Gestational Diabetes Mellitus Impairs Fetal Endothelial Cell Functions Through a Mechanism Involving MicroRNA-101 and Histone Methyltransferase Enhancer of Zester Homolog-2

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Objective—Gestational diabetes mellitus (GDM) produces fetal hyperglycemia with increased lifelong risks for the exposed offspring of cardiovascular and other diseases. Epigenetic mechanisms induce long-term gene expression changes in response to in utero environmental perturbations. Moreover, microRNAs (miRs) control the function of endothelial cells (ECs) under physiological and pathological conditions and can target the epigenetic machinery. We investigated the functional and expression effect of GDM on human fetal ECs of the umbilical cord vein (HUVECs). We focused on miR-101 and 1 of its targets, enhancer of zester homolog-2 (EZH2), which trimethylates the lysine 27 of histone 3, thus repressing gene transcription. EZH2 exists as isoforms α and β.

Approach and Results—HUVECs were prepared from GDM or healthy pregnancies and tested in apoptosis, migration, and Matrigel assays. GDM-HUVECs demonstrated decreased functional capacities, increased miR-101 expression, and reduced EZH2-β and trimethylation of histone H3 on lysine 27 levels. MiR-101 inhibition increased EZH2 expression and improved GDM-HUVEC function. Healthy HUVECs were exposed to high or normal ω-glucose concentration for 48 hours and then tested for miR-101 and EZH2 expression. Similar to GDM, high glucose increased miR-101 expression. Chromatin immunoprecipitation using an antibody for EZH2 followed by polymerase chain reaction analyses for miR-101 gene promoter regions showed that both GDM and high glucose concentration reduced EZH2 binding to the miR-101 locus in HUVECs. Moreover, EZH2-β overexpression inhibited miR-101 promoter activity in HUVECs.

Conclusions—GDM impairs HUVEC function via miR-101 upregulation. EZH2 is both a transcriptional inhibitor and a target gene of miR-101 in HUVECs, and it contributes to some of the miR-101-induced defects of GDM-HUVECs. (Arterioscler Thromb Vasc Biol. 2015;35:664-674. DOI: 10.1161/ATVBAHA.114.304730.)

Key Words: diabetes, gestational | endothelial cells | epigenomics | EZH2 protein human | microRNAs | polycomb repressive complex 2

Epigenetic modifications, including histone marks, DNA modification, and several types of RNA molecules, change chromatin dynamics, thereby affecting gene transcription. In fact, epigenetic changes contribute to the modulation of gene expression in development, cell physiology, and during pathological conditions. Early human epidemiological studies identified that the intrauterine environmental conditions can influence the phenotype of offspring both at birth and at later phases of life. Epigenetic factors have been proposed to play a role in fetal metabolic programming. For example, individuals who were prenatally exposed to famine during the Dutch Hunger Winter in 1944 to 1945, 6 decades later, still exhibited altered DNA methylation patterns in the insulin-like growth factor II gene, unlike their unexposed siblings. Therefore, epigenetics lies at the mechanistic core of the environment–gene interactions.

Gestational diabetes mellitus (GDM) may develop in pregnant women and is characterized by glucose intolerance resulting in maternal hyperglycemia. During pregnancy, maternal ω-glucose can transfer to the fetus by means of the fetal–placental...
circulation, whereas maternal insulin is unable to cross the placental barrier. Consequently, GDM-induced maternal hyperglycemia leads to endothelial dysfunction in the fetal micro- and macrocirculation, fetal hyperglycemia and chronic hyperinsulinemia, ultimately producing adverse long-term consequences for the child, who is more susceptible to developing type 2 DM and cardiovascular disease. It has been suggested that, similar to observations in adult diabetic patients, GDM induces fetal endothelial cell (EC) dysfunction. The persistence of the phenotypic and molecular changes induced by GDM in utero might be responsible for the increased cardiovascular and type 2 DM risk in children. In line with this hypothesis, in vivo or in vitro exposure to high glucose (HG) levels induces epigenetic changes that negatively affect endothelial function.

Differently from epigenetic factors, which modulate gene transcription, microRNAs (miRs) are a class of small, non-coding RNAs that regulate gene expression at the post-transcriptional level. Each miR acts on several target messenger RNAs (mRNAs), which are mostly recognized through semi-complementary oligonucleotide sequences in the miR seed sequence (8 nucleotides) and the 3′-untranslated region of the mRNA. MiRs control vascular development, function, and disease. Moreover, there is an accumulating evidence that DM-associated miR deregulation has negative functional consequences on the endothelium. MiRs and components of the epigenetic machinery have been shown to be reciprocally regulated. Indeed, several examples of mutual regulation between miRs and their target genes have also been reported.

MiR-101 is known to affect endothelial function and angiogenesis under normal glucose conditions and to target the histone methyltransferase enhancer of zester homolog 2 (EZH2) for inhibition.

EZH2 is part of the polycomb repressor complex 2, a multisubunit complex, which initiates and maintains the trimethylation of histone H3 on lysine 27 (H3K27me3), an epigenetic mark associated with heterochromatin formation and gene silencing. We have previously shown that the EZH2 locus generates, by alternative splicing, 2 different isoforms, EZH2-α and EZH2-β, both localized to the cell nucleus, able to generate the polycomb repressor complex 2, and to regulate the H3K27 methylation. The different isoforms can each not only regulate distinct targets but also share gene expression networks, making the regulation of transcription based on the deposition of the H3K27 histone mark more complex than previously anticipated.

