CHAPTER V

Identification of candidate oxidative stress response genes regulated by EZH2 and fluid shear stress

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In preparation
ABSTRACT

The exposure of blood vessels to a variety of risk factors, in particular those that induce oxidative stress, causes endothelial dysfunction and predisposes to cardiovascular disease. In arteries the high level of fluid shear stress suppresses endothelial oxidative stress, while in areas with low or disturbed flow endothelial cells are prone to dysfunction. The epigenetic mechanisms that suppress endothelial dysfunction are poorly understood. As EZH2 methyltransferase is involved in the regulation of endothelial gene expression and plays a role in endothelial response to FSS, we hypothesized that EZH2 is also involved in the regulation of the expression of oxidative stress-responsive genes under FSS. RNA-seq analysis revealed that expression of genes within the GO cluster “Response to oxidative stress” is regulated by EZH2 or by FSS. Of those, 32 genes were regulated by both EZH2 and FSS, in HUVEC. Four genes whose expression increased more than 2-fold upon both EZH2-depletion and FSS-exposure were HMOX1, PTGS1, PTK2B and GPX3. EZH2 was present in the proximal promoter regions of these genes, as determined through the analysis of ENCODE HUVEC ChIP-seq data. The two genes with the relatively lowest expression, PTK2B and GPX3, consistently showed high levels of H3K27me3 around their promoter regions in the ENCODE dataset, suggesting they might be direct targets of activity of EZH2. The 5’-UTRs of these four genes shared putative transcription factor binding sites of the repressive factors ZNF263 and FOXP1.

This study revealed four candidate genes that are under the repressive control of EZH2 and are activated by FSS, including a well-known oxidative stress-responsive gene HMOX1. It further explores the generic epigenetic make-up around promoters of these genes and suggests regulation by two candidate transcription factors. These results shed new light on the mechanisms behind the anti-oxidant effects of high FSS and corroborate the role of EZH2 as an epigenetic regulator of endothelial gene expression.
INTRODUCTION

Endothelium, the inner layer of all blood vessels, serves several functions essential for vascular homeostasis. Chronic endothelial dysfunction contributes to the development of cardiovascular disease (CVD). Many risk factors of CVD are known to also cause endothelial dysfunction by eliciting the oxidative stress; these include cigarette smoke, chronic inflammation, oxidized low density lipoprotein (OxLDL), among others.\textsuperscript{1,2} Reactive oxygen species (ROS) are an integral part of cellular function: they are produced in the metabolic reactions in the cytoplasm and mitochondria and are part of signaling pathways such as NFkB.\textsuperscript{3} However, their excessive production and chronic presence are detrimental to the cellular homeostasis. ROS may damage cellular components through oxidation which may cause mitochondrial dysfunction and cell death. In the cell, multiple biochemical mechanisms counteract excessive ROS,\textsuperscript{4} including the oxidative stress-responsive expression of protective genes, such as heme oxygenase-1 (HMOX1) or superoxide dismutases (SOD). The products of the activated genes are enzymes or scavenger molecules which neutralize ROS.

In the body, endothelial cells (ECs) are constantly exposed to the blood flow, which exerts the force known as fluid shear stress (FSS) on the blood vessel wall. Disturbed blood flow exerts on average low levels of FSS and is associated with endothelial dysfunction.\textsuperscript{5-7} Cells exposed to disturbed flow produce relatively high levels of ROS and are thus exposed to oxidative stress.\textsuperscript{8,9} On the other hand, high FSS protects ECs, preserves endothelial function\textsuperscript{5-7} and associates with reduced levels of oxidative stress.\textsuperscript{8,9} Enhancer of Zeste Homolog-2 (EZH2), the methyltransferase of the Polycomb complex of epigenetic repressors,\textsuperscript{10} regulates a large number of genes that are also affected by FSS in endothelial cells (Maleszewska M et al., submitted). High FSS causes decreased EZH2 expression, which reduces endothelial proliferation and promotes quiescence. In this study, we investigated whether some of the anti-oxidant effects of FSS could be explained by the decrease in EZH2 expression resulting in differential expression of the oxidative stress-responsive genes.
MATERIALS AND METHODS

