Modifications of collagen and chromatin in ECM-related disease
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CHAPTER 6

Enhancer of zeste homolog-2 (EZH2) methyltransferase regulates transgelin/smooth muscle-22 alpha expression in endothelial cells in response to interleukin-1 beta and transforming growth factor-beta 2

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ABSTRACT

Smooth muscle-22α (SM22α), encoded by transgelin (TAGLN), is expressed in mesenchymal lineage cells, including myofibroblasts and smooth muscle cells. It is an F-actin binding protein that regulates the organization of actin cytoskeleton, cellular contractility and motility. SM22α is crucial for the maintenance of smooth muscle cell phenotype and its function. SM22α is also expressed in the processes of mesenchymal transition of epithelial (EMT) or endothelial cells (EndMT). The expression of TAGLN/SM22α is induced by transforming growth factor-β (TGFβ) signaling and enhanced by concomitant interleukin-1β (IL-1β) signaling. We investigated the epigenetic regulation of TAGLN expression by enhancer of zeste homolog-2 (EZH2), the methyltransferase of Polycomb, in the context of TGFβ2 and IL-1β signaling in endothelial cells. We demonstrate that the expression of EZH2 in endothelial cells was regulated by the inflammatory cytokine IL-1β. A decrease in both expression and activity of EZH2 led to an increase in TAGLN expression. Inhibition of EZH2 augmented TGFβ2-induced SM22α expression. The decrease of EZH2 levels in endothelial cells co-stimulated with IL-1β and TGFβ2 correlated with decreased H3K27me3 levels at the TAGLN proximal promoter. Moreover, the SM22α expression increased. Taken together, this suggests that EZH2 regulates the chromatin structure at the TAGLN promoter through trimethylation of H3K27. EZH2 therefore acts as an epigenetic integrator of IL-1β and TGFβ2 signaling, providing an example of how cellular signaling can be resolved at the level of epigenetic regulation. Since IL-1β and TGFβ2 represent the pro-inflammatory and pro-fibrotic conditions during vascular fibroproliferative disease, we surmise that EZH2, as the molecule that integrates their signaling, could also be a promising target for development of future therapy.
INTRODUCTION

Smooth muscle-22α (SM22α2, transgelin), encoded by the gene TAGLN, is an evolutionarily conserved, F-actin binding protein, involved in the structural organization and stabilization of the actin cytoskeleton (1,2). The levels of SM22α determine celluarmotility (3) and contractility (4). SM22α/TAGLN is expressed in mesenchymal lineage cell types, such as fibroblasts or smooth muscle cells (SMC) (5). Its expression is altered upon differentiation processes (6,7), and it is induced by microenvironmental stimuli such as transforming growth factor-β (TGFβ) (8–10).

In particular, evidence shows that SM22α plays a crucial role in the maintenance of the SMC phenotype. Han et al. showed that depletion of SM22α results in a loss of SMC phenotype (1). Knock-out of SM22α in mice, although not lethal, results in decreased actin levels and reduced contractility of SMC (4). This knock-out also leads to an abnormal chondrogenic response of aortic SMC to injury and promotes vascular inflammation (11,12).

Given its necessity for the maintenance of the SMC phenotype, it is not surprising that SM22α is upregulated early in the course of the endothelial to mesenchymal transition (EndMT), a process in which endothelial cells differentiate into myofibroblast-like or SMC-like cells (8–10, 13–15). Postnatally, EndMT is a pathophysiological phenomenon that occurs in a pro-fibrotic environment (16–19). We previously investigated the influence of pro-fibrotic microenvironment on the progression of EndMT (10). Two major components of this environment are profibrotic factors from the TGFβ family and pro-inflammatory cytokines, such as interleukin-1β (IL-1β) (20,21). We demonstrated that IL-1β and TGFβ induce EndMT in vitro and that their signaling synergizes in the induction of expression of mesenchymal genes/proteins, an effect which was the most prominent in case of SM22α (10).

