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Epigenetic suppression of iNOS expression in human endothelial cells: A potential role of Ezh2-mediated H3K27me3

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ABSTRACT

Objective: Cytokines strongly induce expression of the inducible nitric oxide synthase (iNOS) in rodent but not in human endothelial cells. We recently identified *NOS2* as a potential target of the histone methyltransferase enhancer of zeste homolog 2 which mediates trimethylation of histone 3 at lysine 27 (H3K27me3).
Methods and results: Compared to an unspecific IgG control, chromatin immunoprecipitation using a H3K27me3-specific antibody followed by DNA quantification by PCR showed a strong DNA enrichment — indicating that *NOS2* is associated with H3K27me3 in human umbilical vein endothelial cells (HUVEC). siRNA-mediated knock down of Ezh2 diminished *NOS2* DNA enrichment — suggesting that the association of *NOS2* with H3K27me3 is mediated by Ezh2. Ezh2 knock down, however, was not sufficient to increase iNOS expression after stimulation of HUVEC.
Conclusion: NOS2 is associated with Ezh2-mediated H3K27me3 in HUVEC. This might contribute to an epigenetic suppression of iNOS inducibility in human endothelial cells.

1. Introduction

Nitric oxide (NO) is both a central regulator of endothelial function and part of the innate immune response. In the vasculature, NO is generated at low concentrations by the endothelial NO synthase (eNOS) and responsible for vasodilation through cyclic guanosine monophosphate-dependent relaxation of vascular smooth muscle cells. eNOS is primarily expressed in endothelial cells and mainly regulated by phosphorylation. For antimicrobial defense, NO is produced in high concentrations by the inducible NO synthase (iNOS). In contrast to eNOS, iNOS is regulated by cytokine-dependent transcriptional activation and primarily expressed in macrophages, monocytes and hepatocytes [1–3]. Of note, iNOS is highly inducible in many cell types from rodents but not from humans — despite conserved *cis*-elements for transcription factors such as NFκB, IRF-1, AP-1, STAT3, and HIF1 [4]. As a possible explanation for this species-specific inducibility, a previous study demonstrated hypermethylation of the human iNOS promoter and an association of the promoter with trimethylated lysine 9 in histone 3 (H3K9me3) in endothelial cells [4]. In macrophages, a recent report confirmed a strong inducibility of iNOS in murine cells — and a lack thereof in cells of human origin. This correlated with an inverse presence of activating and repressive histone modifications at the iNOS promoter in both cell types, respectively. Specifically, the iNOS promoter was predominantly associated with activating trimethylated lysine 4 in histone 3 (H3K4me3) in murine macrophages. In contrast, human macrophages showed an inverse relationship favoring trimethylated lysine 27 in histone 3 (H3K27me3) [5].

In a recent project, we identified *NOS2*, the gene encoding iNOS, as a putative target gene of the histone methyltransferase enhancer of zeste homolog 2 (Ezh2) in human umbilical vein endothelial cells (HUVEC) [6]. Ezh2 belongs to the Polycomb group (PcG) proteins which are highly conserved regulators of the epigenome first described in Drosophila [7]. As part of the Polycomb repressive complex 2 (PRC2), Ezh2 catalyzes trimethylation of histone 3 at lysine 27 (H3K27me3) which acts as a repressive epigenetic mark [8]. In addition, Ezh2 recruits DNA methyltransferases, which further enhance gene repression [9]. Moreover, it is essential for propagation of the H3K27me3 epigenetic mark in proliferating cells — a defining feature of epigenetic regulation [10].

In human stem cells, Polycomb repressive complexes are essential for maintaining pluripotency by repression of developmental genes [7,11]. Diminished expression of PcG proteins during differentiation results in de-repression of PRC target genes and has been shown to play an important role in heart development, neurogenesis, skeletal muscle differentiation and adipogenesis [12–16]. While little is known about the function of Ezh2 in differentiated cells, a transcriptomic study recently suggested relevant expression of Ezh2 in endothelial cells [17]. We therefore analyzed the effects of siRNA-mediated knock down of Ezh2 in HUVEC and identified 276 putative Ezh2 target genes by combining ChIP-on-chip- and mRNA-expression arrays [6]. Among these genes, *NOS2* was highly enriched for Ezh2-mediated repressive

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methylation marks offering a putative explanation for the lack of inducibility of iNOS in human endothelial cells. Accordingly, the aim of our current study was to confirm *NOS2* as a target gene of Ezh2 in endothelial cells.

2. Methods

2.1. Cell culture

HUVEC from different donors were isolated as described previously [18]. Briefly, HUVEC were cultured in EC medium (MCDB 131, Gibco BRL, Life Technologies GmbH, Karlsruhe, Germany) with the following supplements: 20 μg/mL Endothelial Cell Growth Supplement (PromoCell, Heidelberg, Germany), 1 ng/mL fibroblast growth factor β (Biomol, Hamburg, Germany), 0.1 ng/mL endothelial growth factor (Biomol), 2% fetal calf serum (Biochrom), 2 mmol/L L-glutamine (Gibco), 1 μg/mL hydrocortisone (Sigma-Aldrich, Hamburg, Germany), 1 IU/mL heparin (Biochrom), 5 IU/mL penicillin and 5 μg/mL streptomycin (Biochrom). Cell preparation conformed to the principles outlined in the Declaration of Helsinki and local university ethics guidelines.