Recent studies suggest that EZH2 regulates angiogenesis, although the molecular mechanisms underlying this event are still under intensive investigation. To the best of our knowledge, the regulation of EZH2 by miRs, in the context of DM, is still unexplored. Here, we provide the first evidence that exposure to a diabetic intrauterine environment alters fetal EC function in association with increased miR-101 levels and decreased EZH2 expression in human umbilical vein ECs (HUVECs), which are of fetal origin. We observed that a particular EZH2 isoform, EZH2β, is reduced in GDM-HUVECs. MiR-101 inhibition improves functional capacity and restores the level of EZH2 in GDM-HUVECs and in HG-stimulated control HUVECs in vitro. Moreover, our data suggest that EZH2 binds to the miR-101 locus, resulting in trimethylation of H3K27 and transcriptional inhibition. This epigenetic regulatory response is blunted by HG and in HUVECs from GDM pregnancies, potentially contributing to a fetal endotheliopathy, which could favor the appearance of cardiovascular disease in the offspring of mothers with GDM. Thus, our data provide the evidence implicating miR-101 and EZH2 in regulating fetal EC functions in the context of GDM. Because miR inhibitors are in clinical trials, our findings not only have mechanistic importance but also should be considered for the future development of potential therapies aimed at washing out the epigenetic imprinting of a GDM pregnancy from the offspring vasculature.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

**GDM Induces Phenotypic Alterations in HUVECs**

Flow cytometry for the endothelial marker CD31 confirmed the endothelial phenotype of HUVECs from both healthy (control) and GDM-complicated pregnancies. In fact, ≥95% of GDM-HUVECs and healthy HUVECs were CD31 positive (data not shown). In comparison with healthy HUVECs controls, GDM-HUVECs showed an increased rate of apoptosis (Figure 1A), a lower proliferative index after stimulation with fetal bovine serum (Figure 1B), a reduced capacity to form cellular networks on Matrigel (Figure 1C), and an impaired migration capacity (measured using a scratch assay) (Figure 1D). These GDM-induced phenotypic alterations were observed after 5 to 6 passages in GDM-HUVECs cultured under normal glucose conditions, which suggests that they likely mimic long-term effects of the GDM-induced in utero environment.

### Exposure to HG Impairs Survival and Functional Capacity of Control HUVECs

The deleterious effects of HG on cultured HUVECs have been widely reported. To seek confirmation of these effects, in our own experimental system, cultured healthy HUVECs were exposed to 2 different concentrations of increased glucose: 25 or 12.5 mmol/L for 12 to 48 hours. Normal glucose (5 mmol/L) was combined with 20 mmol/L of -glucose and used as an osmotic control. Figure I in the online-only Data Supplement illustrates the results of these experiments. In the first 12 hours, 25 and 12.5 mmol/L -glucose did not influence the HUVEC angiogenic...
capacity, whereas an increased angiogenic response was observed after 24 hours in 12.5 mmol/L of d-glucose. A longer exposure to 25 mmol/L d-glucose impaired the angiogenic capacity.

GDM Is Associated With Increased MiR-101 Expression That Affects HUVECs Survival and Functional Capabilities

MiR-101 expression was higher in GDM-HUVECs when compared with healthy HUVECs (Figure 2A). To elucidate the effect of miR-101 on GDM-HUVECs survival and angiogenic capacities, we altered intracellular miR-101 levels by transfecting with pre-miR-101 (to increase the miR-101 expression) or anti-miR-101 (to inhibit miR-101 expression), or a scramble sequence as control. We used healthy HUVECs transfected with scramble for reference. Figure 2B shows miR-101 changes in response to successful transfection. We found that anti-miR-101 exerted an antiapoptotic effect on GDM-HUVECs (Figure 2C). By contrast, the proliferation capacity of GDM-HUVECs was unresponsive to the forced miR-101 expressional changes (Figure 2D). Although an antiangiogenic property of miR-101 on normal HUVECs has already been reported, its effect on GDM-HUVECs remained to be investigated. We observed that anti-miR-101 restored the GDM-HUVEC functional angiogenic capacity, measured as capillary-like tube formation on Matrigel (Figure 2E). Moreover, pre-miR-101 reduced the migratory capacity in GDM-HUVECs, whereas anti-miR-101 elicited no effect (Figure 2F).
EZH2 Is Downregulated in GDM-HUVECs

MiR-101 reportedly targets EZH2 for inhibition.\textsuperscript{20,21,27} Consistently, the mRNA levels of EZH2 were reduced in GDM-HUVECs in comparison with control healthy-HUVECs (Figure 3A). Interestingly, we noticed that the EZH2-β isoform was decreased in GDM-HUVECs, whereas...
the EZH2-α level was unchanged (Figure 3A). EZH2 protein levels were also reduced in GDM-HUVECs when compared with that in healthy HUVECs (Figure 3B). Accordingly, trimethylation of histone H3 on lysine 27 (H3K27me3), derived by EZH2 enzymatic activity, was decreased in GDM-HUVECs (Western blotting, with blot densitometry normalized to H3; C). Adenovirus (Ad)-induced EZH2-β overexpression increased H3K27me3 levels in GDM-HUVECs, whereas Ad.EZH2-α was ineffective (D). All data are presented as mean±SEM. *P<0.05 vs healthy HUVECs; +P<0.05 vs Ad.Null and ^P<0.05 vs Ad.EZH2-α in GDM-HUVECs. The number of donors used to prepare the tested HUVECs is reported in each panel.