Polycomb is a family of chromatin modifying complexes, essential in the development and in adult life. Enhancer of zeste homolog-2 (EZH2) methyltransferase of the Polycomb repressive complex-2 (PRC2) is responsible for the deposition of the tri-methylation epigenetic mark on the lysine 27 of histone 3 (H3K27me3), which is associated with the maintenance of transcriptional repression (22,23). Polycomb function has been associated with inflammatory signaling. Tumor necrosis factor-α (TNFα) stimulation causes increased interaction between yin yang-1 (YY1) and PRC2 which, in satellite cells, enhances the formation of repressive chromatin on the developmentally important Pax7 promoter (24). On the other hand, depletion of suppressor of zeste-12 homolog (SUZ12), another component of PRC2, enhances inflammatory response to IL-1β in epithelial cells (25).

The expression of SM22α was shown to be regulated at the epigenetic level by histone acetylation (26,27). We hypothesized that histone methylation by Polycomb family members epigenetically regulates SM22α expression in endothelial cells. EZH2 modulates endothelial gene expression and is important for endothelial function (28,29). As SM22α is synergistically induced by IL-1β and TGFβ2 in endothelial cells, we investigated the influence of IL-1β and TGFβ2 on the expression of EZH2 and the downstream role of EZH2 and H3K27me3 in mediating the effects of IL-1β and TGFβ2 and in the regulation of TAGLN/SM22α expression in endothelial cells.
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MATERIALS AND METHODS

Cell culture

Human Umbilical Vein Endothelial Cells (HUVEC, Lonza) were used between passages 5 and 8. Cells were cultured in gelatin-coated dishes in endothelial cell medium (ECM) prepared as described before (10), but with 5.5 mM glucose and 10% heat-inactivated fetal calf serum (FCS; Lonza) for regular culture, and 5% heat-inactivated FCS in all experiments involving stimulations. IL-1β and TGFβ2 (Peprotech, #200-01B and #100-35B, respectively) were used at concentration of 10 ng/ml each. The specific EZH2 inhibitor GSK126 (30) (Cellagen Technology, C4126-2s) was dissolved in DMSO and used at 1 μM final concentration. Equal volume of DMSO was used in control medium. All stimulations were performed for 4 days, and media were refreshed daily.

Lentiviral transduction

Human Embryonic Kidney (HEK) cells were cultured in 10% FCS DMEM (Lonza), 2 mM L-glutamine (Lonza), 1% penicillin/streptomycin (Gibco). HEK were transfected with following plasmids: pLKO.1-shEZH2 or pLKO.1-SCR, pCMVΔR8.91 (gag-pol 2nd generation packaging plasmid) and pVSV-G (envelope plasmid) using Endofectin™-Lenti (Gene Copoeia, EFL-1001-01). A day after transfection, virus collection was commenced in 10% FCS ECM medium. Supernatants were collected 2 times at 24 h intervals, filtered through 0.45 μm filters and applied to 30% confluent HUVEC cultures. Every first transduction was performed with addition of polybrene at concentration of 4 μg/ml. Cells were allowed to proliferate for another 3 days before they were selected in 10% FCS ECM with 2 μg/ml of puromycin (Invitrogen). Surviving cells were allowed to proliferate for another 24 h and were used for downstream analyses at day 7 from the first transduction.

