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RESEARCH ARTICLE | *Making Cell Culture More Physiological*

Glucose-induced, duration-dependent genome-wide DNA methylation changes in human endothelial cells

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Aref-Eshghi E, Biswas S, Chen C, Sadikovic B, Chakrabarti S. Glucose-induced, duration-dependent genome-wide DNA methylation changes in human endothelial cells. *Am J Physiol Cell Physiol* 319: C268–C276, 2020. First published May 27, 2020; doi:10.1152/ajpcell.00011.2020.—DNA methylation, a critical epigenetic mechanism, plays an important role in governing gene expressions during biological processes such as aging, which is well known to be accelerated in hyperglycemia (diabetes). In the present study, we investigated the effects of glucose on whole genome DNA methylation in small [human retinal microvascular endothelial cells (HRECs)] and large [human umbilical vein endothelial cells (HUVECs)] vessel endothelial cell (EC) lines exposed to basal or high glucose-containing media for variable lengths of time. Using the Infinium EPIC array, we obtained 773,133 CpG sites (probes) for analysis. Unsupervised clustering of the top 5% probes identified four distinct clusters within EC groups, with significant methylation differences attributed to EC types and the duration of cell culture rather than glucose stimuli alone. When comparing the ECs incubated for 2 days versus 7 days, hierarchical clustering analyses [methylation change >10% and false discovery rate (FDR) <0.05] identified 17,354 and 128 differentially methylated CpGs for HUVECs and HRECs, respectively. Predominant DNA hypermethylation was associated with the length of culture and was enriched for gene enhancer elements and regions surrounding CpG shores and shelves. We identified 88 differentially methylated regions (DMRs) for HUVECs and 8 DMRs for HRECs (all FDR <0.05). Pathway enrichment analyses of DMRs highlighted involvement of regulators of embryonic development (i.e., *HOX* genes) and cellular differentiation [transforming growth factor- β (TGF- β) family members]. Collectively, our findings suggest that DNA methylation is a complex process that involves tightly coordinated, cell-specific mechanisms. Such changes in methylation overlap genes critical for cellular differentiation and embryonic development.

diabetes; DNA methylation; endothelial cells

INTRODUCTION

Vascular endothelial cell (EC) dysfunction is a major factor in the development of chronic diabetic complications (58). Secondary to hyperglycemia, one of the major mechanisms leading to EC dysfunction is an increase in the formation of reactive oxygen species (46). Oxidative stress-induced DNA damage promotes alterations in the transcriptional states of ECs (46), which can be observed in both aging and diabetes (38, 42, 71). Consistently, some of the changes seen at the cellular and tissue levels in diabetes are similar to those seen in

normal aging (17, 21, 51), and these changes are typically reached at an accelerated rate (20).

Transcriptional states in the cells are regulated by epigenetic mechanisms, the most comprehensively studied of which include DNA methylation and posttranslational histone tail modifications. We have previously shown that glucose-induced oxidative stress causes histone acetylation by p300, a transcriptional coactivator with intrinsic histone acetyltransferase activity (20). We have also demonstrated that glucose-induced changes are associated with reduced expression of sirtuins, a class of histone deacetylases required for balancing the acetylation of histone tail residues (49). Increasing evidence has also highlighted glucose-induced EC changes in the transcription of genes involved in inflammation. Activation of nuclear factor- κ B (NF- κ B)-dependent signaling in ECs exposed to hyperglycemia has been observed through histone modification changes in the *NFKB3* promoter region, including increased monomethylation of histone 3 lysine 4 (H3K4), reduced H3K9 methylation, and histone H3K9 hyperacetylation (15). Activation of other inflammatory genes [e.g., heme oxygenase 1 (*HMOX1*), *IL8*, *TNFA*, *COX2*, and matrix metalloproteinase 10 (*MMP10*)], through promoter H3K9 hyperacetylation, has also been frequently observed in vascular and immune cells of patients with diabetes (44, 52).

In addition to histone modifications, the methylation of DNA CpG dinucleotides has gained interest in studying complex diseases (1, 3, 18, 41, 53, 68). DNA methylation in gene promoters regulates transcription through an inverse association with gene expression. In noncoding areas, however, DNA methylation is involved in the regulation of enhancers, *cis*- and *trans*-acting elements, and maintaining the genomic integrity and silencing of transposons (2). Although the role of epigenetic mechanisms in chronic diabetic complications is just beginning to be unraveled (53), the detailed role of DNA methylation and its alterations in response to glucose in EC dysfunction are poorly investigated. Of note, however, emerging evidence has recently shown that aberrant DNA methylations are associated with some chronic diabetic complications including diabetic retinopathy (33, 47, 48, 55).