Figure 3. The enhancer of zester homolog-2 (EZH2) is downregulated in gestational diabetes mellitus-human umbilical vein endothelial cells (GDM-HUVECs). The relative mRNA levels of total EZH2 and EZH2-β were reduced in GDM-HUVECs in comparison with healthy HUVECs, whereas the relative expression of EZH2 α was not (A). EZH2 protein expression was also reduced in GDM-HUVECs (measured Western blotting, with blot densitometry normalized to lamin A/C; B). Accordingly, trimethylation of histone H3 on lysine 27 (H3K27me3), derived by EZH2 enzymatic activity, was decreased in GDM-HUVECs (Western blotting, with blot densitometry normalized to H3; C). Adenovirus (Ad)-induced EZH2-β overexpression increased H3K27me3 levels in GDM-HUVECs, whereas Ad.EZH2-α was ineffective (D). All data are presented as mean±SEM. *P<0.05 vs healthy HUVECs; +P<0.05 vs Ad.Null and ^P<0.05 vs Ad.EZH2-α in GDM-HUVECs. The number of donors used to prepare the tested HUVECs is reported in each panel.

Mir-101 Downregulates EZH2 Expression in GDM-HUVECs
In line with the hypothesis that miR-101 directly targets EZH2 for inhibition in GDM-HUVECs, we found that both pre-miR-101 and anti-miR-101 affected EZH2 mRNA expression (Figure 4A). When looking specifically at the effect of anti-miR-101 on each of the 2 EZH2 isoforms, we found that both were increased (Figure 4B). In addition, pre-miR-101 decreased EZH2 protein (Figure 4C) and trimethylation of histone H3 on lysine 27 (Figure 4D) expression. Finally, anti-miR-101
increased EZH2 in GDM-HUVECs (Figure 4C), but it did not change the overall H3K27me3 expression (Figure 4D).

**GDM Reduces EZH2 and H3K27me3 Occupancy at the miR-101 Locus**

EZH2 has been previously proposed to regulate miR-101 in non-ECs.21 Notably, as described above, we here provide new evidence that miR-101 regulates EZH2 in GDM-HUVECs. We also hypothesized that EZH2 is required for the silencing of miR-101 expression and that lower EZH2-β levels found in GDM-HUVECs may contribute to explain the increased miR-101 levels found in these cells. Consequently, to further characterize the mechanisms by which EZH2 regulates miR-101 in HUVECs, we looked at EZH2 and H3K27me3 occupancy within the promoter of the *miR-101* locus. A scheme of the miR-101 promoter is presented as Figure 5A.

By performing chromatin immunoprecipitation with antibodies targeting EZH2 and H3K27me3, followed by polymerase chain reaction for different *miR-101* promoter regions (Table II in the online-only Data Supplement), we found that EZH2 and H3K27me3 occupy the *miR-101* promoter in both healthy HUVECs and GDM-HUVECs (Figure 5B and 5C). Moreover, in GDM-HUVECs, we observed decreased EZH2 and H3K27me3 occupancy at a regulatory sequence beginning −396 bp upstream of the transcriptional start site (Figure 5B and 5C). Taking into consideration current paradigms, it is expected that reduced EZH2 occupancy on the *miR-101* promoter results in a higher accessibility of chromatin, thereby increasing *miR-101* transcription in GDM-HUVECs.

**EZH2 β Inhibits miR-101 Transcription in HUVECs**

In line with the data above, we found increased miR-101 expression after EZH2 silencing in GDM-HUVECs (Figures 5D). To complement these studies, we performed luciferase reporter assays on healthy HUVECs overexpressing either EZH2-α or EZH2-β. Figure 5E shows that Ad-mediated EZH2-β overexpression inhibits the transcriptional activity of the *miR-101* promoter. Taken together, these results suggested that in HUVECs miR-101 transcription is regulated by EZH2, in particular by the EZH2-β isoform.

**In Vitro Exposure of Control HUVECs to HG Lowers EZH2 Binding to the miR-101 Promoter and Increases miR-101 Expression**

Next, we wanted to investigate whether exposure of normal HUVECs to HG in vitro may recapitulate the molecular changes observed in HUVECs from GDM pregnancies. To this
end, healthy HUVECs were exposed to HG or normal glucose concentration (with osmotic control) for 48 hours. Chromatin immunoprecipitation-quantitative polymerase chain reaction was used to measure EZH2 binding to the aforementioned miR-101 promoter region beginning at around −396 bp upstream of the transcriptional start site. Indeed, similar to GDM, we found that in vitro exposure to HG reduced the presence of EZH2 at this miR-101 promoter regulatory region (Figure 6A). Moreover, congruent with the results from chromatin immunoprecipitation assays, HG increased miR-101 levels in healthy HUVECs (Figure 6B). Taken together, our data suggest that EZH2 and miR-101 are reciprocally regulated in HUVECs and that an imbalance of this equilibrium in response to either in vivo GDM or in vitro exposure to HG might result in a lower EZH2 occupancy of the miR-101 locus, thus leading to increased miR-101 transcription and further phenotypic alterations of ECs.

**Effect of EZH2 Overexpression on Phenotypic Alterations in GDM-HUVECs**

To see whether restoring EZH2 levels in GDM-HUVECs could rescue the cell survival and functional capabilities, we used Ad.EZH2-α or Ad.EZH2-β (control: Ad.Null). Ad.EZH2-β decreased the relative apoptotic activity (Figure 7A) and increased the migratory capacity (Figure 7C) of GDM-HUVECs. In contrast, this treatment did not rescue impaired proliferation (Figure 7B) and tube formation (Figure 7D) of GDM-HUVECs. Ad.EZH2-α did not alter the behavior of...
GDM-HUVECs (Figures 7A–7D). Taken together, these data suggest that a deficit in EZH2-β contribute to some of the functional defects induced by GDM in fetal ECs.