Real-time PCR

Cells were lysed either with TriZOL (Invitrogen) or RNA-Bee (TELTEST, Inc.) reagents. RNA was isolated in accordance to standard procedures. Briefly, chloroform was added and samples were shaken and incubated on ice for 10 min, then centrifuged. The aqueous phase was collected and RNA was precipitated with 2-propanol, washed 2 times with ice-cold 75% ethanol, dried and suspended in RNase-free water. Concentrations were determined by spectrophotometry (NanoDrop, ThermoScientific). cDNA synthesis was performed using RevertAid™ First Strand cDNA Synthesis Kit (ThermoScientific). 10 ng of cDNA, calculated based on the RNA input, was used per a single Real-time PCR reaction (20 ng per reaction in the IL1B PCR). Real-time PCR was performed using SYBR-Green chemistry (BioRad or Roche) with the ViiA7 Real Time PCR system (Applied Biosystems), and data were analyzed using ViiA7 software (Applied Biosystems). Downstream analysis was performed in Excel. Geometrical mean of ACTB and GAPDH Ct values, or only GAPDH Ct values (consistent within an experiment) were used for normalization (ΔCt). Fold change over control samples was calculated using ΔΔCt method. The following primers were used (5’ to 3’): ACTA2 Forward: CTGTTCAGCCAATCTCTTCAT, Reverse: TCATGATGCTGTTGAGGTGGT; ACTB Forward: CCAACCGCGAGAAGATGA, Reverse: CCAGAGGCGTACAGGGATAG; CNN1
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Forward: CCAACCATAACAGTGAG, Reverse: TCACCTTGTTCCTTCTGTT; EZH2
Forward: GCGAAGGATACAGCCTGTGACA, Reverse: AATCCAATGGTCACCCGAAAC; FN1 Forward: TCACTCACAGTCTCCTGAAA, Reverse: TTGATCCAAACACAAATCTT; IL1B Forward: AAGCTGGATAATGTCTCTGC, Reverse: ACACAAATGGCATGGTGAAG; GAPDH Forward: AGCCATCGTCTCAGACAC, Reverse: GCCCAATACGACCGAATCC; TAGLN Forward: CTGAGGACTATGGGGTCATC, Reverse: TAGTGCCATCCTTCTGTT.

**Western blotting**

Cells were lysed with RIPA buffer (ThermoScientific) supplemented with proteinase inhibitor cocktail and phosphatase inhibitor cocktails-2 and -3 (all from Sigma Aldrich) and stored at −80 °C. Prior to Western blotting, lysates were thawed, sonicated and centrifuged. Protein concentration in the supernatants was measured using Bio-Rad DC™ protein concentration assay (Bio-Rad). Samples were loaded onto 10% polyacrylamide gels and electrophoresis was performed. Transfer was performed onto nitrocellulose membranes at 100 V. Membranes were blocked with Odyssey Blocking Buffer (Li-COR Biosciences) diluted 1:1 with Tris-buffered saline (TBS) for 1 h at room temperature (RT), then incubated with primary antibodies dissolved in Odyssey Blocking Buffer diluted 1:1 with TBS for 4 °C overnight, at a rocking platform. Next day the membranes were washed 3 times with TBS 0.1% Tween-20 and incubated with secondary antibodies in Odyssey Blocking Buffer diluted 1:1 with TBS 1 h at RT, mixing. Then they were washed 3 times with TBS 0.1% Tween and 3 times with TBS, and scanned using the Odyssey scanner (Li-COR Biosciences). Digital images of the membranes were converted into grayscale images using the Odyssey software (Li-COR Biosciences). These images were used in the densitometry analysis with TotalLab 120 software (Nonlinear Dynamics). The following antibodies were used: EZH2 (1:1000, Cell Signaling, 5246), GAPDH (1:1000, Abcam, ab9485 or ab9484), phospho-SMAD2 (1:500, Cell Signaling, 3108), SM22α (1:1000, Abcam, ab14106), antirabbit IgG IRDye-680LT (1:10 000, Li-COR Biosciences, 926-68021), anti-mouse IgG IRDye-800CW (1:10 000, Li-COR Biosciences, 926-32210).

