

## Evidence for MicroRNA-Mediated Regulation of Steroidogenesis by Hypoxia

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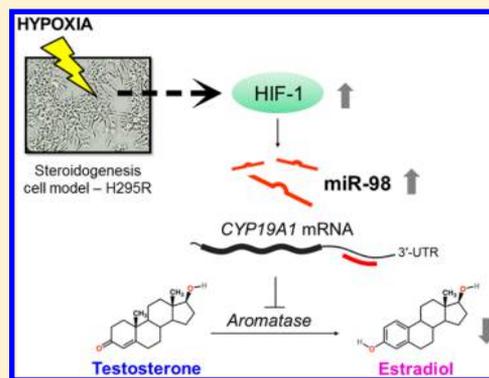
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**ABSTRACT:** Environmental hypoxia can occur in both natural and occupational environments. Over the recent years, the ability of hypoxia to cause endocrine disruption via perturbations in steroid synthesis (steroidogenesis) has become increasingly clear. To further understand the molecular mechanism underlying hypoxia-induced endocrine disruption, the steroid-producing human cell line H295R was used to identify microRNAs (miRNAs) affecting steroidogenic gene expression under hypoxia. Hypoxic treatment of H295R cells resulted in the downregulation of seven steroidogenic genes and one of these, *CYP19A1* (aromatase), was shown to be regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1). Using bioinformatic and luciferase reporter analyses, miR-98 was identified to be a *CYP19A1*-targeting miRNA from a subset of HIF-1-inducible miRNAs. Gain- and loss-of-function analysis suggested that under hypoxia, the increased expression of miR-98 led to the downregulation of *CYP19A1* mRNA and protein expression and that it may have contributed to a reduction in estradiol (E2) production. Intriguingly, luciferase reporter assays using deletion constructs of a proximal 5'-flanking region of miR-98 did not reveal a hypoxia-responsive element (HRE)-containing promoter. Overall, this study provided evidence for the role of miRNAs in regulating steroidogenesis and novel insights into the molecular mechanisms of hypoxia-induced endocrine disruption.



### INTRODUCTION

Environmental hypoxia is a state of reduced oxygen availability. Typically, it occurs in eutrophic waters (aquatic hypoxia), at high altitudes (hypobaric hypoxia) or in low-oxygen workplace environments, such as fire-protected rooms (normobaric hypoxia). Modulation of steroid synthesis (steroidogenesis) by hypoxia has been widely observed in various tissues, such as the gonads,<sup>1–6</sup> placenta<sup>7–9</sup> and adrenal glands.<sup>10,11</sup> Because the steroidogenesis pathway is catalyzed by a number of oxygen-sensitive cytochrome P450 enzymes (CYPs), hypoxia is often thought to hinder steroidogenesis through enzymatic inhibition. However, recent evidence has suggested that it can also modulate the expression of steroidogenic genes, leading to either stimulation or inhibition of steroid production.<sup>3–9,12–14</sup>

From invertebrates to mammals, the transcriptional response to hypoxia is primarily mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimeric protein that consists of an oxygen-sensitive HIF-1 $\alpha$  subunit and a constitutively expressed HIF-1 $\beta$  subunit.<sup>15</sup> Under hypoxic

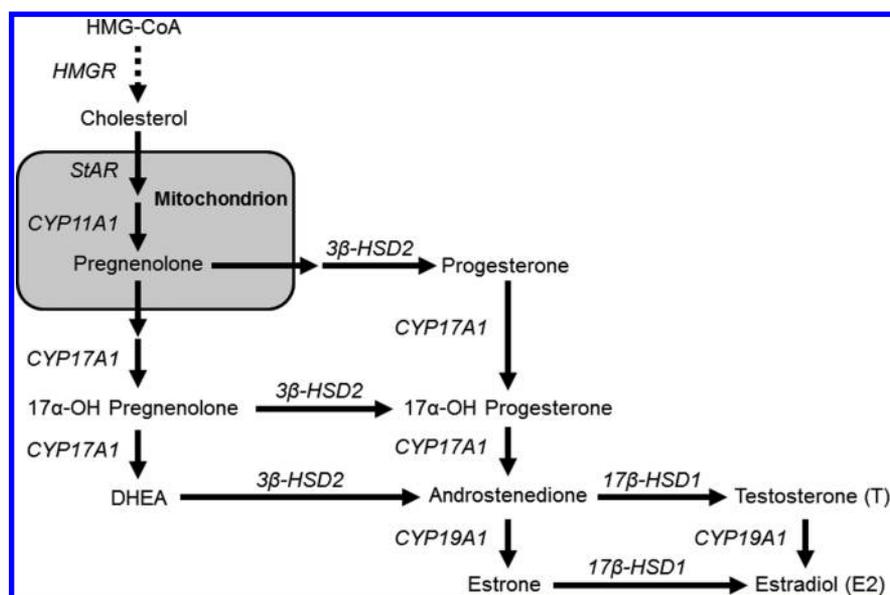
conditions, HIF-1 $\alpha$  accumulates and dimerizes with HIF-1 $\beta$  to activate expression of HIF-1 target genes through binding to their hypoxia-responsive elements (HREs). To date, there are over 100 genes that are known to be regulated by HIF-1, including those involved in oxygen delivery (erythropoietin, *EPO*), angiogenesis (vascular endothelial growth factor, *VEGF*) and glucose uptake (glucose transporter 1, *GLUT-1*).<sup>16</sup> The involvement of HIF-1 in the regulation of steroidogenic gene expression has been previously suggested by a number of studies. For instance, exposure to cobalt chloride (CoCl<sub>2</sub>, a chemical inducer of HIF-1) decreases *CYP11A1* mRNA expression and hence progesterone production in testicular Leydig cells.<sup>17</sup> Other studies have demonstrated that HIF-1 can bind to and activate the gene promoters of  $\beta$ -HSD1 and

Received: September 24, 2014

Revised: December 10, 2014

Accepted: December 12, 2014

Published: December 12, 2014



**Figure 1.** Pathway for the synthesis of estradiol (E2) in H295R cells. Steroidogenic proteins are in italics.  $3\beta$ -HSD2,  $3\beta$ -hydroxysteroid dehydrogenase type 2;  $17\beta$ -HSD1,  $17\beta$ -hydroxysteroid dehydrogenase type 1; CYP11A1, cholesterol side-chain cleavage; CYP19A1, aromatase; CYP17A1,  $17\alpha$ -hydroxylase/ $17,20$ -lyase; DHEA, dehydroepiandrosterone; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; StAR, steroidogenic acute regulatory protein.

CYP19A1 in Leydig cells<sup>3</sup> and breast adenocarcinoma cells,<sup>14</sup> respectively. Despite these new insights, it remains to be determined whether hypoxia or HIF-1 regulates steroidogenic expression via any post-transcriptional mechanisms, such as microRNA (miRNA) regulation.