In response to glucose and subsequent oxidative stress, ECs demonstrate responses that are similar to the changes seen in aging (49); however, these are observed to variable degrees in different cell types. Large vessel ECs, for instance, change at a slower pace compared with microvascular ECs (49). Hence, it is important to examine both macrovascular and microvascular ECs in the context of glucose-induced duration-dependent changes. To explore such alterations due to prolonged glucose

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culture, in the present study, we examined human umbilical vein endothelial cells (HUVECs), representing ECs from the macrovasculature, and human retinal microvascular endothelial cells (HRECs), representing the microvasculature. We exposed the two cell populations for variable lengths of time to basal and high glucose-containing media, enabling us to examine the effects of glucose and aging on whole genome DNA methylation. We then identified the genomic regions and neighboring/overlapping genes highly influenced in this process and evaluated the differences and similarities of the glucose-induced changes in DNA methylation between the microvascular and macrovascular ECs.

MATERIALS AND METHODS

Cell treatment and culture. For our experiments, we used both human retinal microvascular ECs (HRECs; mature microvascular ECs, cat. no. ACBRI 181; Cell Systems, Kirkland, WA) and human umbilical vein ECs (HUVECs; relatively immature large vessel ECs, cat. no. C2519A; Lonza, Walkersville, MD). As described previously (10, 11, 20, 29, 49, 63, 64), these cells were grown in complete EBM-2 medium (Lonza, Kirkland, WA) supplemented with EGM-2 SingleQuots (Lonza) and 10% fetal bovine serum. Prior to experimentation, cells were plated at a density of 4.3×10^5 cells/mL and used between passages 4 and 5 to minimize variability. At 80% confluence, cells were cultured in serum- and growth-free medium overnight before exposure to a final concentration of 5 mM glucose (to mimic euglycemia) or 25 mM glucose (to mimic hyperglycemia). Cells were then examined following either 2- or 7-day incubation. For our methylation array, three independent samples were used for each group, which amounted to a total sample size of $n = 12$ independent samples per cell line.

DNA extraction. DNA extractions were performed using Blood & Cell Culture DNA Mini Kit (Qiagen) as per the manufacturer's instructions. Briefly, $\sim 10^7$ ECs were lysed, and proteins were simultaneously denatured. Proteinase K was then added. Following incubation, lysates were loaded onto the columns. DNA binds to the column, while other cell constituents pass through. Following washing, pure, high-molecular weight DNA is eluted and precipitated with isopropanol. The extracted DNA was quantified spectrophotometrically.

Methylation array and quality assessment. Methylation assessment, bioinformatics, and statistical analysis were performed using a modification of previously published methods (4–9, 57). Following bisulfite conversion, DNA methylation analysis was performed using the Illumina Infinium MethylationEPIC BeadChip (San Diego, CA), according to the manufacturer's protocol, at the London Health Sciences Molecular Genetics Laboratory. This array covers above 850,000 human genomic methylation CpG sites, including 99% of the Reference Sequence Database (RefSeq) genes and 96% of CpG islands (CGIs). All of the samples were processed in a single experiment to avoid batch effect. Methylated and unmethylated intensity data were generated as IDAT files and imported into R 3.5.2 for analysis. Normalization was performed using Illumina normalization method with background correction using the minfi package in R 3.5.2. Probes with a detection P value >0.01 were excluded from the downstream analysis. For further quality improvement, probes located on chromosomes X and Y, probes known to contain single-nucleotide polymorphisms (SNPs) at the CpG interrogation or the single-nucleotide extension, and probes known to cross-react with sex chromosomes were removed. All of the samples were examined for genome-wide methylation density, and it was ensured that none of the samples deviate from representing a bimodal distribution. Following the normalization and removal of nonspecific probes, a principal component analysis was performed to identify outliers or poor quality samples. The methylation levels for each probe were measured as beta value

(β), calculated from the ratio of the methylated signals versus the sum of unmethylated and methylated signals, ranging between 0 (no methylation) and 1 (full methylation). This value was used for biological interpretation and visualization. For statistical analysis, wherever a normal distribution was required, beta values were transformed to M values using the following equation: $\log_2 [B/(1 - \beta)]$.

Clustering and dimension reduction and identification of differentially methylated CpGs. Hierarchical clustering was performed using Ward's method on Euclidean distance by the *gplots* package. Multiple-dimensional scaling was performed by scaling of the pairwise Euclidean distances between the samples. The analyses were performed using the top 5% highly variable probes and probes selected to be significantly differentially methylated. Subsequently, the analysis was repeated using pairwise comparison of the methylation levels across the samples with different duration and concentrations of the treatment, separately for each cell type. A methylation difference of >0.1 and a false discovery rate (FDR) of <0.05 (limma univariate regression modeling) were considered significant (54). For significant probes in each comparison (e.g., between different durations of treatment), we adjusted the analysis for the other factor (i.e., the concentration of glucose), using limma multivariate regression modeling, to evaluate their potential confounding effect on the identified CpGs.

