The Long Non-Coding RNA HOTAIR Is a Critical Epigenetic Mediator of Angiogenesis in Diabetic Retinopathy

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PURPOSE. Diabetic retinopathy (DR) remains a pressing issue worldwide. Abnormal angiogenesis is a distinct vascular lesion in DR, and research has established that vascular endothelial growth factor A (VEGF-A) is a primary mediator of such changes. However, limitations in current anti-VEGF therapies suggest that our understanding of molecular networks underlying ocular angiogenesis remains far from complete. Based on our long non-coding RNA (lncRNA) array analyses, HOX antisense intergenic RNA (HOTAIR) was identified as one of the top upregulated lncRNAs in high glucose-cultured human retinal endothelial cells (HRECs). Given the well-documented roles of HOTAIR in cancer, no studies have examined the epigenetic implications of HOTAIR in DR, and we investigated such relationships herein.

METHODS. We used HRECs exposed to various glucose concentrations and epigenetic modulators to examine HOTAIR, angiogenic, and DR-related molecular markers. Oxidative stress, angiogenesis, and mitochondrial dysfunction were assessed. Retinal tissues of diabetic rodents and the vitreous humor and serum of patients with proliferative DR were also investigated.

RESULTS. Hyperglycemia significantly augmented HOTAIR expression in HRECs and promoted angiogenesis, oxidative damage, and mitochondrial aberrations. Similarly, vitreous humor and serum from proliferative DR patients and retinas from diabetic animals demonstrated increased HOTAIR expression compared to non-diabetic controls. HOTAIR knockdown protected against glucose-induced increases of angiogenic and diabetes-associated molecules in the retina. Mechanistically, we showed that HOTAIR exerts its capabilities by preventing oxidative stress and modulating epigenetic pathways involving histone methylation, histone acetylation, DNA methylation, and transcription factors.

CONCLUSIONS. Our findings suggest that HOTAIR is a critical lncRNA in the pathogenesis of DR and may potentially be important for diagnostic and therapeutic targeting.

Keywords: LncRNA, HOTAIR, epigenetics, angiogenesis, diabetic retinopathy
the expression of genes involved in biological processes, including DR.\textsuperscript{12–14} However, detailed characterization of the functions of certain lncRNAs in DR is lacking.

We initially carried out a lncRNA microarray using human retinal endothelial cells (HRECs; GEO ID: 122189), and such analyses demonstrated a glucose-induced increase in HOX antisense intergenic RNA (HOTAIR) expression by 2.7-fold. Therefore, in this research, we directed our efforts toward thoroughly characterizing the epigenetic implications of HOTAIR in DR. Using both in vitro and in vivo models, we confirmed glucose-induced increases of HOTAIR and demonstrated its role in angiogenesis and glucose-induced mitochondrial and DNA damage through related epigenetic pathways.

METHODS

Cell Culture

HRECs (Cell Systems, Kirkland, WA, USA) and mouse retinal microvascular endothelial cells (Applied Biological Materials, Richmond, BC, Canada) were cultured in Endothelial Basal Medium-2 (Lonza, Walkersville, MD, USA), as described previously, and were used between passages 3 and 6 (see Supplementary Materials).\textsuperscript{9,13,14} At 80% confluence, ECs were cultured in serum-free medium overnight before exposure to various glucose levels, epigenetic blockers, or siRNA treatments. All in vitro or ex vivo experiments were independently repeated at least three times and performed with six replicates, unless specified.

Endothelial Tube Formation Assay

Approximately $1.5 \times 10^4$ HRECs were used, as described (see Supplementary Materials).\textsuperscript{9,14} Images were captured at 6 hours ($n = 8$ independent samples per group). The total number of tubules and branching points was assessed using WimTube Image analyzer software (Wimasis, Córdoba, Spain).

RNA Fluorescence In Situ Hybridization

RNA fluorescence in situ hybridization (FISH) was performed, as described (see Supplementary Materials),\textsuperscript{9} using Stellaris FISH Probes, Human HOTAIR, with Quasar 570 Dye (5 nmol; Biosearch Technologies, Petaluma, CA, USA).