MiRNAs have emerged as a novel class of post-transcriptional regulators of gene expression. They are small noncoding RNAs (20–22 nucleotides) that bind imperfectly to the 3'-untranslated regions (3'-UTRs) of their target mRNAs, causing mRNA degradation and/or translational repression. It has been estimated that over one-third of human genes are regulated by miRNAs.<sup>18</sup> Previously, a genome-wide screen has implicated approximately 50 miRNAs in human ovarian steroidogenesis.<sup>19</sup> Notably, most of the miRNAs have been shown to inhibit, and not to stimulate, the release of steroids. This raises the possibility that under hypoxia, some of these or other miRNAs may be induced by HIF-1 and be responsible for the hypoxic repression of certain steroidogenic genes.

The main goals of this study were to examine the effects of hypoxia on the expression of a subset of steroidogenic genes essential for estradiol (E2) synthesis, including *HMGR*, *StAR*, *CYP11A1*, *CYP17A1*,  $3\beta$ -HSD2,  $17\beta$ -HSD1, and *CYP19A1* (Figure 1), and to identify HIF-1-mediated miRNAs that contribute to the observed differential gene expression. To accomplish these goals, we employed the human adrenocortical carcinoma cell line H295R as an *in vitro* steroidogenesis model. This cell line expresses all of the key enzymes involved in adrenal and gonadal steroidogenesis, overcoming the limitation associated with the frequent tissue- and developmental stage-specific expression of these genes *in vivo*. Its versatility is exemplified by its use in the recently OECD validated H295R Steroidogenesis Assay for the screening and detection of endocrine disrupting chemicals.<sup>20</sup> Our results have indicated that miR-98 is an HIF-1-regulated miRNA that attenuates *CYP19A1* expression and E2 production in hypoxic H295R cells.

## MATERIALS AND METHODS

**Cell Culture.** H295R cells were obtained from the ATCC and cultured as described previously.<sup>21</sup> Exposure of H295R cells ( $1.2 \times 10^6$ ) to normoxia (20% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>) was carried out at 37 °C for 24 h. The normoxic or hypoxic conditions were created in a CO<sub>2</sub> incubator by mixing 20% O<sub>2</sub>, 75% N<sub>2</sub> and 5% CO<sub>2</sub> or 1% O<sub>2</sub>, 94% N<sub>2</sub> and 5% CO<sub>2</sub>, respectively, using a gas mixer (WITT). The O<sub>2</sub> levels were monitored using a GasAlertMax XT II gas detector (BW Technologies) throughout the experiment.

**qRT-PCR.** qRT-PCR for quantifying expression of steroidogenic genes was performed as described previously.<sup>21</sup> miR-98 expression was quantified using a TaqMan MiRNA Reverse Transcription Kit and a TaqMan Gene Expression Assay (both from Applied Biosystems). U6 snRNA was used as a normalization control. The relative expression levels of steroidogenic genes and miR-98 were analyzed using the comparative threshold cycle ( $C_T$ ) method.<sup>22</sup>

**Western Blot Analysis.** Western blot analysis was performed as previously described<sup>23</sup> using anti-HIF1 $\alpha$  (BD Biosciences, #610959), anti-CYP19A1 (Santa Cruz, #sc-30086) and anti- $\beta$ -actin (Sigma, #A2228) antibodies.

**Quantification of Hormones.** Extraction and quantification of E2 and testosterone (T) in the cell culture medium were performed as described by Hecker et al.<sup>24</sup>

**Overexpression and Knockdown of miR-98.** Overexpression and knockdown of miR-98 were conducted using precursor (pre-miR-98) and inhibitor (anti-miR-98) molecules (Ambion), respectively. These molecules were transfected into H295R cells using a Neon electroporation system (Invitrogen;  $3 \times 1600$  V, 10 ms) at final concentrations of 100 and 150 nM for the pre-miR-98 and anti-miR-98, respectively. After 24 h, fresh medium was added to the electroporated cells, and they were incubated under hypoxic conditions for an additional 24 h. The cells were then harvested for qRT-PCR and Western blot analyses.

**Preparation of Lentiviral Constructs for Overexpression and Knockdown of HIF-1 $\alpha$ .** Under normoxia, the HIF-1 $\alpha$  protein is rapidly degraded via the ubiquitin-proteasome pathway that is initiated by the hydroxylation of two conserved proline residues ( $P^{402}$  and  $P^{564}$ ) present in the oxygen-dependent degradation (ODD) domain of HIF-1 $\alpha$ .<sup>25,26</sup> To express the normoxia-resistant HIF-1 $\alpha$  protein, we generated an HIF-1 $\alpha$  mutant construct (HIF1 $\alpha$ : $\Delta$ ODD), in which  $P^{402}$  and  $P^{564}$  were substituted with alanine residues using a GeneArt Site-Directed Mutagenesis System (Invitrogen). The mutated HIF-1 $\alpha$  ORF was then cloned into a pENTR/D-TOPO entry vector (Invitrogen) and subsequently transferred to a pLenti6/V5-DEST lentiviral destination vector (Invitrogen) to produce an expression construct, pLenti-HIF1 $\alpha$ . To accomplish the siRNA-mediated knockdown of HIF-1 $\alpha$ , two complementary ssDNA oligonucleotides targeting HIF-1 $\alpha$  were designed using BLOCK-iT RNAi Express software (Invitrogen). The ssDNA oligonucleotides were annealed to generate dsDNA oligonucleotides (HIF-1 $\alpha$ i956), which were then cloned into a pcDNA 6.2-GW/EmGFP vector (Invitrogen). Subsequently, an entry clone was generated via a pDONR221 donor vector (Invitrogen). The target sequence was finally transferred to a pLenti6/V5-DEST vector (Invitrogen) to generate an expression construct, pLenti-HIF1 $\alpha$ i956, which was used for HIF-1 $\alpha$  knockdown.

**Lentivirus Production and Transduction.** Lentiviruses for overexpression and knockdown of HIF-1 $\alpha$  were produced using a ViraPower Lentiviral Gateway Expression Kit (Invitrogen). Briefly,  $3 \times 10^6$  293FT cells were seeded 1 day before transfection. A total of 3  $\mu$ g of lentiviral constructs and 9  $\mu$ g of packing mix were cotransfected into the 293FT cells using Lipofectamine 2000 (Invitrogen). The transfected cells were then incubated at 37 °C for 48 h. Next, the cells were incubated with Lentivirus Concentrator (Clontech) at 4 °C for 4 h to concentrate the viruses. Viral particles were recovered by centrifuging the medium at 3000g for 5 min at 4 °C, and the pellet was resuspended in ice-cold DMEM/F12 complete medium and stored at -80 °C until use. H295R cells ( $1 \times 10^6$ ) were seeded 1 day prior to transduction. A viral suspension containing either pLenti-HIF1 $\alpha$ , pLenti-HIF1 $\alpha$ i956 or empty pLenti6/V5-DEST (control) was applied to the H295R cells in the presence of Polybrene (6  $\mu$ g/mL; Sigma). The transduced cells were incubated at 37 °C for 24 h. After incubation, the cells were fed with fresh medium and kept under normoxia or hypoxia at 37 °C for another 24 h before harvesting.