Identification of the differentially methylated regions. To identify genomic regions harboring methylation changes [differentially methylated regions (DMRs)], the DMRcate algorithm was used. First, the P values were calculated for every probe using multivariable limma regression modeling. Next, these values were kernel smoothed to identify regions with a minimum of three probes no more than 1 kb apart and an average regional methylation difference $>10\%$. We selected regions with a Stouffer-transformed FDR <0.05 across the identified DMRs. A pathway enrichment analysis was conducted using a hypergeometric model, implemented in the ReactomePA package, on the list of genes found to be overlapping the identified DMRs.

RESULTS

DNA methylation profiling of HRECs and HUVECs cultured in basal or high-glucose media. Human retinal microvascular endothelial cells (HRECs) and human umbilical vein endothelial cells (HUVECs) were incubated with 5 mM glucose (mimicking euglycemia) or 25 mM glucose (mimicking hyperglycemia), for durations of 2 and 7 days. Following a genome-wide DNA methylation experiment using Infinium EPIC arrays and quality controls, methylation levels at 773,133 CpG sites (probes) were obtained for analysis. We first performed an unsupervised clustering analysis on the top 5% probes with the greatest variability in their methylation levels across the 24 samples. This analysis identified four distinct clusters within the samples (Fig. 1). The greatest difference was related to the cell types, i.e., between HUVECs and HRECs, which were found to have dramatically different methylation profiles from each other. The next separation occurred on the basis of the duration of incubation. In both HUVECs and HRECs, samples treated for 7 days were placed at distance from those treated for 2 days. This pattern was more prominent in the HUVECs than in HRECs. No difference was observed between the samples that were incubated in different glucose concentrations. Analysis of the data using the entire probes led to similar observations (Supplemental Fig. S1; see <https://doi.org/10.6084/m9.figshare.12200870>).

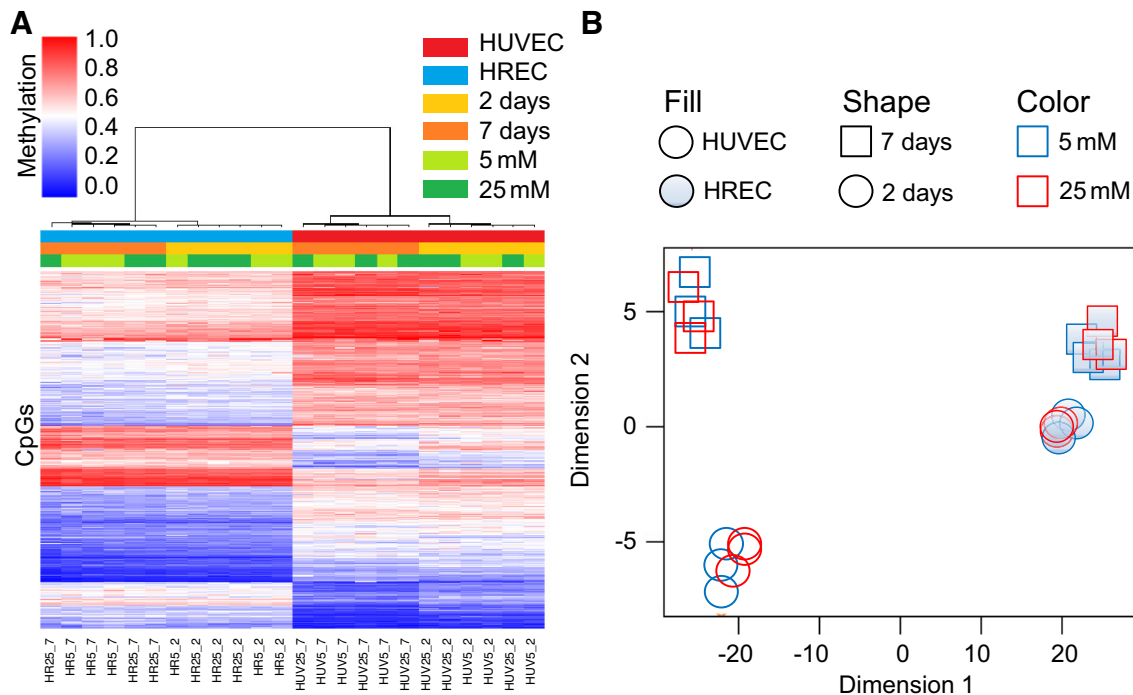


Fig. 1. DNA methylation profiling of human retinal microvascular endothelial cells (HRECs) and human umbilical vein endothelial cells (HUVECs). Unsupervised clustering using the top 5% variable CpGs in HUVECs and HRECs in different culture states shows complete separation based on the cell types (the greatest difference) and the duration of culture. There is no distinction between the cells treated with different concentrations of glucose. *A*: hierarchical clustering with heat map: rows indicate CpGs, and columns show the samples; the color scale from blue to red indicates the level of methylation from 0 to 1. *B*: the top two dimensions from the multidimensional scaling indicate a greater distance for duration of treatment in HUVECs than in HRECs ($n = 3$ independent samples per group).