Cell culture

Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Lonza (Basel, Switzerland) and used between passages 4 and 9. Cells were cultured in endothelial culture medium (ECM) as described before, with 5.5 mM glucose and 10% fetal calf serum (FCS; Lonza, Basel, Switzerland). L-α-lysoosphatidylcholine (LPC; Sigma-Aldrich, St. Louis, MO, USA) was used at the concentration of 25µM in serumfree ECM for 24h; control cells were incubated with the same concentration of L-α-phosphatidylcholine (PC; Sigma-Aldrich, St. Louis, MO, USA). Oligomycin (Sigma-Aldrich, St. Louis, MO, USA) was used at 20µg/ml concentration for 2 days in 5% FCS ECM, control cells were incubated with DMSO. EZH2 inhibitor GSK126 (Cellagen Technology, San Diego, CA, USA, C4126-2s) was used at 1µM concentration for 4 days in ECM with 5% FCS and control cells were incubated with DMSO. Medium was refreshed daily.

Fluid shear stress (FSS) of 20 dyne/cm² was applied to confluent HUVEC cultures in ECM with 5% FCS in Ibidi 0.4 µ-Luer slides with use of the Ibidi pump system (Ibidi, Planegg-Martinsried, Germany), for 3 days, in standard cell culture conditions. Static controls were incubated along in the same incubator and medium was refreshed daily.

Human Embryonic Kidney (HEK) cells were cultured in DMEM (Lonza, Basel, Switzerland) supplemented with 1% penicillin/streptomycin (Gibco/Thermo Fisher Scientific, Waltham, MA, USA), 2mM L-glutamine (Lonza, Basel, Switzerland) and 10% FCS.

ROS measurement

Cells were detached with accutase (PAA Laboratories, Pasching, Austria), collected in culture medium into FACS tubes and centrifuged. Pellets were suspended in 500µl of 20µM solution of 2’,7’-dichlorodihydrofluoresceindiacetate (DCFH-DA; Sigma-Aldrich, St. Louis, MO, USA) in medium. Cells were incubated at 37°C for 30min. Cells were then analyzed at FACSCalibur™ (BD Biosciences, San Jose, CA, USA). Further data analysis was performed using FlowJo software.
ATP measurement

ATP levels were measured using the “ATP Bioluminescence Assay Kit CLS II” (Roche, Basel, Switzerland), following the manufacturer’s protocol. Cells were washed with PBS and detached using accutase. Upon centrifugation, pellets were suspended in medium and cells were counted. Cells were then centrifuged and suspended at 2x10^6 cells/ml (oligomycin experiments) or 1x10^6 cells/ml (LPC experiments). One volume of each cell suspension was combined with 9 volumes of boiling buffer (100mM Tris, 4mM EDTA, pH=7.75). Samples were incubated for 2 min at 100°C, cooled on ice and centrifuged at 1,000x g for 1 min. Supernatants were collected and stored on ice. ATP standard was prepared in Milli-Q water by tenfold serial dilution in the range of 10^-4 to 10^-10 M. Samples or standard solutions were combined with luciferase solution 1:1, in 96-well flat-bottom black plates. Both ATP standard and luciferase were part of the kit and were prepared accordingly with the manufacturer’s protocol. Luminescence was measured with the Luminoskan™ Ascent (Thermo Scientific, Waltham, MA, USA). Linear standard curves were plotted using log-transformed values and the standard curve equations were used to determine the concentration of ATP in the samples.