**Preparation of Luciferase Reporter Constructs.** To assess the interaction between miR-98 and *CYP19A1* mRNA, a 174-bp fragment of the *CYP19A1* 3'-UTR was amplified by PCR using genomic DNA from H295R cells and the following primers: 5'-GCAAAGCTTCATGACCCCAAAGCCAAG-3' (forward) and 5'-CAGACTAGTGC GAATTC-CAAGGTGTC-3' (reverse), which contained *Hind*III and *Spe*I sites (underlined), respectively. The PCR product was double-digested with *Hind*III and *Spe*I and cloned into the *Hind*III-*Spe*I site of the pMIR-REPORT luciferase vector (Ambion) immediately downstream of the CMV-driven firefly luciferase gene. A construct possessing mutations disrupting the putative miR-98 binding sites in the 3'-UTR of *CYP19A1* was prepared using a GeneArt Site-Directed Mutagenesis System (Invitrogen) with the following primers: 5'-AAGTATTTT-TAATCCTAATTCAAATTTAACAGTTAC-3' (forward) and 5'-GTAAGTGTAAATTTTGAATTAGGATTAATAA-TACTT-3' (reverse). Both the wild-type and mutated plasmid

clones (designated as pMIR-19-WT and pMIR-19-MUT, respectively) were verified by DNA sequencing.

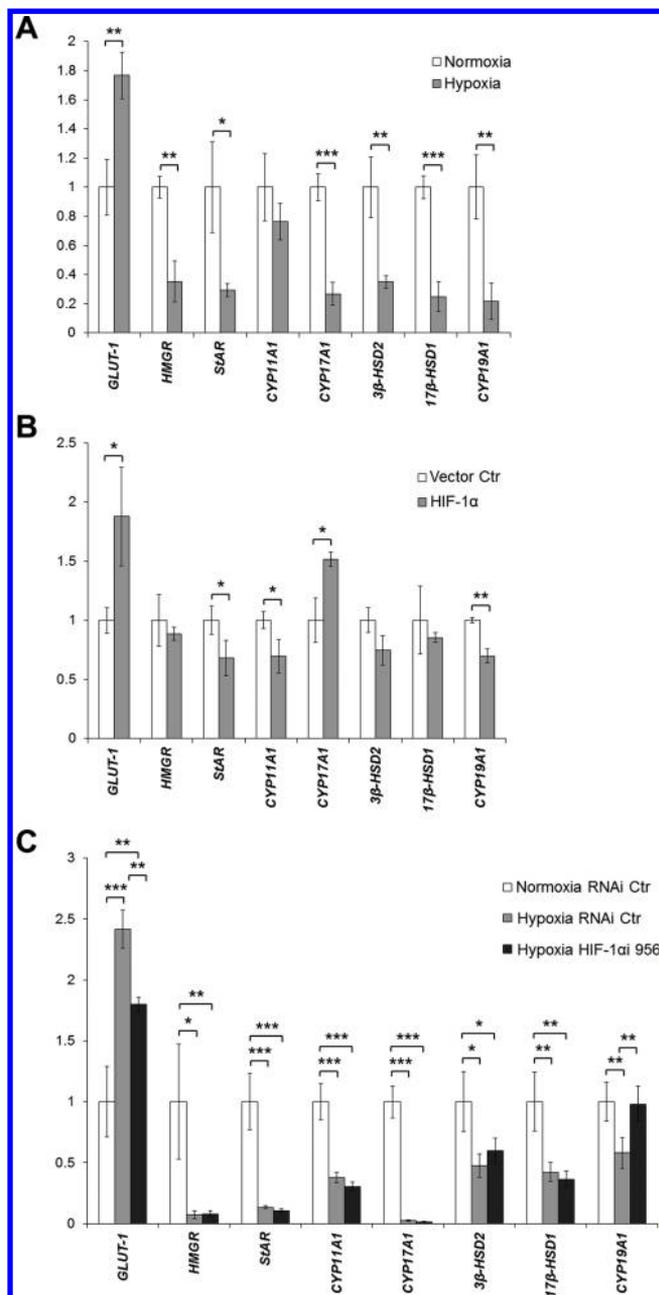
To assess the transactivation potential of HIF-1 on the miR-98 promoter, five deletion constructs of the miR-98 5'-flanking region (with or without putative HREs) were generated from genomic DNA by PCR using the following primers (restriction sites are underlined): forward primers, 5'-ACGTTGC-TAGCCGCTGGACAGAAGAATGCAA-3' for construct pGL-miR98p1 (-2687/+17), 5'-AGGATGCTAGCTT-GACTGTGTCTGCCGTAATGTG-3' for construct pGL-miR98p2 (-2283/+17), 5'-TGAACCTCGAGAGTGAACC-TAGCCTGTGACTGA-3' for construct pGL-miR98p3 (-1473/+17), 5'-ATCCAGCTAGCAACAGAATCTCT-CAGGTAAC-3' for construct pGL-miR98p4 (-1055/+17), 5'-GTCTCGCTAGCTCCTAATTCTGTGGTACC-3' for construct pGL-miR98p5 (-522/+17), and a common reverse primer, 5'-GCATCAAGCTTGGCATGAGCAGAATCCTC-TAA-3'. The PCR products were double-digested with *Nhe*I (or *Xho*I) and *Hind*III and cloned into the *Nhe*I-*Hind*III or *Xho*I-*Hind*III site of a pGL4.10 vector (Promega).

**Transient Transfection and Luciferase Reporter Assay.** Transfection into HeLa cells was carried out using Lipofectamine 2000 (Invitrogen) in a 24-well plate ( $1 \times 10^5$  cells/well). For the miRNA-mRNA interaction assay, cells were cotransfected with pMIR-REPORT constructs with or without pre-miR-98 (100 nM) or scrambled oligos (Ambion). For the miR-98 promoter assay, cells were cotransfected with pGL-miR98p1-5 deletion constructs and HIF-1 $\alpha$  expression vector pCMV-HIF1 $\alpha$ , which contained the full-length HIF1 $\alpha$ : $\Delta$ ODD sequence inserted in pCMV-TNT (Promega). EPO-HRE-luciferase reporter plasmid (pGL-Epo-HRE), which contained four copies of the HRE sequence from the human *EPO* promoter, was used as a positive control. Cells were harvested at 24 h after transfection, and reporter assays were performed using a Dual-Luciferase Reporter Assay Kit (Promega). Luciferase expression was measured with a FLUOstar Optima plate reader (BMG Labtech) and normalized using  $\beta$ -galactosidase expression as an internal control. Data are expressed as the mean of the relative luciferase units (RLUs) measured in three independent experiments.

**Statistical Analysis.** Student's *t*-test or ANOVA followed by Student-Newman-Keuls *post hoc* test was used to test the null hypothesis that there was no significant difference between the values in the control and treatment groups. The results are presented as the mean  $\pm$  SD. Differences were considered to be statistically significant at a *p*-value of  $\leq 0.05$ .

