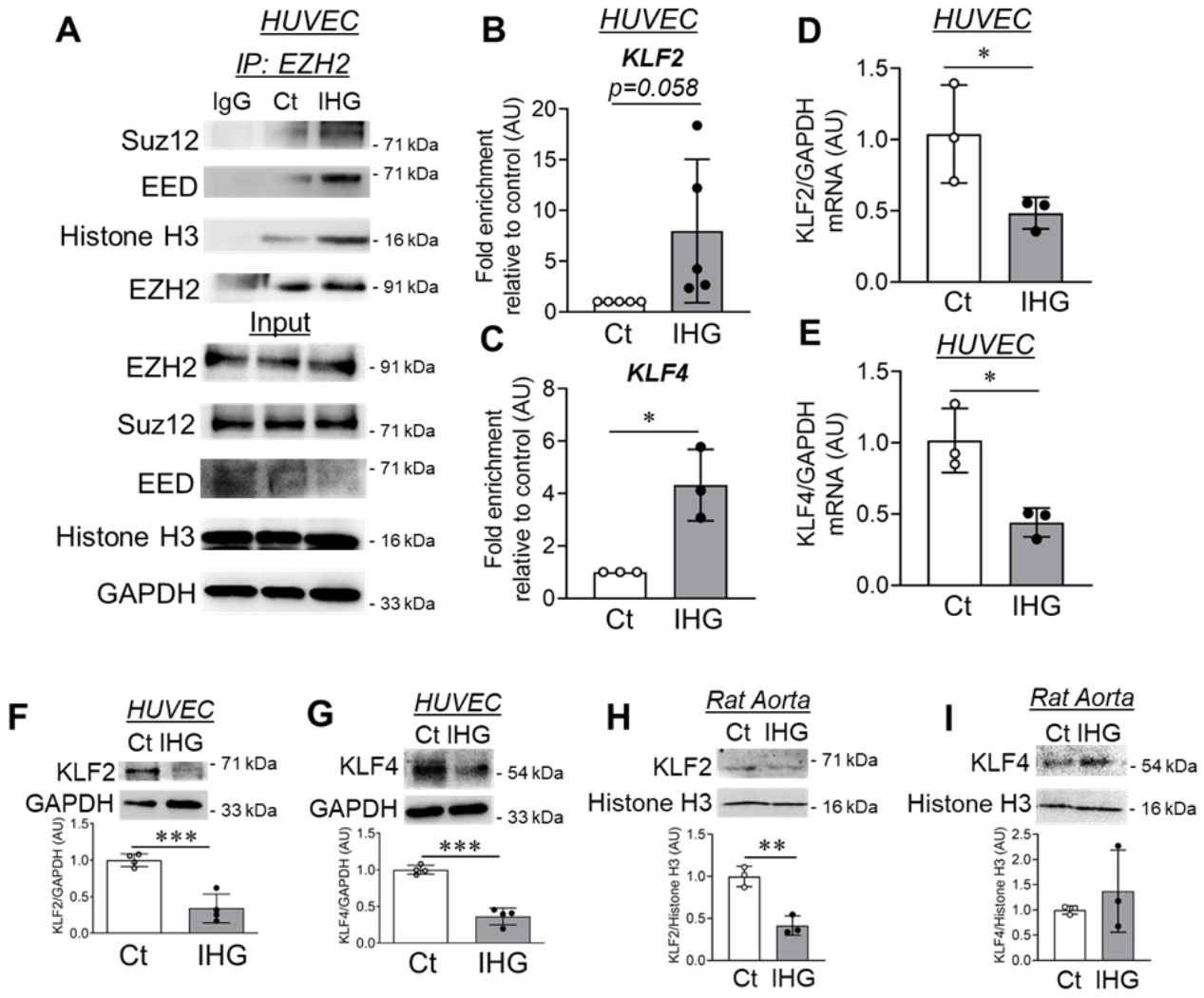


Fig 3.2.3. Intermittent hyperglycemia causes EZH2 coupling with PRC2 proteins, leading to H3K27 trimethylation at promoter regions of the KLF2 and KLF4 genes. *(**A**) Co-immunoprecipitation with EZH2 antibody, followed by immunoblotting for Suz12, EED, and histone H3 in both immuno-precipitated and total cell lysate (input) sample (n = 3). (**B**,**C**) ChIP assay using H3K27me3 antibody, followed by qPCR using primers specific to amplify promoter regions of KLF2 gene ((**B**), n = 5) and KLF4 gene ((**C**), n = 3) in HUVEC cells treated with intermittent high glucose. (**D**,**E**) Transcriptomic quantitation of KLF2 ((**D**), n = 3) and KLF4 ((**E**), n = 3) in HUVEC challenged with intermittent high glucose. (**F**,**G**) Immunoblotting and quantitation for KLF2 ((**F**), n = 4) and KLF4 ((**G**), n = 4) in intermittent high glucose-challenged HUVEC. (**H**,**I**). Tissue lysates of intermittent high glucose-treated rat aortic rings were immunoblotted for KLF2 ((**H**), n = 3) and KLF4 ((**I**), n = 3). Control treatment condition (cells constantly exposed to normal glucose (5.5*

*mM)) represented as "Ct" in the figure. All analyzed data were normalized to the control treatment condition. Values represent the mean _ SD. * p < 0.05, ** p < 0.01, and *** p < 0.001 by unpaired t test.*

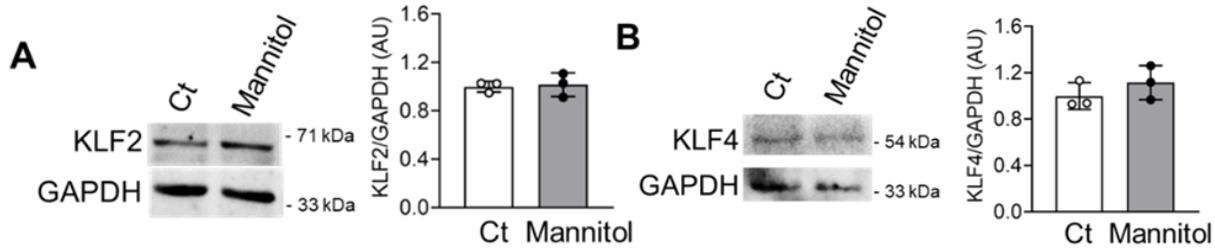


Fig 3.2.3.1. Intermittent mannitol treatment that imparts similar level of osmolality as like intermittent high glucose did not alter homeostatic genes in EC. *(**A-B**) Immunoblotting and quantification of KLF2 (**A**), KLF4 (**B**) in HUVEC under exposure to intermittent high glucose (12 hours normal (5.5mM) and 12 hours high glucose (25mM) in three cycles) n=3.*

3.2.4. Inhibition of EZH2 Methyltransferase Activity or siRNA-Mediated Knockdown of EZH2 Reverses Intermittent High Glucose-Dependent Endothelial Inflammation

Because we established that nuclear EZH2 assembles PRC2 to cause an H3K27me3-dependent decrease in KLF2 and KLF4 level, we therefore investigated whether inhibition of EZH20s methyltransferase activity reverses the endothelial inflammatory phenotype upon intermittent high glucose exposure. To explore this possibility, we took advantage of a selective inhibitor of EZH2, GSK126. We detected no change in viability of HUVEC that were exposed to 10 μM of GSK126 (**Fig 3.2.4.2 A**). To address the non-specific effect of GSK126, we also performed a comprehensive analysis of GSK126 effects on global histone H3 tri-methylation at different locations—lysine 4, lysine 9, lysine 27, lysine 36, and lysine 79—to understand its non-specific effect on different methyltransferases catalyzing tri-methylation in these specific sites. With EZH2, being the specific target of GSK126, we observed a significant reduction in H3K27me3, while the level of H3K4me3, H3K9me3, H3K26me3, and H3K79me3 majorly remained unaltered in both HUVEC (**Fig 3.2.4.2 B–F**) and HUVEC-derived transformed cell line EA.hy926 cells (**Fig 3.2.4.2 G–K**).

GSK126 exposure further caused a complete inhibition of H3K27me3 content in HUVEC exposed to intermittent high glucose (**Fig 3.2.4 A**). Coincident with the reduction in H3K27me3 levels, KLF2 mRNA (Figure 3.2.4 B) and protein level (**Fig 3.2.4 C**) were elevated in HUVEC that were co-treated with intermittent high glucose and GSK126 combination in comparison with only the intermittent high glucose-treated group. Correspondingly, in the presence of GSK126, intermittent high glucose exposure failed to diminish eNOS protein level (**Fig 3.2.4 D**) and to increase in ICAM1 protein level (**Fig 3.2.4 E**). Next, we took an alternative molecular approach to inhibit EZH2 by using specific siRNA and studied the inflammatory effect of intermittent high glucose on HUVEC with diminished EZH2 level. We then detected a significant reduction in EZH2 protein level (**Fig 3.2.4.1 A**) upon EZH2- specific siRNA transfection, with a concurrent reduction in H3K27me3 (**Fig 3.2.4.1 C**). Such reduction in EZH2 protein level was also apparent in cells that were exposed to intermittent hyperglycemia (**Fig 3.2.4.1 B**). Furthermore, intermittent high glucose-dependent reduction in KLF2 (**Fig 3.2.4.1 D**) and eNOS (**Fig 3.2.4.1 E**) protein levels were reversed in HUVEC transfected with EZH2 siRNA. In contrast, elevated ICAM1 protein level in intermittent high glucose-exposed cells was found to be diminished upon EZH2 knockdown (**Fig 3.2.4.1 F**).

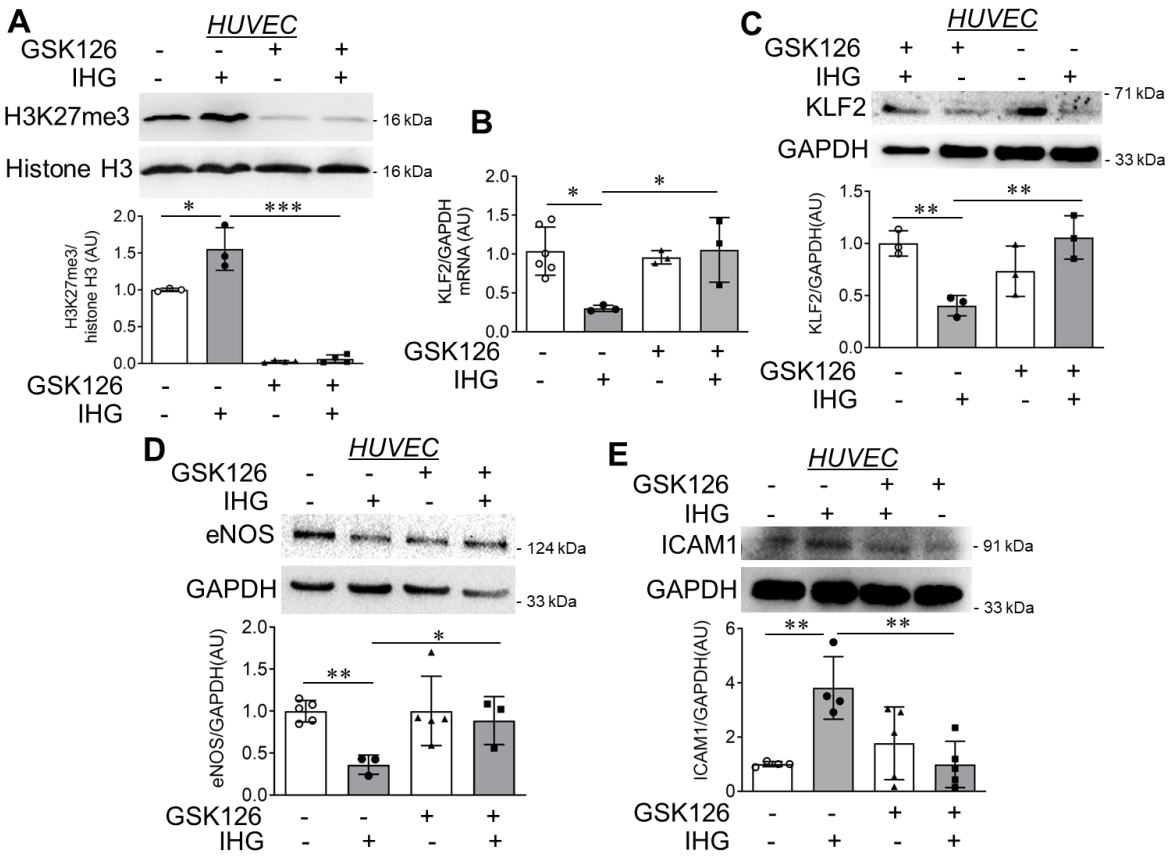


Fig 3.2.4. Small molecule-mediated inhibition of EZH2 in intermittent high glucose-exposed HUVEC decreases H3K27me3 level and blocks the inflammatory switch of EC. *(**A**) Immunoblotting for H3K27me3 (n = 3) in GSK126-treated and intermittent high glucose-exposed HUVEC. (**B**) KLF2 mRNA (n = 3) expression in HUVEC challenged with intermittent high glucose (alternating 5.5 mM (normal glucose) and 25 mM (high glucose) cycle of 12 h for 3 cycles) in combination with GSK126 (10 μM for 72 h). (**C**–**E**) KLF2 (**C**), eNOS (**D**), and ICAM1 (**E**) in cultured HUVEC treated with the EZH2 inhibitor GSK126 (10 μM for 72 h) in combination with intermittent high glucose (n = 3). Cells continuously exposed to normal glucose (5.5 mM) were considered as the non-intermittent high glucose treatment condition. All analyzed data were normalized to the control treatment condition. Values represent the mean ± SD. * p < 0.05, ** p < 0.01, and *** p < 0.001 by unpaired t test.*