RNA isolation and qRT-PCR

Cells were washed with PBS and lysed with either TriZOL (Invitrogen, Carlsbad, CA, USA) or RNA-Bee (Tel-TEST, Inc., Friendswood, TX, USA, Invitrogen, Carlsbad, CA, USA). RNA was isolated with standard chloroform/phenol extraction and precipitated with 2-propanol. RNA pellets were washed 2 times with ice-cold 75% ethanol, dried, and suspended in RNAse-free water. RNA concentrations were measured using Nanodrop (Thermo Scientific, Waltham, MA, USA) and cDNA was synthetized using the RevertAid™ First Strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). Real-time qPCR was performed at the ViiA7 Real Time PCR machine (Applied Biosystems, Foster City, CA, USA). SYBR-Green chemistry was used (Bio-Rad, Hercules, CA, USA), with 10ng cDNA input per reaction. Data were analyzed with the ViiA7 software. Samples were normalized with ΔCt method, using the geometrical mean of β-actin and GAPDH as control. Fold change in gene expression versus control was calculated using ΔΔCt method. The following primers were used (5’-3’; Forward, Reverse):

β-actin: CCAACCGCGAAGAGATGA, CCAGAGGCCTACTACGGGATAG,
EZH2: GCGAAGGATACAGCCTGTGCACA, AATCCAAGTCACTGGTCACC, GAPDH: AGCCACATCGCTAGACAC, GCCCAATAGACAAATCC, HMOX1: GGGTGATAGAAGGAGCCAAGA, AGCTCCTGCAACTCCTCAA, p16: CTTCCCTGGACACGCTG, GCAGGTACCTGCGACAT.

Lentiviral transduction

To produce lentiviral particles, HEK cells were transfected with lentiviral pLKO.1 vectors containing shRNA against EZH2 (shEZH2) or scrambled control shRNA (SCR), along with the 2nd generation lentiviral vector plasmids pCMVΔR8.91 and pVSV-G. To this end, Endofectin™-Lenti (Gene Copoeia, Rockville, MD, USA, EFL-1001-01) was used, according to the manufacturer’s guidelines. Subconfluent HUVEC cultures were transduced 2 times at 24h intervals, using 0.45µm-filtered HEK supernatants, in ECM with 10% FCS. Every first transduction was executed with an addition of 4 µg/ml of polybrene. Cells were selected at day 5 post-first transduction in medium with 2µg/ml of puromycin (Invitrogen). Surviving cells were allowed to regenerate for 24h prior to lysis at day 7 post-first transduction.

Bioinformatical analysis

RNA-seq datasets acquired previously (Maleszewska M et al., submitted) were analyzed here. Details of the experimental set-up and analysis methods were described before (ibidem). The list of genes associated with the Gene Ontology (GO) term “Response to oxidative stress”, GO:0006979, was obtained from the GO repository using AmiGO2 (as of 24.09.2014). The list was used to retrieve the differential gene expression data from our RNA-seq datasets, using CummeRbund (v. 0.1.3) in R-studio 0.98. Network analysis was performed using the String 9.1 database. Intersection of lists of genes was performed with BioVenn and visualized with Venn Diagram R-package. Data presented in Supplementary Table 2 was obtained from Ensembl, using BioMart. The visualization of the selected ENCODE HUVEC ChIP-seq data (Broad Institute, Bernstein lab) was performed with Integrative Genomics Viewer (IGV 2.3). GEO accessors: GSM733688 (H3K27me3), GSM733673 (H3K4me3), GSM733691 (H3K27ac), GSM1003517 (H3K9me3), GSM1003518 (EZH2).
The 5’-UTR sequences of 1,200bp upstream of the first exon of each analyzed gene were obtained from Ensembl (GRCh38). The motif analysis was performed with Multiple Em for Motif Elitication (MEME, v. 4.9.1). The resulting significant motifs (E<0.05) were used in TOMTOM Motif comparison tool (v. 4.9.1) using JASPAR core vertebrate database (as of 20.10.2014) and Uniprobe mouse database (as of 20.10.2014). The most significant hits for each motif are reported.