## RESULTS

**Hypoxia Downregulates *CYP19A1* mRNA Expression via HIF-1.** Exposure of H295R cells to hypoxia (1% O<sub>2</sub>) significantly suppressed mRNA expression of the majority of the steroidogenic genes examined with the exception of *CYP11A1*, which was not significantly affected by hypoxia (Figure 2A). All other genes, including *HMGR*, *StAR*, *CYP17A1*, *3 $\beta$ -HSD2*, *17 $\beta$ -HSD1*, and *CYP19A1*, showed 3–4 fold reductions in expression in the hypoxic cells. The activation of HIF-1 in hypoxic cells was supported by an increase in the expression of *GLUT-1* (a HIF-1 target gene). To investigate the role of HIF-1 in regulating steroidogenic gene expression, gain- and loss-of-function experiments were carried out. Overexpression of HIF-1 $\alpha$  under normoxia significantly decreased mRNA expression of *StAR*, *CYP11A1*, and *CYP19A1* and increased that of *CYP17A1*; however, all of the changes in



**Figure 2.** Regulation of steroidogenic gene expression by hypoxia and HIF-1 in H295R cells. The relative mRNA levels of *HMGR*, *StAR*, *CYP11A1*, *CYP17A1*, *3β-HSD2*, *17β-HSD1*, and *CYP19A1* in H295R cells were quantified by qRT-PCR after (A) exposure to normoxia (20% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>) for 24 h, (B) HIF-1α overexpression (pLenti-HIF1α or control pLenti6 vector) was applied under normoxia and (C) HIF-1α knockdown (HIF-1αi 956) was applied under hypoxia whereas a negative RNAi control was applied under normoxia or hypoxia). *GLUT-1* expression was monitored as an endogenous marker of hypoxia. Data are presented as the mean relative fold change ± SD ( $n = 3$ ) relative to the gene expression level in the normoxic control (arbitrarily set to 1). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

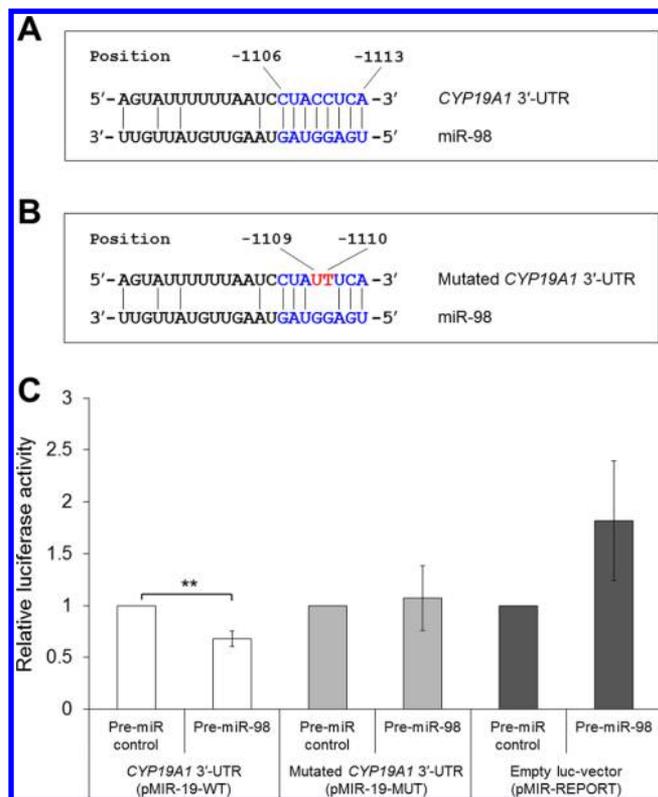
expression were present at low levels (<2-fold) (Figure 2B). On the other hand, knockdown of HIF-1α in the hypoxic cells abolished the inhibitory effect of hypoxia on the mRNA expression of *CYP19A1* but not on that of the other five hypoxia-suppressed genes (Figure 2C). The same treatment

resulted in a significant reduction in hypoxic *GLUT-1* expression, thereby validating the efficacy of HIF-1α knock-down. Overall, these data suggest that although mRNA expression of most steroidogenic genes is suppressed by hypoxia, only the suppression of *CYP19A1* involves the HIF-1 protein, either directly or indirectly.

**MiR-98 is a Hypoxia-Upregulated MiRNA that Targets *CYP19A1*.** Using miRNA profiling, we have previously identified 70 miRNAs that are upregulated in H295R cells following HIF-1α overexpression.<sup>23,27</sup> To determine whether the decrease in *CYP19A1* expression that occurs in hypoxic cells might be mediated by any of these 70 miRNAs, three independent bioinformatics algorithms, TargetScan (<http://www.targetscan.org/>), PicTar (<http://pictar.mdc-berlin.de/>) and MicroCosm Targets (<https://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl>), were used to screen these miRNAs for interaction with the *CYP19A1* mRNA. Intriguingly, only miR-98 was detected by all three algorithms to target this mRNA. Sequence alignment results showed that the 3'-UTR of the *CYP19A1* mRNA contained a consecutive sequence of eight bases (positions 1106–1113) that was perfectly complementary to the miR-98 seed sequence (Figure 3A). Luciferase reporter assays were then performed to test whether miR-98 specifically interacts with this 3'-UT sequence to downregulate *CYP19A1* expression. Two constructs of the pMIR-REPORT vector were generated, including one containing the wild-type *CYP19A1* 3'-UTR (Figure 3A) and another with the *CYP19A1* 3'-UTR, in which the predicted miR-98 binding site was mutated (Figure 3B). These constructs were cotransfected with pre-miR-98 or scrambled oligos (control) into HeLa cells. It was found that overexpression of pre-miR-98 significantly decreased the luciferase activity of the wild-type *CYP19A1*-3'-UTR reporter, while the mutations in the *CYP19A1* 3'-UTR abolished this inhibitory effect (Figure 3C). These data suggest that the hypoxia-upregulated miR-98 could repress *CYP19A1* expression via specific interaction with its target site in the 3'-UTR of the *CYP19A1* mRNA.

**MiR-98 Mediates Hypoxia-Induced Inhibition of *CYP19A1* Expression and Estrogen Synthesis.** To confirm the hypoxia responsiveness of miR-98 and its regulation by HIF-1, miRNA qRT-PCR was performed on H295R cells subjected to hypoxia and gain- and loss-of-function of HIF-1α, respectively. In agreement with our miRNA profiling results,<sup>23,27</sup> miR-98 was found to be significantly upregulated by both hypoxic treatment (Figure 4A) and HIF-1α overexpression (Figure 4B), exhibiting fold increases of approximately 4 and 1.6, respectively. Conversely, knockdown of HIF-1α by siRNA (HIF-1αi 956) abolished the hypoxic induction of miR-98 (Figure 4B), confirming the regulation of miR-98 expression by HIF-1.