JC-1 and 8-OHdG Staining

HRECs were stained with 10 \mu M of JC-1 dye (Abcam, Cambridge, UK), 4′,6-diamidino-2-phenylindole (DAPI), or 8-hydroxy-2′-deoxyguanosine (8-OHdG, 1:50; Santa Cruz Biotechnology, Dallas, TX, USA) counterstains following the manufacturer’s instructions. Images were captured using the Zeiss LSM 410 inverted laser-scanning microscope (Carl Zeiss, Wetzlar, Germany) and were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA) (see Supplementary Materials).\textsuperscript{15}

Animal Models

The Western University Council for Animal Care Committee approved all animal experiments, which were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. As our previous studies were performed in male mice, we selected only male rats (Sprague–Dawley; 174.9 ± 2.96 g; 6 weeks old) and mice (C57/BL6j background; 25.7 ± 1.82 g; 8 weeks old) (Charles River Laboratories, Wilmington, MA, USA) for our study and randomly divided them into control and diabetic groups. Streptozotocin (STZ) was used to generate a type 1 diabetic animal model. Animal monitoring and tissue collection have been described previously.\textsuperscript{9,13}

For the therapeutic in vivo model (4-week duration), mice (with or without diabetes) were randomly divided into four groups ($n = 6$/group). Intravitreal injections of scrambled (SCR) siRNA or siHOTAIR (1 \mu l of 100 nmol/L in In VitroGen Lipoctamine 2000; Thermo Fisher Scientific, Waltham, MA, USA) following isoflurane anesthesia were administered once every week for 3 weeks using a 33-gauge needle as described previously.\textsuperscript{17} Also, to circumvent inflammation and damage from the intravitreal procedure, external regions of the injected eye were immediately treated with a single drop of proparacaine hydrochloride ophthalmic solution (0.5%; Alcon, Geneva, Switzerland) and neomycin and polymyxin B sulfates and bacitracin ophthalmic ointment (Vetoquinol Canada, Lavaltrie, QC, Canada). Toxicity analyses were done after the single injections (see Supplementary Materials).

Clinical Samples

The Western Research Ethics Board at the University of Western Ontario approved this study. Patients provided informed consent prior to the procedure and all samples were handled in accordance with the tenets of the Declaration of Helsinki. Both serum and vitreous humor (VH) were collected from patients undergoing a pars plana vitrectomy (Supplementary Table S3). Specimens were categorized into two groups: control and PDR. Mean age of PDR group was 60.9 ± 10.43 years ($n = 11$; 10 males, one female) and that of the (non-diabetic) control group was 69.2 ± 8.87 years ($n = 10$; two males, eight females). RNA extraction and quantitative reverse transcription PCR (RT-qPCR) assays are described in the Supplementary Materials.

Methylation Analysis of Cpg Sites Across HOTAIR

Methylation patterns of CpG sites across the HOTAIR gene were examined in HRECs using the Illumina Infinium MethylationEPIC BeadChip array (Illumina, Inc., San Diego, CA, USA), following the manufacturer’s instructions and our previously published protocols (see Supplementary Materials).\textsuperscript{16}

Statistical Analyses

Data are presented as mean ± SEM or mean ± SD. Experiments were performed in triplicate ($n = 6$/group), unless specified otherwise. Statistical significance ($P < 0.05$) was assessed by the Mann–Whitney U test or Student’s t-test or one-way ANOVA, followed by Tukey’s post hoc test, as appropriate.

Data and Resource Availability

All data generated or analyzed during this study are included in this published article and its Supplementary Materials.
**RESULTS**

**HOTAIR RNA Expression Is Glucose Dependent**

In our array analyses (GEO ID: 122189) and following stringent filtering criteria (fold change ≤ 2 or ≥ 2), 2669 to 3518 lncRNAs were upregulated and 890 to 1991 lncRNAs were downregulated in high glucose (HG) (Supplementary Figs. S1A–C). RT-qPCR confirmed the glucose-induced expression of HOTAIR (Supplementary Fig. S2A), along with augmented expression of VEGF-A and ET-1 transcripts (Supplementary Figs. S2B, C). We also investigated HOTAIR expression at different time points with various glucose levels. HOTAIR demonstrated significant HG-induced elevations at 48 hours (P = 0.0014). Hence, the 48-hour time point was used for subsequent experiments. No significant differences in VEGF-A, VEGF-A, or ET-1 expression were observed in osmotic controls (25-mM l-glucose) (Supplementary Fig. S2). RNA FISH was used to delineate the subcellular localization of HOTAIR. HOTAIR was present in both the nucleus and perinuclear/cytosolic regions (Fig. 1A). Furthermore, HG significantly elevated the expression of HOTAIR (Fig. 1B). These findings suggest that HOTAIR may be implicated in both nuclear and cytoplasmic processes.