Statistical analysis

Statistical analysis was performed in the GraphPad Prism 5. T-test or one-way ANOVA with Tukey post-hoc comparisons were performed were relevant. P-values < 0.05 were considered cut-off for statistical significance. Graphs depict mean and standard error of the mean, unless specified otherwise.

RESULTS

Selection of the groups of genes involved in the response to oxidative stress, regulated by either EZH2 or FSS

We first set out to identify the genes that play a role in the oxidative stress response and are regulated by EZH2 in endothelial cells. We obtained the list of genes associated with the Gene Ontology term “Oxidative stress response” (Supplementary Table 1) and analyzed their changes in expression upon the knock-down of EZH2. A total of 56 genes showed changes in expression in the absence of EZH2 (Fig. 1A), some of which are known to occur or function together (Fig. 1B).

On the other hand, of the genes associated with the “Oxidative stress response” term, 58 were differentially expressed under FSS, compared to static conditions (Fig. 2A). Some of these FSS-responsive genes are also interconnected, suggesting that they work together (Fig. 2B).

Identification of candidate oxidative stress response genes regulated by the decrease in EZH2 under FSS

Intersecting the lists of genes differentially regulated by either EZH2 or FSS allowed to identify a total of 32 genes regulated both by depletion of EZH2 and by exposure to FSS (Fig. 3A and Supplementary Table 2). Four of these genes were 2-times or more upregulated (Fig. 3B and C), suggesting that they are biologically the most relevant. These genes were HMOX1, PTGS1, GPX3.
and PTK2B, encoding heme oxygenase-1, prostaglandin G/H synthase-1 (also known as COX-1, Cyclooxygenase-1), glutathione peroxidase-3, and protein tyrosine kinase-2β (also known as Pyk2), respectively.

**Regulation of EZH2 by changes in cellular ROS and ATP levels**

We and others found that action of EZH2 can be affected by changes in its expression.$^{23,24}$ To assess the influence of ROS on EZH2 expression, we treated
Regulation of the oxidative stress response genes

HUVEC with LPC, a component of OxLDL species, that induce oxidative stress and cause endothelial dysfunction. Treatment of HUVEC with LPC increased intracellular ROS levels (Supplementary Fig. 1A and B). The gene expression of ROS-responsive HMOX1 and of EZH2 was elevated, while the expression of EZH2 target gene p16 seemed to decrease, however none of these effects reached statistical significance (Supplementary Fig. 1C through E).

High levels of oxidative stress lead to mitochondrial dysfunction and a decreased ATP production. ATP levels tended to decrease in LPC-treated HUVEC.
Figure 3. Identification of genes regulated by both EZH2 and FSS. A – Venn diagram showing the numbers of genes regulated only by the knock-down of EZH2 (24 out of 56 differentially regulated genes in the comparison of static-scrambled control vs. static-SH-EZH2), or by FSS only (26 out of 58 differentially regulated genes in the comparison of static-scrambled control vs. FSS-scrambled control), or by both (the common field including 32 genes). B – Venn diagram showing the numbers of genes that were 2-times or more upregulated by the knock-down of EZH2 only (4 out of 8), the exposure to FSS only (3 out of 7), or by both (the common 4). C – Heatmap representation of relative gene expression of the 4 genes that were upregulated by both the knock-down of EZH2 and the exposure to FSS. Log10(FPKM+1) values are shown in the colour scale, with dark-green indicating high, and light-green/yellow indicating low expression.
Regulation of the oxidative stress response genes

(Supplementary Fig. 1F). Also cells depleted of ATP by means of oligomycin treatment (Supplementary Fig. 2A) had approximately 2-fold higher levels of EZH2 mRNA (Supplementary Fig. 2B).