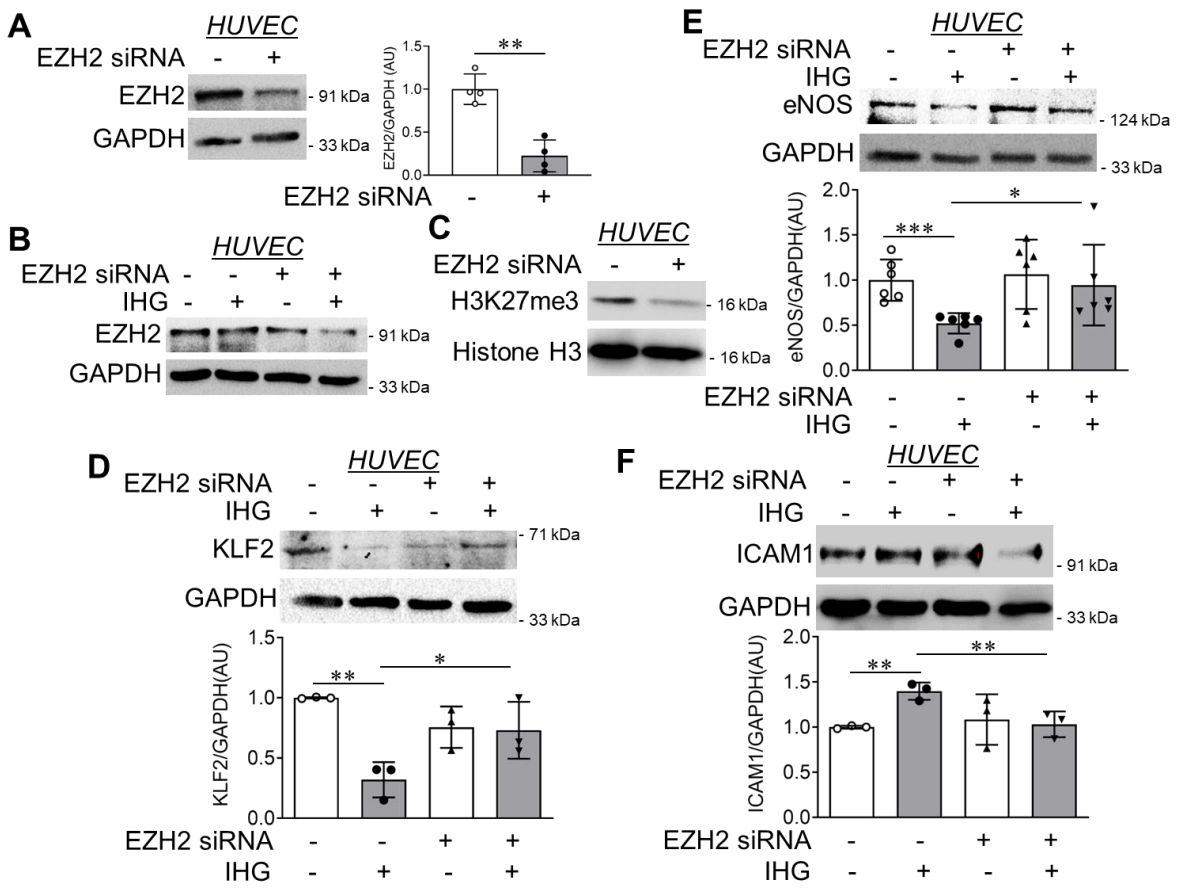


Fig 3.2.4.1. EZH2 knockdown in HUVEC reduces H3K27me3 and reverses intermittent high glucose-driven endothelial inflammation. *(**A**–**E**) Immunoblotting of EZH2 in normal glucose (5.5 mM)-treated HUVEC ((**A**), n = 4), EZH2 in both normal glucose (5.5 mM) and intermittent high glucose-treated HUVEC ((**B**), n = 3), H3K27me3 ((**C**), n = 3) and quantification of KLF2 ((**D**), n = 3), eNOS ((**E**), n = 6), and ICAM1 ((**F**), n = 3) in HUVEC cells transiently transfected with scrambled or EZH2 siRNA, followed by challenging with intermittent high glucose. Cells continuously exposed to normal glucose (5.5 mM) were considered as the non-intermittent high glucose treatment condition. All analyzed data were normalized to the control treatment condition. Values represent the mean ± SD. * p < 0.05, ** p < 0.01, and *** p < 0.001 by unpaired t test.*

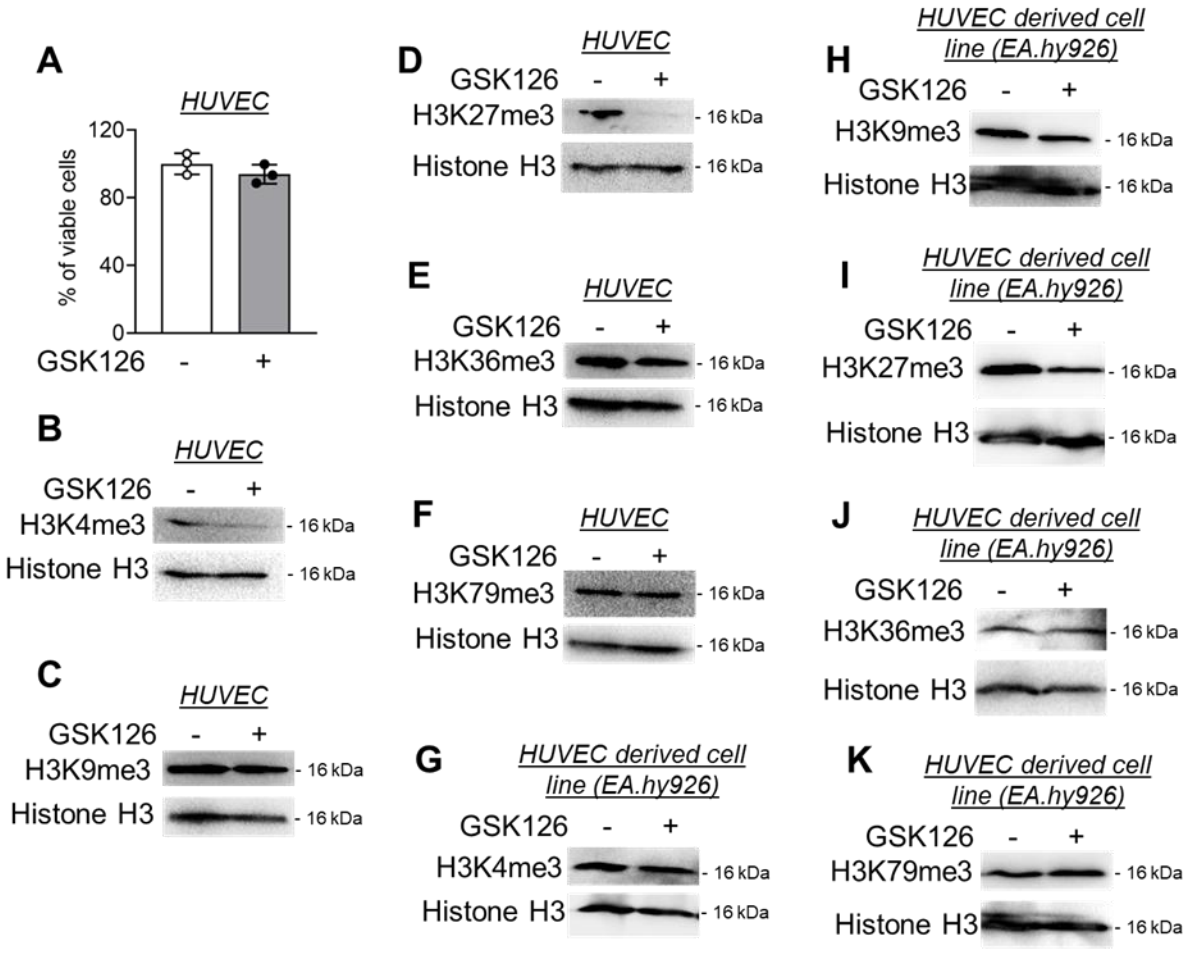


Fig 3.2.4.2. GSK126 exposure to endothelial cells did not affect its viability and specifically caused reduction of H3K27me3. *(**A**) Cell viability of HUVEC was detected using MTT that are exposed to GSK126 (10μM) for 72 hours. (**B-F**) Relative level of H3K4me3 (**B**), H3K9me3 (**C**), H3K27me3 (**D**), H3K36me3 (**E**) and H3K79me3 (**F**) were measured in HUVEC treated with GSK126 (10μM) for 72 hours. (**G-K**) Relative level of H3K4me3 (**G**), H3K9me3 (**H**), H3K27me3 (**I**), H3K36me3 (**J**) and H3K79me3 (**K**) were measured in EA.hy926 treated with GSK126 (10μM) for 72 hours. Values represented as mean ± SD.*

3.2.5. Inhibition of EZH2 Blocks Intermittent High Glucose-Driven Endothelial Inflammation in Ex Vivo Rat Aortic Ring Model

Having found that intermittent high glucose promoted endothelial inflammation in cultured EC, while EZH2 inhibition or knockdown blocked changes in such inflammatory pathways, we next performed an EZH2 small molecule inhibition study to assess its efficacy in attenuating endothelial inflammation in rat aortic rings that were challenged with intermittent hyperglycemia. Treatment with GSK126 robustly inhibited an intermittent high glucose-dependent increase in H3K27me3 level (**Fig 3.2.5 A**). Next, by measuring endothelial inflammatory markers in the treated rat aortic rings, we found that intermittent high glucose significantly abrogated total KLF2 (**Fig 3.2.5 B**) and eNOS (**Fig 3.2.5 C**) protein, while increasing ICAM1 level (**Fig 3.2.5 D**). In contrast, when EZH2 was inhibited in these conditions, intermittent high glucose treatment failed to impart its effect on protein-level expression changes of KLF2, eNOS, and ICAM1 (**Fig 3.2.5 B–D**).

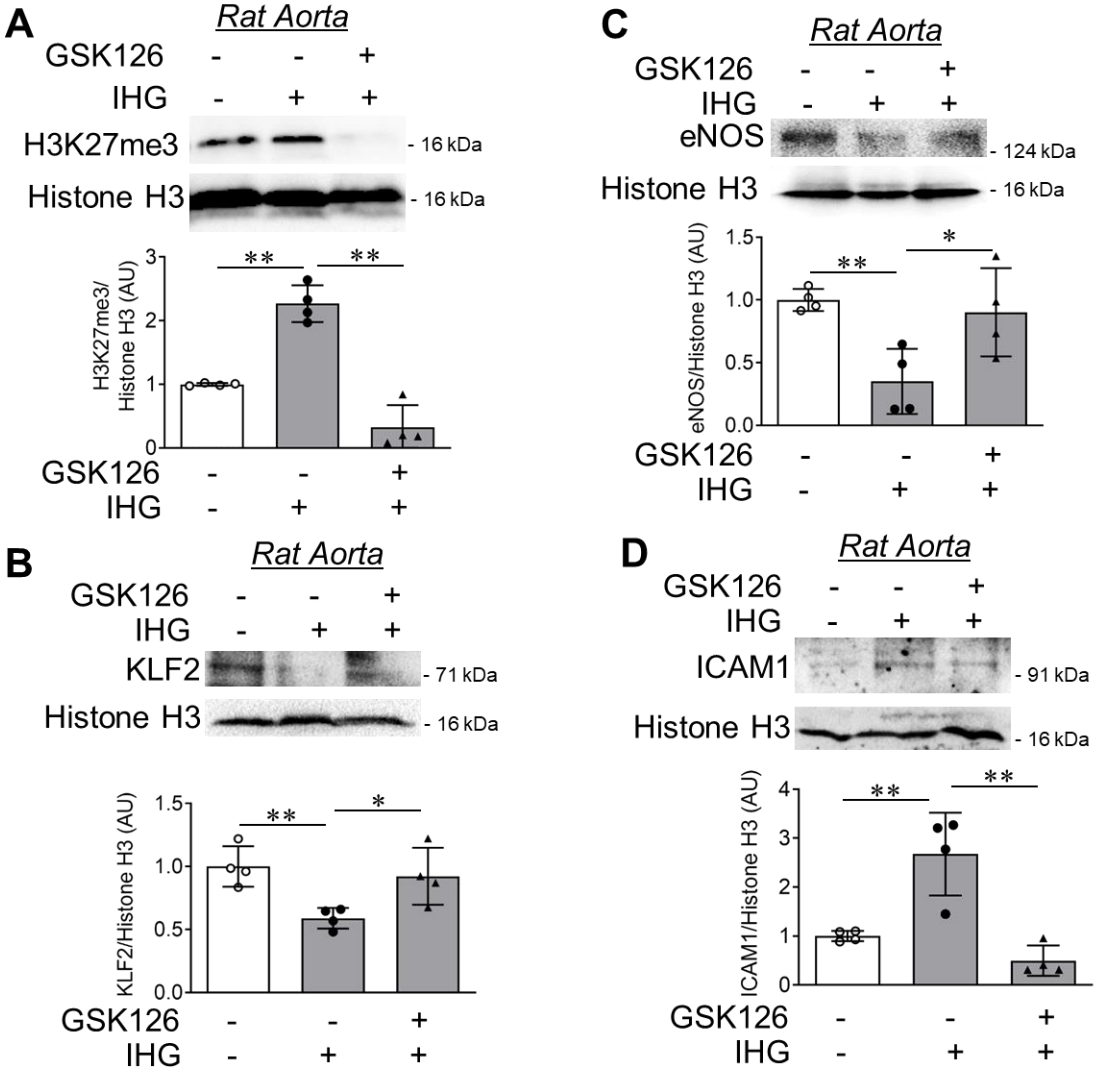


Fig 3.2.5. Inhibition of EZH2 in rat aortic rings exposed to intermittent hyperglycemia reduces H3K27me3 levels and blocks inflammatory signaling ex vivo. *(**A**–**D**) Immunoblotting for H3K27me3 ((**A**), n = 4), KLF2 ((**B**), n = 4), eNOS ((**C**), n = 4), and ICAM1 ((**D**), n = 4) in rat aortic rings challenged with the EZH2 inhibitor GSK126 (10 μM for 72 h) in combination with intermittent high glucose. Tissues continuously exposed to normal glucose (5.5 mM) were considered as the non-intermittent high glucose treatment condition. All analyzed data were normalized to the control treatment condition. Values represent the mean ± SD. * p < 0.05 and ** p < 0.01 by unpaired t test.*

3.3 DISCUSSION

Epigenetic regulation of gene expression has emerged as an essential mechanism for regulating endothelial function during development and in diseases. Post-translational modification of histone proteins is one the many epigenetic mechanisms that contribute to the differential gene expression pattern responsible for endothelial dysfunction. For instance, trimethylation of lysine residue 27 on histone H3 by methyltransferase EZH2 at the promoter region of target genes may lead to transcriptional silencing. EZH2-mediated regulation of gene expression through its H3K27me3 catalytic activity is well known to control the pathogenesis of different diseases; however, how such mechanisms drive endothelial inflammation and its dysfunction upon hyperglycemia challenge is not clearly understood. Here, we describe that intermittent high glucose exposure predominantly causes a robust inflammatory switch of EC through modulation of histone methylation. In specific, it alters the cellular level of H3K27me3 by promoting EZH2 nuclear localization and PRC2 assembly, followed by PRC20s recruitment to histone H3. Such recruitment of PRC2 to histone H3 causes an abrupt enrichment of H3K27me3 in the gene promoters of two important transcription factors essential for endothelial function—KLF2 and KLF4—thereby causing their repression (**Fig 3.3.1**).