**Chromatin immunoprecipitation**

HUVEC treated with IL-1β, TGFβ2, or IL-1β and TGFβ2 (or control) were washed with PBS, harvested with trypsin (MP Biomedicals, LLC), counted and fixed with 1% formaldehyde. The fixing solution was neutralized with glycine solution (125 mM), cells were washed three times with PBS and cell pellets were stored at −80 °C. Prior to ChiP, cell pellets were thawed and lysed on ice for 20 min with SDS lysis buffer (1% SDS, 50 mM Tris–HCl pH 8.0, 10 mM EDTA) supplemented with proteinase inhibitor cocktail and PMSF (Sigma Aldrich). The chromatin was fragmented by sonication with a Bioruptor® device (Diagenode) and cleared by centrifugation at 13000 RPM for 10 min at 4 °C. The chromatin was diluted tenfold with RIPA buffer (0.1% SDS, 0.1% Na-deoxycholate, 1% Triton-X100, 1 mM EDTA, 10 mM Tris–HCl pH 7.5, 140 mM NaCl, 0.5 mM EGTA) supplemented with proteinase inhibitor cocktail and PMSF. Then, 40 μl Dynabeads® Protein-A (Life Technologies) were coated with 5 μg antibodies against H3K27me3 (Millipore, 07-449) or normal rIgG (Abcam, ab46540) as a control, and incubated overnight at 4 °C with diluted chromatin of 0.8×10⁶ cells. The following day,
the beads were washed three times with ice cold PBS and the remaining complexes were eluted with a solution of 100 mM NaHCO3 and 1% SDS. After reversing the crosslinks in the elutes with NaCl at 62 °C and treating with RNAse (Roche) and Proteinase K (Roche), the DNA fragments were purified by using a QIAquick PCR purification kit (Qiagen) and quantified by Real-time PCR with SYBR Green (Roche) and primers that amplify regions in the proximal promoters. Data is represented as fold enrichment over control rIgG values. The following primers were used (5' to 3'): IL1B Forward: GGACATCAACTGCACAACGA, Reverse: ATGGAAGGGCAAGGAGTAGC; TAGLN Forward: TCTCCAAACCATGCAGAGAA, Reverse: GACTCCACACAGGGCCTCCATA.

**Immunofluorescence**

Cells were cultured in 24-well plates. After 4 days of stimulation, cells were washed with PBS and fixed in 2% paraformaldehyde in PBS for 30 min at RT. Cells were washed with PBS, permeabilized with 1% Triton-X100 in PBS for 10 min at RT, washed with PBS and blocked with 10% donkey serum in PBS for 30 min at RT. Subsequently, cells were incubated with primary antibodies diluted in 10% donkey serum in PBS overnight at 4 °C, rocking. Controls were incubated with 10% donkey serum in PBS. Subsequently, cells were washed 3 times in PBS 0.05% Tween-20, 1 time in PBS and then incubated with secondary antibodies in 10% donkey serum in PBS with DAPI (1:5000), for 1 h at RT, rocking. Cells were then washed with PBS 0.05% Tween, and in PBS. Wells were filled with 0.5 ml of PBS and plates were scanned with TissueFAXS microscope (TissueGnostics). Images were further analyzed with TissueQuest 4.0.1.0127 software (TissueGnostics). DAPI nuclear staining was used to identify cells. For better visualization the brightness of the images included in the figures was enhanced in a linear manner and to the same extend in each image (Adobe Photoshop CS6). The following antibodies were used: SM22α (1:500, Abcam, ab14106), anti-rabbit IgG AlexaFluor-555 (1:500, Life Technologies, A31572).

**ELISA**

Three hours prior to sampling, medium of HUVEC was refreshed. Collected culture supernatants were filtered (0.2 μm), aliquotted and stored at −80 °C. The concentration of secreted IL-1β was determined using the human IL-1 beta DuoSet ELISA kit (R&D Systems, Oxon, UK) according to the manufacturer’s protocol. Concentrations of two-fold serially diluted samples were determined by comparison to the IL-1β concentration standard curve, fit using a four parameter logistic (4-PL) curve-fit. The cells were lysed and total DNA was measured with CyQuant kit (Invitrogen). Total amount of IL-1β per well was calculated based on the measured IL-1β concentrations, and was normalized to the amount of DNA in a well, to normalize for differences in cell numbers.

