Molecular mechanisms of Endothelial-Mesenchymal Transition in coronary artery stenosis and cardiac fibrosis
Vanchin, Byambasuren

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Chapter 5

Reciprocal regulation of Endothelial-Mesenchymal Transition by MAPK7 and EZH2 activity in Intimal Hyperplasia and Coronary Artery Disease

Byambasuren Vanchin¹,², Marloes Sol¹,², Gjaltema RAF², Marja Brinker¹, Bianca Kiers³, Alexandre C. Pereira³, Martin C. Harmsen¹, Jan-Renier A.J. Moonen³,⁴, Guido Krenning¹

¹ Laboratory of Cardiovascular Regenerative Medicine, Dept. Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Hanzeplein 1 (EA11), 9713GZ Groningen, The Netherlands
² Epigenetic Editing Research Group, Dept. Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Hanzeplein 1 (EA11), 9713GZ Groningen, The Netherlands
³ Laboratory of Genetics and Molecular Cardiology (LIM13), Heart Institute (InCor), University of São Paulo, Avenida Dr. Eneas C. Aguiar 44, 05403-000 São Paulo, SP, Brazil.
⁴ Center for Congenital Heart Diseases, Dept. Pediatric Cardiology, Beatrix Children’s Hospital, University of Groningen, University Medical Center Groningen, Hanzeplein 1 (CA40), 9713GZ Groningen, The Netherlands.

*Authors contributed equally.

Submitted
ABSTRACT

Endothelial-Mesenchymal Transition (EndMT) is a specific form of endothelial dysfunction wherein endothelial cells acquire a mesenchymal phenotype and lose their endothelial functions. We, and others, recently described that EndMT contributes to intimal hyperplasia and atherosclerosis.

The mitogen activated protein kinase 7 (MAPK7, also known as Erk5) inhibits EndMT. MAPK7 activation decreases the expression of the histone methyltransferase Enhancer-of-Zeste homologue 2 (Ezh2) thereby maintaining endothelial quiescence. This decrease in Ezh2 expression may therefore be responsible for the protective effects of MAPK7 activation. Ezh2 is the catalytic subunit of the Polycomb Repressive Complex 2 that methylates lysine 27 on histone 3 (H3K27me3). It is elusive how the crosstalk between MAPK7 and Ezh2 is regulated in the endothelium and if the balance between MAPK7 and EZH2 is disturbed during intimal hyperplasia.

In this study, we showed that in endothelial cells there is reciprocity between MAPK7 signaling and EZH2 expression and that disturbances in this reciprocal signaling circuit associate with the induction of EndMT and severity of human coronary artery stenosis. The reciprocity between MAPK7 and EZH2 is governed by a complex mechanism involving microRNAs and the phosphatases DUSP-1 and DUSP-6.
INTRODUCTION

Neointimal hyperplasia is characterized by an increasing amount of fibroproliferative cells and extracellular matrix in the neointimal lesion, resulting in vascular lumen narrowing and eventually obstruction of the vessel. Endothelial cells play a pivotal role in the formation of neointimal lesions by the acquisition of a fibro-proliferative phenotype through endothelial-to-mesenchymal transition (EndMT)(1-5). EndMT is characterized by a change from an endothelial phenotype into a phenotype comprising of mesenchymal-like properties, in which the expression of endothelial cells markers, such as eNOS, PECAM-1 and VE-cadherin is lost, and the expression of mesenchymal genes, including SM22α, αSMA and vimentin is gained. Moreover, EndMT-derived fibroproliferative cells secrete extracellular matrix components, which might contribute to the buildup of the neointima(6).

EndMT was originally identified during embryogenesis, where it plays a pivotal role in cardiac valve, septum and endocardial cushion formation(7). In adults, EndMT contributes to fibroproliferative diseases, including atherosclerosis(1-5), cerebral cavernous malformation(8), pulmonary fibrosis(9), kidney fibrosis(10) and cardiac fibrosis(11). Uniform laminar shear stress (LSS) conveys atheroprotective effects to the endothelium, while endothelial cells exposed to disturbed or low oscillatory shear stress are prone to EndMT(12, 13). Uniform LSS activates the mitogen-activated protein kinase 7 (MAPK7) - also known as extracellular signal-related kinase 5 (Erk5) and big-mitogen kinase-1 (BMK-1) - which suppresses EndMT(5, 14, 15). Concurrently, the loss of MAPK7 signaling facilitates EndMT(5, 16). Currently, it is elusive how MAPK7 activity is regulated in fibroproliferative disease.

The histone methyltransferase Enhancer of Zeste Homolog 2 (EZH2), which is the catalytic subunit of the Polycomb Repressive Complex 2, plays a pivotal role in endothelial dysfunction(17-19). EZH2 is responsible for the trimethylation of lysine 27 on histone 3, which silences gene expression and is elevated in endothelial cells in atherosclerotic lesions(20). Serendipitically, we uncovered that uniform LSS reduces the expression of EZH2, whereas the RNAi-mediated repression of EZH2 reciprocally activates MAPK7 signaling in endothelial cells even in the absence of LSS(18). Currently, it is elusive how the crosstalk between MAPK7 and EZH2 is regulated in the endothelium and whether the balance between MAPK7 and EZH2 is disturbed during intimal hyperplasia and coronary artery stenosis.