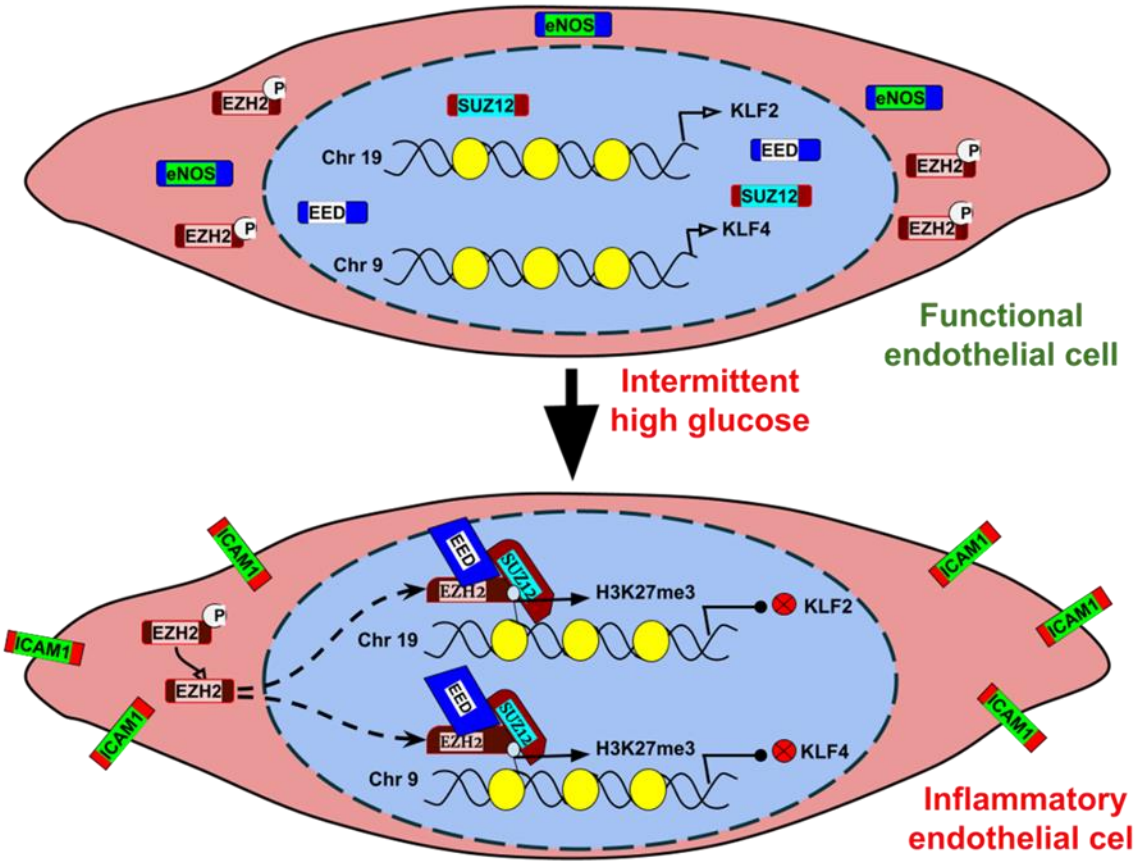


Fig 3.3.1. Mechanism by which elevated H3K27me3 level upon intermittent high glucose exposure promotes inflammatory signaling in endothelial cells. *In normal human endothelial cells, the histone methyltransferase EZH2 localizes primarily in cytosol and thus causes restricted tri-methylation of lysine residue 27 (K27) on histone H3 (H3K27me3). During such condition, the expression levels of essential signaling molecules such as KLF2, KLF4, and eNOS are maintained in endothelial cells to allow physiological function. In human endothelial cells exposed to intermittent hyperglycemia, this causes nuclear localization of EZH2 by de-phosphorylating Thr367 phosphorylation. Once localized in the nucleus, it assembles PRC2 and binds to histone H3 to cause an elevated quantity of H3K27me3. Such gain of H3K27me3 upon intermittent hyperglycemic exposure results in repression of KLF2 and KLF4 through promoter-level*

enrichment of H3K27me3. Repression of KLF2 and KLF4 initiates inflammatory signaling in endothelial cells through downregulation of eNOS and upregulation of ICAM1.

Targeting EZH2 through small molecule inhibitors or siRNA-attenuated intermittent high glucose-mediated increase in H3K27me3 thus restores KLF2 level and reverses the inflammatory phenotype of EC. KLF2 and KLF4—that are essential for endothelial function and maintaining endothelial homeostasis [161]. Although previous findings reported elevation in ICAM1 and E-selectin expression in HUVEC upon constant high glucose exposure [164], previous reports and our current findings clearly indicate a stronger response to intermittent high glucose in enhancing the expression of these adhesion molecules. In addition, clinical studies in type 2 diabetic subjects established a strong correlation between oscillating blood glucose level and pathological cardiovascular events [153,154], indicating that oscillating / Intermittent high glucose is more deleterious to the cardiovascular system. While a transient high glucose condition is reported to cause inflammatory signaling in EC [48], we also found in our current setting and condition that transient high glucose also caused a selective increase in certain adhesion molecules, such as P-selectin.

Hyperglycemia has been implicated in epigenetic modifications such as DNA methylation and histone modifications. Miao et al. showed that high glucose exposure led to increased H3K4me2 and H3K9me2 levels. They reported that the heightened levels of H3K4me2 were associated with increased methylation of nine genes, including ICAM3, FOS, GSTA-4, IL-8, and BCL-9—many of which are associated with inflammation [56]. Floris et al. isolated HUVEC from the umbilical cords collected from healthy or gestational diabetes mellitus-affected human subjects (referred to as GDM-HUVEC) and demonstrated a reduction in cellular H3K27me3 level due to concomitant reduction in EZH2 level via miR101 [152]. In contrast, we observed that intermittent high glucose exposure to HUVEC isolated from healthy individual (as specified by the supplier) elevated cellular H3K27me3 level and that such a hyperglycemic treatment condition preferentially favored an inflammatory state in endothelial cells. Such dissimilarity in the observation could be explained through the fact that in the previously reported work, Floris et al. measured the level of EZH2 and H3K27me3 in HUVEC that were directly harvested from individuals

with gestational diabetes mellitus. In contrast, in the present study, EZH2 and H3K27me3 was measured in HUVEC (isolated from healthy human subjects) that were challenged with intermittent high glucose under a culture condition. Because oscillatory/ intermittent blood glucose level through the day is apparent in people with type 2 diabetes [153,154], our study more robustly complements studies of such high glucose exposure under in vitro conditions. A coordinated alteration by histone methyltransferase SET7 and demethylase LSD1 dynamically alters H3K4 mono-methylation and H3K9me2/3 demethylation, which leads to increased NF-κB p65 activation to support the expression of inflammatory factor monocyte chemoattractant protein-1 (MCP-1) [157]. In addition, Han et al. also demonstrated that EC treated with high glucose exhibited enhanced H3K4me2 and H3K4me3 marks, which caused MCP-1 gene expression via the enrichment of such histone methylation marks on MCP1 promoter [165]. In a separate study, Set7 protein was shown to accumulate in the nucleus in response to hyperglycemia, and such localization activated proinflammatory genes in a Set7-dependent manner [166]. Similarly, in our study, we observed no changes in the expression level of EZH2, while intermittent high glucose caused nuclear localization of EZH2 through its interplay with threonine 367 phosphorylation. Furthermore, nuclear EZH2 assembled PRC2 complex by associating with key PRC2 core proteins; SUZ12 and EED followed by enhancing its recruitment to histone H3. The zinc finger transcription factors KLF2 and KLF4 are required for endothelial cell survival to maintain vascular integrity [162]. Endothelial-specific deletion led to embryonic lethality, suggesting that KLF2 in EC is necessary for vascular function [167]. A finding by Atkins et al. (2008) demonstrated that mice lacking one copy of the KLF2 gene exhibited increased atherosclerosis when mated with apoE-deficient mice and fed with a high fat and cholesterol diet [168]. In physiological settings, KLF2 and KLF4 cause inhibition of inflammatory gene expression and upregulation of vascular protective genes [167,169,170]. However, in diabetes, the underlying epigenetic mechanism of KLF2 and KLF4 regulation in EC still remains elusive. Cancer cells exhibited epigenetic silencing of KLF2 through direct transcriptional repression mediated through the EZH2–H3K27me3 axis [171]. A recent finding described the hyper-methylation of DNA in the KLF4 promoter region, thus regulating KLF4 expression in EC [172]. Parallel to this set of observations,

our findings indicate that elevated H3K27me3 enriched in the promoter regions of KLF2 and KLF4 in EC upon intermittent high glucose exposure further cause repression of these genes. Blocking EZH2 activity through gene silencing or catalytic inactivation caused reversal of H3K27me3 - dependent inhibitory effects on KLF2 in EC exposed to intermittent high glucose. In ApoE$^{-/-}$ mice, high levels of homocysteine (Hcy) that induced EZH2 expression were detected, which led to H3 at lysine 27 (H3K27) tri-methylation. Global increase in trimethylation of H3K27 was observed in atherosclerotic plaques in the late stage of the pathology [173]. However, the role of EZH2 and its associated catalytic product H3K27me3 in hyperglycemia-induced endothelial dysfunction and apoptosis has not yet been explored. Through the present study, we detected an elevated H3K27me3 level in intermittent high glucose-challenged EC, and reducing the level of H3K27me3 through inhibition or knockdown of EZH2 reversed the intermittent high glucose-dependent inflammatory phenotype of EC.

In summary, an elevated level of H3K27me3 in EC exposed to intermittent high glucose is responsible for endothelial inflammation. Intermittent high glucose-challenged EC led to an abrupt increase in H3K27me3 through EZH2 nuclear localization that caused repression of KLF2 and KLF4, resulting in the endothelial inflammatory phenotype. Targeting EZH2 caused re-expression of KLF2 and KLF4 and further revoked the inflammatory state of EC imparted through the intermittent high glucose treatment condition. Changes in histone patterning upon hyperglycemia govern an inflammatory switch of EC through the EZH2–H3K27me3 axis, and inhibition of said epigenetic pathway alters the course of the EC inflammatory switch.

Chapter 4

Regulation of shelterin proteins TERF2IP and TRF2 by H3K4me3-p65 axis drives hyperglycemia dependent endothelial senescence

4.1 INTRODUCTION

Diabetes-associated complications are one of the most common causes of cardiovascular and kidney diseases worldwide. Diabetes-associated complications are mainly manifested by both microvascular and macrovascular anomalies. Endothelial cells (EC) being the inner lining of the vasculature play a major role in maintaining homeostasis in different organs including the cardiovascular system. In patients with diabetes, endothelial dysfunction appears to be a consistent finding and was shown to be responsible for cardiovascular diseases including atherosclerosis, diabetic cardiomyopathy, diabetic retinopathy and diabetic kidney disease. Hyperglycemia, a hallmark of diabetes causes induction of a plethora of pathological pathways leading to endothelial dysfunction. Several studies have reported endothelial senescence in patients with diabetes [172,173] and cultured cells that were exposed to high glucose [174,175]. Indeed, such senescent phenotype is a hallmark of cardiovascular anomalies in diabetes setting both *in vitro* and *in vivo*.

Senescence particularly leads to a phenotype known as senescence associated secretory phenotype. Two types of senescence can be accounted based on the environment and subsequent activity related with cell injury and division of the cell [178]. The first is replicative senescence wherein a cell after dividing for many cycles can no longer propagate majorly because of excessive DNA damage leading to suppression of cell cycle mediators responsible for checkpoints at G1/S phase. The second is damage induced senescence in which stress due to toxic mediators or an injury based inflammation leads to curtailing of cell division. In this case, the cell experiences a total arrest but is metabolically active [179]. In such cases, these stressed cells start secreting various chemokines like TNF-α, IL-6 which lure immune cells such as macrophages and neutrophils towards the site of assault. This occurs preferentially through high amount of ROS activity by the mitochondria. The NF-κB transcriptional pathway along with the cGAS-STING pathway is primarily involved as p65 acts as a mediator for many of the signaling pathways progressing towards senescence [180]. Interestingly, p65 known to interact with TERF2IP, part of shelterin complex, in promoting endothelial senescence. According to recent report, TERF2IP was found to shuttle from the nucleus to the

cytoplasm causing p65 phosphorylation enabling it to translocate to the nucleus where it acts as a transcriptional inducer for endothelial senescence.

Shelterin complex is important as it helps in protecting chromosomal ends from damage thereby regulating cellular senescence. The complex composed of six proteins, namely- TPP1, TERF2IP, TRF1, TRF2, POT1, and TIN2. TRF1 and TRF2 are responsible for recruiting TERF2IP to form a complex by forming a D-loop at the telomeric ends [181]. Loss of these proteins seems to trigger senescence either by hampering cell division or through telomere shortening. In vascular inflammation, shelterin knockouts have been found to progress to senescence, whereas if these were induced for a gain in expression, it was found to reduce inflammation. However, in a clinical setting, the difference could only be seen in high grade inflammation, particularly in lymphocytes [182]. For senescence to proceed, it would involve expression of the cell cycle control proteins – CDK 40, p16, p21, p27, particularly the tumor suppressor gene p53. A normally dividing cell would stop proliferation under two conditions; the first being replicative senescence and the second induced due to stress or deterioration, in this case inflammation [183]. Although endothelial senescence was described in hyperglycemia setting, any role of shelterin protein in such phenotype have never been reported. We previously reported a H3K27me3 dependent repression of *KLF2* and *KLF4* genes resulting in endothelial inflammation [184]. We also described hyperglycemia-dependent induction of partial mesenchyme-like phenotype of EC by elevating H3K4me3 level via MLL2-WDR82 protein of SET1/COMPASS complex which supported Jagged1 and Jagged2 expression and Notch activation [185]. In this study, we report an increase in the expression of TERF2IP and TRF2 in EC upon exposure to intermittent high glucose. We also show that these proteins regulate the expression of classic cellular senescence associated proteins, p53 and p21, thereby causing senescence in EC upon hyperglycemia challenge. Interestingly, the expression of TERF2IP and TRF2 is also regulated by H3K4me3. This is a significant finding as no other study has reported the epigenetic regulation of TERF2IP and TRF2.