The duration of cell culture is the only inducer of DNA methylation changes. The comparison of the samples incubated for 2 days versus 7 days identified 17,354 and 128 differentially methylated CpGs for HUVECs and HRECs, respectively [methylation change $>10\%$ and false discovery rate (FDR) <0.05 ; Supplemental Tables S1 and S2 (all Supplemental Tables are available at <https://doi.org/10.6084/m9.figshare.11551551>) and Fig. 2]. No probe met these cutoffs when applied to the comparison between the samples incubated in different glucose concentrations. Neither was any significant difference found in this analysis when a lower cutoff for methylation difference was applied (down to 5%), indicating that the concentration of glucose at incubation does not induce considerable methylation changes in these cells. Therefore, the rest of the study focused on the differences induced by the duration of incubation. We also questioned whether the observed differences by the duration of treatment could be confounded by the glucose concentrations of the culture. Using multivariable models, we adjusted the analysis for the concentration of the glucose (Supplemental Tables S1 and S2). The results indicated that glucose concentrations have a limited effect on the observed differences by the duration of treatment. As an example, of the total of 17,354 significant probes in HUVECs, only 78 (0.4%) lost the significance of FDR <0.05 , while still maintaining FDR <0.1 .

The examination of the two sets of differentially methylated CpGs using hierarchical clustering revealed that each set alone was capable of separating the samples treated for 7 days from those treated for 2 days in both HUVECs and HRECs (Fig. 2). However, the nominal overlap between them was limited to only 18 probes. Both probe sets mainly represented a gain of

methylation in the seventh day of incubation compared with the second day (76% and 57% of the significant probes for HUVECs and HRECs, respectively). The levels of methylation changes for these probes in both cell types were within the same range (mean difference \pm standard deviation $\sim 13 \pm 3\%$). The CpGs identified in HUVECs were found to be significantly enriched at enhancer elements [odds ratio (OR), 1.21; P value (P) = 1.7×10^{-6}] and the regions surrounding CpG islands, i.e., shores and shelves (OR, 1.23; P = 2.2×10^{-16}), but significantly less likely to occur at CpG islands (OR, 0.34; P = 2.2×10^{-16}) and promoters (OR, 0.27; P = 2.2×10^{-16}) compared with the entire CpGs tested. These estimates were similar for the HREC probe set; however, because of the small number of probes in this list, they did not yield a significant P value (<0.05) in some of the analyses (enhancers: OR, 1.70; P = 0.2; shores and shelves: OR, 1.13; P = 0.5; islands: OR, 0.34; P = 0.0001; promoters: OR, 0.56; P = 0.08).

Differentially methylated regions in HUVECs and HRECs following extended incubation exposures. To identify the target genes affected by the differential duration of incubation, we mapped differentially methylated regions (DMRs) across the genome harboring a minimum of three consecutive CpGs and an average regional methylation difference of $>10\%$ between the cells incubated for 2 versus 7 days (Supplemental Tables S3 and S4). This analysis identified 88 DMRs for HUVECs (Supplemental Fig. S2; see <https://doi.org/10.6084/m9.figshare.11550594>) and 8 DMRs for HRECs (Supplemental Fig. S3; see <https://doi.org/10.6084/m9.figshare.11551461>; all FDRs <0.05). The most significant DMRs in HUVECs in the seventh day compared with the second day of incubation included a hypom-