**HOTAIR Knockdown Prevents Glucose-Mediated Induction of Multiple Angiogenic Factors, Epigenetic Mediators, and EC Dysfunction-Related Molecules**

Based on our findings, we explored additional angiogenic factors, including angiopoietin-like 4 (ANGPTL4), placental growth factor (PGF), hypoxia-inducible factor (HIF), interleukin-1 beta (IL-1β), as well as epigenetic mediators and EC damage-induced molecules, including poly(ADP-ribose) polymerase 1 (PARP1) and cytochrome b, by performing loss-of-function studies using siRNA-mediated knockdown of HOTAIR. Significantly decreased HOTAIR expression was observed across all siRNA transfections, with SMARTpool siHOTAIR showing the largest reduction of HOTAIR RNA levels (~91%) (Supplementary Fig. S3). Hence, we selected SMARTpool siRNA for subsequent analyses.

siHOTAIR-transfected HRECs in HG showed decreased expression of multiple RNA transcripts implicated in angiogenesis (VEGF-A, ET-1, ANGPTL4, PGF, HIF-1α) (Fig. 2); DNA and oxidative damage (PARP-1 and cytochrome b) (Fig. 2); and epigenetic regulation (EZH2, SUZ12, DNMT1, DNMT3A, DNMT3B, CTCF, and P300) (Supplementary Fig. S4), compared to SCR siRNA-transfected cells in HG. To determine whether these changes are reflected at the protein level, we performed ELISAs for the most characterized angiogenic markers, VEGF-A and HIF-1α. As expected, HOTAIR knockdown prevented glucose-induced upregulation of VEGF-A and HIF-1α proteins (Supplementary Figs. S5A, B). We additionally examined the RNA expression of HOXD3 and HOXD10, because HOTAIR transcriptionally represses HOXD. Knockdown of HOTAIR in HG induced upregulation of HOXD3 and HOXD10, compared to SCR HG (Supplementary Figs. S5C, D). We also investigated the viability of HRECs following siHOTAIR treatment and showed that HOTAIR siRNA transfection did not cause increased cell death but instead improved cellular viability compared to SCR controls (Supplementary Fig. S5E). These results suggest that HOTAIR is a possible epigenetic regulator of glucose-induced EC dysfunction and angiogenesis.
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To examine the role of HOTAIR in angiogenesis in hyperglycemia, we used a tube formation assay.21 At 6 hours, cells transfected with SCR in HG showed increased capillary tubules compared to SCR-transfected cells in normoglycemia (NG) (Fig. 3). Following siHOTAIR transfection, the degree of branching and total number of tubules were decreased in both NG and HG (Figs. 3B, C). Interestingly, following siHOTAIR transfection, exogenous VEGF proteins were unable to recover HG-induced changes, implying that HOTAIR knockdown may be further desensitizing ECs to additional angiogenic factors.

Diabetic Rodents Show Increased Retinal HOTAIR Levels

We then examined HOTAIR in the retina during diabetes. We used both STZ-induced diabetic mice and rats after 2 months of diabetes. Diabetic animals showed hyperglycemia, glucosuria, and reduced body weight (not shown). Significant upregulation of HOTAIR in the retinas of both diabetic mice and rats was observed at 2 months (Supplementary Figs. S6A, B). To evaluate the therapeutic potential of HOTAIR, we used a SMARTpool siHOTAIR that specifically targeted mouse HOTAIR. In mouse retinal microvascular endothelial cells in HG, 50 nM and 100 nM of siHOTAIR evoked significant reductions (~79% and ~80%, respectively) of HOTAIR RNA, as well as VEGF-A and ANGPTL4 transcripts (at 100-nM concentration) (Supplementary Figs. S7A–C).

Intravitreal Injections of siHOTAIR Prevent Early DR-Related Retinal Changes and Diabetes-Induced Retinal Vascular Permeability