4.2 RESULTS

4.2.1 Intermittent high glucose selectively elevated the level of p53 and p21 to cause endothelial senescence without causing apoptosis

Induction of ROS upon hyperglycemia exposure was described to be responsible for cellular senescence [186]. eNOS-mediated nitric oxide production caused inhibition of endothelial senescence upon hyperglycemia challenge [28]. We previously reported robust reduction in eNOS transcript and protein levels thereby prompting us to study the effect of intermittent hyperglycemia on senescence [184]. Furthermore, we also reported no alteration in two proliferation markers Cyclin D3 and c-Myc in EC exposed to hyperglycemia [185]. Exit from cell cycle and pause in cell proliferation is generally the end point of cellular senescence. Therefore, we evaluated the changes in cyclin inhibitors like p53, p21, p16 and damage induced senescence marker lamin B1. Protein expression of both p53 (**Fig 4.2.1 A,G**) and p21 (**Fig 4.2.1 B,F**) increased significantly upon intermittent hyperglycemia challenge indicating hyperglycemia did lead to changes in cell proliferation. No robust change was observed in expression of other senescence markers such as p16 (**Fig 4.2.1 C**), TNF-α (**Fig 4.2.1 D**), lamin B1 and MMP3 (**Fig 4.2.1 E**). We also confirmed the increase in p53 level in intermittent hyperglycemia-exposed rat aorta (**Fig 4.2.1 H**). As p53 and p21 were well established to halt cell cycle at G1/S checkpoint and our previous findings revealed no alteration in level of Cyclin D3 and c-Myc [185], we wondered that intermittent hyperglycemia is leading to cell cycle arrest at G0/G1 phase. To test this, we performed cell cycle analysis using PI staining followed by flow cytometric analysis to determine the total DNA content of each cell. In doing so, we detected significant number of intermittent hyperglycemia exposed EC arrested at G0/G1 phase than normal glucose treated group (**Fig 4.2.1 I**). Moreover, five days of intermittent hyperglycemia exposure caused a significant increase in cells having G0/G1 arrest (**Fig 4.2.1 I**). Because cellular senescence is responsible for cell apoptosis, we therefore detected the relative fraction of apoptotic cells after intermittent hyperglycemia exposure. No changes in percentage of apoptotic cell (**Fig 4.2.1 J**) was detected when EC were challenged with intermittent hyperglycemia, thus disclosing absence of senescence-dependent endothelial apoptosis phenotype in the present experimental settings. To exclude the possibility that the intermittent hyperglycemia effect was due to osmolality changes, we simultaneously analyzed these senescence markers in HUVEC exposed to intermittent high mannitol, which generates a comparable osmolality that is similar to

intermittent hyperglycemia. In so doing, we detected no changes in p53 and p21 level in EC exposed to IHM (**Fig 4.2.1.1**).

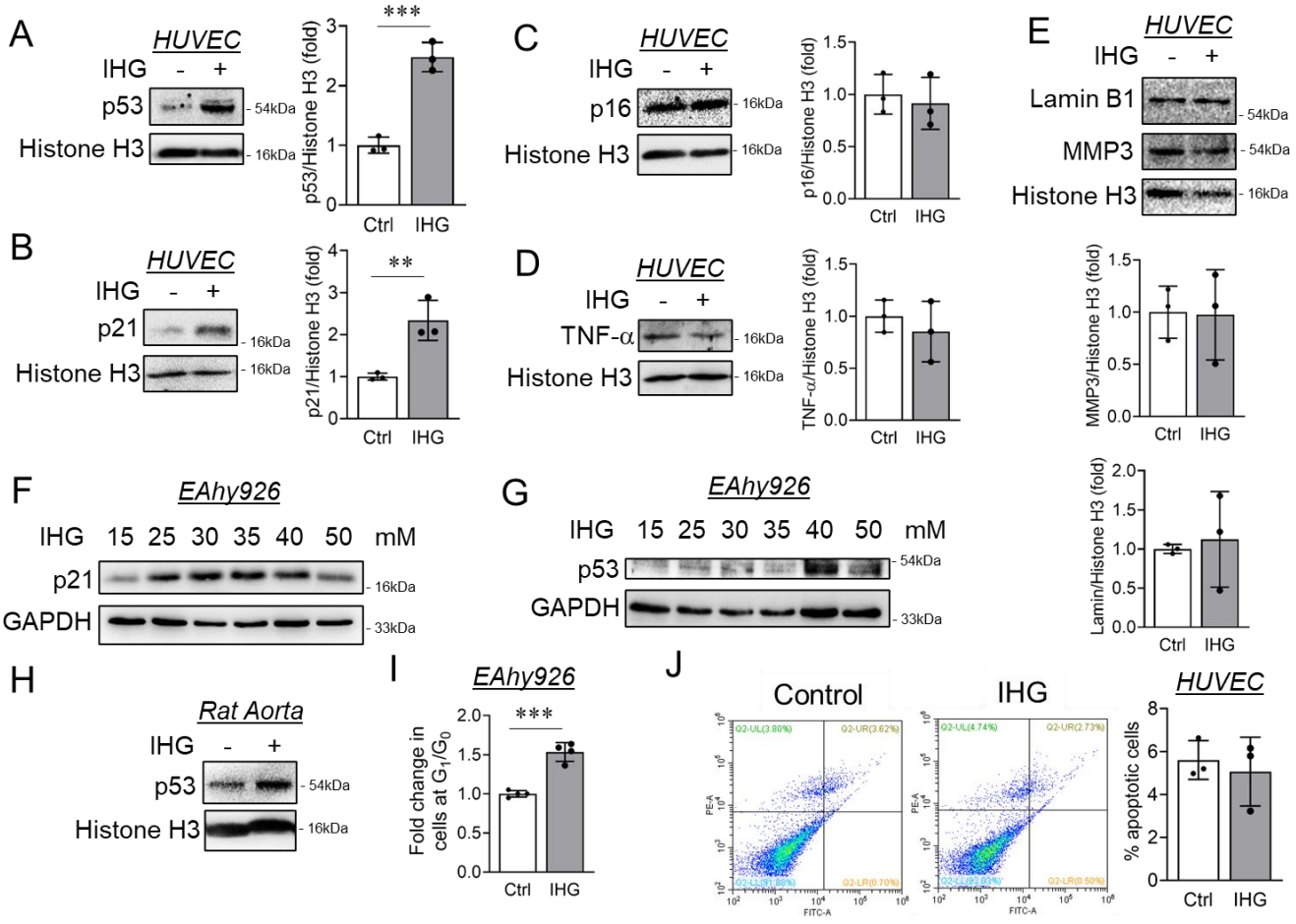


Fig 4.2.1. Intermittent high glucose exposure enhanced endothelial senescence without altering apoptosis by inducing selective expression of p53 and p21. *(**A-E**) Immunoblot analysis of HUVEC exposed to intermittent high glucose to detect the level of p53 (**A**), p21 (**B**), p16 (**C**), TNF-α (**D**), Lamin B1 and MMP3 (**E**) (n=3). (**F-G**) Level of p21 (**F**) and p53 (**G**) were detected in EA.hy926 challenged with intermittent high glucose (n=3). Densitometric quantification of the blots were represented. (**H**) Immunoblot analysis of p53 in tissue lysate collected from rat aorta exposed to intermittent high glucose ex vivo (n=3). (**I**) Cell cycle analysis of intermittent high glucose challenged EA.hy926 cells using PI staining followed by flow cytometry analysis to quantify cells at G_1/G_0, S-phase and G_2/M phase (n=4). Data represented as fold change relative to*

*control (normal glucose cells) for cells at G_1/G_0 phase. Values represent the mean ± SD. **P < 0.01, and ***P < 0.001, by unpaired t test. (J) Measuring the level of apoptosis in HUVEC treated with intermittent high glucose using Annexin V-PI staining followed by flow cytometry analysis; distribution plots of early and late apoptotic cells and quantified data that include both early and late apoptotic cell population. (n=3).*

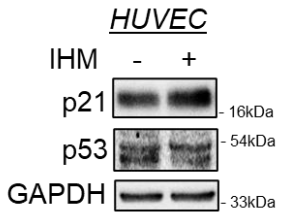


Figure 4.2.1.1. Intermittent high mannitol exposure did not alter p21 and p53 level in HUVEC. *Immunoblot analysis of HUVEC exposed to intermittent high mannitol (IHM) to detect the level of p21 and p53. (n=3)*

4.2.2 Intermittent high glucose elevated the level of p65 and Shelterin complex proteins TERF2IP and TRF2

Oxidative stress and inflammation disrupt shelterin in vascular cells causing telomere dysfunction and cellular senescence [182]. Moreover, deletion of shelterin complex protein like TERF2IP inhibited d-flow-induced EC senescence, apoptosis, and activation [187]. Hence, we evaluated the expression level of proteins related to shelterin complex in EC challenged with intermittent hyperglycemia condition. Transcript and protein level expression analysis revealed significant increase in TERF2IP (**Fig 4.2.2 A,E,M**) and TRF2 (**Fig 4.2.2 B,F,L**) in EC exposed to intermittent hyperglycemia. In contrast, the level of other two components of the shelterin complex; TRF1 (**Fig 4.2.2 C,G**) and TPP1 (**Fig 4.2.2 D,H**) remain to be unaltered upon intermittent hyperglycemia challenge. Analysis of TERF2IP (**Fig 4.2.2.1 A**) and TRF2 (**Fig 4.2.2.1 B**) in intermittent high mannitol challenged EC exhibited no alteration in these proteins, thus excluding any contribution of osmolality in intermittent hyperglycemia dependent increase in TERF2IP and TRF2 levels. The canonical NF-κB pathway has been shown to occur in many chronic diseases including obesity, inflammation, cardiovascular damage and type 2 diabetes. In addition, NF-kB signaling plays a pivotal role in endothelial senescence [188]. p65 is an important transcription factor as it activates the downstream genes responsible for cell cycle arrest.

Hyperglycemia and supported senescence mechanisms having p65 involvement as a mediator are widely known. We therefore analyzed the expression level of proteins involved in NF-κB analysis. We found a profound increase in expression of p65 in intermittent hyperglycemia as opposed to untreated samples (**Fig 4.2.2 I,M**). Expression of IkB-α (**Fig 4.2.2 J,M**) and IKK-α (**Fig 4.2.2 K**) remain unchanged in EC treated with intermittent hyperglycemia condition. Therefore, induction of p65 expression upon intermittent hyperglycemia exposure in EC was likely to be independent of IkB-α and IKK-α action on p65 activity. Intermittent high mannitol exposure to EC did not alter the level of p65 (**Fig 4.2.2.1 B**) suggesting osmolality changes was not responsible for increase in p65 level upon IHG exposure. To validate these findings in more relevant *in vivo* model, we detected the changes in these genes in isolated rat aortas which are exposed to intermittent high glucose treatment condition *ex vivo*. We detected a significant increase in level of TRF2 and p65 (**Fig 4.2.2 N**) in tissue lysates of aortas that undergone intermittent hyperglycemia treatment condition while no alteration in IkB-α (**Fig 4.2.2 N**) was detected in these tissue.

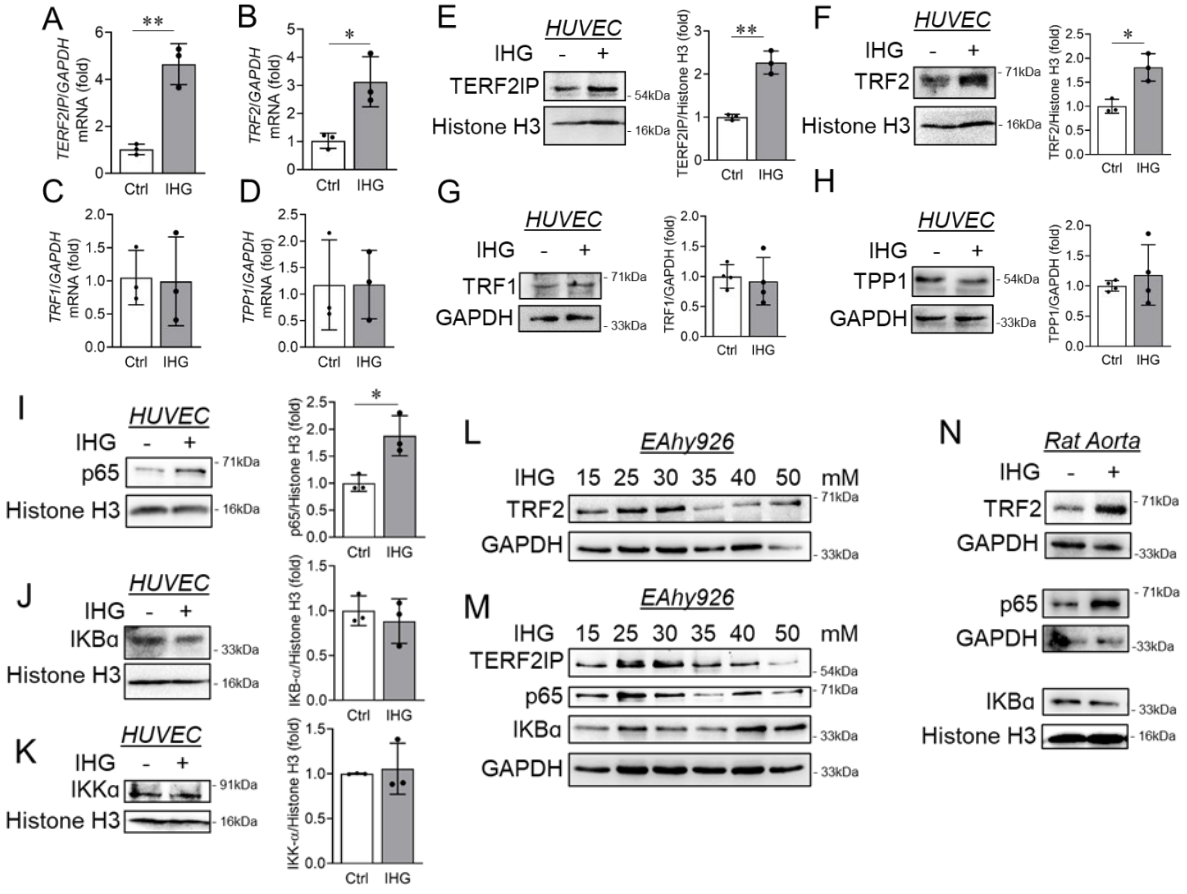


Figure 4.2.2. Shelterin proteins TERF2IP-TRF2 and p65 are elevated in EC exposed to intermittent high glucose. *(**A-D**) Transcript-level expression of TERF2IP (**A**), TRF2 (**B**), TRF1 (**C**) and TPP1 (**D**) measured through RT-qPCR technique in HUVEC challenged with an intermittent high-glucose condition (n=3). (**E-H**) Immunoblot analysis of HUVEC exposed to intermittent high glucose to detect the level of TERF2IP (**E**), TRF2 (**F**), TRF1 (**G**) and TPP1 (**H**) (n=3). Densitometric quantification of the blots were represented. (**I-K**) The level of p65 (**I**), IkBα (**J**) and IKKα (**K**) were quantified in HUVEC challenged with intermittent high glucose (n=3). Densitometric quantification of the blots were represented. (**L-M**) Level of TRF2 (**L**), TERF2IP (**M**), p65 (**M**), and IkBα (**M**) were detected in EA.hy926 challenged with intermittent high glucose (n=3). (**N**) Immunoblot analysis of TRF2, p65 and IkBα in tissue lysate collected from rat aorta exposed to intermittent high glucose ex vivo (n=3). Values represent the mean ± SD. *P < 0.05, and **P < 0.01, by unpaired t test.*