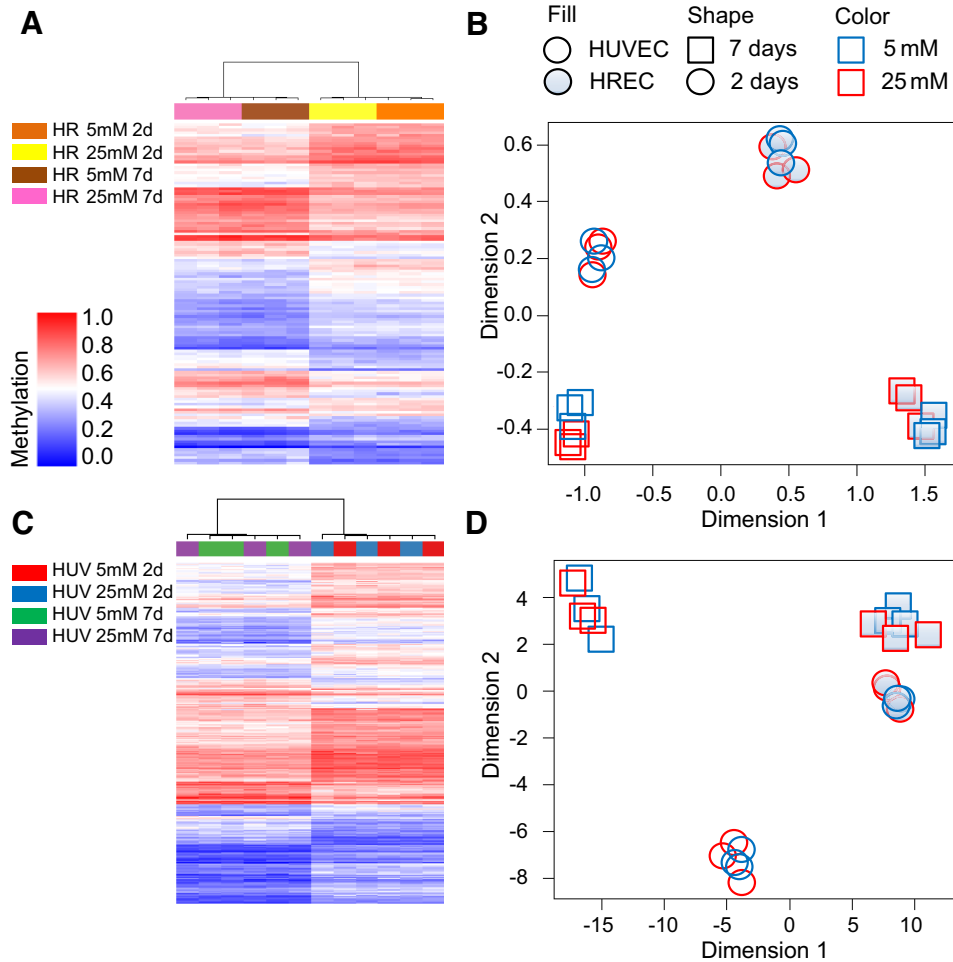


Fig. 2. DNA methylation profile of human umbilical vein endothelial cells (HUVECs) and human retinal microvascular endothelial cells (HRECs) by duration of culture. *A*: DNA methylation pattern of probes differentially methylated in HRECs treated for 2 days vs. HRECs treated for 7 days. *B*: in addition to HRECs, the same probes generate a distinct pattern between the HUVECs treated for 2 days vs. HUVECs treated for 7 days as shown using multidimensional scaling. *C* and *D*: the same pattern is observed for HUVECs' specific probes ($n = 3$ independent samples per group). Here, d, days; HR, HREC; HUV, HUVEC.

ethylation of an extended region annotating to the promoters of *HOXA2* and *HOTAIRM1* (33 CpGs, 11% methylation difference; FDR, 1.7×10^{-46}), as well as a hypomethylation in the *SMAD3* promoter (11 CpGs, 11% methylation difference; FDR, 7.3×10^{-26}). In HRECs, loss of methylation in the promoter of Meis homeobox 1 (*MEIS1*) was the most significant event (13 CpGs, 11% methylation difference; FDR, 6.5×10^{-6}). Two regions were shared across the two DMR lists including the hypermethylation of the promoters of *HOXC4–6* and *SMAD6* (11% methylation change for both DMRs in both HUVECs and HRECs).

Enrichment of *HOX* and transforming growth factor- β signaling pathway genes among the DMRs. Within both of the identified DMRs were numerous genes encoding the members of *HOX* proteins including various *HOX* gene family members and *MEIS1*. Enrichment analysis was performed to identify the pathways whose members were overrepresented within the genes in each of the two DMR lists (Fig. 3 and Supplemental Table S5). In the DMRs of HUVECs, the most enriched pathways included activation of the *HOX* genes followed by TGF- β signaling. Both of these were also present as the most significant pathways in the DMRs of HRECs. Bone morphogenetic protein (BMP) signaling, complement cascade, and runt-related transcription factor 2 (*RUNX2*) transcriptional regulation were additional identified pathways to be enriched in the DMRs of HRECs (Fig. 3 and Supplemental Table S5). Therefore, despite a moderate overlap across the two DMR

lists, both cell lines represented members of shared functional pathways.

DISCUSSION

DNA methylation is a critical epigenetic mechanism that plays an important role in governing gene expressions during biological processes (i.e., embryonic development; 43). In the context of disease, DNA methylation can be greatly altered, which is evident by the large number of DMRs documented in various cancers (25, 32, 34, 50) and genetic/developmental conditions (4–8, 57). Although the rapid advent of sequencing technologies provides the opportunity to detect aberrant DMRs in human diseases, the development of these technologies also offers great potential for elucidating the roles of DNA methylation in in vitro cellular systems—which remain largely unexplored. In the present study, we investigated genome-wide DNA methylation differences in two different EC lines (HUVECs and HRECs) cultured in basal or hyperglycemic environments for different durations and identified differentially methylated regions in these cells, which have not been reported previously.