To investigate the role of miR-98 in regulating *CYP19A1* expression, *CYP19A1* mRNA and protein levels were measured after gain- and loss-of-function of miR-98. Overexpression of pre-miR-98 decreased the mRNA expression of *CYP19A1* by approximately 1.5-fold (relative to the scrambled control miRNAs), while knockdown of miR-98 (via overexpression of anti-miR-98) reversed the inhibitory effect of hypoxia on *CYP19A1* mRNA expression (Figure 4C). *CYP19A1* protein levels were greatly reduced in response to either hypoxia or overexpression of the anti-miR control under hypoxia, and HIF-1 induction was demonstrated in both treatments (Figure 4D). Overexpression of pre-miR-98 (under normoxia) led to a substantial decrease in *CYP19A1* protein levels, while the anti-



**Figure 3.** miR-98 regulates *CYP19A1* expression via binding to its 3'-UTR. (A) *CYP19A1* contains a conserved 3'-untranslated sequence (positions 1106–1113) that perfectly complements the miR-98 seed sequence (both are shown in blue). (B) The mutant *CYP19A1* 3'-UTR contains mutations in the miR-98 binding site that disrupt base pairing (positions 1109 and 1110; indicated by red). (C) Effect of miR-98 on *CYP19A1*-3'-UTR-driven luciferase activity. HeLa cells were cotransfected with wild-type (pMIR-19-WT) or mutant *CYP19A1*-3'-UTR (pMIR-19-MUT) luciferase constructs plus pre-miR-98. Empty luciferase vector (pMIR-REPORT) and scrambled oligos (pre-miR control) were applied as negative controls. The results are expressed as the mean relative luciferase activity  $\pm$  SD ( $n = 3$ ) with respect to the activity of the wild-type *CYP19A1*-3'-UTR plus the pre-miR control. \*\* $p < 0.01$ .

miR-98 treatment (under hypoxia) partially restored normal *CYP19A1* protein levels (Figure 4D).

Because *CYP19A1* converts T to E2, the effect of miR-98 on the production of these sex steroids was studied using the same gain- and loss-of-function approach. In hypoxic cells, E2 concentrations were reduced by half compared to those in normoxic cells, albeit statistically insignificant (Figure 4E). Similar decreases were observed for cells overexpressing either pre-miR-98 (1.9-fold relative to the pre-miR control) or the anti-miR control under hypoxia (2.4-fold relative to the anti-miR control under normoxia). Intriguingly, miR-98 knockdown (using anti-miR-98) in hypoxic cells failed to restore normal E2 production. The production of T was altered in a similar manner as that observed for E2 (Figure 4F). The major differences were 2-fold: (i) the extent of inhibition of T production caused by hypoxia was greater than that observed for E2 (3.8- and 2-fold relative to the normoxic control, respectively); and (ii) miR-98 overexpression (under normoxia) did not significantly alter T concentrations compared to the pre-miR control.

Overall, these results suggest that (i) miR-98 is a HIF-1-regulated miRNA that downregulates *CYP19A1* expression in

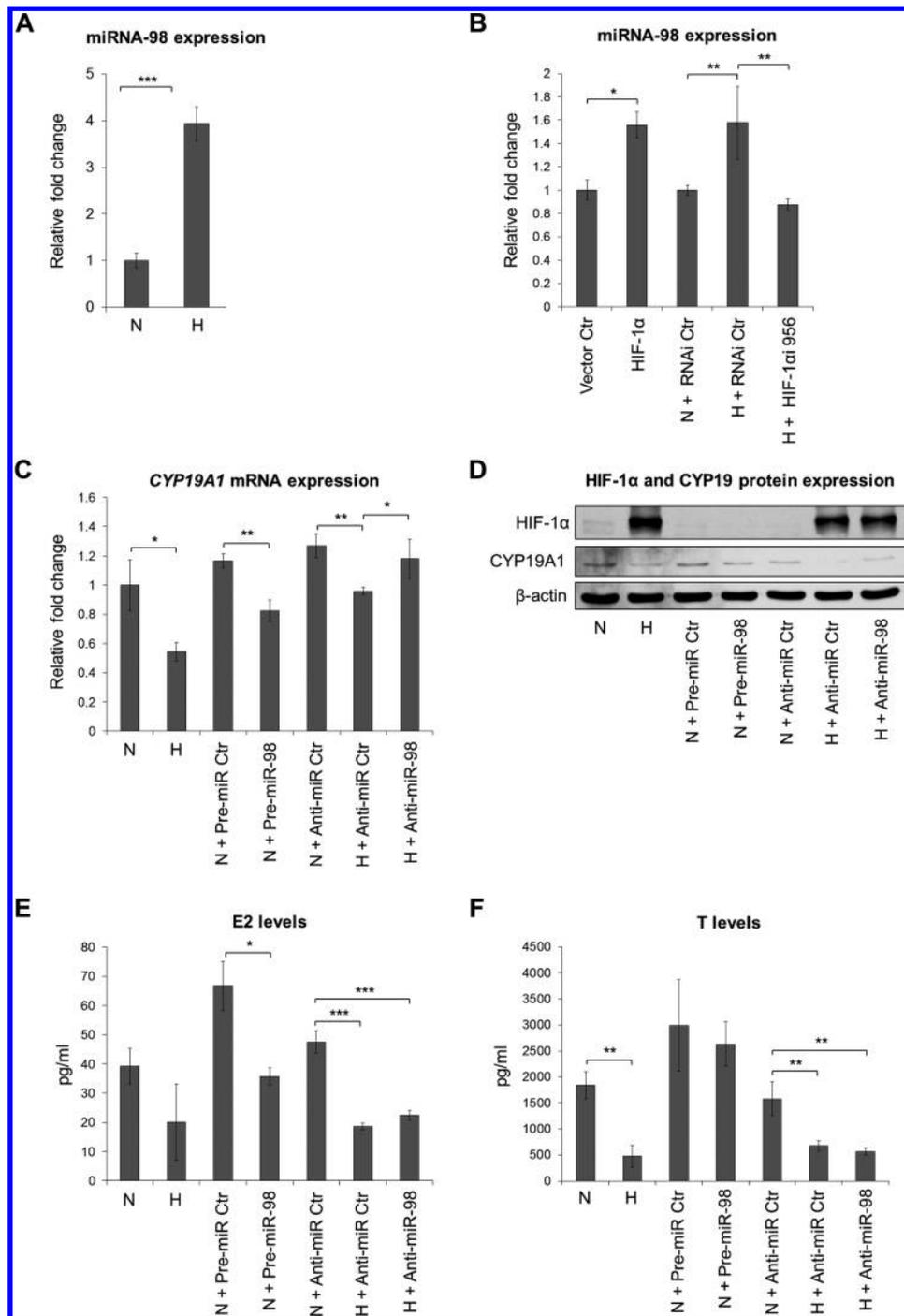
hypoxic cells; and (ii) the reduction in *CYP19A1* expression may account, in part, for the reduction in E2 production in hypoxic cells.