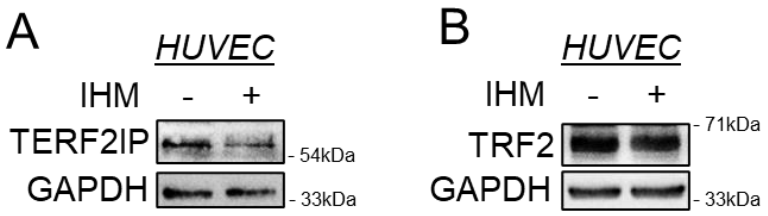


Fig 4.2.2.1. Intermittent high mannitol exposure did not alter TERF2IP and TRF2, level in HUVEC. *Immunoblot analysis of HUVEC exposed to intermittent high mannitol (IHM) to detect the level of TERF2IP (**A**) and TRF2, (n=3).*

4.2.3 Increased level of H3K4me3 upon intermittent high glucose epigenetically regulated the expression of TERF2IP, TRF2 and p65

Because we observed an increased level of TERF2IP, TRF2 and p65, we therefore decided to decipher the underlying mechanism responsible for such selective increase in gene expression. We previously showed that intermittent high glucose led to increase in H3K4 trimethylation in EC through preferential increase in MLL2 and WDR82 level [185]. With this in mind, we decided to understand the role of H3K4me3 in intermittent hyperglycemia dependent increase in TERF2IP, TRF2 and p65 level. ChIP-qPCR

analysis revealed heightened enrichment of H3K4me3 in the promoter region of *p65* (**Fig 4.2.3 A**), *TERF2IP* (**Fig 4.2.3 B**) and *TRF2* (**Fig 4.2.3 C**) genes.

To further ascertain that elevated promoter level enrichment of H3K4me3 was the reason of enhanced expression of TERF2IP, TRF2 and p65, we further analyzed the expression of these genes in EC incubated with selective pharmacological inhibitors of SET1/COMPASS methylation complex or specific siRNA to MLL2. Selective inhibition of the activity of SET1/COMPASS complex neutralized the IHG driven enhanced expression of TERF2IP (**Fig 4.2.3 D**), TRF2 (**Fig 4.2.3 E**) and p65 (**Fig 4.2.3 D**). In parallel, we detected that selective knockdown of MLL2 reversed intermittent hyperglycemia dependent increase in expression of TERF2IP (**Fig 4.2.3 I**), TRF2 (**Fig 4.2.3 J**) and p65 (**Fig 4.2.3 I**). Because we suspected that elevated level of p65, TERF2IP and TRF2 was responsible for enhanced level of senescence associated biochemical changes, we therefore detected the level of p53 and p21 in EC treated with selective inhibitors of SET1/COMPASS complex activity or MLL2 specific siRNA and exposed to intermittent hyperglycemia. In so doing, we reported attenuation of intermittent hyperglycemia induced increase in the expression of p21 (**Fig 4.2.3 G,I**) and p53 (**Fig 4.2.3 F,K**) in EC. As we reported previously [185], we confirm the reduction in the level of H3K4me3 in EC that were exposed to intermittent hyperglycemia in combination with selective inhibitors of SET1/COMPASS complex activity or MLL2 specific siRNA (**Fig 4.2.3 H,L**). In addition, we also showed no changes in the level of H3K4me3 in EC treated with intermittent high mannitol condition thereby eliminating the role of osmolality in the catalysis of H3K4me3 (**Fig 4.2.3.1**).

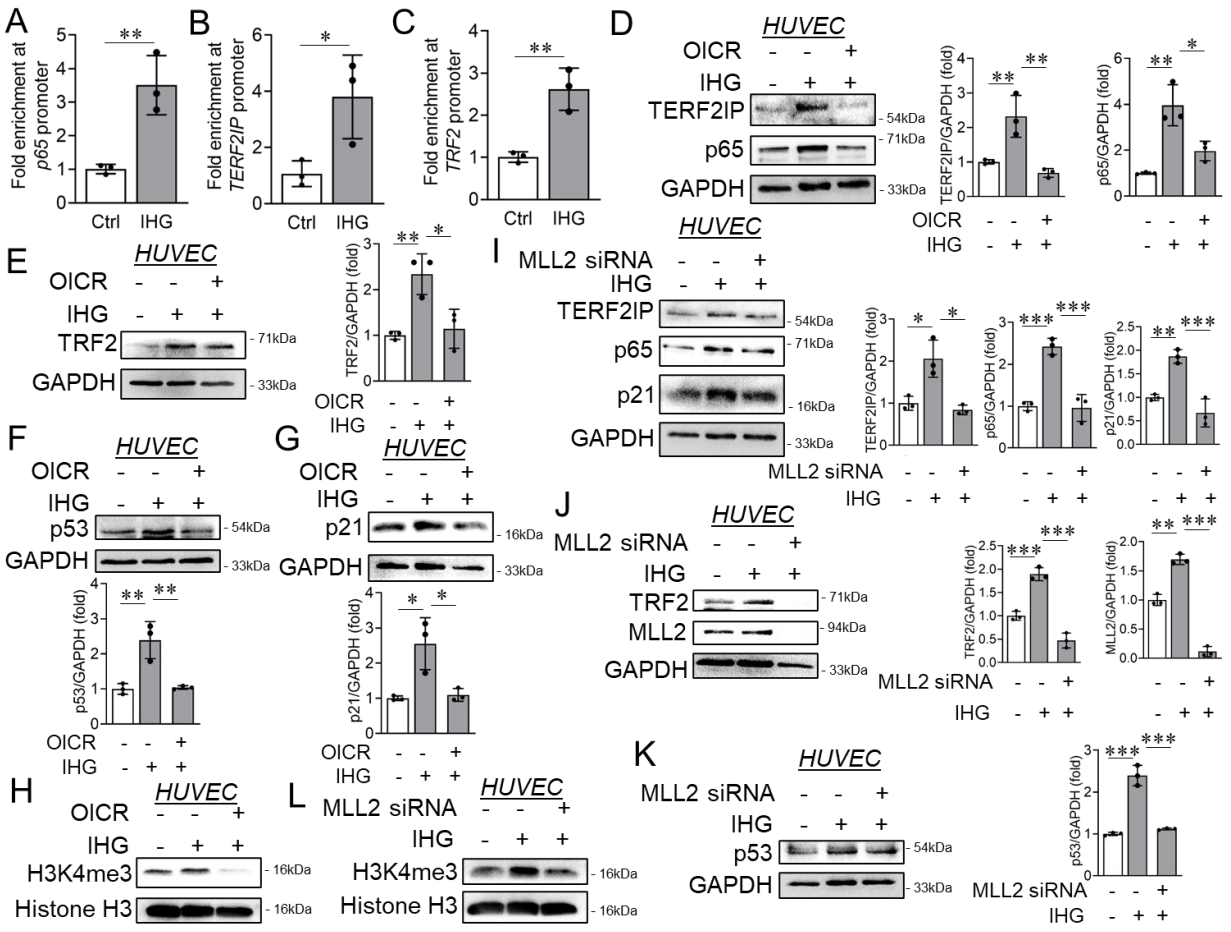


Fig 4.2.3. Increased H3K4me3 was responsible for intermittent high glucose dependent increase in p65, TERF2IP and TRF2 levels. *(**A-C**) ChIP-qPCR for p65 (**A**), TERF2IP (**B**) and TRF2 (**C**) gene promoters following enrichment with H3K4me3 antibody in HUVEC challenged with intermittent high glucose treatment condition (n = 3). (**D-H**) Immunoblot analysis of HUVEC lysate collected from cells pre-treated with OICR-9429 (10 μM) followed by exposing to intermittent high glucose. Immunoblots were probed with antibodies against TERF2IP and p65 (**D**), TRF2 (**E**), p53 (**F**), p21 (**G**) and H3K4me3 (**H**) proteins/modifications along with densitometry quantification of the blots (n=3). (**I-L**) Immunoblot analysis of HUVEC lysates collected from cells transfected with MLL2 specific siRNA followed by challenging with intermittent high glucose and probed for TERF2IP, p65, and p21 (**I**), TRF2 and MLL2 (**J**), p53 (**K**), and H3K4me3 (**L**) proteins/modifications along with densitometry quantification of the blots (n=3). Values represent the mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001, by Tukey's multiple comparisons test of one-way ANOVA.*

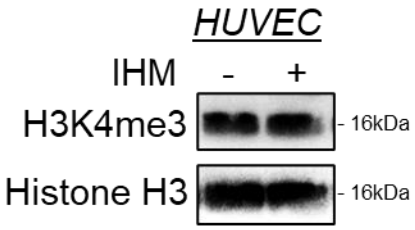


Fig 4.2.3.1. Intermittent high mannitol exposure did not alter H3K4me3 level in HUVEC. *Immunoblot analysis of HUVEC exposed to intermittent high mannitol (IHM) to detect the level of H3K4me3 (n=3).*

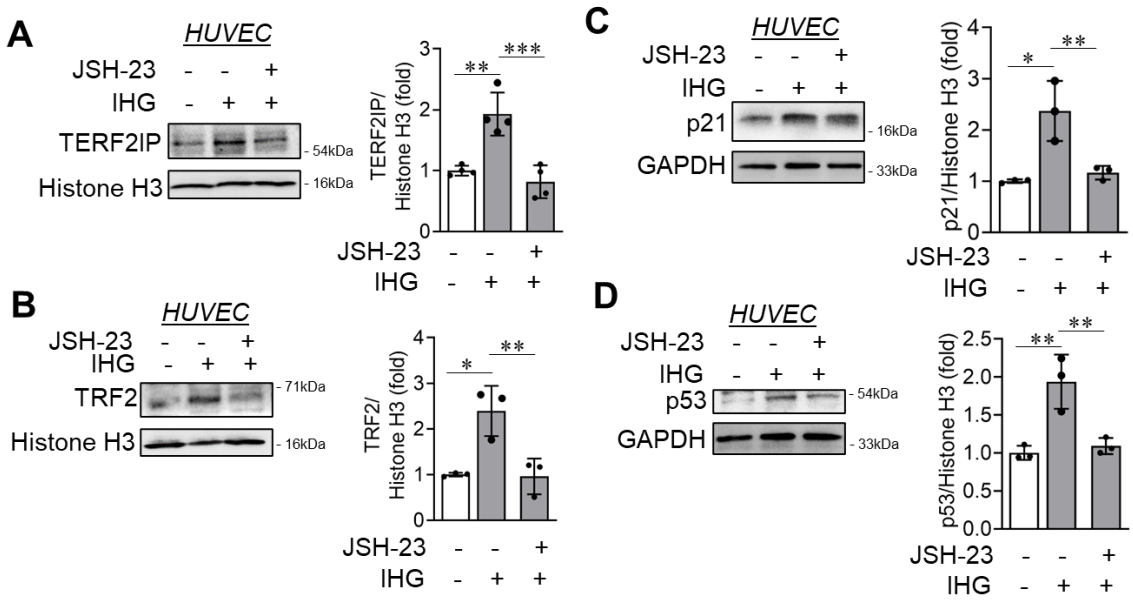
4.2.4 p65 in conjunction with MLL2 was responsible for intermittent high glucose-dependent increase in TERF2IP and TRF2

A relatively recent study indicated TERF2IP is able to translocate from nucleus to cytoplasm during endothelial inflammation and associate with p65 to cause activation of NF-κB signaling in EC [189]. Therefore, we studied the effect of intermittent high glucose exposure on the subcellular localization of TERF2IP and TRF2. In so doing, we found both TERF2IP (**Fig 4.2.4.1 A**) and TRF2 (**Fig 4.2.4.1 B**) were primarily localized in the nucleus while no changes in the subcellular localization of these proteins were detected between control and intermittent hyperglycemia-treated cells. To further validate, we performed a cell fractionation assay followed by immunoblot analysis. Although a significant increase in both nuclear and cytosolic localization in TERF2IP was detected due to an elevation in total TERF2IP level, relative distribution remained constant (**Fig 4.2.4.1 C**). In contrast, TRF2 did not show any changes in sub-cellular localization (**Fig 4.2.4.1 D**). The association of TERF2IP with p65 is well documented [187], we thus performed a co-immunoprecipitation study to evaluate TERF2IP association with p65 upon intermittent hyperglycemia challenge. Interestingly, as reported earlier in EC, we detected an association of p65 with TERF2IP, however, a modest reduction in the association of TERF2IP and p65 was detected upon intermittent high glucose exposure (**Fig 4.2.4.1 E**).