Discussion

Endothelial dysfunction is a common feature in many diseases of pregnancy, as well as under the different forms of DM. In particular, fetal endothelial dysfunction is involved in the abnormal fetal development and growth caused by GDM, and it might be responsible for the increased cardiovascular risk and incidence of type 2 DM observed later in life in the offspring from GDM mothers.10,11

Large-scale clinical trials and epidemiological studies of type 1 and type 2 DM patients have revealed that the exposure to prolonged hyperglycemia leaves a long-lasting impression on vascular cells and progressively induces the development of vascular complications, which persist after glycemic control is achieved.34 Nonetheless, the same studies also demonstrated that good glycemic control can delay the onset of diabetic vascular complications.35 Both lines of evidence are compatible with an underlying implication of epigenetic mechanisms. The epigenome is a complex system, which shapes the chromatin conformation of the different loci, thus ultimately profoundly affecting gene transcription. Epigenetic modifications allow cells and organisms to respond to changing internal and environmental stimuli and additionally confer the ability of the cell to memorize these environmental situations. According to an early definition, epigenetics is associated with chromatin changes, which were transmitted from parents to offspring. However, this concept has been revisited and it is now suggested that epigenetic changes have a long-lasting effect, which can be transmitted at cell division, but that are not necessary perpetual. In other words, the epigenetic modifications (and their transcriptional result), once written, can still be diluted and possibly erased over time under particular circumstances, such as with the use of drugs or lifestyle changes.36 The concept of a changeable epigenetic status is important because it confers therapeutic hope at different stages of prenatal and postnatal life. Several laboratories, including ours, have hypothesized that well-targeted epigenetic drugs will be one of the big things of future medicine. Before we can reach the stage of designing optimal agents, we should note that the pattern of specific chromatin modifications in vascular ECs under hyperglycemia (as well as different adverse environmental conditions) is not as yet fully understood and necessitates more research. Human vascular ECs reportedly respond to even weak or transient increases in glucose concentration by changing their chromatin status, which results in altered gene expression.37 Histones are important regulators of the chromatin status. In vascular ECs, the H3 has been reported to be particularly responsive to in vitro exposure to high glucose.37 Different H3 modifications have been shown to command either transcriptional inhibition or activation.38 A balance of these H3 modifications, together with other regulatory systems (including transcription factors and some classes of long noncoding RNAs) acting at gene promoters, contributes to the switching on or off of transcription under physiological and pathological conditions. Additional layers of gene expression regulation include miRs, which act by post-transcriptional inhibition of their targeted mRNAs.39

HUVECs represent a good cell model allowing for non-invasive analyses of the effect of the uterine environment on the fetal endothelium. For instance, molecular abnormalities, especially in the nitric oxide production machinery and the adenosine transport system, have been described in HUVECs from GDM pregnancies.38,39 However, to the best of our knowledge, our study has provided a functional characterization of the effect of GDM on HUVECs. In fact, here, we report that cultured GDM-HUVECs demonstrated decreased survival and functional capacity in comparison with HUVECs obtained from healthy nondiabetic mothers who gave birth at the same obstetric unit. Notably, these analyses were performed after cells were expanded in normal α-glucose concentrations for 5 to 6 passages, suggesting that the cells were...
still affected by the chronic hyperglycemia sensed in utero, which is consistent with the existence of a metabolic memory or legacy. Moreover, we have identified a novel underlying mechanism involving miR-101 and EZH2, the catalytic subunit of the polycomb repressor complex 2 in GDM-elicited endothelial dysfunction. In particular, we have identified that in HUVECs, EZH2 is both a target of, and a transcriptional repressor for, miR-101. Moreover, we have shown that among the 2 recently identified EZH2 isoforms, \( \text{EZH2-} \beta \) is the only one which is both a target of GDM and a mediator of some of the GDM damaging effects on HUVECs.

Next, we have used chromatin immunoprecipitation-polymerase chain reaction to provide evidence that EZH2 targets the \( \text{miR-101} \) promoter for inhibition in HUVECs, and that this mechanism is impaired in both HUVECs from GDM pregnancies and in HUVECs exposed to HG in culture. In fact, our data suggest that increased \( \text{miR-101} \) expression in both GDM-HUVECs and HG-HUVECs is associated with reduced EZH2 binding at transcriptional regulatory regions of the \( \text{miR-101} \) gene promoter and reduced H3K27 methylation (within 0.5 kb of transcription starting site), which should result in higher accessibility to transcription factors and consequently to increased \( \text{miR-101} \) transcription.

In line with the knowledge that miRs and their target genes are often linked in a regulatory feedback loop, which maintains their homeostatic expressional balance, we propose a model where \( \text{miR-101} \) is both a transcriptional target and a post-transcriptional inhibitor of EZH2 in HUVECs. In GDM-HUVECs, \( \text{miR-101} \) transcription seems regulated by EZH2-\( \beta \), only. We have been the first to detect diabetes-induced changes in the levels of \( \text{miR-101} \) and EZH2. A recent report indicated a proangiogenic role for EZH2 in HUVECs and identified 946 genes upregulated \( \geq 2 \)-fold because of EZH2 knockdown, in accordance with the EZH2 transcriptional repressive function. Importantly, many of these EZH2-targeted genes are implicated in endothelial dysfunction and cardiovascular disease, which is in line with the hypothesis that a decrease in EZH2 may confer an increased cardiovascular risk profile in the offspring from pregnancies complicated by GDM. If validated in population studies allowing for the follow-up of the children to maturity, this could help scientists understand whether there is any association between what is sensed in HUVECs and the incidence of vascular diseases. Moreover, in future studies, it would be worth attempting to correlate \( \text{miR-101} \) and EZH2 levels in HUVECs with the glucose levels in the maternal