2.2. Chromatin immunoprecipitation (ChIP)

ChIP was performed using an antibody directed against H3K27me3 (ab6002, Abcam, Cambridge, UK) or an unspecific mouse IgG (sc2025, Santa Cruz, Heidelberg, Germany) as described recently [6]. Purified DNA was either used for ChIP-PCR experiments or further analyzed by promoter chip arrays (ChIP-on-chip, NimbleGen 2.1 M Deluxe Promoter, imaGenes, Berlin, Germany). H3K27me3 marks were identified using the data from three independent experiments (NimbleScan 2.3, NimbleGen, Waldkraiburg, Germany) [19].

2.3. Quantification of DNA enrichment by ChIP (ChIP-PCR)

DNA enrichment by ChIP was quantified using the SYBR Green method and a specific primer (FW CATGGTGCCCACCACGAGGC, RV AAGGGACATGGTGCGGGGCT) set in the region close to the *NOS2* transcription start site which was previously identified as associated with H3K27me3 in ChIP-on-chip experiments. Diagrams depict fold DNA enrichment by the H3K27me3-specific antibody compared to the unspecific IgG.

2.4. siRNA transfections

25 nmol/L siRNA directed against Ezh2 (s4918, Ambion, Austin, TX) or negative control siRNA (Silencer Select Negative Control siRNA, Ambion) were transfected using Oligofectamine (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol. Total mRNA was harvested for qRT-PCR using TRIzol reagent (Invitrogen) 72 h after transfection as described previously [18].

2.5. mRNA expression analysis by qRT-PCR

Total RNA was prepared and reverse transcribed as described before [20]. Primers were obtained from Applied Biosystems, Carlsbad, CA (Ezh2 Hs00172783_m1, RPL19 Hs02338565_gH). mRNA expression was quantified using the TaqMan method, standardized to the housekeeping gene RPL19 and normalized to expression after transfection of control siRNA using the comparative Ct method ($2^{-\Delta\Delta ct}$) [6,20].

2.6. Western blot analysis

Protein expression of Ezh2 after transfection with siRNA was quantified by Western blotting using antibodies against Ezh2 (#3147, Cell Signaling, Danvers, MA) and β-actin as a loading control (cell signaling) as described previously [6]. A pan-NOS antibody from cell signaling (#2977) yielded the best technical results and was used to analyze iNOS protein expression with amido black staining as loading control. 48 h after transfection, HUVEC were stimulated for 8 h with 250 IU/mL interferon γ (IFNγ), 10 ng/mL tumor necrosis factor α (TNFα), 8 ng/mL interleukin 1β (IL1β) (R & D Systems, Minneapolis, MN) and 500 ng/mL lipopolysaccharide (LPS, Sigma). As a positive control for iNOS, murine RAW264.1 macrophages were treated with 500 ng/mL LPS for 24 h. Bands were detected using ECL plus (GE Healthcare, Munich, Germany). ImageJ (U.S. National Institutes of Health, http://rsb.info.nih.gov/ij/) was used for densitometric analysis.

2.7. Statistics

Statistical significance was calculated using Student's *t*-test or Analysis of Variance (Holm–Sidak's method) as appropriate (SigmaStat 3.0, SPSS, Inc.). An error probability of $p < 0.01$ was considered significant.

3. Results

3.1. Association of NOS2 with H3K27me3

ChIP-on-chip experiments demonstrated an association of the region around the *NOS2* transcription start site with H3K27me3 as indicated by a significant DNA enrichment after chromatin immunoprecipitation using an antibody specific for H3K27me3 (Fig. 1A). To specifically confirm these results, we designed DNA primers for the region

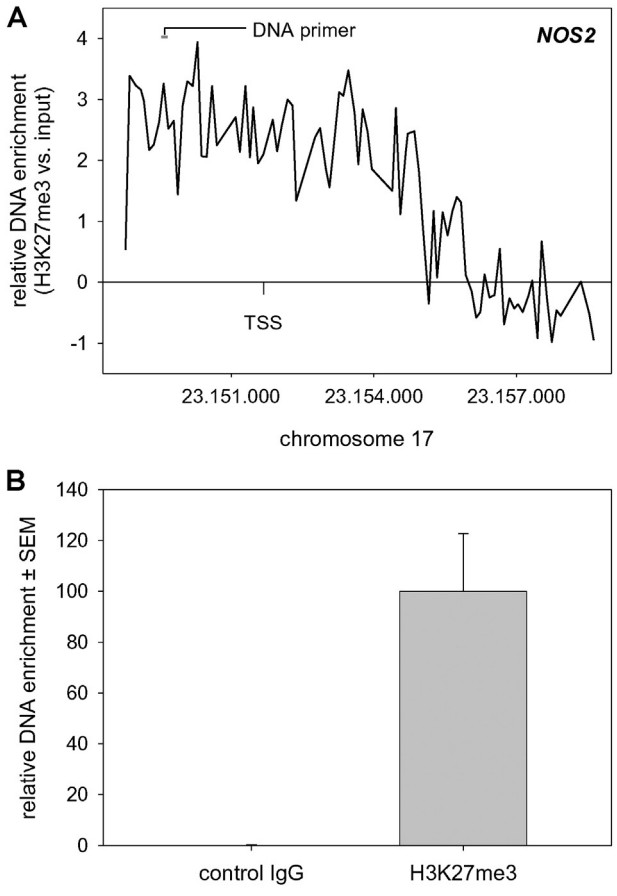

Fig. 1. A) DNA enrichment in proximity of the *NOS2* transcription start site (TSS) after chromatin immunoprecipitation using a H3K27me3-specific antibody compared to input quantified by promoter chip arrays (ChIP-on-chip). B) DNA enrichment after chromatin immunoprecipitation with a H3K27me3-specific antibody or an unspecific IgG quantified by polymerase chain reaction (ChIP-PCR) using a specific DNA primer set in the region marked in panel A ($P = 0.029$).