We next investigated the role of p65 in the regulation of IHG-dependent induction of TERF2IP and TRF2. We next inhibited p65 using the pharmacological inhibitor, JSH-23

and evaluated its effect on intermittent high glucose-induced TERF2IP and TRF2 expression. Inhibition of NF-κB activation by JSH-23 completely neutralized intermittent hyperglycemia-dependent elevation in TERF2IP (**Fig 4.2.4 A**) and TRF2 level (**Fig 4.2.4 B**). Moreover, inhibition of p65 also reversed intermittent hyperglycemia-supported increase in p21 (**Fig 4.2.4 C**) and p53 (**Fig 4.2.4 D**) levels.

As per our findings, both inhibitions of MLL2 or p65 caused a reversal of intermittent hyperglycemia-induced TERF2IP and TRF2 expression, we next wondered whether MLL2 and p65 work in tandem with each other to regulate the expression of TERF2IP and TRF2. As MLL2 and p65 were never reported to associate with each other in any cell type, we questioned whether intermittent hyperglycemia can confer such an association between p65 and MLL2. Co-immunoprecipitation analysis confirmed the association of MLL2 and p65 in EC, moreover, such association is enhanced upon the intermittent high glucose challenge (**Fig 4.2.4 E**). We confirmed such association of MLL2 and p65 in the nucleus of EC using immunofluorescence analysis. (**Fig 4.2.4 F**).



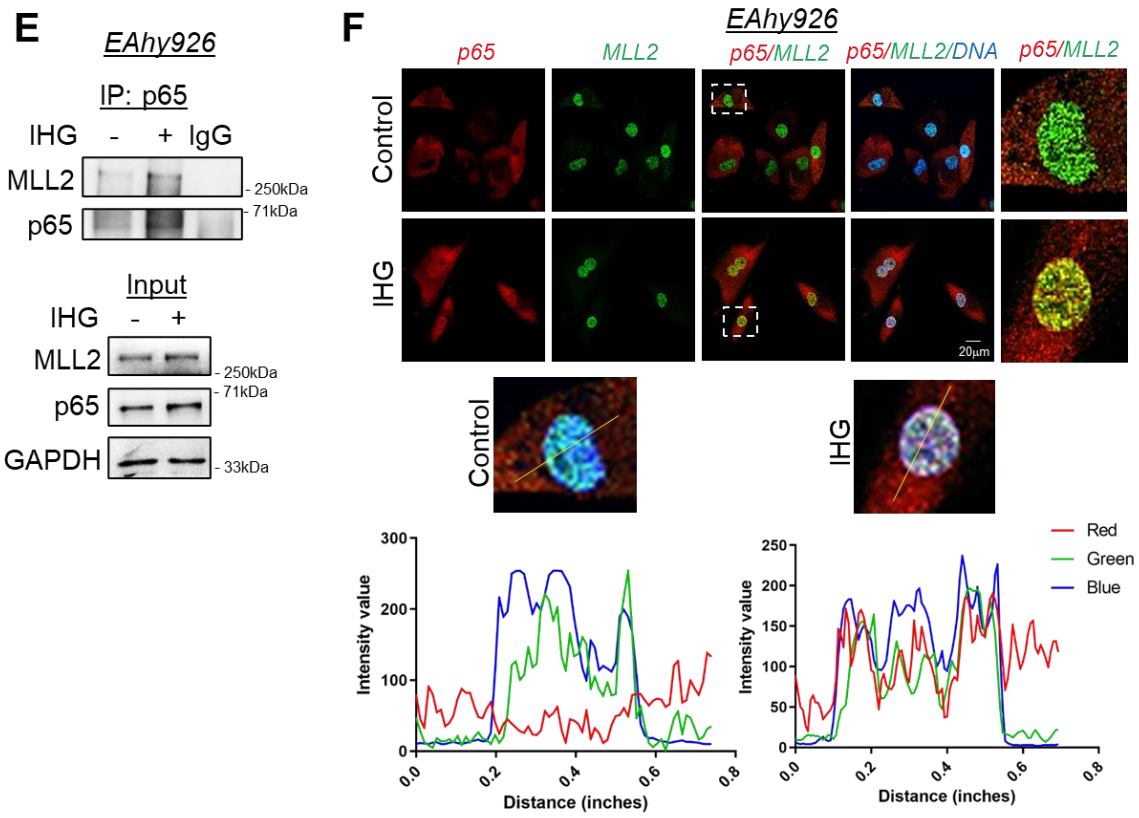


Figure 4.2.4. p65 in conjunction with MLL2 was responsible for intermittent high glucose dependent increase in shelterin proteins TERF2IP-TRF2 level and cellular senescence markers p21-p53. *(**A-D**) Immunoblot analysis of HUVEC lysate collected from cells pre-treated with JSH-23 (10 μM) followed by exposing to intermittent high glucose. Immunoblots were probed with antibodies against TERF2IP (**A**), TRF2 (**B**), p21 (**C**) and p21 (**D**) proteins along with densitometry quantification of the blots (n=3). (**E**) Co-immunoprecipitation with p65 antibody, followed by immunoblotting for MLL2, p65, and GAPDH in either immuno-precipitated or total cell lysate (Input) sample (n=3). (**F**) Immunofluorescence analysis and co-staining of EA.hy926 exposed to intermittent high glucose for p65 and MLL2. DAPI staining to visualize the nucleus is shown in blue (n = 3). Line graph indicating pixel-wise intensities of green (MLL2) and red (p65) fluorescence in the yellow line highlighted areas within the image. Values represent the mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001, by Tukey's multiple comparisons test of one-way ANOVA.*

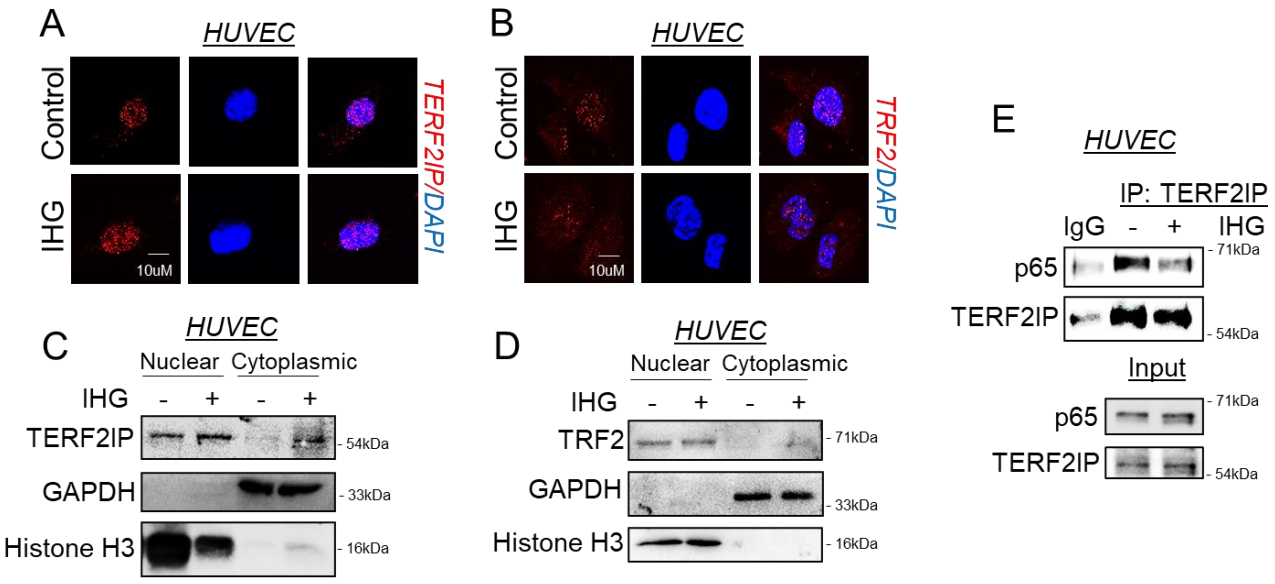


Fig 4.2.4.1. Relative distribution of TERF2IP and TRF2 upon intermittent high glucose remain unaltered while p65 invariably associate with TERF2IP independent of intermittent high glucose challenge. *(**A-B**) Immunofluorescence analysis and co-staining of HUVEC exposed to intermittent high glucose for TERF2IP (**A**) and TRF2 (**B**). DAPI staining to visualize the nucleus is shown in blue (n=3). (**C-D**) Subcellular fractionation, immunoblotting, and quantitation of nuclear and cytosolic level of TERF2IP (**C**) and TRF2 (**D**) in intermittent high glucose-challenged HUVEC (n=3). (**E**) Co-immunoprecipitation with TERF2IP antibody, followed by immunoblotting for p65, and TERF2IP in either immuno-precipitated or total cell lysate (Input) sample (n=3).*

4.2.5 Pharmacological inhibition of MLL2 reversed the level of TRF2, p65 and senescence marker p53 in IHG exposed rat aortic ring model *ex vivo*

Upon confirming MLL2 and p65 regulation of shelterin protein TEFR2-IP and TRF2 and further intermittent hyperglycemia effect on cellular senescence marker p53, we next assessed the effect of such inhibition in ex vivo model. Blocking SET/COMPASS complex activity using pharmacological inhibitor OICR-9429 in *ex vivo* model of rat aortic rings caused a reversal of intermittent hyperglycemia-induced expression of TRF2 (**Fig 4.2.5 A**). Furthermore, such inhibition of H3K4me3 catalysis also neutralized intermittent high

glucose-dependent increase in p65 level and cellular senescence marker p53 (**Fig 4.2.5 B**).

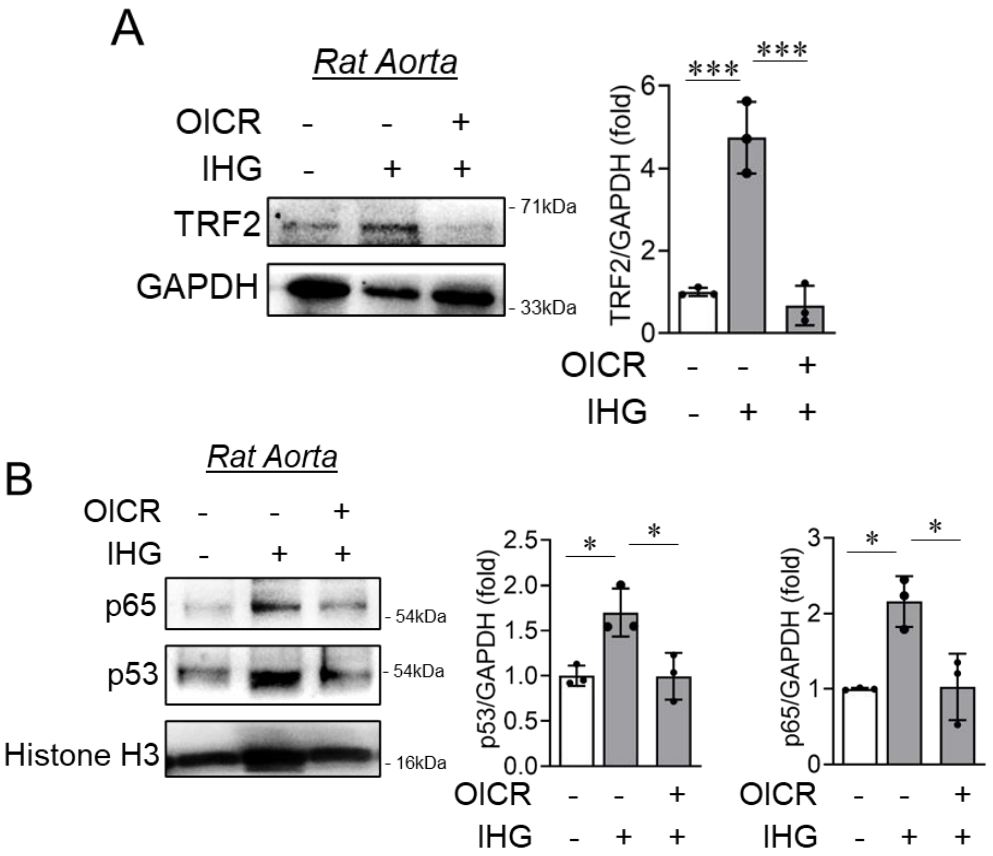


Fig 4.2.5 Catalytic inhibition of MLL blocked intermittent high-glucose-dependent increase in TRF2, p65 and p53 in rat aortic rings ex vivo. *(**A**-**B**) Immunoblot analysis of tissue lysates collected from rat aorta treated with intermittent high glucose in combination with OICR-9429 (10μM) and probed for TRF2 (**A**), p65 (**B**), and p53 (**B**) along with densitometry quantification (n=3). Values represent the mean ± SD. *P < 0.05, and ***P < 0.001, by Tukey's multiple comparisons test of one-way ANOVA.*

4.2.6 Knockdown of TERF2IP and TRF2 reversed p53 and p21 level and associated endothelial senescence imparted by intermittent high glucose

Induction of senescence state in cells is dependent on the relative expression of TERF2IP [190] and TRF2 [191]. Through a recent study, aberrant expression of TERF2IP has been known to cause aging-related phenotype [192]. Therefore, we wondered whether upregulation in the level of shelterin protein TERF2IP and TRF2 upon intermittent high glucose exposure caused changes in senescence-associated biochemical markers, p21 and p53. To this end, we transfected cells with TERF2IP siRNA or TRF2 siRNA and exposed them to intermittent hyperglycemia treatment condition. TERF2IP knockdown as confirmed through immunoblot (**Fig 4.2.6 A**) completely reversed intermittent high glucose-induced increase in the expression of p21 (**Fig 4.2.6 A**) and p53 (**Fig 4.2.6 B**). In parallel, TRF2 knockdown (**Fig 4.2.6 C**) in EC overrode the changes observed in p21 (**Fig 4.2.6 C**) and p53 (**Fig 4.2.6 D**) level upon intermittent high glucose challenge. Knockdown of TERF2IP or TRF2 also completely abrogated intermittent high glucose induced cell cycle arrest of EC at G1/G0 phase (**Fig 4.2.6 E**).