**Statistical analysis**

Downstream calculations were performed using Excel, statistical analysis and plotting were executed in GraphPad Prism 4 or 5 (GraphPad Software, La Jolla, CA). Graphs present mean values with standard error of the mean of at least 3 independent experiments. t-test or 1-way ANOVA with Tukey post-hoc comparisons between all pairs of means were performed where appropriate. Probability values lower than 0.05
were considered to indicate significant difference between means.

RESULTS

**IL-1β suppresses expression of EZH2 in endothelial cells**

Previously, we demonstrated that co-stimulation of endothelial cells with IL-1β and TGFβ2 leads to a synergistical induction of gene expression of TAGLN within day 5 of stimulation. Here, we confirmed that a similar synergistical upregulation of SM22α occurred at day 4 of stimulation (Fig. 1a). We evaluated the expression of EZH2 at the same time point. Stimulation with IL-1β, or with IL-1β and TGFβ2 together, decreased the gene expression of EZH2 to a similar level (Fig. 1b), however TGFβ2 alone did not affect EZH2 expression, which shows that the change in gene expression of EZH2 depended solely on IL-1β signaling. The protein expression of EZH2 also decreased under IL-1β treatment, but not under TGFβ2 treatment (Fig. 2a and b).

![Figure 1. Regulation of the expression of TAGLN and EZH2 by IL-1β and TGFβ2.](image)

Cells were treated daily with 10 ng/ml of IL-1β, TGFβ2 or both. a: Fold change in gene expression of TAGLN. b: Fold change in gene expression of EZH2. *p<0.05, **p<0.01, ***p<0.001.

Treatment of HUVEC with IL-1β upregulated *IL1B* gene and protein expression (Fig. 3a and c). As *IL1B* is a putative EZH2-regulated gene (28), we speculated that IL-1β and EZH2 would form a regulatory feedback loop in endothelial cells. The decrease of EZH2 in IL-1β-stimulated HUVEC could lead to decrease of H3K27me3 at *IL1B* promoter and hence to an increase in IL1B/IL-1β expression. However, the inhibition of EZH2 activity with GSK126 increased IL1B expression to a lower extend than IL-1β-stimulation (Fig. 3b). Furthermore, despite a significant increase in *IL1B* gene expression, the protein levels of IL-1β only tended to increase upon inhibition of EZH2 (Fig. 3d). Finally, IL-1β, TGFβ2, or the combination, did not affect the abundance of H3K27me3 at the IL1B promoter (Fig. 3e). Altogether, this suggests that EZH2 activity does not directly regulate the activity of the IL1B promoter in response to IL-1β and/or TGFβ2.
Figure 2. Regulation of the expression of EZH2 protein by IL-1β and TGFβ2.
Cells were treated daily with 10 ng/ml of IL-1β, TGFβ2 or both. a: Representative Western blotting images showing the expression of EZH2. GAPDH serves as loading control. b: Fold change in protein expression of EZH2. *p<0.05.

Figure 3. IL-1β positive feedback regulation does not require EZH2.
HUVEC were stimulated daily for 4 days with 10ng/ml IL-1β, 10ng/ml TGFβ2, or both, or with the GSK126 EZH2 inhibitor. a and b: Fold change in IL1B expression. c and d: IL-1β protein levels determined by ELISA. e: H3K27me3 abundance levels at the proximal promoter of IL1B, presented as fold enrichment over respective rabbit IgG control pull downs. *p<0.05.
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Next, we evaluated the putative contribution of EZH2 to the regulation of TAGLN/SM22α expression. Both inhibition of EZH2 activity with GSK126 and knock-down of EZH2 using shRNA increased the expression of TAGLN in HUVEC (Fig. 4a and b). We also observed upregulation of other mesenchymal genes ACTA2, CNN1 and FN1 upon inhibition of EZH2 activity (Suppl. Fig. 1). However, shRNA-mediated EZH2-depleted cells had an increased expression of ACTA2, but the expression of CNN1 and FN1 was not increased (Suppl. Fig. 2). Protein expression analysis of EZH2-depleted HUVEC confirmed the knock-down of EZH2 and the increase of SM22α expression (Fig. 4c, d and e).