Here, we describe the reciprocity that exists between MAPK7 and EZH2 in the regulation of EndMT and in human coronary artery stenosis. In uniform LSS-stimulated endothelial cells, activation of MAPK7 increases the expression of microRNA (miR)-101, which represses the expression of EZH2. Reciprocally, the loss of EZH2 coincides with a decreased expression of the Dual Specificity Phosphatase (DUSP)-1 and DUSP-6 – the phosphatases responsible for the dephosphorylation of MAPK7(21) - which facilitates the activation of MAPK7. Disbalances in this reciprocal signaling circuit culminate in the induction of EndMT and associate to the severity of human coronary artery stenosis.
CHAPTER 5

MATERIALS AND METHODS

HUMAN CORONARY ARTERY SAMPLES

Human coronary arteries were obtained from autopsy specimens from 10 patients (age 59.1±2.6 years, range 39-69) that died from an acute coronary episode at the Heart Institute (InCor), Sao Paulo, Brazil. Hypertension was resent in 9 subjects, and diabetes in 6. Five individuals were active smokers. Next-of-kin gave informed consent and the investigation was performed according to institutional guidelines (InCor, Sao Paulo #SDC 3723/11/141 and #CAPPesq 482/11) and the Declaration of Helsinki. During necropsy each dissected coronary artery was fixed in neutral-buffered formalin with constant perfusion at a quasi-normal perfusion pressure before paraffin embedding.

DETERMINATION OF INTIMA-MEDIA THICKNESS

Four micron-thick sections were prepared from human coronary artery samples and deparaffinized using Xylol and rehydrated using a series of EtOH solutions of decreasing concentration. Samples were stained in Verhoef’s solution (92 mM hematoxylin, 137 mM FeCl₃, 27 mM KI, 4 mM I₂ in 55% EtOH) at room temperature for 1 hour. Samples were differentiaed in FeCl₃ (123 mM in dH₂O) for 1 minute and treated with Sodium Thiosulphate (316 mM in dH₂O) at room temperature for 1 min. Samples were dehydrated using increasing concentrations of EtOH and cleared in 100% xylene. Samples were mounted in Permount resinous mounting medium. The intimal thickness was determined as the distance between the inner elastic lamina and the lumen and the medial thickness was determined by measuring the distance between the inner elastic lamina and the outer elastic lamina at 10 spots within 1 samples. Intimal/Medial thickness was calculated by dividing the average intimal thickness by the average medial thickness.

HUMAN UMBILICAL VEIN ENDOTHELIAL CELL CULTURE AND SHEAR STRESS EXPERIMENTS

Human umbilical vein endothelial cells (HUVEC, Lonza #C2519) were cultured in endothelial cell culture medium (ECM) as described previously(5, 22). EndMT was the addition of 10 ng/ml TGFβ1 to the culture medium as described before(5, 23). For shear stress experiments, HUVEC (60 000 cells/cm²) were seeded on 0.1% gelatin-coated µ-Slides I 0.4 Luer (Ibidi GmbH, Martinsried, Germany) and allowed to adhere under standard culture conditions overnight. Slides with a confluent endothelial cell monolayer were exposed to uniform laminar shear stress (20 dyne/cm²) for 24 hours. Where indicated 5µM of the small molecule inhibitor to DUSP-1/6 (BCI, Axon Medchem, Groningen, The Netherlands) was applied.

VIRAL TRANSDUCTION OF ENDOTHELIAL CELLS

pLKO.1-shEZH2 and pLKO.1-SCR were kindly provided by Prof.dr. J.J. Schuringa (dept. Hematology, UMCG). HEK293 cells were co-transfected with pLKO.1-shEZH2 or pLKO.1-SCR, pVSVG (envelope plasmid) and pCMV-R8.91 (gag-pol 2nd generation packaging
plasmid) using Endofectin-Lenti (Gene Copoeia, Rockville, MD, USA). At 48 and 72 hours post-transfection, viral supernatants were collected.

A retroviral construct encoding the constitutively active rat MEK5-α1 (pBabePuro-MEK5D) and empty vector controls were kindly provided by Prof.dr. M. Schmidt (Dept. Dermatology, University Würzburg, Germany). Retroviral transduction of HUVEC was performed as detailed before(24). In brief, virus-producing Phoenix cells were cultured until 70% confluency, after which basal medium was replaced by ECM after which viral supernatants were collected twice at 24 hours intervals.

Viral supernatants were supplemented with polybrene (6µg/ml; Sigma, St.Louis, MO) and applied to 30% confluent HUVEC for two consecutive rounds of 24h exposure. Transduced HUVECs were passaged twice and transduced cells were selected by puromycin (4µg/ml; Invitrogen, Carlsbad, CA, USA).