Following the results from clustering analysis of the CpG probes, the examination of the CpG methylation patterns between HUVECs and HRECs revealed that different EC types exhibited distinct methylation profiles; specifically, four sepa-

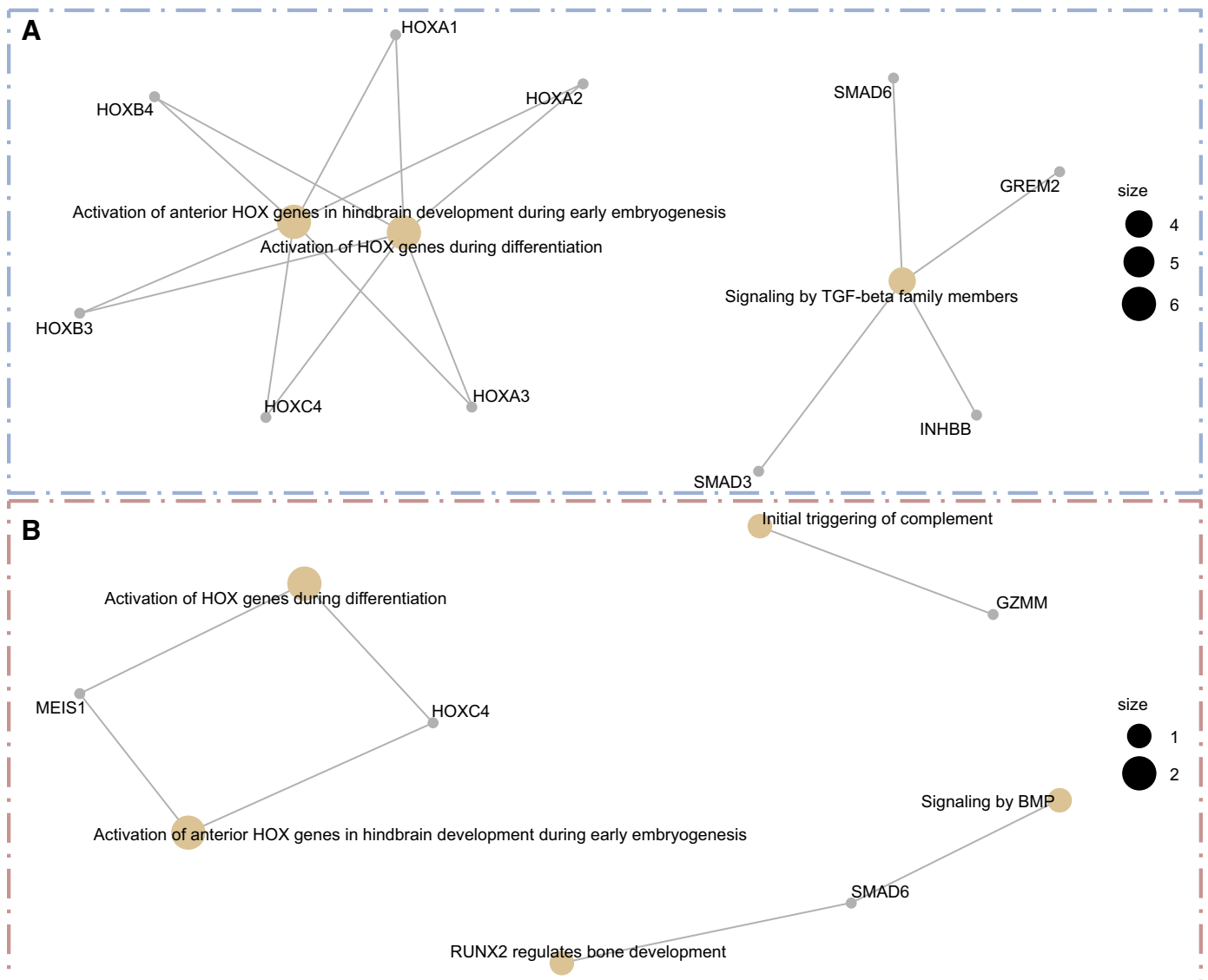


Fig. 3. Pathways enriched in differentially methylated regions (DMRs) of human retinal microvascular endothelial cells (HRECs) and human umbilical vein endothelial cells (HUVECs) treated for different durations. *A*: pathways enriched in HRECs cultured for 2 vs. 7 days. Pathways are indicated with tan circles, and the related DMR genes are shown using gray dots. *B*: the same for HUVECs.