**Figure 7.** Effect of enhancer of zester homolog-2 (EZH2) on survival and functional capacities of gestational diabetes mellitus-human umbilical vein endothelial cells (GDM-HUVECs). GDM-HUVECs were infected with adenovirus carrying EZH2-\( \alpha \), EZH2-\( \beta \), or an empty vector (Ad.Null). Ad.EZH2-\( \beta \) rescued GDM-HUVEC survival (A) and showed no effect on cell proliferation (B). Moreover, Ad.EZH2-\( \beta \) improved the migration capacity of GDM-HUVECs (C), whereas it exerted no effect on tube formation (presented here as tube length; D). In all tests, Ad.EZH2-\( \beta \) produced similar effect as Ad.Null. C and D, Scale bars, 0.5 mm. Data are presented as mean±SEM. *P<0.05 vs Ad.Null. The number of donors used to prepare the tested HUVECs is reported in each panel.
and fetal blood. In addition, we correlate the level of miR-101 in the HUVECs and the mother’s blood at different stages of pregnancy. This could lead to the development of miR-101 as a circulating marker of fetal endothelial damage induced by GDM.

We recently found that increased EZH2 after hypoxia and in vivo limb ischemia in mice negatively controls the angiogenic response and that EZH2 targets 2 proangiogenic genes for transcriptional repression.30 We also showed that Ad-mediated EZH2-α overexpression impairs the angiogenic capacity of healthy HUVECs in vitro.30 The results of our 2 studies can be reconciled if we speculate that hypoxia/ischemia and HG/GDM regulate different EZH2 isoforms and other RNA-binding molecules in ECs and that changes in EZH2-α and EZH2-β expression differently affect gene regulation.

Our data show that GDM increases miR-101 in fetal ECs and additionally alters the homeostatic regulatory feedback loop between EZH2 and miR-101. The altered miR-101/ EZH2 equilibrium could continue to influence vascular gene expression and function well after birth. Our data also suggest that some defects of GDM are mediated by miR-101 independently of EZH2. In fact, although miR-101 inhibition rescues most of the studied cell functions in GDM-HUVECs, Ad-mediated EZH2-β only improved the cell survival and migration. MiR-101 acts post-transcriptionally on multiple target genes, including EZH2 and vascular endothelial growth factor A.40 We did not find vascular endothelial growth factor A expression in HUVECs to be altered by GDM (data not shown). A possible limitation of our study is that we have not identified all the miR-101 target genes, which are involved with the GDM-induced vascular defects. Moreover, because EZH2 regulates the transcription of many different genes, the rescue effect of EZH2-β can be mediated by the inhibition of a plethora of damaging genes.

A recent study has analyzed the methylation profile of placenta and cord blood and described DNA methylation differences between GDM and healthy pregnancies,41 but this study has not selectively worked on ECs. A more extended profiling of epigenetic modifications should be performed and linked to follow-up data. These studies would help understand whether epigenetic changes in HUVECs could be developed as predictive biomarkers of future vascular disease, in line with the concept of personalized medicine through risk-stratified monitoring and treatment guided by biomarkers. It would be also important to understand (and develop a marker allowing understanding) at what phase of the GDM pregnancy these changes on the fetal endothelium start developing to better design the timing for initiating possible therapeutic interventions. With the focus on the observed changes in EZH2 levels, it would appear conceivable to target miR-101 for inhibition in GDM. MiR-targeting drugs are becoming increasingly appealing for clinical translation. Consequently, it is conceivable to hypothesize that miR-101 inhibition at a certain phase of pregnancy, or soon after birth, could be exploited to restore normal EZH2 levels in the endothelium of the fetus or child.

Acknowledgments
For the duration of this study, I. Floris and A. Vardeu were visiting PhD students (PhD scholarships for I. Floris and A. Vardeu has been cofounded by University of Sassari and Sardinia Region within the P.O.R. SARDEGNA, F.S.E 2007–2013—Human Capital Objective—Line of Activity 1.3.1.) at the Bristol Heart Institute (supervised by C. Emanueli and G. Pintus); T. Mitić received an Early Career Grant from the British Society for Endocrinology; SS is a British Heart Foundation (BHF) PhD student; R. Urrutia is supported by DK52913, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-funded Center for Cell Signaling in Gastroenterology, and the Epigenomics Translational Program, CIM, Mayo Clinic; C. Emanueli is a British Heart Foundation Senior Research Fellow.

Sources of Funding
This study was supported by the European Foundation for the Study of Diabetes (to C. Emanueli); the Leducq Transatlantic Network in vascular microRNAs (to C. Emanueli); the British Heart Foundation (to C. Emanueli), the Bank of Sardinia Foundation (to G. Pintus), and the National Institute for Health Research (NIHR) through the Bristol Biomedical Research Unit in Cardiovascular Medicine (to C. Emanueli).

Disclosures
None.

References
microRNA-therapeutics might be able to combat the metabolic memory associated with diabetes mellitus. The gestational diabetes mellitus-human umbilical vein endothelial cells phenotype, suggesting the hypothesis that epigenetic drugs and more or less accessible to the transcription machinery. MicroRNAs represent a different regulatory layer of gene expression, acting post-risk in individuals previously exposed to hyperglycemia. Histone covalent modifications affect the chromatin architecture, thus making a gene Large population studies suggest that glycemic control can improve, but not reverse, the metabolic memory, which increases cardiovascular blood vessel formation through reduced repression of EZH2. Würdinger T. Down-regulation of miR-101 in endothelial cells promotes protein complex containing the Enhancer of Zeste protein.