MICRORNA TRANSFECTIONS IN ENDOTHELIAL CELLS

HUVEC or COS7 cells were seeded in antibiotic free medium at a density of 20 000/cm². Cells were transfected with 50 pmol of microRNA mimics (miR-101 (#PM11414), miR-200a (#PM10991), miR-200b (#PM10492), miR-200c (#PM11714), miR-141 (#PM10860), miR-429 (#PM10221) or scrambled control (#AM17110, all Ambion/Life Technologies, Carlsbad, CA) using the siRNA reagent system (Santa Cruz, #sc-45064, Santa Cruz, CA) according to manufacturer’s instructions.

IMMUNOFLUORESCENCE

Cells were fixed using 2% paraformaldehyde in PBS at room temperature for 30 minutes. For the analysis of intracellular proteins, cells were permeabilized by 0.05% Triton X-100 solution for 10 minutes. Blocking of non-specific antibody binding was performed using 5% donkey serum in PBS for 10 minutes. Samples were incubated with antibodies to VE-Cadherin (1:200, R&D #9381, Minneapolis, MN) or SM22α (1:200, Abcam #14106, Cambridge, UK) in PBS containing 2% donkey serum at 4°C overnight. Samples were washed extensively with 0.05% Tween-20 in PBS and incubated with Alexa Fluor® 594-conjugated antibodies to Rabbit IgG (Life Technologies, Carlsbad, CA, #A21207) in DAPI/PBS with 2% human serum at RT for 1 hour. Image analysis was performed on TissueFAXS (TissueGnostics, Vienna, Austria) in fluorescence mode, in combination with Zeiss AxioObserver Z1 microscope. Data analysis was performed using TissueQuest fluorescence (TissueGnostics, Vienna, Austria) software.

IMMUNOBLOTTING

Cells were harvested in RIPA buffer (Thermo Fisher Scientific, Waltham, MA) supplemented with 1% v/v protease inhibitor cocktail (Sigma Aldrich, St Louis, MO) and 1% v/v phosphatase inhibitor cocktail (Sigma Aldrich, St Louis, MO). Samples were sonicated and protein concentration was determined with a DC protein assay (BioRad, Hercules, CA). Equal amount of protein were separated by electrophoresis on 10% polyacrylamide gels after which proteins were blotted onto nitrocellulose membranes using the semi dry Transblot Turbo system (Bio-rad, Hercules, CA). Membranes were blocked with Odyssey
Blocking buffer (Li-COR Biosciences, Lincolin, NE) at RT for 1 hour, and incubated with antibodies against β-actin (1:2000, Cell Signaling, Danvers, MA, USA), EZH2 (1:1000, Cell Signaling, Danvers, MA, USA), MAPK7 (1:1000, Merck Millipore, Billerica, MA, USA), MKP-1 (DUSP-1, 1:1000, Abcam #195261) or MKP-3 (DUSP-6, 1:500, Santa cruz, #sc377070) at 4°C overnight. Membranes were washed in TBS Tween (0.1%) and developed using IRDye-conjugated antibodies to rabbit IgG (1:10,000, #926-68021), mouse IgG (1:10,000, #926-32210, both Li-COR Biosciences) or AP-conjugated antibodies to rabbit IgG (1:2000, #7054S, Cell Signaling) at RT for 1 hour. Protein detection was done using the Odyssey Infrared Imaging System (Li-COR Biosciences). The development of AP-conjugated antibodies, membranes were incubated with AP-detection buffer (100nM NaCl, 100mM Tris, 50mM MgCl₂, pH 9.5) supplemented with nitro-blue tetrazolium chloride (NBT) (330µg/mL) and 5-bromo-4-chloro-3’-indolyphosphate p-toluidine salt (BCIP) (165µg/mL). Densitometry analysis was performed using Totallab 120 (Nonlinear Dynamics, Newcastle upon Tyne, England).

RNA ISOLATION AND GENE EXPRESSION ANALYSIS

RNA was isolated using the TRIzol reagent (Invitrogen Corp, CA, USA) according to the manufacturer’s protocol. RNA concentration and purity were assessed using UV spectrometry (Nanodrop 1000, Thermo Scientific MA, USA) and RNA integrity validated on 1% agarose gels. For gene expression analysis, cDNA synthesis was performed using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, MA, USA), according to the manufacturer’s protocol. For microRNA transcript analysis, 10ng of total RNA was reversely transcribed using the ABI Taqman microRNA reverse transcription kit (#4366597, ThermoFisher Scientific) according to manufactures instructions using 1.0 µM microRNA-specific stemloop primers (table 1). For all transcript analyses, the cDNA was amplified on a VIIA7 thermal cycling system (Applied Biosystems, Carlsbad, CA) in a reaction containing 0.6 µM primers (table 2) using SYBR Green chemistry (Bio-Rad, VA, USA). Cycle threshold (Cₜ) values for individual reactions were determined and normalized against GAPDH/ACTB (gene transcript analysis) or RNU6 (microRNA transcript analysis). All cDNA samples were amplified in triplicate. Relative expression was calculated using the ΔCₜ method. Data are presented as fold change compared with control.