We initially performed a toxicology study involving siHOTAIR. Wild-type C57Bl/6] mice were subjected to a one-time intravitreal injection of either SCR siRNA (100 nM) or siHOTAIR at varying concentrations (25, 50, or 100 nM) and were monitored for 7 days when tissues were collected. After 7 days, retinal HOTAIR expression appeared to be the lowest following a 100-nM dose of siHOTAIR (~50% reduction) when compared to SCR controls (Supplementary Fig. S8A). No behavioral changes were observed, and analysis of H&E stained sections showed no structural abnormalities across retinal, heart, lung, liver, or kidney tissues (Supplementary Figs. S8B–F). Hence, for therapeutic assessment, 100 nM of siHOTAIR or SCR was intravitreally injected weekly in diabetic mice showing hyperglycemia and loss of body weight (Supplementary Figs. S9A, B), and HOTAIR knockdown did not affect these parameters. However, at 4 weeks, elevated RNA expression of HOTAIR, Vegf-a, Ctgf, Et-1, Angptl4, Mcp-1, Il-1β, Pgf, Hif-1α, p300, polycomb repressive complex 2 (Prc2) components (Ezh2, Suz12, and Eed), Hoxd3, and Parp1 were seen in the retina of diabetic mice administered SCR siRNAs (Fig. 4; Supplementary Figs. S9C–I). Knockdown of HOTAIR (~58% reduction) reduced diabetes-induced upregulations of most of these transcripts, suggesting that HOTAIR knockdown protects against early diabetes-induced molecular aberrations in the retina. However, we did not observe differences in retinal expression of Il-1β, Pgf, and Hoxd3 between

FIGURE 2. HOTAIR knockdown prevents glucose-mediated induction of several angiogenic factors and diabetes-related molecules in vitro. RT-qPCR analyses of expression of (A) HOTAIR, (B) VEGF-A, (C) ET-1, (D) ANGPTL4, (E) PGE, (F) IL-1β, (G) HIF-1α, (H) PARP1, and (I) cytochrome b following the administration of SCR siRNA or siHOTAIR in HRECs subjected to 48 hours of NG or HG culture. Data are expressed as a ratio to β-actin and normalized to SCR NG. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey’s post hoc test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; n.s., not significant). Data represent the mean ± SEM of three independent experiments (n = 6/group).
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SCF and siHOTAIR-treated animals (Figs. 4G; Supplementary Figs. S9F, I). Retinal vascular permeability was also assessed by immunohistochemical staining for immunoglobulin G (IgG). Our findings demonstrated that IgG was mainly localized within the capillaries, without any significant staining of retinal tissues in non-diabetic animals treated with SCF or HOTAIR siRNAs (Fig. 4I, score 1). Conversely, diffuse extravascular IgG staining was observed in the retinal tissues of diabetic animals treated with SCF siRNAs, signifying increased extravasation in the neural retina (Fig. 4I, score 3), whereas HOTAIR knockdown protected diabetic animals from such changes (Fig. 4I, score 1).

We further measured retinal VEGF-A proteins. VEGF-A was increased in the retinas of diabetic mice treated with SCF siRNAs, but siHOTAIR reduced retinal VEGF-A proteins in diabetic mice (Supplementary Fig. S9J). Moreover, we did not find any observable toxic effects in retinal, cardiac, lung, liver, kidney, and brain tissues following 1 month of siHOTAIR injections (Supplementary Fig. S10).

HOTAIR Is Upregulated in the Vitreous and Serum of Patients With PDR

To determine the role of HOTAIR as a potential biomarker, we examined HOTAIR expression in the serum and VH of patients with PDR. HOTAIR expression was significantly upregulated in both vitreous and serum of patients with PDR compared to that of non-diabetic patients (Supplementary Fig. S11). A positive correlation for HOTAIR levels between serum and vitreous samples ($R^2 = 0.482$) (Supplementary Fig. S11C) was seen, suggesting that HOTAIR may have potential to serve as a biomarker for DR.

HOTAIR Knockdown Prevents Glucose-Induced DNA and Mitochondrial Damage and Endothelial Cell Junction Disruptions

To understand molecular mechanisms for HOTAIR and its role in mitochondrial dysfunction, we assessed the mitochondrial transmembrane potential ($\Delta \Psi_M$) in HRECs after HOTAIR knockdown by detecting JC-1 signals. HG significantly evoked mitochondrial depolarization compared to SCF NG controls (Fig. 5), whereas the knockdown of HOTAIR in NG increased mitochondrial activity. Interestingly, HOTAIR knockdown partially reduced HG-induced mitochondrial dysfunction/depolarization when compared to SCF HG.

As HG induces abnormalities in several metabolic pathways causing oxidative DNA damage, we examined such relationships using 8-OHdG levels (a biomarker for oxidative DNA damage). In comparison to SCF NG, SCF HG cells demonstrated increased 8-OHdG. Conversely, HOTAIR knockdown reduced glucose-induced increases in 8-OHdG expression (Fig. 6). Furthermore, the loss of endothelial junctions was investigated electronmicroscopically. HG-induced disruptions of EC junctions were prevented by HOTAIR knockdown (Supplementary Fig. S12). Collectively, these results suggest that HOTAIR contributes to oxidative stress and EC dysfunction in DR.