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Arterioscler Thromb Vasc Biol. 2015;35:664-674; originally published online January 22, 2015; doi: 10.1161/ATVBAHA.114.304730

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Gestational diabetes mellitus impairs fetal endothelial cell functions through a mechanism involving microRNA-101 and the histone methyltransferase EZH2.

Floris et al: miR-101 and EZH2 in fetal diabetic endotheliopathy

SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Human study groups and ethics
Umbilical cords were collected upon delivery from 24 full-term healthy and 22 full-term gestational diabetic pregnancies (see Supplementary Table I for donor characteristics). The investigation conforms to the principles outlined in the Declaration of Helsinki. Approval was granted by the ethics committee of the Faculty of Medicine, University of Sassari, and all mothers were fully informed and provided consent. Patients at 24-28 weeks gestation with basal glycemia>95 mg/dL (i.e. following an overnight fast) and >155 mg/dL two hours after a 75 gram oral glucose load were diagnosed as having gestational diabetes mellitus (GDM). Human Umbilical Vein Endothelial Cells (HUVECs) were prepared (see below) at the University of Sassari, Italy, and exported to the University of Bristol, UK, where all experiments were performed. Import, storage and use of HUVEC samples in Bristol for this study were approved by NRES Committee West Midlands (REC reference: 12/WM/0366; IRAS Project Code: 113869).

Human endothelial cell extraction
Primary cultures of HUVECs were extracted from the umbilical cord vein, as previously described1, 2. Briefly, umbilical cord veins were flushed with PBS and gently massaged to expel the blood. Endothelial cells (ECs) were subsequently detached from the lumen of the vein by treatment with 0.05% (w/v) collagenase type II from Clostridium histolyticum (Sigma) in M199 medium (Invitrogen) containing 100 U/ml of penicillin G sodium salt and 100μg/ml streptomycin sulfate (Sigma), followed by incubation for 10 min at 37°C. A cell suspension was collected and centrifugated at 1000xg for 10 min. The ECs were then resuspended in 5 ml of M199 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 10% (v/v) newborn calf serum (Invitrogen, Carlsbad, CA), 2mM glutamine and antibiotics, then plated in 25 cm² tissue culture flasks (Falcon, Oxnard, CA) pre-treated with 0.1 % gelatin and cultured in an atmosphere of 5% carbon dioxide and 95% air. From passage 2, HUVECs were cultured in endothelial cell basal medium following the addition of a SingleQuots™ Kit (EGM-2, Lonza/Cambrex, UK) and with 10% FBS. The endothelial identity of the cultured cells was tested by flow cytometry (FACS Canto II, BD) using mouse anti-human PE-CD31 antibodies (1:100, eBioscience) as an EC marker. Propidium iodide (PI, 1:400, Invitrogen) incorporation allowed recognition of dead cells.

Exposure to high glucose experiments
HUVECs obtained from the healthy donors were cultured in high D-glucose (Sigma), at two different concentrations (12.5 mM and 25 mM), to mimic hyperglycemic conditions. L-glucose (Sigma) served as an osmotic control for each concentration of D-glucose. Cells were treated for 12, 24 and 48 hours. For each time point the phenotypic properties and functional characteristics of HUVECs were analyzed. For experiments involving EZH2 binding to miR-101 promoter regions (see below), HUVECs were exposed for 48 hours to D-glucose at 25 mM (or 5 mM D-glucose with 20mM L-glucose added as an osmotic control).
Cell transfection
HUVECs were seeded on a 6-well plate at a density of 2 x 10^5 cells per well in 2 mL complete EGM-2 medium and grown for 24 hours until 70–80% confluent. Cells were then maintained in OPTIMEM medium (Invitrogen) for 2 hours prior to transfection with 12.5 nM of scramble control (Ambion), pre-miR-101 (Ambion), anti-miR-101 (Ambion), EZH2 siRNA (Qiagen) or siRNA-AF (negative control, Qiagen). Oligonucleotides were transfected into HUVECs using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. After 5 hours, the transfection medium was replaced by EGM-2 (10% FBS) until further analysis.

Adenoviral transduction
HUVECs were seeded on a 12-well plate at a density of 1 x 10^5 per well in 1 mL complete EGM-2 medium and grown 24 hours until 70–80% confluent. Cells were then infected overnight with adenoviruses (Ad) carrying human EZH-2-alpha, human EZH2-beta or an empty vector (Null) (all already extensively described in^3 at 150 multiplicity of infection (MOI) in EGM-2 (10% FBS).