Figure 4. EZH2 regulates the expression of TAGLN/SM22α.

a: Cells were treated with 1µM EZH2 inhibitor GSK126 daily for 4 days. Fold change in gene expression is depicted. b through c: Cells were depleted of EZH2 by means of lentiviral overexpression of shRNA. b: Fold change in gene expression of TAGLN. c: Representative Western blotting images showing the expression of EZH2 and SM22α protein, GAPDH serves as loading control. d and e: Densitometry results of the Western blotting data showing the protein expression of EZH2 and SM22α, normalized to GAPDH levels. **p<0.01.
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**Inhibition of EZH2 activity potentiates TGFβ2-induced expression of SM22α**

TGFβ signaling induces SM22α expression in fibrotic tissue and during the process of EndMT. We assessed if the inhibition of EZH2 enhanced the increase of SM22α expression induced by TGFβ2. Separate treatment with the EZH2 inhibitor GSK126 or with TGFβ2 both tended to increase SM22α expression. The combination of both GSK126 and TGFβ2 additively increased the expression of SM22α (Fig. 5a and b). Treatment with GSK126 and TGFβ2 together also increased the phosphorylation levels of mothers against decapentaplegic homolog-2 (SMAD2) (Suppl. Fig. 3). Immunofluorescent staining confirmed the induction of SM22α expression by the co-treatment with GSK126 and TGFβ2 (Fig. 5c and d).

![Figure 5. Inhibition of EZH2 activity enhances the TGFβ2-induced expression of SM22α.](image)

HUVEC were treated with the EZH2 inhibitor GSK126 (1μM), or 10ng/ml TGFβ2, or combination of both, daily for 4 days. a: Representative Western blotting results of SM22α expression; GAPDH served as loading control. b: Expression of SM22α derived through densitometry of Western blotting data, normalized to the GAPDH loading control. c: Expression of SM22α based on the measurement of mean fluorescence intensity (MFI) of SM22α staining. d: Representative images of SM22α staining (red), 20x magnification, bars indicate 100μm. Blue – DAPI. *p<0.05, **p<0.01.

**The IL-1β and TGFβ2-co-induced SM22α expression is associated with decreased H3K27me3 levels at the TAGLN/SM22α promoter**

We next checked if the decrease in EZH2 expression upon IL-1β (and concomitant TGFβ2) treatment would result in changes in H3K27me3 levels at TAGLN promoter and
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coincide with the change in \( \text{TAGLN}/\text{SM22}\alpha \) expression. Co-treatment with IL-1\( \beta \) and TGF\( \beta \)2 decreased H3K27me3 levels at the \( \text{TAGLN} \) promoter (Fig. 6a). The lowest levels of H3K27me3 at the \( \text{TAGLN} \) promoter corresponded with the highest level of SM22\( \alpha \) expression under co-treatment conditions (Fig. 6b and c).

**Figure 6. The IL-1\( \beta \) and TGF\( \beta \)2 decrease H3K27me3 levels at the \( \text{TAGLN}/\text{SM22}\alpha \) promoter.**

HUVEC were stimulated daily for 4 days with 10ng/ml IL-1\( \beta \), 10ng/ml TGF\( \beta \)2, or both. a: H3K27me3 abundance levels at the proximal promoter of \( \text{TAGLN} \), presented as fold enrichment over respective rabbit IgG control pull downs. b: Representative images of SM22\( \alpha \) staining (red), 20x magnification, bars indicate 100\( \mu \)m. Blue – DAPI. c: Levels of SM22\( \alpha \) expression, derived from mean fluorescence intensity of its staining. *\( p<0.05 \), **\( p<0.01 \), ***\( p<0.001 \).