**Validation of HMOX1 regulation**

Of the four genes upregulated by EZH2-depletion and FSS-exposure, *HMOX1* had the relatively highest expression in control HUVEC, yet it was further upregulated by EZH2-depletion, FSS-exposure, and both (Fig. 3C). Independent validation confirmed that *HMOX1* gene was upregulated upon the knock-down of EZH2 (Fig. 4A) and the exposure to FSS (Fig. 4B). Also the inhibition of EZH2 activity in HUVEC led to an increase in HMOX1 expression (Fig. 4C).

**Chromatin landscape of HMOX1, PTGS1, GPX3 and PTK2B genes**

To better understand the regulation of *HMOX1, PTGS1, GPX3* and *PTK2B* gene expression, we used the ENCODE ChIP-seq data from HUVEC cells, which can be regarded as equivalent to our control condition (i.e. to the scrambled shRNA static control cells). In the ENCODE dataset, EZH2 was present in proximity of the gene body and 5’-UTR of *HMOX1*, but the H3K27me3 was absent, likely replaced by high levels of H3K27ac (Fig. 5A). The high level of marks associated with active transcription (H3K4me3 and H3K27ac) and low level of repressive marks (H3K27me3) suggests that the *HMOX1* gene is actively expressed already in control HUVEC, which corroborates the relatively high expression of *HMOX1* in our experiment in control conditions (Fig. 3C). Interestingly, another gene with relatively high expression in control conditions, PTGS1 (Fig. 3C), had no H3K27me3 present in its proximity, similarly to *HMOX1*, despite the presence of EZH2 peaks (Fig. 5B). On the

![Figure 4. HMOX1 gene expression is regulated by EZH2 and FSS. A – Gene expression of HMOX1 upon 7-day knock-down of EZH2 in HUVEC. B – Gene expression of HMOX1 upon 3-day exposure to FSS, 20 dyne/cm2. C – Gene expression of HMOX1 upon treatment with EZH2 inhibitor GSK126 for 4 days. *p<0.05, **p<0.01, t-test.](image-url)
Regulation of the oxidative stress response genes
Figure 5. Chromatin landscape around the gene body and 5'-UTR of HMOX1, PTGS1, GPX3 and PTK2B genes (A, B C and D, respectively). The tracks depict the ChIP-seq peaks (P) and signal (S) in HUVEC cells, from the ENCODE data (Broad Institute), visualized with IGV. The signal (S) is the read density of the experimental sample, while the peak (P) is the region where the signal is significantly enriched (compared to the input sample), and therefore can be considered the “footprint” of EZH2 or the histone modification in question.
other hand, the genes with the lowest basal expression (Fig. 3C), GPX3 and PTK2B, had both EZH2 and H3K27me3 in their proximity (Fig. 5C and D, respectively), indicative of a repressed state.

**Identification of shared putative TFBSs in 5’-UTRs of HMOX1, PTGS1, GPX3 and PTK2B**

Genes regulated in response to the same stimuli are likely to share similarities in their transcriptional control, including common transcription factors driving their expression. The four candidate genes identified here are regulated in response to oxidative stress. We therefore checked whether they share any common motifs within their 5’-UTRs (1200bp upstream of the first exon), which could be potential transcription factor binding sites (TFBSs). The most significantly enriched motif, motif A (Fig. 6A) was found in the 5’-UTRs of all four genes (Fig. 6C and Supplementary Fig. 3A) and was significantly similar to the motif bound by the transcription factor ZNF263 (Fig. 6E). The second identified motif, motif B (Fig. 6A), was only shared by HMOX1 and GPX3 (Fig. 6D and Supplementary Fig. 3B), and was similar to the motif bound by FOXP1 transcription factor (Fig. 6F).

**DISCUSSION**

**Main findings**

In this study we identify four candidate genes associated with the response to oxidative stress, which can be regulated by the decrease in EZH2 under FSS in endothelial cells: HMOX1, PTGS1, GPX3 and PTK2B. We further propose two candidate transcription factors which can be involved in the regulation of these genes based on the shared sequence motifs in the 5’-UTRs: ZNF263 and FOXP1.