HUVEC functional assays
The HUVECs were analyzed for their endothelial phenotypic properties using functional assays. The following assays were performed: 1) BrdU incorporation assays were used to test the proliferative capacity of HUVECs. The assay was performed in a 96-well format using a commercially-available colorimetric enzyme-linked immunosorbent assay (ELISA) kit (Roche). The HUVECs were maintained for 1 hour without FBS, then cultured for 24 hours with 0.5% or 10% FBS, in the presence of BrdU. Cells were fixed and the BrdU incorporation was measured. The index of proliferative capacity in response to FBS was calculated. The ratio between the average proliferative capacity of 0.5% FBS and 10% FBS cultures gave an indication of FBS-stimulated proliferation. 2) Scratch assays were performed to test HUVEC migratory capacity. The cells were cultured in a 12-well plate (1 x 10^5 cells/well) to confluence. Then, the cell monolayer was manually scratched with a pipette tip, and the medium changed to EGM-2 (10% FBS) with 2mM hydroxyurea (Sigma) added to arrest growth. Images were taken at 0, 6 and 10 hours at 5x magnification using a brightfield inverted microscope (Zeiss). Image J (NIH) was used to calculate the wound area at the three time points. The final result obtained was the average speed of closure of the wound (in mm^2/h). 3) Matrigel assays were used to test the cell angiogenic capacity. Cells (1.5 x 10^5 cells/well) were placed on 40 μl reduced growth factors matrigel (Corning), in triplicate, on a 96-well plate. Images were taken after 6 and 24 hours at 5x magnification using a bright field inverted microscope. The total tube length (mm) was calculated as a parameter of angiogenic capacity in vitro.

Apoptosis
Apoptosis was studied using the Cell Death Detection ELISA kit (Roche). HUVECs were cultured on 6-well plates (1 x 10^6 cells/per condition). The ELISA specifically detects the mono- and oligonucleosome in the cytoplasmic fraction of the cell lysate. The enrichment of mono- and oligonucleosomes in the cytoplasm occurs in apoptotic cells secondary to DNA degradation. For the ELISA assay, the cellular pellet was incubated in Incubation Buffer (a lysis buffer provided in the kit) for 30 minutes; then centrifuged at 20,000xg for 10 min to obtain the cytosolic fraction of the supernatant. This was collected and used for the photometric enzyme immunoassay. The values of absorbance are reported as a fold change (vs. controls).

RT-qPCR analysis
Total RNA was isolated using QIAzol Lysis Reagent (Qiagen), and reverse transcription (RT) performed using the QuantiTect Reverse Transcription kit (Qiagen), according to the manufacturers’ protocols. cDNA
(equivalent to 300 ng total RNA) was incubated in triplicate with gene-specific primers for EZH2 (Sigma), EZH2-α, EZH2-β and Power SYBR Green qPCR Master Mix (ABI Life Technologies), and quantified using the LightCycler480 qPCR detection system (Roche). mRNA levels were normalized to beta-actin, chosen as the housekeeping gene. The primer sequences used were as follows: beta-actin (forward 5′-TGGACATCGCAAGCCTGTA-3′; reverse 5′-GGGAGTGTACCTTGTCAT-3′); EZH2 (forward 5′-GGGAGTGTACCGACATCGGAAGAAG-3′; reverse 5′-GTGCATAACGCTGACACACCTGACT-3′), EZH2 (forward 5′-CGAGCTCTGAAGACCTGTA-3′; reverse 5′-AACCTAGCAATTGACACGAA-3′), EZH2 alpha (forward 5′-GCGGGACTAGGGAGGTGGAAGA-3′; reverse 5′-GAGCTCTGAAGACCTGTA-3′; reverse 5′-AAAAGCTTTGATCATCTTCCA-3′), EZH2 beta (forward 5′-GGGAGTGTACCGACATCGGAAGAAGAAG-3′; reverse 5′-GCATCCAAAACACTCCCAAA-3′)

For miRNA analyses, reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed using the TaqMan universal master mix with the LightCycler480 qPCR detection system (Roche). Individual microRNA primers for both RT and qPCR were included with TaqMan MicroRNA assay sets (Life Technologies). SnU6B was used as a reference for normalization. Relative levels of mRNAs and miRNAs were defined from threshold cycle (Ct) values calculated using the 2^ΔΔCt method.

**Western blot assay**

Cell nuclear extracts were prepared using a previously published protocol. Briefly, cells were washed twice with cold PBS then resuspended in buffer A (10mM Hepes pH 7.5, 10mM KCl, 1.5mM MgCl2, 0.34M Sucrose, 10% Glycerol, 0.1% TritonX-100) supplemented with protease inhibitors (1mM DTT, 0.5mM PMSF, 2 µl/ml Protease Inhibitors Cocktails, Sigma) and incubated at 4°C on a rotator for 30 minutes to improve lysis. Nuclei were isolated by centrifugation at 6500g for 5 minutes at 4 °C, washed once with buffer A (depleted of Triton X-100) and subsequently resuspended in buffer B (3 mM EDTA, 0.2 mM EGTA, plus protease inhibitors). Nuclear protein fractions extracted from HUVECs were loaded, subjected to SDS–PAGE and blotted on PVDF membranes. Blots were incubated overnight at 4°C with primary antibodies against EZH2 (Active Motif, 1:2000); EZH2α (1:500), EZH2-alphaβ (1:500), EZH2 beta (1:500), anti-trimethyl-Histone H3 (Lys27) (UPSTATE Cell Signaling, 1:2000); anti-Histone H3 (Active Motif, 1:2000); anti-LaminA/C (Active Motif, 1:1000) and an HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by chemiluminescence detection (ECL prime, GE Healthcare). Bands were visualized on X-ray film (GE Healthcare) and quantified using Image J (NIH).