**Proximal 5'-Flanking Region of MiR-98 Lacks an HRE-Containing Promoter.** To determine whether HIF-1 regulates miR-98 expression via binding to HRE(s) within the miR-98 promoter, a 3-kb 5'-flanking sequence of miR-98 was searched by computer for the HRE core motif (A/G)CGTG. This search revealed three putative HREs in the most upstream 1 kb portion of the analyzed region (Figure 5A). To test the functionality of these putative HREs, serial promoter deletion constructs in a pGL4.10 luciferase reporter vector were generated (namely pGL-miR98p1-5; Figure 5A) and transiently cotransfected with the HIF-1 $\alpha$  expression vector (pCMV-HIF1 $\alpha$ ) into HeLa cells. An additional luciferase reporter construct, pGL-Epo-HRE, which contained four copies of the human *EPO* HRE, was used as a positive control. Our results indicated that neither the full-length nor the truncated miR-98 promoter constructs resulted in luciferase activities that were higher or significantly different from that of the empty vector control (pGL4.10) following cotransfection with HIF-1 $\alpha$  (Figure 5B). Because the luciferase activity of the positive control was increased by approximately 3-fold in the cells overexpressing HIF-1 $\alpha$ , the negative results observed for the miR-98 promoter constructs are unlikely to be due to a defect in the reporter system used. These data imply that the 3-kb proximal 5'-flanking region examined may lack an HRE-containing promoter.

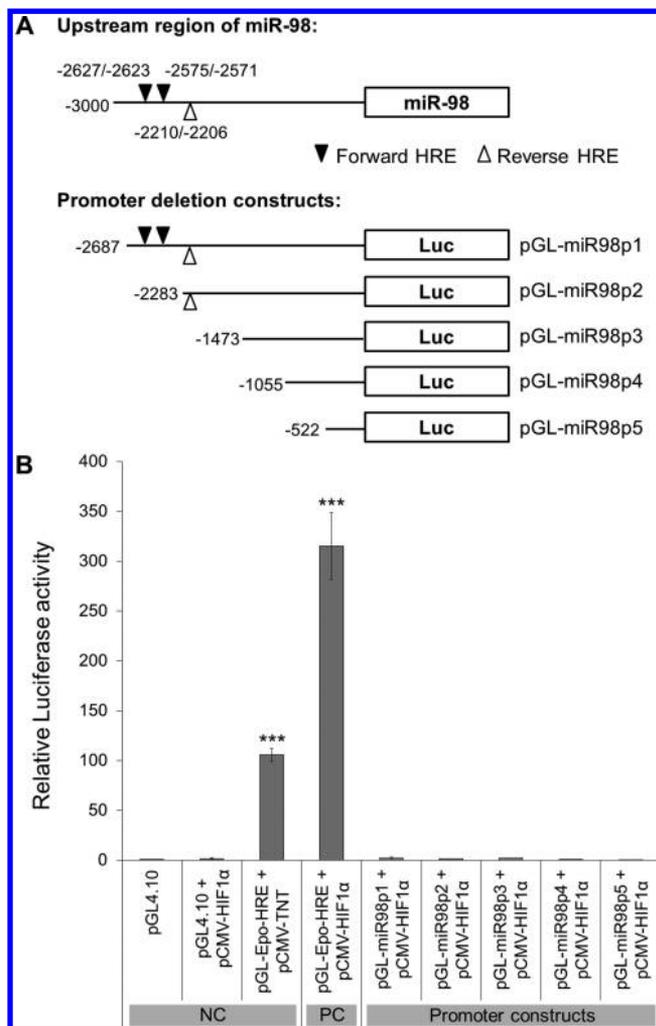
## DISCUSSION

Inhibition of steroidogenesis by hypoxia has been described in a number of tissues from various species.<sup>1,2,4–13</sup> Similar inhibition has also been observed after treatment with the HIF-1 inducer  $\text{CoCl}_2$ ,<sup>17</sup> suggesting a possible role of HIF-1 in modulating steroidogenic gene expression under hypoxia. HIF-1 is a transcription factor that binds to HREs, leading to upregulation (major) or downregulation (minor) of target genes. In contrast with the prediction that HIF-1 downregulates steroidogenic gene expression, there is evidence that it can bind to and activate the promoters of *3 $\beta$ -HSD1* and *CYP19A1* in testicular Leydig<sup>3</sup> and breast adenocarcinoma<sup>14</sup> cells, respectively. In the present study, all examined steroidogenic genes with the exception of *CYP11A1* were shown to be repressed in hypoxic H295R cells (Figure 2A). Our gain- and loss-of-function analysis indicated that HIF-1 is involved in the repression of *CYP19A1* (Figures 2B and C). Assuming that this repression is not driven by HRE(s) in *CYP19A1*, other mechanisms, such as HIF-1-mediated post-transcriptional regulation, might be involved in its repression under hypoxia. Therefore, we postulated that HIF-1 activates the expression of specific miRNA(s) that mediate degradation and/or translational repression of *CYP19A1* mRNA.

From the bioinformatic analysis of the subset of 70 miRNAs that have been found to be upregulated in HIF-1-overexpressing H295R cells,<sup>23,27</sup> we determined that miR-98 potentially interacted with *CYP19A1* mRNA (Figure 3A). Although the copy number of miR-98 in H295R cells was not measured in this study, the Taqman-based qPCR for miR-98 yielded an average  $C_T$  value of ca. 25, which indicated that miR-98 is expressed at a moderate level ( $25 < C_T < 30$ ) and is likely to be biologically relevant in H295R cells. Furthermore, our luciferase reporter assay confirmed that miR-98 directly interacts with the 3'-UTR of this mRNA, causing its post-