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RECIPROCITY OF MAPK7 AND EZH2 ACTIVITY IN CORONARY ARTERY STENOSIS

Table 1. Primer sequences for microRNA expression analysis

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Table 2. Primer sequences for gene expression analysis

3'UTR BINDING ASSAYS

3'UTR fragments were isolated from a cDNA pool of various human tissues using specific primers for EZH2-3'UTR (sense 5'-CATCTGCTACCTCTCCCCC-3', antisense 5'-GACAAGTCTCAAGTATTCTTTT-3'), DUSP-1-3'UTR (sense 5'-AAGGGCACGGGAGTGAGGC-3', antisense 5'-CAATAGAAATGCCATAATT-3'), and DUSP-6-3'UTR (sense 5'-AAGACCCCACACCCCTCCTT-3', antisense 5'-CAATAGCCAAATAGTATT-3', all 0.6 µM, Biologio, Leiden, The Netherlands). Sense and antisense primers were extended with SgfI (GCGATCGC) and NotI (GCGGCCGC) restriction sequences, respectively. Amplification was performed using the DyNAzyme EXT PCR kit (Finnzymes, Vantaa, Finland) according to manufacturers’ instructions. Amplicon size was validated by gel electrophoresis on 1% agarose gels prior to purification using the QIAquick PCR Puriﬁcation kit (Qiagen, Venlo, The Netherlands) and cloning into the dual luciferase reporter vector psiCHECK-2 (Promega, Madison, WI) using T4 DNA Ligase (Fermentas/Thermo Fisher Sci., Waltham, MA) according to standard protocols.

COS7 cells were transfected with 100 ng UTR reporter plasmid and 50 pmol microRNA mimics as detailed above. 48 hours post-transfection, luciferase activity was assayed using the DualGlo Luciferase assay system (Promega, Madison, WI) and recorded for 500 ms on a Luminoskan ASCENT (Thermo Scientific, Waltham, MA) according to manufacturer’s instructions. Relative luciferase activity was calculated by dividing the luminescence from Renilla luciferase activity by the luminescence from firefly luciferase activity and normalized to control samples.

HISTONE CHROMATIN-IMMUNOPRECIPITATION (CHIP)

Cells were harvested using accutase, pelleted and the chromatin crosslinked using 1% formaldehyde (37% F1268 Sigma-Aldrich) for 8 minutes. Crosslinking activity was quenched using 125mM glycine (104201 Merck). Cell pellets were lysed on ice with SDS
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Table 3. Primers used for histone CHIP RT-PCR assays
lysis buffer (1% SDS, 50mM Tris HCl pH 8.0, 10mM EDTA) supplemented with freshly added 100mM protease inhibitor cocktail (Sigma Aldrich P8340) for 15 minutes. The chromatin was fragmented by Biorupter (Diagenode, Seraing, Belgium) with 5 cycles of (30’ ON/OFF). The sonicated sample was centrifuged and chromatin containing supernatant was kept for further analysis. The chromatin was diluted 10 times with RIPA buffer (0.1% SDS, 0.1% Sodiumdeoxycholate, 1% Triton-X100, 1mM EDTA, 10mM Tris-HCl pH 7.5, 140mM NaCl, 0.5mM EGTA) supplemented with 100 mM protease inhibitor cocktail. Immunoprecipitation was performed by 4µg H3K27Me3 antibody (Merk Millipore 07-449) or IgG control (Abcam ab46540) added to the 40µL Dynabeads Protein-A (Life technologies, 10002D) coated tubes. Subsequently, the chromatin of 0.8*10^6 cells was added to antibody bound beads and incubated overnight at 4°C while rotating. The beads were washed 3 times with ice cold PBS and the remaining complexes were eluted with 100mM NaHCO₃ and 1% SDS in PBS. 5M NaCl and RNAse (Roche #11119915001) were added to the eluted samples and incubated at 62°C to reversing the crosslink for 4 hours. 2µL Proteinase K (Roche #03115828001) was added and incubated at 62°C for 1 hour to liberate the DNA from the histones. DNA fragments were purified using a QiAquick PCR purification kit (Qiagen) according to manufacturers’ instructions. Precipitated DNA was analyzed by qPCR using 7 sets of primers for each promoter area. (Table 3; all 0.6µM Biolegio) Enrichment of promoter sequences in the precipitate was calculated relative to the percentage of input.

ANGIOGENIC SPROUTING ASSAY

10µL Matrigel (BD Corning, 356230) was added into the bottom compartment of µslide Angiogenesis (81501, Ibidi GmbH, Martinsried, Germany) and incubated at 37°C, 5% CO₂ for 1 hour. Cells were diluted to 2*10^6 cells/ml. 50µl cell suspension was added on top compartment. After 6 hours incubation at 37°C, 5% CO₂, light microscopy images were obtained and complete octamer niches were counted by eye.