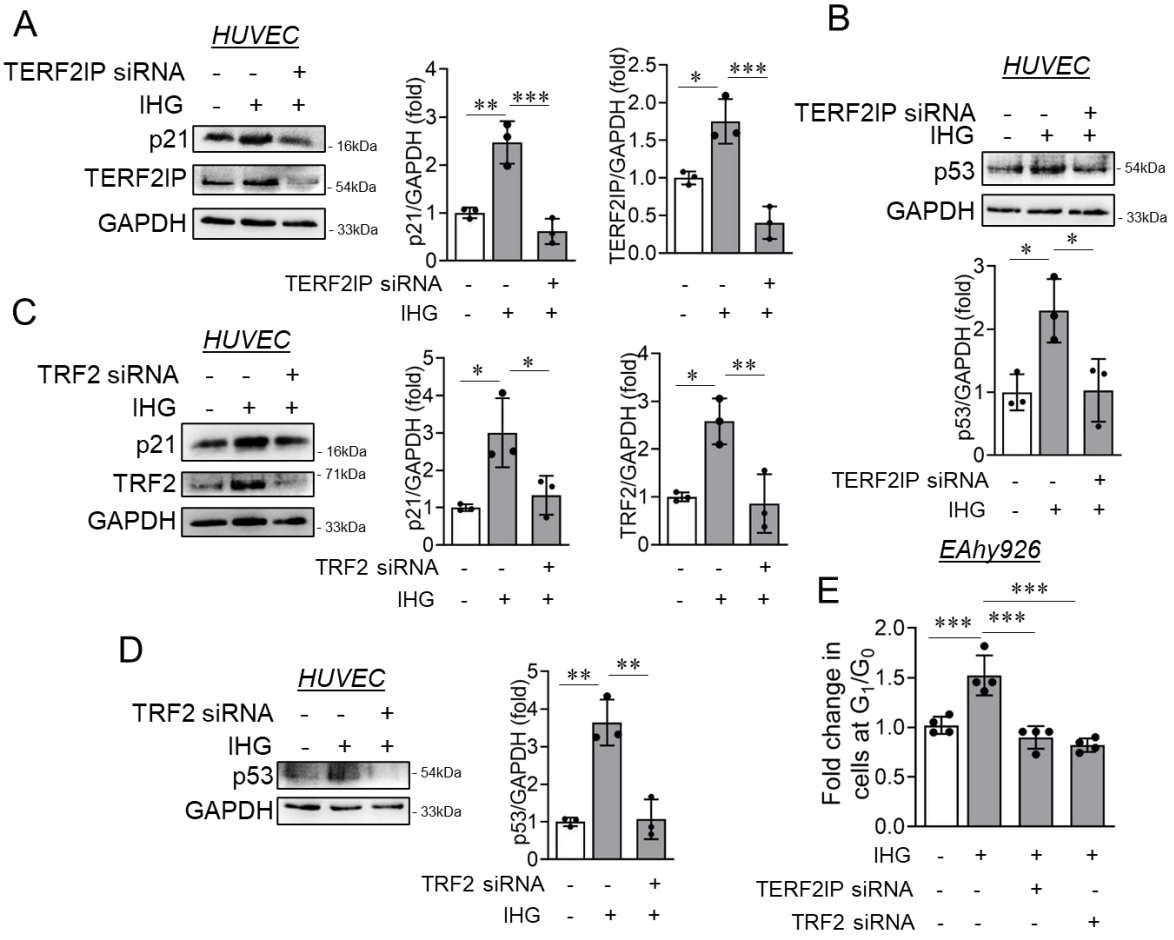


Fig 4.2.6. Knockdown of either TERF2IP or TRF2 reversed p53 and p21 level including endothelial senescence imparted by intermittent high glucose exposure. *(**A-B**) Immunoblot analysis of HUVEC lysates collected from cells transfected with MLL2 specific siRNA followed by challenging with intermittent high glucose and probed for p21 (**A**), TERF2IP (**A**) and p53 (**B**). Densitometry quantification of the blots were plotted as graph (n=3). (**C-D**) HUVEC transfected with TRF2 specific siRNA were challenged with intermitted high glucose treatment condition followed immunoblot analysis with the cell lysate for p21 (**C**), TRF2 (**C**), and p53 (**D**) along with densitometry quantification of the blots (n=3). (**E**) Cell cycle analysis of TERF2IP or TRF2 knockdown and intermittent high glucose challenged EA.hy926 cells using PI staining followed by flow cytometry analysis to quantify cells at G_1/G_0 (n=4). Data represented as fold change relative to control (normal glucose incubated cells) for cells at G_1/G_0 phase. Values represent the mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001, by Tukey's multiple comparisons test of one-way ANOVA.*

4.3 DISCUSSION

Senescence can occur as a result of DNA damage, cytokine stress or replicative dysfunction. Moreover, senescence has been known to cause inflammation through induction of ROS and increase in NADPH oxidases [193], [194]. Such phenomenon is dependent on replication of progeny through induction of cell cycle or be a pseudo enhancement damage induced senescence. Transcriptional regulation through histone modifications like H3K9me3, H3K4me3 or H3K27me3 at specific foci can also act to promote senescence in cells. However, many such mechanisms of regulation are studied based on progression of senescence towards inflammation. Furthermore, this is not always the case as senescence activated inflammatory cells is a long term response and because of chronic manifestation of such phenotype, these cells could no longer be senescent in nature. Cells existing in senescence or growth arrest are more detrimental as they can no longer maintain a constant metabolic state. Through the present study we describe the effect of intermittent high glucose on induction of a senescent response in endothelial cells through transcriptomic regulation of p53 and p21 by two shelterin proteins TERF2IP and TRF2. We report that intermittent high glucose causes MLL2-

H3K4me3-mediated transcriptional regulation of p65 which in turn binds and recruits MLL2 to cause enhanced transcriptional activity of TERF2IP and TRF2 genes. Targeting either NF-kB or MLL2 reversed high glucose induced expression of TERF2IP and TRF2, further reverting the senescence-associated changes in biochemical markers such p53 and p21. Moreover, knockdown of either TERF2IP or TRF2 abrogated hyperglycemia-dependent increase in p53 and p21 levels including reversal G1/G0 arrest of the EC (**Fig 4.3.1**).

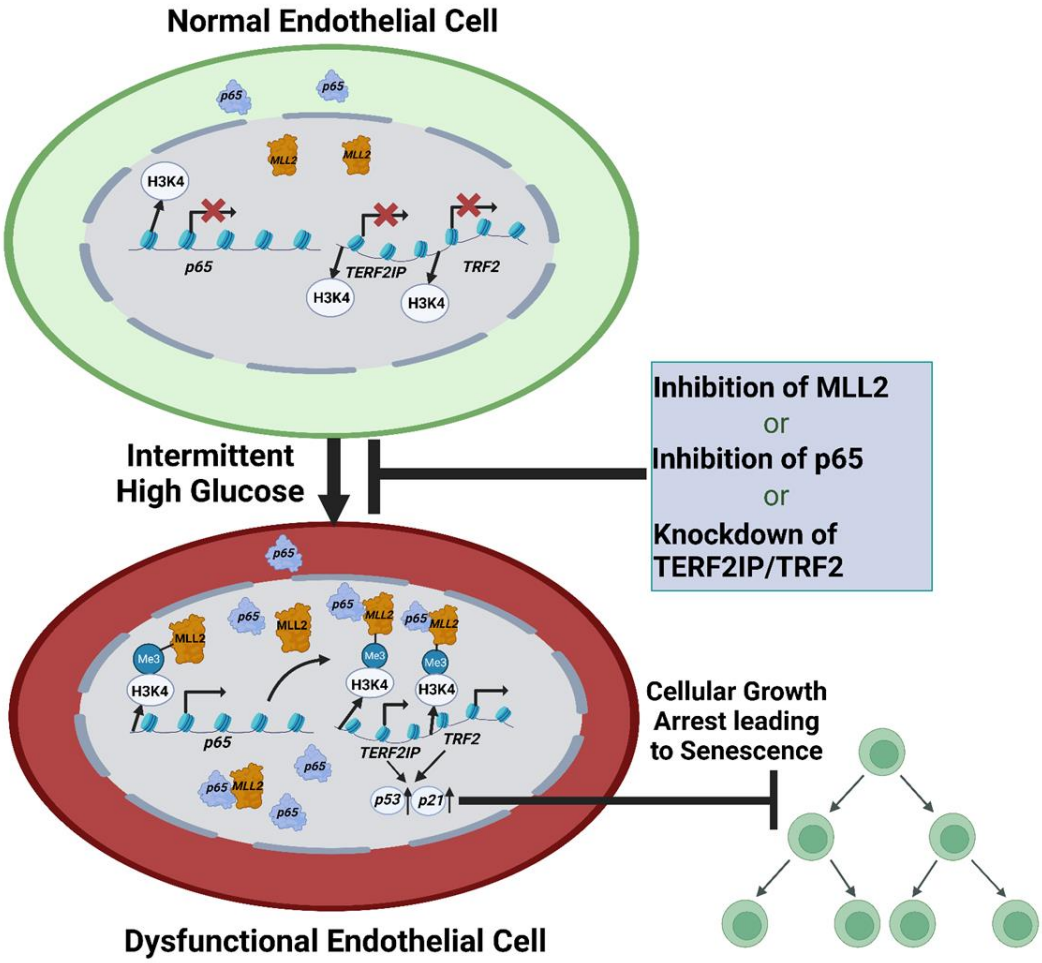


Fig 4.3.1. Schematic illustration of the intermittent high-glucose-driven induction of TERF2IP and TRF2 through H3K4me3-p65 pathway to cause endothelial senescence. *In normal glucose condition, limited level of p65 and MLL2 exist within endothelial cells, thereby supporting minimum expression of shelterin associated genes TERF2IP and TRF2. In such condition, a basal level of cellular senescence associated genes p21 and*

p53 are expressed, thus maintaining normal endothelial turn-over. Upon intermittent high-glucose challenge, endothelial cells exhibit a heightened level of H3K4me3 by MLL2 induction supporting transcriptional derepression of p65 gene and increasing its relative abundance. Further, MLL2 in conjunction with p65 caused H3K4me3 dependent elevation in TERF2IP and TRF2 level which supported the expression of cellular senescence associated genes p53 and p21 leading to endothelial senescence upon hyperglycemia shock. In contrast targeting either MLL2 or p65 or TERF2IP or TRF2 either by pharmacological inhibitor or siRNA reversed intermittent high glucose dependent increase in p21 and p53 level and cellular senescence. The schematic diagram was prepared using BioRender (Agreement number: EX25CEZQD).

Shelterin proteins are responsible for preventing telomeric damage leading to telomeric shortening, further causing associated phenotype [195]. However, in many reports, it has been observed that cells experience senescence even though they have long telomeres, and differing levels of TERF2IP and TRF2 proteins [188,195,196]. Indeed, TERF2IP and TRF2 were shown to have non telomeric activity and can respond to oxidative stress in many cells like fibroblasts, mesenchymal cells, smooth muscle cells, skin cells[189], [198], [199]. A telomeric independent pathway has been shown in which both TERF2IP and TRF2 can act as transcription factors in angiogenesis, maintaining pluripotency in stem cells [200,201]. Both can also act independently as opposed to telomeric capping in which TRF2 recruits TERF2IP and binds to telomeric ends in a D loop. TERF2IP knockout mice were shown to be incapable of atheroma progression while ApoE knockout mice when introduced with TRF2 were shown to have a greater amount of senescence in liver [202]. However, in humans, senescence is inconsistent and is different in various organs as they age. Both mice and rats have longer telomeres than human, yet they are more likely to manifest senescence phenotype due to fast developmental maturity decline [203]. In such cases, senescence also depends on the stage at which the cell is in and its current environment. Therefore, the presence of shelterin complex and associated proteins to stabilize the telomere could not be the sole determinant for senescence or growth arrest phenotype of different cells. In addition, these studies clearly indicated shelterin independent functions of TERF2IP/TRF2 including working as a transcriptional regulator

or causing induction of cellular senescence when overexpressed. In parallel, we observed that induction of TERF2IP and/or TRF2 in a hyperglycemic setting caused induction of cellular senescence/growth arrest supported by enhanced level of TERF2IP and/or TRF2. Furthermore, gene specific knockdown of TERF2IP or TRF2 reversed high glucose-dependent endothelial senescence/growth arrest indicating heightened expression of these genes are likely responsible for the manifestation of growth arrest phenotype.

p65 subunit of the NF-κB pathway is a transcriptional modulator and has been shown to regulate both p53 and p21 expression which are key part of cellular senescence pathways [204]. Published work by Wang et al 2009, embryonic fibroblasts isolated from p65 knockout mouse showed to escape senescence at a faster rate than embryonic fibroblasts collected from p65 overexpressing mice [205]. Such observation indicated a key role of p65 in imparting cellular senescence response. In an independent study forced expression of c-Rel, a member of the NF-κB transcription factor family was sufficient to induce senescence in fibroblasts by an oxidative-stress-related mechanism [205]. Furthermore, p65 has also been shown to activate cell cycle genes responsible for G1-phase entry during cell division [206]. We observed that intermittent hyperglycemia led to an increase in the p65 subunit with no difference in levels of IKK-α. Because we observed heightened level of shelterin proteins, TERF2IP and TRF2, we were curious to understand the crosstalk of TERF2IP/TRF2 and p65 pathways. TERF2IP in humans is responsible for inhibiting phosphorylation of the p65 subunit of NF-κB, through IKK-α making p65 competent for transcriptional activation [189]. Such activation of p65 by TERF2IP was dependent on cytosolic translocation of TERF2IP to associate with p65 for further transactivation of the NF-κB signaling. However, our data did not indicate changes in the relative abundance of TERF2IP in cytosolic fraction of EC upon hyperglycemia exposure. Although, we detected association of TERF2IP with p65, such association remained unaltered upon hyperglycemia challenge. In contrast, inhibition of p65 translocation to nucleus by JSH-23 led to a repression in protein levels of both TERF2IP and TRF2 in endothelial cells in a hyperglycemia setting.

Many global histone modifications particularly H3K9me3 and H3K27me3 which lead to repression of Ras signaling, DNA damage response pathways, eventually lead to a stress induced premature senescence. The epigenetic regulator SET1/COMPASS particularly

the MLL complex has been implicated in senescence. For instance, targeting MLL1 as a therapeutic strategy to deal with both oncogene induced senescence and senescence activated secretory phenotype as it activates the cell cycle genes namely p16 and p27. In another study, H3K4 methylation through SETD1A caused mitotic switch of cells and DNA-damage responses through regulation of different genes. Indeed, depletion of SETD1A caused chromosome misalignment and segregation defects including induction of senescent phenotype by transcriptional suppression of SKP2, which degrades p27 and p21 [207]. Although SET/COMPASS proteins or H3K4me3 have been shown to repress the expression of senescence associated genes in cancer microenvironment, however, our study in hyperglycemia settings with EC strongly indicated MLL2 and its catalytic product H3K4me3 caused heightened expression of p65 and shelterin protein TERF2IP-TRF2 which in turn induced the expression of p53 and p21. Downregulation of the H3K4me3 mark via pharmacological inhibition or gene knockdown of MLL2 reversed the expression of p65, TERF2IP, TRF2, p53 and p21 including countermanding hyperglycemia-driven cellular senescence.