**Figure 4.** miR-98 represses *CYP19A1* expression via HIF-1 in hypoxic H295R cells. (A) Cells were exposed to either normoxia (N, 20% O<sub>2</sub>) or hypoxia (H, 1% O<sub>2</sub>) for 24 h. At the end of the treatment, total RNA was extracted for qRT-PCR analysis of miR-98. The data are presented as the mean relative fold change  $\pm$  SD ( $n = 3$ ) with respect to the normoxia control (arbitrarily set to 1). \*\*\* $p < 0.001$ . (B) Cells were transfected with either pLenti-HIF1 $\alpha$  (HIF-1 $\alpha$  overexpression) or HIF-1 $\alpha$  956 (HIF-1 $\alpha$  knockdown) followed by exposure to normoxia or hypoxia, respectively. The empty pLenti6 vector (under normoxia) and RNAi control (under normoxia or hypoxia) were used as negative controls in the HIF-1 $\alpha$  overexpression and knockdown experiments, respectively. The data are presented as the mean relative fold change  $\pm$  SD ( $n = 3$ ) with respect to the empty pLenti6 vector control (arbitrarily set to 1). \* $p < 0.05$ , \*\* $p < 0.01$ . (C) Regulation of *CYP19A1* mRNA expression by miR-98. Cells were electroporated with either pre-miR-98 (miR-98 overexpression) or anti-miR-98 inhibitor (miR-98 knockdown), followed by exposure to normoxia or hypoxia, respectively. The pre-miR (scrambled) control was used under normoxia, while the anti-miR control was used under normoxia or hypoxia. Unelectroporated cell controls grown under normoxia or hypoxia were also used. The data are presented as the mean relative fold change  $\pm$  SD ( $n = 3$ ) with respect to the unelectroporated normoxia control (arbitrarily set to 1). \* $p < 0.05$ , \*\* $p < 0.01$ . (D) Regulation of *CYP19A1* protein expression by hypoxia and miR-98. Western blot analysis of *CYP19A1* and HIF-1 $\alpha$  was performed using cells treated as above.  $\beta$ -actin was used as a loading control. (E) and (F) The role of miR-98 in modulating sex steroid synthesis in hypoxic H295R cells. Concentrations of (E) estradiol (E2) and (F) testosterone (T) were measured in the culture medium using commercial ELISA kits. The data are presented as the mean steroid concentration  $\pm$  SD ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 5.** Functional analysis of the proximal 5'-flanking region of miR-98. (A) Schematic representation of the location and orientation of the three putative HREs identified in the 3'-kb region upstream of miR-98. The HREs in the forward orientation are shown as inverted triangles, and the reverse HRE is shown as the upright triangle. A series of deletion constructs containing the indicated portions of the 5'-flanking region were synthesized in the luciferase reporter vector pGL4.10. (B) HeLa cells were cotransfected with the illustrated deletion constructs and pCMV-HIF1 $\alpha$  expression vector. The reporter construct containing four copies of the HRE from the *EPO* gene (pGL-Epo-HRE) was used as a positive control. Negative controls included empty pGL4.10 vector and empty pCMV-TNT vector. The results are expressed as the mean relative luciferase activity  $\pm$  SD ( $n = 3$ ). Luciferase activities that are significantly different from that of the empty vector control (pGL4.10) are indicated by asterisks (\*\*\*)  $p < 0.001$ .

transcriptional repression (Figure 3C). Because miRNAs can have multiple targets, it is possible that in addition to *CYP19A1*, miR-98 may also target other transcripts that are directly or indirectly involved in steroidogenesis. In a recent study, the effects of 80 miRNAs on the release of major ovarian steroid hormones were examined in ovarian granulosa cells.<sup>19</sup> The results demonstrated that miR-98 overexpression by transient transfection inhibited not only the level of E2 but also the levels of two other hormones preceding the *CYP19A1* (aromatization) step, T and progesterone. Together with our findings, this suggests that miR-98 may concomitantly regulate *CYP19A1* and other steroidogenic targets. To explore this possibility, we

sought for all potential miR-98 targets using miRNA target prediction softwares, including TargetScan, MicroCosm and PicTar. However, our results indicated that *CYP19A1* is the only steroidogenic target of miR-98. Therefore, it appears that in ovarian granulosa cells, miR-98 may have other non-steroidogenic targets that are indirectly involved in the regulation of steroidogenesis.

This study is the first report of the post-transcriptional regulation of estrogen steroidogenesis by hypoxia, showing that miR-98 is an HIF-1-regulated miRNA that represses *CYP19A1* expression (both at the mRNA and protein levels) in H295R cells (Figures 4B, C and D). Although aromatase activity was not measured in this study, based on the strong correlation between *CYP19A1* expression and aromatase activity previously reported in normal adrenal tissues and adrenocortical tumors,<sup>28</sup> it is not unreasonable to assume that the reduction in *CYP19A1* mRNA and protein expression would correspond to a reduced aromatase activity. This assumption is corroborated by the concomitant reduction in E2 levels in miR-98-overexpressing and hypoxic H295R cells (Figure 4E). Despite these observations, miR-98 activation (or *CYP19A1* inhibition) *per se* is insufficient to fully explain the decline in E2 levels observed under hypoxia because miR-98 knockdown failed to restore normal E2 concentrations in hypoxic cells (Figure 4E). It is worth noting that in addition to *CYP19A1*, other genes that are located upstream of *CYP19A1* in the steroidogenesis pathway (i.e., *HMGR*, *StAR*, *CYP17A1*, *3 $\beta$ -HSD2*, and *17 $\beta$ -HSD1*) were also found to be downregulated in the hypoxic cells (Figure 2A). Therefore, it is tempting to speculate that the decrease in E2 observed in these cells could have been due to the reduced availability of precursor steroid metabolites (such as T; Figure 4F). However, as the final step in estrogen synthesis, *CYP19A1* can also affect E2 levels independent of upstream effect and even change the levels of its substrates. For example, it was shown that both E2 and *CYP19A1* mRNA levels were decreased in hypoxic ovarian tissues even when levels of upstream precursor steroid metabolites are unaffected or increased.<sup>5</sup> More importantly, it was found in the same study that reduced E2 levels are able to in turn decrease T levels by increasing the conversion of T to 11-ketotestosterone (11-KT).<sup>5</sup> Due to the interplay between E2 and T, it is difficult to evaluate the substrate effect on E2 production based on the changes in T levels.

Although the role of miR-98 as a hypoxia-inducible repressor of *CYP19A1* was demonstrated at the post-transcriptional level, the possible contribution of other mechanisms to transcriptional repression of *CYP19A1* cannot be ruled out. One of these mechanisms may involve the hypoxia-inducible transcription factor c-Jun.<sup>29</sup> Our unpublished microarray data indicated that *c-Jun* mRNA was significantly increased by 3.1-fold in H295R cells when exposed to the same hypoxic conditions used in this study (data not shown). In human granulosa cells, c-Jun represses *CYP19A1* transcription by binding to the tissue-specific promoter PII.<sup>30</sup> Since PII is also specific to the adrenal cortex, it is possible that an increase in c-Jun may also repress *CYP19A1* transcription in hypoxic H295R cells. On this premise, it is possible that the reduction of *CYP19A1* mRNA in hypoxic H295R cells might be due, in part, to both transcriptional (via c-Jun) and post-transcriptional (via miR-98) repression. Besides, it should be noted that *CYP19A1* transcription can be altered under the influence of the second messenger cAMP. Our previous studies revealed that *CYP19A1* mRNA and E2 levels were significantly increased in H295R

cells treated with the cAMP inducers, 8Br-cAMP and/or forskolin.<sup>20,31</sup> Although the effect of miR-98 in cAMP-stimulated H295R cells was not tested in this study, we predict that E2 production will also be suppressed by miR-98 in the stimulated cells since miR-98 negatively regulates *CYP19A1* expression at the post-transcriptional level.