COLLAGEN CONTRACTION ASSAY

Cells were dissociated using trypsin-EDTA, pelleted and suspended at a concentration of 22.5*10^6 cells/ml ECM. 45µL cell suspension was added to a collagen solution (3.3mg/ml rat tail collagen type I (#354236, BD, San Jose, CA), 100mM Na₂HPO₄ and 5mg/ml NaHCO₃) of neutral pH. The cell/collagen mixture was immediately aliquoted into 50ul droplets and allowed to polymerize at 37°C, 5% CO₂ for 30 min. Polymerized gels were released and 1mL of ECM was added. At time points t=0 hours and t=24 hours, gels were visualized using a regular flatbed scanner and the gel surface area quantified using with ImageJ (NIH). Gel contraction was calculated as the relative reduction in gel surface area at 24 hours.

PERMEABILITY ASSAY

Cells (5*10⁴/cm²) were cultured on polycarbonate cell culture inserts strips (pore size 0.4µm, porosity, 0.9*10⁶/cm² Fisher Scientific, #15639536) coated with 0.1% gelatin for 72 hours to establish a monolayer. Monolayer permeability was assessed by the addition of 5 µg/mL FITC dextran (Sigma) in upper compartment. Fluorescence was measured
in the bottom compartment on fluorescence reader at Ex485/Em519 30 minutes after the addition of dextran. Relative permeability levels were calculated using the fluorescence signal of a naked strip (100% permeability) or the fluorescence signal from the culture medium (0% permeable). Permeability was calculated by following formula: 
Permeability=\((Em_{519}(\text{sample})\,-\,Em_{519}(\text{ECM}))/\,Em_{519}(\text{Empty well})\)*100.

DATA REPRESENTATION OF STATISTICAL ANALYSES

Data are expressed as mean ± s.e.m. from at least five independent experiments. Where the mean of two groups were compared, p-values were calculated using student t-tests. Otherwise, p-values were calculated using the one-way analysis of variance (ANOVA) followed by Bonferroni’s post-hoc comparisons tests using Prism Graphpad (Graphpad Software, La Jolla, CA, USA). P < 0.05 was considered statistically significant.

RESULTS

RECIROCITY BETWEEN MAPK7 AND EZH2 IN CORONARY ARTERY STENOSIS

Human coronary artery samples were stratified into three groups based on their intima/media thickness, ranging from mild stenosis (IMT <1 µm·µm\(^{-1}\)), medium (IMT 1-3 µm·µm\(^{-1}\)) and severe stenosis (IMT >3 µm·µm\(^{-1}\); fig.1a-d). Coronary artery stenosis is characterized by progressively increasing intima-media thickness (fig.1a-d), wherein coronary artery MAPK7 expression decreases with an increasing stenosis (\(r^2=0.2517, p=0.04\); fig.1f). In contrast, EZH2 expression is elevated in coronary artery stenosis (fig.1g,h) and increasing stenosis associates with increased EZH2 expression (\(r^2=0.4417, p=0.004\), fig.1h),
Figure 1. Reciprocity between MAPK7 and EZH2 in human coronary artery stenosis. (a-c) Representative pictures of Verhoef-stained human coronary artery samples with mild (IMT<1, a), medium (IMT 1-3, b) or severe (IMT >3, c) stenosis. Intima-media thickness was measured (µm/µm) and divided in groups based on their intima-media thickness (d). MAPK7 expression levels were determined by qPCR and normalized to mild stenosis (e). MAPK7 decreases with increasing stenosis (f). EZH2 expression levels were determined by qPCR and normalized to mild stenosis (g). EZH2 expression increased with stenosis severity (h). *p<0.05, **p<0.001.

RECIPROCAL SIGNALING BETWEEN MAPK7 AND EZH2 IN ENDOTHELIAL CELLS

We recently uncovered that disturbed fluid shear stress contributes to intima hyperplasia by the induction of endothelial-mesenchymal transition (EndMT)(5), partially mediated by EZH2(18). Atheroprotective uniform laminar shear stress (LSS) decreases EZH2 expression at both the gene (2.2-fold, p<0.001; fig.2a) and protein (1.9-fold, p=0.028; fig.2b) level. Uniform laminar shear stress does not change the expression of MAPK7, neither on transcript (fig.2c) nor on protein level, however, FSS increases the activity of MAPK7 as indicated by the increased phosphorylation (3.5-fold, p=0.036, fig.2d). Knockdown of EZH2 does not alter its expression, whereas MAPK7 activity is increased upon EZH2 reduction (1.9-fold, p=0.049; fig. 2d). Moreover, protein expression levels of EZH2 associate with MAPK7 activation ($r^2=0.7723$, $p=0.021$; fig.2e) proving evidence of the reciprocity between EZH2 expression levels and MAPK7 activity.
Figure 2. Reciprocal signaling between MAPK7 and EZH2 in endothelial cells. EZH2 expression levels were determined by qPCR in HUVEC exposed to FSS (20dyne/cm²) compared to static controls (a). EZH2 protein levels were determined by western blot in HUVEC exposed to FSS and compared to static control (b). MAPK7 expression levels were determined by qPCR in HUVEC exposed to FSS, and HUVEC that are deficient in EZH2 (shEZH2)(c). MAPK7 activation (pMAPK7) levels were determined by immunoblotting and normalized to total MAPK7 protein levels (d). Protein expression of EZH2 and MAPK7 activation were associated in endothelial cells (e). ***p<0.001.