**DISCUSSION**

We demonstrated that, in endothelial cells, the expression of \( \text{TAGLN}/\text{SM22}\alpha \) is regulated at the epigenetic level by the Polycomb methyltransferase EZH2. Both expression and activity of EZH2 co-determine the expression levels of \( \text{TAGLN}/\text{SM22}\alpha \) in endothelial cells. Our *in vitro* investigation suggests that *in vivo* EZH2 might partake in the regulation of \( \text{TAGLN} \) in response to its physiological pro-fibrotic and pro-inflammatory inducers such as TGF\( \beta \) and IL-1\( \beta \). The decrease in EZH2 levels upon IL-1\( \beta \) and TGF\( \beta \)2 stimulation, and the resulting decrease of repressive modification H3K27me3 at the \( \text{TAGLN} \) promoter, could be the mechanism for activation of \( \text{TAGLN} \) promoter leading to the enhanced expression of \( \text{TAGLN}/\text{SM22}\alpha \).
EZH2 can therefore be a downstream effector that integrates the signaling pathways of IL-1β and TGFβ2. This mechanism could be crucial in the regulation of the SM22α expression in a pro-fibrotic inflammatory microenvironment and during the process of EndMT.

Several epigenetic mechanisms involving Polycomb play a role in mediating the transcriptional effects of pro-inflammatory signaling. Hahn et al. reported changes in DNA methylation of Polycomb target genes in epithelial cells in an inflammatory bowel disease model (31). De Santa et al. linked inflammation to the function of an H3K27me3 demethylase Jmjd3 (32), which could be a complementary mechanism (to EZH2-mediated H3K27me3 deposition) for modulation of H3K27me3 levels. Palacios and co-workers have shown that TNFα changes the activity of EZH2, but not EZH2 expression itself (24). Therefore, to the best of our knowledge, the repressive effect of IL-1β on EZH2 expression, which we observed, has not been reported before.

We showed previously that the synergistic increase in TAGLN/SM22α expression upon IL-1β and TGFβ2 co-stimulation depends on NFκB activation (10). However, we did not observe a rescue of the EZH2 expression upon inhibition of the canonical NFκB pathway in our initial experiments (data not shown), therefore the effects of IL-1β and TGFβ2 on EZH2 activity did not seem to depend on NFκB signaling.

TGFβ2 signaling could also contribute to the regulation of EZH2 activity, as the H3K27me3 levels at the TAGLN promoter decreased the most upon IL-1β and TGFβ2 co-stimulation. Wang et al. proposed that TGFβ-activated SMAD2 and SMAD4 displace EZH2 from Il9 locus in Th9 T-cells (33). This seems to corroborate our results, as the TGFβ2-activated SMAD2 (pSMAD2) could possibly facilitate the removal of EZH2 from different nuclear loci, including the promoter region of TAGLN (which is a TGFβ/SMAD2-inductive gene). Therefore, the TGFβ2-signaling, in particular through SMAD2 activation, could add to the IL-1β-mediated decrease of EZH2 expression and thereby explain the lowest levels of the H3K27me3 histone mark and the highest expression levels of SM22α under IL-1β and TGFβ2 co-stimulation conditions.

Moreover, by inhibiting EZH2 activity we were able to enhance the TGFβ2-induced expression of SM22α. Interestingly, EZH2 inhibition had similar effect on activation of SMAD2. Vella et al. observed increased expression of TGFβ in tissue with decreased EZH2 levels and in cells upon inhibition of EZH2 (34). Such autocrine production of TGFβ could explain our observation of increased SMAD2 activation by TGFβ2 upon inhibition of EZH2 in HUVEC.

Our study therefore describes a new level of integration of IL-1β and TGFβ2 signaling, which occurs at the level of EZH2. Even if the mechanisms of IL-1β- and of TGFβ2-exerted regulation of EZH2 are distinct (decrease in expression versus displacement), eventually they could act cooperatively, as illustrated by the decreasing levels of H3K27me3 at TAGLN promoter. This interaction in a pro-fibrotic milieu might be required to efficiently alter histone methylation, and to thereby change the promoter activity of fibrosis-related genes, such as TAGLN.