**Figure 6. Identification of common motifs in the 5’-UTR regions of human HMOX1, GPX3, PTGS1 and PTK2B genes.** A and B – Sequence logos of the two significantly enriched motifs from the 5’-UTRs of HMOX1, GPX3, PTGS1 and PTK2B. C – The localization of the motif A within the 1200bp of 5’-UTRs of HMOX1, GPX3, PTGS1 and PTK2B, obtained from MEME. D – The localization of the motif B within the 1200bp of 5’-UTRs of HMOX1 and GPX3, obtained from MEME. E – The sequence logo (upper) of the ZNF263 transcription factor, the most significantly similar to the motif A (lower), derived through TOMTOM analysis using JASPAR database. F – The sequence logo (upper) of the FOXP1 transcription factor, the most significantly similar to the motif B (lower), derived through TOMTOM analysis using JASPAR database.
The role of HMOX1, PTGS1, GPX3 and PTK2B in oxidative stress and endothelial cells

GPX3 is connected to the resolution of the oxidative stress, as it is an enzyme facilitating the reaction of glutathione with ROS. GPX3 is normally excreted to plasma and is required for the antithrombotic properties of the endothelium. PTGS1, well known as COX-1, is one of the enzymes in the prostaglandin synthesis, converting arachidonic acid to prostaglandin H2. It is crucial for prostacyclin (PGI2) production in endothelial cells in response to oxidative challenge. In addition, endothelial secreted PGI2 is important to maintain vascular homeostasis as it inhibits platelet activation. PTK2B seems to be the least directly associated with the oxidative stress, yet it is activated by ROS. Moreover, PTK2B mutations are associated with hypertension in the Japanese population, but little is known on its own anti-oxidant properties. HMOX1 is an oxidative stress-responsive gene, crucial for cellular resistance to ROS. The HMOX1 enzyme is responsible for catabolism of heme to biliverdin, ferrous ion and carbon monoxide. In the condition of oxidative stress, free heme can catalyze reactions creating free radicals, and the induction of HMOX1 expression is pivotal to counteract this harmful phenomenon. This role of HMOX1, along with the protective properties of the products of its enzymatic reaction, is essential for cellular homeostasis, and yields HMOX1 as an antiapoptotic and cytoprotective molecule. HMOX1 is also crucial for the function of endothelial cells. The single known human case of HMOX1 deficiency demonstrated with severe endothelial damage due to unresolved oxidative stress. Genetic variation in HMOX1 promoter, reflected by differential expression of HMOX1, affects some aspects of the CVD, and directly modulates the endothelial function.

The repressive regulation of HMOX1, PTGS1, GPX3 and PTK2B

The HMOX1, PTGS1, GPX3 and PTK2B genes are regulated by EZH2. It is corroborated by the fact that the depletion of EZH2 correlates with an increase in their expression. Moreover, the ENCODE results show that EZH2 is present around their promoter regions, and the low-expressed GPX3 and PTK2B also bear the repressive H3K27me3 mark. The relatively higher expressed HMOX1 and PTGS1 have low levels of H3K27me3, but high levels of H3K27ac, indicative of active transcription. Interestingly, both of the candidate transcription factors identified here, ZNF263 and FOXP1, are in fact transcriptional repressors. This reinforces
the concept that the promoters of *HMOX1*, *PTGS1*, *GPX3* and *PTK2B* are regulated by repression.