HOTAIR-Induced Production of DR-Related Molecules Depends on Glycolytic Metabolism

To delineate the regulatory mechanisms, we employed 2-deoxy-D-glucose (2-DG, a glycolytic pathway inhibitor).
Accordingly, 2-DG treatment significantly blocked HG-induced expression of HOTAIR and its downstream molecules, VEGF-A, ET-1, ANGPTL4, MCP-1, IL-1β, CTCF, and cytochrome b (Supplementary Fig. S13). Furthermore, 2-DG evoked reductions in epigenetic molecules including EZH2, SUZ12, EED, and DNMT1, but not DNMT3A or DNMT3B (Supplementary Fig. S14). Interestingly, even at a 5 mM concentration, 2-DG treatments did not reduce PARP1 and P300 expression and did not augment cytochrome b expression, suggesting that there may be additional non-mitochondrial mechanisms leading to oxidative stress.24 However, additional studies are needed to establish such a notion. Moreover, HOXD3 and HOXD10 expression was upregulated in 2-DG-treated HRECs in HG (Supplementary Figs. S14H, I), highlighting the inverse relationship between HOTAIR and HOXD expression. In keeping with a previous study that confirms the antiangiogenic activity of 2-DG,25 our findings indicate that glucose works upstream of HOTAIR and causes cellular damage through both glycolysis and direct oxidative stress.

**Histone Methylation Regulates HOTAIR and Its Downstream Targets**

As HOTAIR and PRC2 (a histone methyltransferase) may share a relationship,26 we investigated histone methylation using a global histone methylation inhibitor, 3-deazaneplanocin A (DZNep).27 DZNep reduced glucose-induced increases of PRC2 components: EZH2, SUZ12, and EED transcripts (Supplementary Figs. S15A–C). Significant reductions following DZNep treatment of HG cells were also evident for HOTAIR, VEGF-A, ANGPTL4, CTCF, PARP1, P300, and cytochrome b transcripts (Supplementary Fig. S16). DZNep pretreatment, however, augmented the expression of ET-1, MCP-1, IL-1β, HOXD3, and HOXD10 transcripts (Supplementary Figs. S15, S16). These observations, as seen before,9,28 suggest that DZNep-mediated methylation blockade disrupts cellular cross-talk in ECs in HG.

To confirm these findings, we targeted EZH2 (catalytic subunit of PRC2)28 and CTCF (transcription factor maintaining chromosome organization and regulating HOTAIR)29 for knockdown. Following siRNA transfections, significant reductions of EZH2 and CTCF were observed (Supplementary Figs. S17E, F). EZH2 inhibition reduced HOTAIR, VEGF-A, ET-1, ANGPTL4, CTCF, SUZ12, PARP1, MCP-1, IL-1β, cytochrome b, and DNMT1 RNA expression and increased P300, HOXD3, and HOXD10 transcript levels (Supplementary Figs. S17, S18). No differences in expression were observed for DNMT3A and DNMT3B following siEZH2 transfection. These findings imply that EZH2 is directly involved in the transcriptional regulation of HOTAIR and several other downstream genes in hyperglycemia.

Knockdown of CTCF in HG-cultured HRECs produced differential expression of several genes, including significant increases in HOTAIR, ANGPTL4, EED, IL-1β, Cytochrome B,
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**Figure 5.** HOTAIR knockdown partially prevents glucose-induced mitochondrial depolarization/dysfunction. (A) Images captured from the JC-1 assay, where HRECs were pretreated with SCR siRNAs or siHOTAIR and subsequently cultured in NG or HG for 48 hours. JC-1 stains demonstrated HG-induced mitochondrial dysfunction (evidenced by low ∆ψM and more green and less red fluorescence when compared to SCR NG) and were partially corrected by HOTAIR knockdown (siHOTAIR). Cells were also counterstained with DAPI. Original magnification, 20×. Scale bars: 100 μm. (B) The JC-1 red/green fluorescence ratio was quantified using ImageJ. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey’s post hoc test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; n.s., not significant). Data represent the mean ± SEM of 20 cells captured per sample (n = 8 independent samples per group).