**Chromatin immunoprecipitation (ChIP)**

ChIP assays were performed as previously described. Chromatin was sonicated (3x9 cycles 30″ON/30″OFF) to an average DNA length of 400–500bp using a Bioruptorsonicatior (Diagenode, Belgium). Immunoprecipitation was performed in 1ml volume with the addition of 3-5µg of the EZH2 antibody (AC22 clone, mouse monoclonal, Active motif, Lot 2023363), H3K4me3 antibody (Millipore, catalog number 07-449) and control IgGs (Merck Millipore). The following day, immune-complexes were collected with purified protein A-magnetic beads (Invitrogen) for 3 hours at 4 °C with rotation, washed and DNA eluted by reverse-crosslinking. Aliquots of immunoprecipitated DNA were analyzed in triplicate by real-time quantitative PCR analysis with Power SYBR Green qPCR Master Mix (ABI Life Technology) and primers specific for the putative regulatory domain of mir-101 gene (UCSC browser, Supplementary Table II) bound by EZH2 and H3K27me3 within the mir-101 transcription start site (Chr1: 65532194). ChIP-enriched DNA on the target genomic regions was assessed relative to the input DNA and IgG control.
Molecular clone and luciferase reporter assay

The putative regulatory element of the mir-101 promoter was cloned by PCR (KOD polymerase, MERCK-Millipore) from human genomic DNA. A larger fragment was initially cloned using primers (forward 5’-CTCGGTTTCTCTGCACGTTCG-3’ and reverse 5’-GGATTAAAGCTAGGAGGCT-3’) and a second nested PCR was performed to add clamp and restriction endonuclease NheI and BglII recognition sites respectively using GCACGCTAGCCTCGGTTTCTCTGCACGTTCG and GCACAGATCTGACAGCAGATTCCATTAG. The second fragment including nucleotides 65532188 to 65531494 (Homo Sapiens Chromosome1, GRCh37.p13 primary assembly) was cloned upstream (NHE1-BglII sites) of the SV40 promoter of the pGL3-Promoter Luciferase Reporter Vector (Promega).

For vectors transfection healthy HUVECs were seeded in 24-well plates at a density of 6 x 10^4 cells per well in 0.5 mL complete EGM-2 medium and grown for 24 hours. Cells were then co-transfected with 0.075µg renilla(pRLTK, internal control), 0.75µg pGL3 (control vector) or 0.75µg of pGL3 mir-101 promoter, along with 200µl Opti-MEM. Luciferase reporter vectors were transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA USA) for 5 hours in OptiMem. Cells were then transduced with adenovirus overnight. Three days after transfection, cells were harvested and assayed with the Dual-Luciferase Assay system (Promega, Madison, WI, USA), accordingly to the manufacturer’s protocol. Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity.

Statistical analysis

Data are expressed as mean ± SEM from at least three independent experiments. Data were assessed for normality using the Shapiro-Wilk test, and differences in two-group comparisons were tested using the unpaired or paired Student’s t-test, where relevant (for parametric data) or the Mann-Whitney U test (for non-parametric data). Where there were three or more groups, a one-way ANOVA was performed with a Tukey post-hoc test. All statistical analyses were performed using GraphPad Prism for Windows v6.0 (GraphPad Software Inc.). P values < 0.05 were considered statistically significant.
Supplemental References


**SUPPLEMENTAL TABLES AND FIGURES**

**Supplementary Table I.** Characteristics of the healthy and gestational diabetes mellitus (GDM) mothers who donated the umbilical cords for this study

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>GDM</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(n=24)</td>
<td>(n=22)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>31.12 ± 6.54</td>
<td>35.13 ± 7.41</td>
</tr>
<tr>
<td>Smokers (n)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>77.00 ± 15.48</td>
<td>73.73 ± 14.74</td>
</tr>
<tr>
<td>Obese (n)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Hypertensive (n)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Epidural delivery (n)</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Pregnancy at risk (n)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Baby birth weight (Kg)</td>
<td>3.18 ± 0.43</td>
<td>3.45 ± 0.60</td>
</tr>
</tbody>
</table>

Quantitative data are expressed as mean ± standard deviation. No statistically significant differences were found between the two groups for any of the parameters reported above.
**Supplementary Table II.** PCR primers used for amplification of miR-101 promoter regions

<table>
<thead>
<tr>
<th>miR-101 promoter region (distance from the transcriptional start site)</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>-705 / -682 bp</td>
<td>CTTTTTCTCCCTGGGTACG</td>
<td>TCCTTCACCTCATGGGAG</td>
</tr>
<tr>
<td>-562 / -476 bp</td>
<td>AATAACTCTCCCTATGCCC</td>
<td>GCCATCTACCTCCCATCAG</td>
</tr>
<tr>
<td>-396 / +3 bp</td>
<td>TCAGCTCACCTCTCCTCAA</td>
<td>GAGGGTGCTTTGCTGTAAT</td>
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</table>
Supplementary Figure SI. Long exposure to D-Glucose impairs the angiogenic capacity of HUVECs in vitro

Graphs and representative images show the effect of glucose on angiogenic capacity in human umbilical vein endothelial cells (HUVECs), which were tested in Matrigel assays. HUVECs were exposed to normal (5 mM) or high (12.5 and 25 mM) levels of D-glucose, with L-glucose used as an osmotic control (osm.ctr), for different time periods (12, 24, 48h). The scale bar represents 0.5 mm. Results are expressed as the total length of networks (“tubes”) formed by the cells. Data are presented as mean±SEM. *p<0.05 and **p<0.01 vs. 5mM D-glucose; # p<0.05 vs. the osmotic control to 25 mM D-Glucose.
Supplementary Figure SII: Overexpression of EZH2α and EZH2β in GDM-HUVECs

GDM-HUVECs have been infected with adenoviruses (Ads) carrying either EZH2-α or EZH2-β, using a control (Ad.Null) as reference, as specified in the methods. The relative expression of EZH2-α and EZH2-β were altered as expected, as shown by qPCR results (A). Data are presented as mean±SEM. ***p<0.001 vs. Ad.Null.