Moreover, this regulation mechanism of TAGLN promoter by EZH2 under influence of IL-1β and TGFβ2 further shows that not only global, but also local activity of EZH2 at promoters of specific genes can be guided by signaling pathways.

Decreased EZH2 expression has been observed before in the course
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of differentiation of adult stem/progenitor cells and correlated with changes in expression of EZH2-regulated genes (35,36). On the other hand, increased expression of EZH2 is often seen in highly proliferating cells (37). EZH2 expression levels also influence the differentiation decisions in human bone marrow-derived mesenchymal stem cells (38). In differentiating murine embryonic stem cells (mESCs) the expression of developmental genes is regulated by PRC2 activity and its localization to specific loci through interaction with JARID2 (39). Recent report showed that in mESCs PRC2 binds the nucleosome-free CpG islands in proximity of transcriptionally inactive genes, to maintain their repression (23). These reports show that EZH2 and Polycomb function can be regulated by changes in their abundance and activity, to help to guide the differentiation processes of the cells. This suggests that the decrease in EZH2 expression upon IL-1β could have similar functions in EndMT.

The expression of SM22α/TAGLN is regulated at the epigenetic level through histone acetylation (26,27). Here we show that EZH2-mediated histone methylation (H3K27me3) is involved in the regulation of TAGLN promoter and TAGLN expression. TAGLN has been used by others as a model gene to study the regulation of smooth-muscle cell (SMC)-specific genes (26,27). SM22α/TAGLN is also necessary for the maintenance of the phenotype and for proper function of SMC (1,11). In our previous work, increased expression of SM22α/TAGLN was an early-induced indicator of EndMT (10). Our results therefore suggest that EZH2, by integrating the IL-1β and TGFβ2 signaling at the level of TAGLN regulation, could contribute to the progression of transdifferentiation process of EndMT.

Our results suggest that SM22α/TAGLN expression in response to IL-1β and TGFβ2 in endothelial cells is co-regulated at the chromatin level through histone methylation (H3K27me3), most likely through regulation of the abundance of the Polycomb methyltrasferase EZH2. EZH2 appears to integrate and mediate the IL-1β and TGFβ signaling at the epigenetic level. This further suggests a role for EZH2 in the responses of endothelial cells to the microenvironment, in particular to the pro-fibrotic and pro-inflammatory cues driving the process of EndMT.

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REFERENCES


5. Lawson, D., Harrison, M. and Shapland, C. (1997) Fibroblast transgelin and smooth muscle SM22alpha are the same protein, the expression of which is down-regulated in many cell lines. Cell motility and the cytoskeleton, 38, 250-257.


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SUPPLEMENTAL INFORMATION

Supplementary Figure 1. Expression of mesenchymal genes upon inhibition of EZH2.
HUVEC were treated with 1µM EZH2 inhibitor GSK126 daily for 4 days. a through c: Fold change in gene expression of CNN1, ACTA2 and FN1. **p<0.01, ***p<0.001

Supplementary Figure 2. Expression of mesenchymal genes upon knock-down of EZH2.
HUVEC were depleted of EZH2 by lentiviral overexpression of anti-EZH2 shRNA. a through c: Fold change in gene expression of CNN1, ACTA2 and FN1. **p<0.01, ***p<0.001
Supplementary Figure 3. Phospho-SMAD2 levels. HUVEC were treated with the EZH2 inhibitor GSK126 (1μM), or 10ng/ml TGFβ2, or combination of both, daily for 4 days. a: Representative Western blotting results of phosphor-SMAD2 (pSMAD2) expression; GAPDH served as loading control. b: Expression of pSMAD2 derived through densitometry of Western blotting data, normalized to the GAPDH loading control. **p<0.01.