**HOTAIR Regulates VEGF by Binding With Histone-Modifying Enzymes**

To examine direct relationships between HOTAIR and histone-modifying enzymes, we performed a RNA immunoprecipitation assay. In comparison to IgG controls, HOTAIR RNA levels were enriched in the precipitated anti-EZH2 and P300 antibody fractions obtained from HRECs in HG (Supplementary Fig. S19), suggesting that HG promotes HOTAIR binding associations to EZH2 and P300. We also performed chromatin immunoprecipitation qPCR using antibodies for RNA polymerase II (indicative of transcriptional activity), H3K27me3 (indicative of transcriptional repression), and pan-H3K9/14/18/23/27 acetylation (indicative of transcriptional activation) and employed primers spanning the proximal and distal promoter regions of VEGF-A. Compared to NG, RNA polymerase II levels were significantly enriched in both distal and proximal promoter regions of VEGF-A in HG (Supplementary Figs. S20A, D), which were prevented by HOTAIR knockdown. Conversely, HG caused significant reductions of H3K27me3 enrichment in both VEGF-A distal and proximal promoter regions, and siHOTAIR treatment reversed such reductions (Supplementary Figs. S20B, E). Moreover, when compared to NG controls, HG augmented the enrichment of H3K9/14/18/23/27 acetylation in both VEGF-A promoter regions, and knockdown of HOTAIR prevented glucose-induced increases in pan-acetylation levels (Supplementary Figs. S20C, F). Hence, a dynamic interplay exists among HOTAIR, histone-modifying enzymes, and RNA polymerase II in the transcriptional regulation of VEGF-A in hyperglycemia.

**Glucose Does Not Cause Duration-Dependent CpG Methylation Alternations in HOTAIR**

To investigate DNA methylation, we performed a genome-wide DNA methylation analysis following incubation of HRECs in NG or HG for 2 and 7 days. Following the detection of >860,000 CpG sites/probes, we interrogated CpG sites that spanned the HOTAIR gene (5 kb upstream to 1 kb downstream, corresponding to 59 probes). The average methylation intensity was lower for the majority of the probes (β < 0.3), except for seven probes for which the methylation intensities were greater (0.2 < β < 0.5; corresponding to north/south shelf and north/south shore regions) (Fig. 7). A stable DNA methylation pattern persisted in all

**HOXD3, and HOXD10 and decreases in ET-1, EZH2, PARP1, MCP-1, DNMT1, and P300 transcripts, compared to SCR HG cells. No differences were observed for VEGF-A, SUZ12, DNMT3A, and DNMT3B transcripts following CTCF transfection in HG-cultured HRECs (Supplementary Figs. S17, S18). These results suggest diverse roles of CTCF in gene regulation; for example, depletion of CTCF may augment glucose-induced gene expression, possibly through the inability of CTCF to block interactions between enhancers and promoters, or repress gene expression, possibly through chromatin architectural changes.**

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FIGURE 6. Knockdown of HOTAIR significantly prevents glucose-induced oxidative damage. (A) Images captured from the 8-OHdG assay, where HRECs were pretreated with SCR siRNAs or siHOTAIR and subsequently cultured in NG or HG for 48 hours. 8-OHdG stains showed that HG-induced nuclear and mitochondrial oxidative DNA damage (indicated by stronger green fluorescence) was prevented by siHOTAIR. Cells were also counterstained with DAPI. Original magnification, 20×. Scale bars: 100 μm. (B) Mean integrated densities of 8-OHdG expression were quantified using ImageJ. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey’s post hoc test (*P < 0.05, ****P < 0.0001). Data represent the mean ± SEM of 20 cells captured per sample (n = 8 independent samples per group).

groups across the HOTAIR genomic region (Supplementary Fig. S21A). HRECs in HG also displayed a slight trend toward a reduction of DNA methylation in the HOTAIR promoter compared to NG (Supplementary Fig. S21B). These findings suggest stable epigenetic DNA methylation across HOTAIR in HRECs during hyperglycemia.16

DNA Methyltransferases Differentially Regulate HOTAIR and Some of Its Targets

To examine the cause-and-effect relationship of DNA methylation on the expression of HOTAIR and its targets, we used 5-aza-2'-deoxycytidine (5-aza-dC; a demethylating agent). Following 5-aza-dC administration, DNMT1, DNMT3A, and DNMT3B RNA levels were reduced compared to HG (Supplementary Figs. S22A–C). Simultaneously, we observed elevations of HOTAIR, ET-1, CTCF, cytochrome b, PARP1, IL-1β, HOXD3, and HOXD10 transcripts, whereas no significant differences were observed for ANGPTL4, P300, or PARP1 expression (Supplementary Figs. S22, S23). In keeping with previous observations,31 global inhibition of DNA methyltransferases prevented glucose-induced increases in VEGF-A mRNA (Supplementary Fig. S23B).