To summarize, our study revealed the role of TERF2IP and TRF2 in controlling the expression of cellular senescence associated genes p53 and p21 further causing endothelial senescence upon hyperglycemia challenge. This in overall been governed by the regulation of H3K4me3 and in downstream p65 when EC were exposed to high glucose conditions. Telomeric erosion and uncapping is a major cause for concern encompassing senescence, however in many studies, telomere length has been shown to be unchanged. Regardless, independent to its function in telomere stability, shelterin proteins have also been observed to act as transcription factors and regulators of protein activity such as p65 activation, thus ruling out a probability of telomere shortening as the only driver of shelterin regulation dependent cellular senescence response in hyperglycemia settings. Also, TERF2IP and TRF2 have been known to regulate cell cycle proteins during genotoxic stress. Concomitantly, p21 and p53 were both found to be downregulated on knockdown of TERF2IP and TRF2 gene transcripts, suggesting that IHG promotes activation of TERF2IP and TRF2, thereby regulating levels of p21 and p53 and leading to senescence. Exploring the role of shelterin proteins in diabetes could allow

greater understanding of their role in onset and progression of diabetes and associated complications including cardiorenal diseases.

Chapter 5

Conclusion, Limitations and Future Perspectives

5.1 THESIS CONCLUSION

Vascular inflammation occurring due to hyperglycemia in diabetic patients is always had a poor prognostic. This is because even in treated patients, the endothelial lining of blood vessels in such individuals is always damaged even after medical therapy. Moreover, they are exposed to alternating glycemic shock (low and high) throughout a day which further can have an effect on the metabolic functioning of various organs, particularly the liver, pancreatic beta cells and nephron functioning. The so called metabolic memory in which cells still experience inflammation and disruption of basement membranes due to an imprint of earlier dysfunction experienced is more deleterious than a chronic stress. However, studies rarely deal with fluctuations in glucose levels and tell us only about a chronic hyperglycemic shock to which the cells are exposed. Our study therefore deals with different time kinetics for glucose levels in one day, metabolic memory and a chronic regime. Ultimately, a 12hrs high/12 hrs low regime was decided as an intermittent treatment which showed a favourably high inflammation than a chronic regime. Key endothelial homeostatic genes KLF2, KLF4, eNOS showed a significant downregulation under exposure to intermittent hyperglycemia. In addition, ICAM1, P-selectin and VCAM1, necessary for binding of immune cells were found to be significantly upregulated in intermittent hyperglycemia. As discussed earlier, the metabolic memory imprint can only be possible when the cell has epigenetic sites enriched with modifications on its chromatin. This landscape recurrence proves to be a major site of study to control and hereby provide a diagnostic for future therapy. In this case, we therefore sought to analyse repressive mark for histone modification, particularly the trimethylation occurring on H3 at lysine 27 position in promoter regions of KLF2 and KLF4. Even though we observed an increase in their levels in IHG, no change was observed in levels of EZH2. Nuclear accumulation of EZh2 was observed in cell fractionation, while lower levels were observed in cytoplasmic fraction. Immunofluorescence data showed translocation of EZH2 from cytoplasm to nucleus. For shuttling, protein modifications are necessary without which specialized functions like translocation are deemed possible. EZH2 having a threonine modification at 367 position necessary for translocation, we decided to observe a possibility for it occurring in IHG. We found basal level to remain the same, only observing an increase in expression of phosphorylated threonine. Furthermore, ChiP

analysis showed enrichment of H3K27me3 at promoter region of KLF2, thereby leading to its repression. Knockdown and catalytic inhibition of EZH2 also reversed the inflammatory phenotype of endothelial cells. Herein this study depicts the inflammatory potential of IHG associated with KLF2 enrichment and its repression in endothelial cells.

Inflammatory events, mainly in vascular inflammation are always preceded by senescence as any amount of damage to the cell will inadvertently lead to genotoxic stress, DNA damage response arrest of the cell cycle. Proliferation of cell depend on telomeric damage and uncapping at chromosomal ends. Shelterin proteins responsible for capping and protecting the telomeric DNA from damage form an important part in a stress induced senescence potential. Western blot analysis for IHG regime showed increase in levels of two shelterin proteins TERF2IP and TRF2, with no change being observed for TRF1 and TPP1. Studies dealing with senescence always signify an endpoint of inflammation called as secretory phenotype, however the sole mechanism of events leading to senescence, particularly in IHG is not yet properly studied. Further data also showed an increase in the cell cycle proteins p53 and p21 in IHG, as would be the case if shelterin expression was increasing. p65 protein is always observed at initiation of inflammation, we however decided to study it as a sole transcriptional factor independent of inflammation for other proteins, particularly shelterin proteins. Nuclear masking of p65 by an inhibitor JSH-23 led to an upregulation of TERF2IP and TRF2 in IHG. This hereby accounts for an independent role of p65 apart from NF-κB inflammatory pathway. Downstream, p53 and p21 were both found to be downregulated in p65 masking suggesting a role for p65 mediated senescence. From our previous study, we had concluded deposition of repressive histone mark in IHG, however in senescence, active mark particularly H3K4me3has been greatly observed. Its reader MLL1 has been implicated in senescence associated phenotype and regulates the cell cycle entry genes needed during the DNA damage response. Knockdown of MLL1 and its inhibition led to a reversal in expression of p53, p21, TERF2IP and TRF2. ChIP-qPCR analysis showed enrichment of H3K4me3 at promoter regions of TERF2IP, TRF2 and p65. However, as p65 had already been implicated in shelterin regulation, for deposition of a positive epigenetic mark, MLL2 binding would be necessary. A pulldown of p65 showed an increased binding of MLL2 in IHG, along with an imaging analysis for colocalization of

p65 with MLL2 in nucleus. Hence this is p65-MLL2 mediated response for senescence. siRNA analysis for TERF2IP and TRF2 also showed a reversal of expression for cell cycle proteins. Overall, we conclude that IHG can induce senescence through p65 translocation to nucleus and binding with MLL2 to activate expression of shelterin proteins ultimately leading to a cell cycle arrest as envisioned by flow cytometry. It could also be a case wherein apoptotic cells would also induce senescence; however, this was not a case. Annexin V-PI analysis showed negligible apoptotic cells, both early and late, thereby ruling out apoptosis.

5.2 Limitations

- The treatment regime dealt with only 5.5mM and 25mM glycemic shock, other pulsatile levels between this range need to be further explored for their effect in vasculature.

- The study mainly focused on histone methylation and its role in context to inflammation and senescence. Role of other chromatin modifications needs to be analyzed to assess alteration of epigenome.

- All experiments were performed *in vitro* and *ex vivo*, however to lay a strong basis for these mechanisms, *in vivo* experiments can provide better understanding for future consideration.

- Various other components play a role in inflammation and senescence both downstream and upstream. Crosstalk of these mechanisms also needs to be explored for a better understanding of these pathways.

5.3 FUTURE PERSPECTIVE

In a hyperglycemic context, we observed higher chronic inflammation when endothelial cells were exposed to intermittent hyperglycemia. Downregulation of endothelial KLF2

and KLF4 (two key transcription factors to suppress endothelial inflammation) by intermittent hyperglycemia were observed to be regulated by enrichment of H3K27me3 on their promoter regions. We also observed a reversal of KLF2 expression and endothelial inflammation when cells were treated with pharmacological inhibitor of enzyme catalyzing H3K27me3. Therefore, in future study, a more refined experiment would be to induce diabetes in mice either treated with pharmacological inhibitor of EZH2 or use endothelial specific knock out mice with a high fat diet and observe its outcome on arterial injury and dysfunction. As inhibition of EZH2 catalytic action was observed to block inflammatory switch of EC, a complementary drug based approach can be carried out along with diabetic medication in preclinical trials and clinical animal models. This would be a good therapeutic approach to reduce the vascular injury that is still seen even after a strong glucose lowering drug regime in individuals with diabetes. Hyperglycemia was also observed to lead to senescence in endothelial cells via a telomeric independent process. p65 inhibition led to a repression of the shelterin proteins TERF2IP and TRF2. As their upregulation led to progression of senescence, p65 plasmid with a mutated DNA binding motif can be experimented with to observe any activation of sequences coding for TERF2IP and TRF2. A possibility for connecting TERF2IP/TRF2 to cellular senescence also exists via its role as regulators of transcription. Therefore, researching on a possible drug that blocks its DNA binding ability could prove to be a therapeutic approach in preventing inflammation and promoting cell repair through proliferation.

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APPENDIX

Appendix A1. List of publications

From Thesis –

1) **Intermittent High Glucose Elevates Nuclear Localization of EZH2 to Cause H3K27me3- Dependent Repression of KLF2 Leading to Endothelial Inflammation**
   Sumukh Thakar, Yash T. Katakia, Shyam Kumar Ramakrishnan, Niyati Pandya Thakkar, Syamantak Majumder Biology, Cells, 26 September 2021

2) **Regulation of shelterin proteins TERF2IP and TRF2 by H3K4me3-p65 axis drives hyperglycemia dependent endothelial senescence**
   Sumukh Thakar, Ritobrata Bhattacharya, Yash T Katakia, Srinjoy Chakraborty, S Ramakrishnan, Niyati Pandya Thakkar, Smita Dey, Shibasish Chowdhury, Syamantak Majumder. Preprint, posted on bioRXiv, doi: 10.1101/2023.05.12.540614

Other publications –

1) **Dynamic alterations of H3K4me3 and H3K27me3 at ADAM17 and Jagged-1 gene promoters cause an inflammatory switch of endothelial cells**
   Yash T. Katakia, Niyati P Thakkar, Sumukh Thakar, Ashima Sakhuja, R. Goyal, Harshita Sharma, Rakshita Dave, Ayushi Mandloi, Sayan Basu, Ishan Nigam, B. V. Kuncharam, S. Chowdhury, Syamantak Majumder, Journal of cellular physiology,14 September 2021

2) **Elevated H3K4me3 Through MLL2-WDR82 upon Hyperglycemia Causes Jagged Ligand Dependent Notch Activation to Interplay with Differentiation State of Endothelial Cells**
   Niyati Pandya Thakkar, B. M. V. Pereira, Yash T. Katakia, Shyam Ramakrishnan, Sumukh Thakar, Ashima Sakhuja, Gayathry Rajeev, S. Soorya, Karina Thieme, Syamantak Majumder, Frontiers in Cell and Developmental Biology, 22 March 2022

3) **Engineering a light-driven cyanine based molecular rotor to enhance the sensitivity towards a viscous medium.**

Vishal Kachwal, Abhilasha Srivastava, Sumukh Thakar, Maria Zubiria-Ulacia, Diplesh Gautam, Syamantak Majumder, V. K. P., D. Casanova, Rajdeep Chowdhury, N. Rath, S. Mukherjee, P. Alemany, I. R. Laskar, Materials Advances, 9 June 2021

Appendix A2 – List of Conferences

1) **Virtual Symposium – ROLE OF EPIGENETICS IN HEALTH: CHALLENGES AND OPPORTUNITIES, Birla Institute of Technology and Science, Pilani, Pilani Campus, February 25-26,2022**
   Title - Intermittent High Glucose Elevates Nuclear Localization of EZH2 to Cause H3K27me3- Dependent Repression of KLF2 Leading to Endothelial Inflammation
   Sumukh Thakar, Yash T. Katakia, Shyam Kumar Ramakrishnan, Niyati Pandya Thakkar, Syamantak Majumder

2) **Poster presentation – 45th All India Cell Biology Conference, Banaras Hindu University, Varanasi, 20-22 January 2023**
   Title - Intermittent High Glucose caused H3K27me3 dependent repression of IKB-α leading to p65 Mediated Upregulation of TERF2IP to Induce Endothelial Senescence
   Sumukh Thakar, Ritobroto Bhattacharya, Srinjoy Chakraborty, S. Ramakrishnan, Yash Katakia, Syamantak Majumder

Appendix A3 – List of biographies

Brief biography of supervisor

Dr. Syamantak Majumder received his PhD from Anna University, Chennai, India (2007-2011) in field of Cell and Vascular Biology and moved to University of Rochester, Rochester, NY, USA as a Post Doctoral Fellow (2011-2014) followed by another Post Doctoral Training at St Michael's Hospital, University of Toronto, Toronto, ON, Canada (2014-2017). Dr. Majumder has more than 17 years of experience in the field of vascular biology including diabetic complications. Dr. Majumder has authored 57 (5 as corresponding author and 20 as the first author) published research articles, reviews, editorials and book chapters including 49 research articles in peer-reviewed journals with a Google Scholar h-index of 24, about 1920 Citations and i-10 index of 42. His team is trying to identify and target the epigenetic mechanisms driving endothelial inflammation (J Cell Physiol. 2021, IF 6.6; Communication Biology 2022, IF 6.6) during diabetes (Cells 2021, IF 7.7; Frontiers in Cell and Developmental Biology, IF 6.1) that lead to cardiovascular and kidney diseases. He is currently supervising six PhD students. Dr. Majumder's work has been funded by multiple grants from DST, SERB, DBT, ICMR and DRDO including high value projects as PI or Co-PI such as DBT BUILDER and DST-PURSE.

Brief biography of the candidate

Mr. Sumukh Thakar has graduated with a Bachelor degree in Industrial Microbiology from Abasaheb Garware College, Pune University in 2015. He later Graduated with Masters in Molecular and Human genetics from Jiwaji University, Gwalior in 2017. As part of dissertation, he has worked as a research assistant for 6 months in gallbladder cancer thesis entitled as ‘PROMOTER METHYLATION ANALYSIS OF CXC MOTIF CHEMOKINE RECEPTOR 4 GENE IN GALLBLADDER CARCINOMA'. He later joined for his PhD in 2017 under supervisor Prof. Syamantak Majumder. He has published 4 research articles in peer reviewed journals. Alongside Research, he has also been involved in teaching and has taken courses for first degree students in BITS Pilani, Pilani Campus.