MAPK7 DECREASES EZH2 THROUGH MICRORNA-101

As MAPK7 decreases EZH2 post-transcriptionally(18), we investigated whether miRNA-101 - a known translational repressor of EZH2 in endothelial cells(25) - is regulated by MAPK7 signaling. FSS increased the expression of miR-101 in a MAPK7-dependent manner (2.3-fold, p<0.01; fig. 3a). In luciferase reporter assays, miR-101 binds to the 3’UTR of EZH2, reducing the luminescence signal (1.9-fold, p<0.001; fig.3b). In endothelial cells, ectopic expression of miR-101 decreases EZH2 expression at both the gene (2.6-fold, p=0.002; fig. 3c) and protein (2.9-fold, p=0.008; fig. 3c) level, whereas miRNA-101 has no effect on MAPK7 gene expression (fig. 3e) or MAPK7 protein expression level (fig.3f). In human coronary artery stenosis, miR-101 expression is decreased (p<0.01, fig.3g) and increased stenosis associates with a progressive decrease in miR-101 (r²=0.5109, p=0.001, fig.3h). Moreover, the expression level of miR-101 associates with MAPK7 (r²=0.4262, p=0.005; fig.3i) and EZH2 (r²=0.2304, p=0.051; fig.3j) in coronary artery stenosis, where a negative association between MAPK7 and EZH2 expression (r²=0.2568, p=0.038; fig.3kj) is present. Collectively, these data suggest that in coronary artery stenosis, the reciprocity between MAPK7 activity and EZH2 expression is regulated by miR-101.
Figure 3. MAPK7 decreases EZH2 through miRNA-101. MiR-101 expression levels were determined by qPCR in HUVEC exposed to FSS (20dyne/cm²) with or without the MAPK7 inhibitor BIX02189 and normalized to the level of static controls (a). Luciferase reporter binding assays were performed for the 3'UTR of EZH2 in HUVEC with ectopic expression of miR-101 or scrambled control sequences (scr). Luciferase activity was normalized to non-transfected cells (b). EZH2 and MAPK7 expression levels were determined by qPCR in HUVEC with ectopic expression of miR-101 or SCR and normalized to control (c, e). EZH2 and MAPK7 protein levels were determined by western blot in HUVEC with ectopic expression of miR-101 and control (d, f). MiR-101 expression levels were determined by qPCR in HUVEC with ectopic expression of miR-101 and control (d, f). MiR-101 expression levels were determined by qPCR and normalized to mild stenosis (g). MiR-101 decreases with stenosis severity (h), and associates with MAPK7 (i) and EZH2 (j) expression levels. In coronary artery stenosis, MAPK7 expression negatively correlates to EZH2 expression (k). *p<0.05, **p<0.01, ***p<0.001.

EZH2 REGULATES DUSP-1 AND DUSP-6 EXPRESSION THROUGH MIR200A-C

However, EZH2 is a transcriptional repressor that cannot directly regulate the activity of a kinase. MAPK7 activity is regulated by the Dual Specificity Phosphatases (DUSP)-1 and DUSP-6(21), yet a reduction in EZH2 expression is associated with a decreased expression of DUSP-1 and DUSP-6(26, 27). Therefore, we investigated alternative mechanisms that might decrease DUSP expression upon the reduction of EZH2. In silico analysis, using Targetscan.org(28), putatively identifies the microRNA-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) as regulators of DUSP-1 and DUSP-6. Therefore, we investigated if the expression of the miR-200b/a/429 cluster on chromosome 1 and the miR-200c/141 cluster on chromosome 12 are under control of EZH2. Uniform LSS increased the expression of miR-200b (2.5-fold, p=0.008) and miR-200c expression (2.5-fold, p=0.001; fig.4a,e). Moreover, knockdown of EZH2 increased the expression of miR-200b and miR-200c to a similar extend (fig.4a,e).
Figure 4. EZH2 regulates DUSP-1 and DUSP-6 expression through miR200a-c. MiR-200b (a) and miR-200c (e) expression levels were determined by qPCR in HUVEC exposed to FSS (20 dyne/cm²), transduced HUVEC with scr or shEZH2 and normalized to static or scr control cells. H3K27me3 enrichment around the transcription start site (TSS) of the miR-200b/a/429 cluster was determined using ChIP in shEZH2-HUVEC and scr-HUVEC (b) and MEKSD-HUVEC and EV-HUVEC (c). H3K27me3 enrichment is shown as area under the curve (AUC) compared to input samples (d). H3K27me3 enrichment at the around the TSS of miR-200c/141 cluster was determined using ChIP in scr-HUVEC and shEZH2 (f) and in MEKSD-HUVEC and EV-HUVEC (g). H3K27me3 enrichment is shown as AUC compared to input samples (h). Luciferase reporter assays for microRNA binding were performed for the 3’UTR of DUSP-1 in HUVEC with ectopic expression of miR-200a, -200b, -200c, -141, -429 or scr (i). Luciferase reporter assay identified miR-200a and miR-141 to target DUSP-6 (k) and ectopic expression of miR-200a or miR-141 decreases DUSP-6 expression in endothelial cells (l). *P<0.05, **P<0.01, ***P<0.001.