To confirm these findings, we silenced the constitutively expressed DNMT1. Accompanying a ∼71% knock-down following siDNMT1 transfection in HG-cultured cells (Supplementary Fig. S24), DNMT3A and DNMT3B transcripts were significantly reduced. In parallel, significant increases in RNA expression were observed for HOTAIR, ET-1, CTCF, cytochrome b, PARP1, IL-1β, HOXD3, and HOXD10 following DNMT1 knockdown in HG (Supplementary Figs. S24, S25). Although no differences were observed for ANGPTL4, P300, or MCP-1 after such knockdown, significant reductions in VEGF-A transcripts still remained in siDNMT1-transfected HRECs in HG (Supplementary Fig. S25B), confirming the observations from 5-aza-dC experiments. It is possible that depending on the genomic location, inhibition of DNA methylation has varying effects on distal or intragenic regulatory elements with different degrees of CpG density, subsequently regulating gene expression.32

DISCUSSION

Angiogenesis is critical for vascular homeostasis.2,33 In DR, hyperglycemia promotes increased permeability and angiogenesis, which leads to vision-threatening complications.2 Although angiogenesis and DME are primarily mediated by VEGF-A,2,34 limitations in current anti-VEGF therapies suggest that our understanding of the pathobiology of DR remains incomplete. Deciphering novel molecular mediators may allow for the development of biomarkers and better...
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FIGURE 7. DNA methylation profiling of HRECs. (A) Human HOTAIR is located on chromosome 12:54,356,092–54,368,740 (hg19), and its approximate size (for transcript variant 1) is 12,649 nucleotides containing a total of six exons (UCSC database). (B) Unsupervised hierarchical clustering with heatmap using all the CpGs in HRECs that span across HOTAIR, which amounted to 59 probes. No distinctions were noted between cells treated with different concentrations of glucose (5 mM vs. 25 mM) or the duration of culture (2 vs. 7 days). Rows indicate CpGs and columns show the samples; the color scale from blue to red indicates the level of methylation from 0 to 1 (with 0 indicating no methylation and 1 indicating maximum methylation) (n = 3 independent samples per group, indicated by the top panel colors). HR5_2, HRECs cultured in 5-mM glucose for 2 days; HR25_2, HRECs cultured in 25-mM glucose for 2 days; HR5_7, HRECs cultured in 5-mM glucose for 7 days; HR25_7, HRECs cultured in 25-mM glucose for 7 days.

targeted therapies. Recently, lncRNAs have garnered attention due to their critical regulatory capabilities in several diseases; however, very few lncRNAs have been comprehensively characterized.

Here, we provide evidence that the lncRNA HOTAIR mediates angiogenesis and other pathologic changes in DR through complex epigenetic mechanisms (Fig. 8). Targeted knockdown of HOTAIR showed that siHOTAIR can protect against glucose-induced increases of several angiogenic factors, diabetes-associated molecules, and epigenetic mediators. HOTAIR contributes to glucose-induced mitochondrial and DNA damage and facilitates the epigenetic activation of VEGF-A by recruiting RNA polymerase II and acetylators (P300) to the VEGF-A promoter, causing VEGF-A overexpression and angiogenesis. Furthermore, HOTAIR and its target molecules are differentially regulated through histone and DNA methyltransferases and transcription factors.

As noted above, the subcellular localization of lncRNAs provides insights into their functions. Cytoplasmic lncRNAs cause post-transcriptional modifications governing mRNA stability and translation, whereas nuclear-retained lncRNAs are involved in transcriptional regulation, organization of nuclear architecture, and alternative splicing. Certain lncRNAs can also be found in both nucleus and cytoplasm, where they shape the epigenome, regulate organelle formation and function, and influence transcription and translation. In keeping with previous findings, we showed that HOTAIR is present in both nuclear and cytoplasmic compartments of ECs. It is possible that HOTAIR regulates the expression of several transcripts while simultaneously targeting enzymes involved in histone modification and DNA methylation. For example, HOTAIR depletion in fibroblasts causes loss of HOXD silencing and H3K27me3 by PRC2, whereas HOTAIR overexpression promotes cellular...
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**FIGURE 8.** Schematic of hyperglycemia-induced regulation of IncRNA HOTAIR in retinal endothelial cells. HOTAIR can regulate the transcription of several genes implicated in the pathogenesis of diabetic retinopathy through direct and indirect mechanisms involving alterations in the PRC2–P300–DNMT–CTCF epigenetic axis. HOTAIR can also contribute to both mitochondrial and DNA oxidative damage. ROS, reactive oxygen species.

invasiveness through selective retargeting of PRC2 and H3K27me3, enabling the expression of invasion-related genes. In the context of DR, a recent study has also reported that HOTAIR can bind to lysine-specific histone demethylase 1 and subsequently inhibit VE-cadherin transcription by reducing H3K4me3 levels on its promoter, which further facilitates HIF-1α-mediated transcriptional activation of VEGF-A. Hence, HOTAIR may function as a molecular scaffold to regulate gene expression through interactions with epigenetic mediators in DR.