Next to the repressive H3K27me3 mark, these promoters also bore the H3K4me3 mark, indicative of active transcription. In budding yeast, H3K4me3 is found at the 5’ end of genes, often along with the polymerase II in its active state. In mammalian cells, K4me3 decorates histones 3 close to transcription start sites, mapping right after the DNAse I hypersensitivity sites (DHSs), and thereby marking the transition points between a gene promoter and transcribed region. The ENCODE data used here showed that the active H3K4me3 mark is present along with EZH2 and H3K27me3 repressive mark in case of GPX3 and PTK2B. It suggests that the removal of H3K27me3 could lead to activation of transcription. The concomitant presence of H3K4me3 (active mark) and H3K27me3 (repressive mark) is characteristic for so called bivalent domains, regulating many developmentally important genes. It is believed to facilitate the timely activation of their transcription during development and differentiation. Genes regulated by bivalent domains remain in a poised state, where the balance between H3K27me3 and H3K4me3 can be modulated, fine-tuning the gene expression. The concomitant presence of H3K4me3 and H3K27me3 at the promoters of *HMOX1*, *PTGS1*, *GPX3* and *PTK2B* might serve a similar purpose, i.e. constitute the mechanism which holds these genes poised for upregulation in case of occurrence of oxidative stress.

FSS-induced decrease in EZH2 abundance would therefore contribute to the decrease in H3K27me3 levels at the promoters of these genes, allowing for H3K27 acetylation and activation of transcription of *HMOX1*, *PTGS1*, *GPX3* and *PTK2B*, altogether resulting in more resistant endothelial phenotype (Fig. 7).

While the ENCODE data we used can be considered equivalent to our data from the control conditions, it might also differ because of different batch of HUVEC cells used and differences in the cell culture routines, including cell culture medium. Future verification of the ENCODE-based observations, included in this chapter, in our culture system will be required to confirm our conclusions.
CONCLUSIONS

This study identifies a group of candidate genes of high importance in the response of endothelial cells to FSS. The regulation of these genes depends on EZH2 (RNA-seq data), and likely on its epigenetic mark H3K27me3 (based on the integrated ENCODE ChIP-seq data). Besides exploring the similarities in the epigenetic landscape of the promoters of HMOX1, PTGS1, GPX3 and PTK2B, we further propose two transcriptional factors, ZNF263 and FOXP1, which could be involved in the repression of these genes, alongside with EZH2. These findings further corroborate the role for EZH2 in the repression of FSS-responsive genes in endothelial cells.
ACKNOWLEDGEMENTS

We would like to thank Dr. V. van den Boom and Prof. Dr. J.J. Schuringa (Dept. Experimental Hematology, University Medical Center Groningen) for the lentiviral plasmids (pLKO.1-SCR and pLKO.1-shEZH2). The authors would like to acknowledge the financial support of the Netherlands Institute for Regenerative Medicine (NIRM, #FES0908; M.C.H). This work was additionally supported by the Jan-Kornelis de Cock foundation (M.M.), the Groningen University Institute for Drug Exploration (GUIDE; G.K. and M.C.H.) and the ZonMW/Netherlands Organization for Scientific Research (NWO) Innovational Research Incentive (#916.11.022; G.K.). The authors declare no conflicts of interests.

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SUPPLEMENTARY MATERIAL

Supplementary Figures

Supplementary Figure 1. EZH2 expression is not regulated by LPC-induced ROS.
A – Representative histogram showing the shift in MFI between cells treated with L-α-phosphatidylocholine (PC) and L-α-lysophosphatidylocholine (LPC), corresponding to the increase in the ROS levels in the cells. B – Increase in ROS levels in HUVEC treated with LPC as compared to control conditions (PC). ***p<0.001. C, D and E – Gene expression of HMOX1, EZH2 and p16, respectively, in response to the treatment with LPC. F – ATP concentration in cells treated with PC or LPC. In C through F, p-values are derived from t-tests.
Supplementary Figure 2. Cellular ATP levels might affect the expression of EZH2. A – The ATP concentration in control (DMSO) cells and oligomycin-treated cells. B – Gene expression of EZH2 in control cells and cells treated with oligomycin. N=2, error bars depict the range.
Supplementary Figure 3. The alignments depicting the sites where the motif A (A) or motif B (B) (compare Figure 7A and B, respectively) are present in the 5'-UTR sequences of HMOX1, PTGS1, GPX3 and PTK2B genes, as obtained from MEME.