INHIBITION OF DUSP ACTIVITY INCREASES MAPK7 ACTIVITY AND DECREASES EZH2 EXPRESSION

H3K27Me3, the repressive histone mark placed by EZH2, is abundantly present in the promoter regions of the miR-200b/a/429 and miR-200c/141 gene clusters (fig.4b,f). Endothelial cells deficient in EZH2 have reduced levels of H3K27Me3 at these gene promoters (fig.4b,f), which associates with the increased expression of miR-200b and miR-200c. In endothelial cells with constitutively active MAPK7 signaling (MEKSD), the enrichment of H3K27Me3 at the promoter regions of miR-200b/a/429 (1.6-fold, p=0.034; fig.4c,d) and miR-200c/141 (1.9-fold, p=0.035; fig.4g,h) is decreased, suggesting that
MAPK7 activation results in the increased expression of miR-200 family members through the decrease in EZH2 activity.

In luciferase reporter assays, all miR-200 family members were able to bind to the 3’UTR of DUSP-1 (fig.4i), but only miR-200a and miR-141 were able to bind the 3’UTR of DUSP-6 (fig.4k). Corroboratively, exogenous expression of all miR-200 family members in endothelial cells decreased DUSP-1 expression (fig.4j), whereas only miR-200a and miR-141 decreased the expression of DUSP-6 (fig.4l). Collectively, these data imply that the activation of MAPK7 by uniform LSS decreases the expression of DUSP-1 and DUSP-6 expression via the EZH2-dependent regulation of miR-200b/a/429 and miR-200c/141 expression.

Figure 5. Inhibition of DUSP activity increases MAPK7 activity and decreases EZH2 expression. MAPK7 (a) and EZH2 (b) protein expressions were determined using western blotting, in HUVEC treated with 5µM of the DUSP-1/6 small molecule inhibitor BCI and normalized to untreated control cells. DUSP-1 expression levels were determined in human coronary artery stenosis samples by qPCR and normalized to mild stenosis (c). DUSP-1 expression increases with increasing stenosis severity (d) and is associated to EZH2 expression levels (e). DUSP-1 levels show a negative correlation with MAPK7 levels in stenosis (f). DUSP-6 expression levels were determined by qPCR and normalized to mild stenosis (g). DUSP-6 expression is elevated in coronary artery stenosis, but does not associate to the severity of stenosis (h), the level of EZH2 expression (i), nor the level of MAPK7 expression (j). **p<0.01, ***p<0.001.
We investigated if the pharmacological inhibition of DUSP-1 and DUSP-6 activity in endothelial cells would activate MAPK7 signaling and decrease the expression of EZH2. BCI-treated endothelial cells increased MAPK7 phosphorylation (1.9-fold, p=0.007; fig.5a) and decreased EZH2 expression (1.8-fold, p=0.002; fig.5b). In human coronary artery stenosis, DUSP-1 is increased (p<0.001, fig. 5c) and increased stenosis associates with increased DUSP-1 expression ($r^2=0.2767$, p=0.0301; fig.5d). Moreover, the increase in DUSP-1 expression associates with increased EZH2 expression in stenosis ($r^2=0.4541$, p=0.0030; fig.5e) and increase in DUSP-1 expression seems to correlate with decreased MAPK7 expression ($r^2=0.1686$, p=0.1016; fig.5f), although not significantly. Also DUSP-6 seems to be increased in coronary artery stenosis (p<0.1, fig.5g), the increase in DUSP-6 expression does not associate with the severity of stenosis ($r^2=0.0681$, p=0.3116; fig.5h), nor do the expression levels of DUSP-6 associate with EZH2 ($r^2=0.0734$, p=0.2929; fig.5i) and MAPK7 expression ($r^2=0.0163$, p=0.6251; fig.5j).

Figure 6. Ectopic expression of miRNA-101, miRNA-141 and miRNA-200a inhibits endothelial dysfunction and EndMT. MAPK7 (a) and EZH2 (b) protein expressions were determined using western blotting, in HUVEC treated with 10ng/ml TGFβ1 with ectopic expression of miRs-101, -200a and -141 and normalized to untreated control cells. The expression of VE-Cadherin (c,d) and SM22a...
(c,e) were assessed by immunofluorescence and quantified using TissueFAXs analyses. Endothelial cell permeability was assessed using transwell FITC-dextran leakage (f) and collagen gel contraction (g) was assessed as a mesenchymal cell function. The angiogenic sprouting behavior of endothelial cells was assessed using the Matrigel assay (h). *p<0.05, **p<0.01, ***p<0.001.