We demonstrated that HOTAIR contributes to glucose-induced mitochondrial and oxidative DNA damage in ECs. Other studies have shown that HOTAIR can mediate DNA damage response through the regulation of NF-κB activation and maintain mitochondrial function, where HOTAIR knockdown results in the dysregulation of several proteins associated with mitochondrial function in HeLa cells. We observed that HOTAIR knockdown protects the mitochondria. Although cell-specific regulations may exist for HOTAIR, our results could be attributed to stress-induced mechanisms involving mitophagy and autophagy. For example, hyperglycemia evokes oxidative stress, membrane depolarization, and mitochondrial fragmentation. Increases in reduced cytochrome b and specific mitophagy factors (including autophagy-related genes, ATG11 and ATG32) can then trigger non-specific autophagy. A recent study has also demonstrated that HOTAIR promotes autophagy by upregulating ATG7.

Microvasculature aberrations represent one of the earliest pathological manifestations of DR. Hyperglycemia compromises the function and integrity of the endothelium, leading to increased permeability. Coupled with retinal capillary non-perfusion, endothelium damage and activation of several biochemical pathways further stimulate production of inflammatory cytokines and vasoactive factors. We have shown both in vitro and in vivo that a large number of these molecules are HOTAIR dependent, and our findings are in keeping with a recent study that confirmed the direct implications of HOTAIR in retinal EC dysfunction and increased permeability. Similar data have also been reported in some malignancies, where HOTAIR promotes angiogenesis in nasopharyngeal carcinoma via direct and indirect mechanisms (involving glucose regulated protein 78) that activate the transcription of VEGF-A and angioptietin-2. Additionally, another study reported that HOTAIR affects angiogenesis by regulating VEGF-A expression in glioma cells and can be transmitted into endothelial cells through glioma cell-derived extracellular vesicles. The knockdown of HOTAIR can also restrict proliferation and invasion of retinoblastoma cells via the Notch signaling pathway. Given our findings, we recognize the limitations that only male animals were used in the experiments and that other non-retinal endothelial cell types may have been possibly targeted following siHOTAIR administration. Moreover, as we planned to avoid additional confounding variables by maintaining diabetic animals without external insulin, we were unable to assess advanced retinal microangiopathy such as acellular capillaries in our short-term animal model. We further noted that HOTAIR blockade, although increasing cell survival, prevented tube formation; thus, it is tempting to speculate that a differential effect of HOTAIR on cellular proliferation and differentiation may be responsible in DR. However, such hypotheses should be validated by additional experiments.
With HOTAIR demonstrating potential involvement in DR, we addressed the clinical utility of HOTAIR as a biomarker. Compared to non-diabetic controls, HOTAIR levels were significantly upregulated in the VH and serum of PDR patients. These results are in keeping with our previously published findings for another lncRNA, MALAT1, in DR. The demonstrated correlations in HOTAIR RNA levels between serum and VH of PDR patients raises the possibility of using HOTAIR as a potential biomarker for DR. Our data are in keeping with another study that alludes to the diagnostic and prognostic roles of HOTAIR in DR.

Furthermore, with regard to its potential as a therapeutic target, targeting HOTAIR in DR may offer significant advantages over existing anti-VEGF compounds (protein-based), as RNA-based drugs have shown to silence target genes with high efficiency and specificity. They are chemically synthesized without the variability of biologics and exhibit low immunogenicity, and chemically modified siRNAs can even persist for many months in humans, suggesting that RNA therapeutics could be administered less frequently. Undoubtedly, additional experimentation is warranted to further validate the pharmacokinetic profile, clinical durability, and cell specificity of siHOTAIR in DR, as well as its impact on various VEGF isoforms.

In summary, this report demonstrates that the lncRNA HOTAIR critically mediates angiogenesis in DR by directly regulating the expression of multiple DR-associated molecules through a complex epigenetic axis. HOTAIR also has implications in glucose-induced oxidative mitochondrial and DNA damage, and HOTAIR expression in the VH and serum correlates with PDR. Collectively, this study has identified the potential for HOTAIR to serve as a diagnostic and therapeutic target in DR.

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