rate clusters were observed within the samples. Previous reports have demonstrated the existence of functional DNA methylation differences between cell types, between tissues, and across individuals (31, 40, 74). Our findings are in line with these data and demonstrate that the two EC lines would also manifest distinct methylation profiles. Intriguingly, it was only the duration of cell culture, and not glucose concentration, that induced significant methylation changes in both large and small vessel ECs. More specifically, at the 7-day mark, both HUVECs and HRECs demonstrated comparable increases in CpG methylation compared with cells cultured for 2 days. Although the genome-wide DNA methylation patterns for ECs have not been reported previously, other studies have demonstrated that long-term culture of mesenchymal stem cells and fibroblasts can induce senescence-associated methylation changes at specific CpG sites and that methylation states (i.e., either hypermethylation or hypomethylation) were primarily dependent on the cell type (14, 35, 56). Another study dem-

onstrated significant CpG island (CGI)-dependent correlations between methylation and age across nonpathological human tissues from different anatomical sites in which CGI regions within gene loci had a greater propensity to gain methylation with age, whereas nonisland CpGs lost methylation with age (23). Furthermore, from a compendium of publicly available genome-wide DNA methylation data, age-related gain of DNA methylation in 16 different tissues was also shown to accumulate at CGIs and their flanking regions, whereas age-related loss of DNA methylation was present in active regions that included enhancers (60). In our study, we observed a greater degree of CpG enrichment at enhancer elements and regions within CpG shores and shelves in both cultured HRECs and HUVECs, whereas enriched CpGs were significantly less likely to occur at CpG islands and promoters. Such DNA methylation patterns may be attributed to potential cell-specific differences or an *in vitro* culture phenomenon. Moreover, other earlier studies have documented global reductions in the level

of DNA methylation in senescing fibroblasts (69) and in the tissues from aging rats (67) and humans (19, 70), which alludes to the complexity of DNA methylation across tissues.

Although the high concentration of glucose (25 mM) has been extensively used by others and us (10–12, 20, 28, 29, 39, 49, 63, 64) and has also been shown to cause cellular aging and downregulation of sirtuins in cellular models of chronic diabetic complications (39, 49), we did not find significant high glucose-induced alterations in DNA methylation in the present study. One possible explanation for this result may be attributed to the stable epigenetic nature of DNA methylation marks during hyperglycemic events. For example, Chen et al. recently profiled whole genome DNA methylation and the transcriptome of peripheral blood mononuclear cells (PBMCs) from a human volunteer over a 3-yr period and demonstrated that active global methylation changes take place before the physiological elevation of glucose levels (19). More specifically, analysis of MethylC sequencing results revealed that differentially methylated genes were significantly enriched in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways related to glucose and diabetes at 80–90 days before the manifestation of elevated glucose, whereas acute health events, such as viral infections, mainly contributed to dynamic changes in the transcriptome that were greatly associated with immunological-related pathways and terms in KEGG (19). When examining the cell culture model used in our study, it may be possible that active DNA methylation changes could also be occurring at other time points that were not examined, which warrants additional investigation. Similarly, there is also a possibility that the lack of significant results with respect to glucose concentration may be attributed to power limitations. Another alternative explanation for our observations could be that once maximal glucose saturation is reached in culture, the methylation levels of individual CpG sites may reach a plateau. For instance, in our study, certain CpG sites across *TNIP2* (Supplemental Table S1) displayed comparable methylation intensities between ECs cultured in high glucose for 2 days and ECs cultured in basal glucose for 7 days. Such findings suggest that the duration of glucose concentration should still be considered in the experimental design and continuously monitored when examining DNA methylation patterns. Moreover, integrating other omics data, such as transcriptomic analyses, with our DNA methylation findings would provide unique insights into the relationship between DNA methylation and other epigenetic molecular markers. Of note, our previous studies have demonstrated that certain long noncoding RNAs are differentially expressed at the 48-h mark in high glucose-treated ECs (10, 29, 63, 64), and DNA methyltransferases (DNMTs) were also shown to actively participate in the transcriptional regulation of several diabetes-related molecules (10, 11). More specifically, we and others have demonstrated increased mRNA levels of *DNMTs* and subsequent alterations of methylation activity in HRECs exposed to high glucose and in the retinas of diabetic rats (10, 73). These alterations of DNMTs could possibly allude to one of the main mechanisms that facilitate glucose-induced methylation changes in ECs (10, 27, 47, 48, 73). Interestingly, demethylases such as ten-eleven translocation (TET) and Jumonji C (JmjC) enzymes, which facilitate the demethylation of DNA and histones, respectively, can also balance such methylation statuses (30). Although the roles of TETs and JmjCs have not been well studied in the

present context, it appears that a dynamic interaction of such processes can ultimately govern the methylation status of distinct genomic regions. Accordingly, future studies should continue incorporating integrative epigenetic experimental approaches that will help elucidate the molecular underpinnings of glucose-induced cellular damage. It will also be important to examine specific mechanisms that study the upstream pathways for glucose-induced alterations of DNA methylation. For example, some studies have demonstrated that oxidative stress mechanisms can significantly alter the DNA methylation process during the progression of diabetic complications (16, 27, 48). Therefore, examining these dynamic interactions using novel genomic technologies may further illuminate additional functional elements of the DNA methylation landscape in hyperglycemic environments.