As coronary artery stenosis is associated with EndMT(1, 29), we investigated if the ectopic expression of miR-101 or miR-200 family members could preclude EndMT. Endothelial cells transfected with only a single microRNA were susceptible to TGFβ1-induced EndMT (data not shown), however, when miR-101, miR-200a and miR-141 were transfected in combination, endothelial cells increased their MAPK7 activity (fig.6a) and showed reduced expression levels of EZH2 (fig.6b). Corroborating the protective effects of MAPK7 signaling in the preclusion of EndMT(5), TGFβ1 stimulation did not decrease VE-Cadherin expression (fig.6c,d) nor induce the expression of the mesenchymal marker protein SM22α (fig.6c,e) in endothelial cells transfected with miRs-101/200a and -141. Moreover, the ectopic microRNA expression reduced the TGFβ1-induced increase in endothelial permeability by ~40% (fig.6f) and precluded the TGFβ1-induced collagen contraction (fig.6g) – two functional adaptations associated with EndMT – and maintained the endothelial angiogenic sprouting capacity (fig.6h).

**DISCUSSION**

In this study, we show that reciprocity exists between the atheroprotective MAPK7 activation and the expression of histone methyltransferase EZH2 in endothelial cells. The reciprocity is regulated by the MAPK7-induced silencing of EZH2 expression by miR-101 and the EZH2-mediated silencing of the miR-200 family, which increases DUSP-1 and DUSP-6 expression and inhibits MAPK7 activation. The reciprocity between MAPK7-EZH2 might reflect an autoregulatory feedback loop in endothelial cells that ensures endothelial homeostasis. As such, disturbances in this reciprocity leading to increased EZH2 expression can induce endothelial dysfunction and EndMT. In contrary artery stenosis - a condition associated with EndMT(1, 29) – the reciprocity between MAPK7 and EZH2 is disturbed, resulting in elevated expression of DUSP-1 and EZH2 and the decreased expression of MAPK7. Restoring the reciprocity by ectopic expression of miR-101/200a/141 precludes EndMT and might offer therapeutic benefit in coronary artery stenosis.

EndMT contributes to intimal hyperplasia during coronary artery stenosis(1-5), wherein MAPK7 signaling plays a protective role(5, 16). Yet, during intimal hyperplasia the signaling activity of MAPK7 is rapidly lost (Vanchin et al., J. Pathol, under revision). DUSP-1 and DUSP-6 expression levels are elevated in a number of cardiovascular diseases and DUSP-1 deficient mice are protected from atherosclerosis development (30, 31). The elevated expression of DUSPs might explain the loss in protective MAPK7 signaling activity during coronary artery stenosis, which is corroborated by our finding that pharmacological inhibition of DUSP-1/6 is sufficient to activate MAPK7 signaling. The expression of DUSP-1 and -6 is associated with high expression of EZH2 in various oncology’s(26, 27), albeit by a currently unknown mechanism. We found that EZH2 silences the expression of the microRNA-200 family, which posttranscriptionally regulate the
expression of DUSP-1 and DUSP-6 (fig.7). The loss of EZH2 expression by fluid shear stress therefore might increase the expression of miR-200 family members and decrease the expression of the DUSPs culminating in atheroprotective MAPK7 activation. Interestingly, the endothelial cell-specific overexpression of miR-200b precludes EndMT and alleviates diabetic cardiomyopathy in mice(32). In coronary artery stenosis, EZH2 expression levels are elevated and high EZH2 expression is associated with endothelial dysfunction(18, 19).

**Figure 7. Schematic representation of the reciprocity between MAPK7 activity and EZH2 expression.** Hemodynamic force (laminar shear stress) induces the activation of MAPK7 signaling, resulting in the expression of microRNA-101 that posttranscriptionally silences EZH2 expression. This culminates in the hypomethylation of H3K27 at the promoters of the miR-200 family and the miR-200-mediated silencing of DUSP-1/6. In the absence of laminar shear stress – e.g. during coronary artery stenosis – EZH2 expression is increased resulting in the reduction of miR-200 expression and increase in DUSP-1/6 expression. DUSP-1/6 dephosphorylate MAPK7 culminating in a decrease in its activation.

In combination, our current data might explain these observations and unifies them into a single mechanism, linking endothelial mechanotransduction to the epigenetic regulation of MAPK7 activity through DUSP-1/6 (fig.7). This double negative feedback loop might resemble a sensitive autoregulatory mechanism that ensures endothelial homeostasis, which when disturbed culminates in EndMT and possibly coronary artery stenosis.

In summary, we show that in endothelial cells there is reciprocity between MAPK7 signaling and EZH2 expression and that disturbances in this reciprocal signaling circuit associate with the induction of EndMT and severity of human coronary artery stenosis. The reciprocity between MAPK7 and EZH2 is governed by a complex mechanism involving microRNAs and the phosphatases DUSP-1 and DUSP-6 (fig.7). Our study contributes to a better understanding of the molecular and epigenetic cascades that underlie EndMT during coronary artery stenosis and might identify novel targets for therapy.
REFERENCES