Several specific loci have been previously documented to exhibit differential methylation patterns during aging in vitro, including *HOX* genes (14, 35) and *RUNX2* (35). In line with these studies, we also observed DMRs in similar genes encoding specific transcription factors involved in cellular differentiation and embryonic development. Notably, among the most significant DMRs, hypomethylation patterns were observed in the promoters of *HOXA2*, *HOXB3*, and *SMAD3* in HUVECs at the 7-day mark, whereas *HOXC4–6* and *SMAD6* were hypermethylated. In a similar manner, HRECs cultured for 7 days also exhibited significant hypermethylation in the promoter regions of *SMAD6*, *HOX4*, and *HOX6*, whereas the most significant DMR belonged to a hypomethylated promoter encoding *MEIS1*. To further determine the pathways of the genes identified from the DMRs in ECs, we performed enrichment analysis that found several pathways involved in development (i.e., *HOX* activation), TGF- β signaling, BMP signaling, *RUNX2* transcriptional regulation, and the complement cascade. Given that ECs have critical implications in angiogenesis (66) and vasculogenesis (62), the differentially methylated loci (and their respective pathways) identified in our study may be reflecting the positional identity (65) and the proliferative and migratory capabilities of ECs. Indeed, both angiogenesis and vasculogenesis can also be regulated through several molecules, including TGF- β (36), *SMAD6* (72), *HOX* proteins (24), *MEIS1* (45), and *RUNX3* (22), which alludes to the dynamic and overlapping cellular networks present in different EC subtypes. Moreover, in the context of aging, ECs from aged mice have been reported to show diminished functional, migratory, and proliferative capacities (61), whereas endothelial precursor cell (EPC)-like mononuclear cells were found to be significantly decreased in the peripheral blood and bone marrow of aged mice subjected to hind limb ischemia compared with wild type (59). Such findings support the notion that both angiogenesis and vasculogenesis can be impaired with aging. It is further important to note that *SMAD* proteins are important mediators of the TGF- β signaling pathway, which plays a key role in the increased production of extracellular matrix proteins during chronic diabetic complications (13, 37). Furthermore, members of the *HOX* family are regulated by promoter DNA methylation and are shear-sensitive endothelial genes that can be implicated in vascular remodeling, angiogenesis, and extracellular matrix modulation (26). On the basis of our findings, we conclude that the methylation alterations of specific genes observed in this study may also contribute to transcriptional alterations of those genes in ECs, which may or may not

produce effector proteins during hyperglycemic states. It is also possible that such processes may be influenced by additional epigenetic factors (ranging from noncoding RNAs to histone modifications); however, further investigation is warranted. Nonetheless, it would be quite interesting to examine in vivo whether specific methylomic changes exist across ECs in diabetes at various durations of the disease.

Conclusions. Taken together, our study provides novel insights into the DNA methylation profiles associated with glucose-induced and time-dependent effects in cultured ECs. We present for the first time the finding that cell culture duration is a strong and more significant inducer of DNA methylation compared with glucose stimuli alone. As well, the statistically significant CpG probes in both large and small vessel ECs mainly demonstrated a hypermethylation during long-term culture compared with short-term culture, with significant enrichments in enhancer elements and regions surrounding CpG shores and shelves. Such DMRs were enriched in genomic loci involved in embryonic development and cellular differentiation. Collectively, our findings suggest that DNA methylation is a complex process that involves tightly coordinated cell-specific changes and such DNA methylation changes overlap genes critical for cellular differentiation and embryonic development. This further highlights the importance of understanding epigenetic mechanisms underlying chronic glucose stimulation, as part of the complex molecular mechanisms implicated in related conditions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

E.A.E., S.B., C.C., B.S., and S.C. conceived and designed research; E.A.E., S.B., and C.C. performed experiments; E.A.E., S.B., B.S., and S.C. analyzed data; E.A.E., S.B., B.S., and S.C. interpreted results of experiments; E.A.E. and S.B. prepared figures; E.A.E., S.B., and B.S. drafted manuscript; E.A.E., S.B., C.C., B.S., and S.C. edited and revised manuscript; E.A.E., S.B., C.C., B.S., and S.C. approved final version of manuscript.

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