So far, the induction of miR-98 by hypoxia has been observed only in H295R cells (this study) and the nonsteroidogenic head and neck squamous cell carcinoma (HNSCC) cells.<sup>32</sup> Transcriptional activation of hypoxia-regulated miRNAs (HRMs) could be mediated by the binding of HIF-1 to HREs in their promoter regions.<sup>33</sup> To identify functional HRE(s) in miR-98, luciferase reporter constructs containing the 3-kb upstream region of miR-98 (where three putative HREs were located) and their deletions (Figure 5A) were cotransfected with HIF-1 $\alpha$  expression vector into HeLa cells. HeLa cells were used because H295R cells could not be efficiently transfected. Curiously, none of the tested constructs showed significant luciferase activity compared to the promoterless vector control (Figure 5B). These negative results could be explained in at least two ways: (i) the functional HRE(s) (or even the promoter) reside outside of the 3-kb upstream region examined; and (ii) HIF-1 regulates miR-98 transcription not directly but indirectly through other HIF-1-regulated factors that are not expressed in HeLa cells (i.e., an HRE-independent mechanism). miR-98 is embedded within intron 59 of the host *HUWE1* gene. Previously, Ting et al.<sup>34</sup> used a ChIP assay to identify three cis-acting vitamin D receptor response elements (VDREs) within 1–4 kb upstream of the 5' terminus of the miR-98 precursor in human prostate adenocarcinoma LNCaP cells, suggesting that the miR-98 promoter could be located in a proximal upstream region. However, considering the high variability in the genomic location of miR-98 promoters observed in different cell types (the two most distant promoter sites are up to 40 kb apart from each other),<sup>35,36</sup> it is plausible that the 3'-kb upstream region examined here may have lacked a miR-98 promoter specific for H295R cells. In the future, it may be worthwhile to determine the positions of functional HREs by data mining the ChIP-seq/ChIP-on-chip databases available for H295R cells.

In conclusion, this study demonstrates the involvement of miR-98 as an HIF-1-regulated miRNA in the regulation of estrogen synthesis under hypoxia. Because estrogen is not the major steroid product of either the adrenal cortex or H295R cells, it remains to be determined whether the present findings can be extrapolated to other estrogen-producing cells and tissues such as ovarian granulosa cells, and placental and adipose tissues. Notwithstanding this, several lines of evidence suggest that miR-98 may play a role in controlling steroidogenesis in certain estrogen-producing tissues. For example, (i) miR-98 is expressed in normal ovarian<sup>37</sup> and placental<sup>38</sup> tissues; (ii) overexpression of miR-98 decreases E2 production in primary ovarian granulosa cells;<sup>19</sup> and (iii) hypoxia was shown to decrease E2 production in primary cultures of porcine granulosa cells<sup>39</sup> and human placental cells,<sup>40</sup> and in zebrafish interrenal tissues (fish homologue of the mammalian adrenal cortex).<sup>13</sup> Further research on whether and how miR-98 mediates hypoxic regulation of estrogen synthesis in these endocrine tissues is therefore worth pursuing in future studies. Furthermore, it would be of great interest to investigate whether miR-98 is regulated by estrogen in steroidogenic tissues. It has been revealed that in breast cancer cells, E2 can induce miR-98 expression, which likely occurs via an ER $\alpha$ -

mediated mechanism.<sup>41</sup> Intriguingly, a similar observation has also been reported in zebrafish, in which expression of let-7h (the miR-98 homologue in zebrafish) was increased in gills and skin after exposure to E2 by immersion (major steroidogenic tissues were not examined in this study).<sup>42</sup> If miR-98 is truly upregulated by E2 in estrogen-producing tissues, then it is reasonable to postulate that it may be a part of an evolutionarily conserved negative regulatory loop to control estrogen production. Resolution of the above questions will advance our understanding of the role of miRNAs in estrogen homeostasis and hypoxia-induced endocrine disruption.

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### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This work was supported in part by a Seed Collaborative Research Fund (SCRF) (PJ9369101) from the State Key Laboratory in Marine Pollution (SKLMP) and a grant from the General Research Fund (PJ9041727) from the Research Grants Council of Hong Kong Special Administrative Region, People's Republic of China. We acknowledge the support of an instrumentation grant from the Canada Foundation for Infrastructure. Prof. Giesy was supported by the Canada Research Chair program, an at-large Chair Professorship at the Department of Biology and Chemistry and SKLMP, City University of Hong Kong, and the Einstein Professor Program of the Chinese Academy of Sciences.

## REFERENCES

- (1) Basini, G.; Bianco, F.; Grasselli, F.; Tirelli, M.; Bussolati, S.; Tamanini, C. The effects of reduced oxygen tension on swine granulosa cell. *Regul. Pept.* **2004**, *120* (1–3), 69–75 DOI: 10.1016/j.regpep.2004.02.013.
- (2) Nishimura, R.; Sakumoto, R.; Tatsukawa, Y.; Acosta, T. J.; Okuda, K. Oxygen concentration is an important factor for modulating progesterone synthesis in bovine corpus luteum. *Endocrinology* **2006**, *147* (9), 4273–4280 DOI: 10.1210/en.2005-1611.
- (3) Lysiak, J. J.; Kirby, J. L.; Tremblay, J. J.; Woodson, R. I.; Reardon, M. A.; Palmer, L. A.; Turner, T. T. Hypoxia-inducible factor-1 $\alpha$  is constitutively expressed in murine Leydig cells and regulates 3 $\beta$ -hydroxysteroid dehydrogenase type 1 promoter activity. *J. Androl.* **2009**, *30* (2), 146–156 DOI: 10.2164/jandrol.108.006155.
- (4) Martinovic, D.; Villeneuve, D. L.; Kahl, M. D.; Blake, L. S.; Brodin, J. D.; Ankley, G. T. Hypoxia alters gene expression in the gonads of zebrafish (*Danio rerio*). *Aquat. Toxicol.* **2009**, *95* (4), 258–272 DOI: 10.1016/j.aquatox.2008.08.021.
- (5) Hala, D.; Petersen, L. H.; Martinovic, D.; Huggett, D. B. Constraints-based stoichiometric analysis of hypoxic stress on steroidogenesis in fathead minnows, *Pimephales promelas*. *J. Exp. Biol.* **2012**, *215* (10), 1753–1765 DOI: 10.1242/jeb.066027.
- (6) Thomas, P.; Rahman, M. S. Extensive reproductive disruption, ovarian masculinization and aromatase suppression in Atlantic croaker in the northern Gulf of Mexico hypoxic zone. *Proc. Biol. Sci.* **2012**, *279* (1726), 28–38 DOI: 10.1098/rspb.2011.0529.
- (7) Jiang, B.; Kamat, A.; Mendelson, C. R. Hypoxia prevents induction of aromatase expression in human trophoblast cells in culture: Potential inhibitory role of the hypoxia-inducible transcription factor Mash-2 (mammalian achaete-scute homologous protein-2). *Mol. Endocrinol.* **2000**, *14* (10), 1661–1673 DOI: 10.1210/me.14.10.1661.

- (8) Jiang, B.; Mendelson, C. R. USF1 and USF2 mediate inhibition of human trophoblast differentiation and CYP19 gene expression by Mash-2 and hypoxia. *Mol. Cell. Biol.* **2003**, *23* (17), 6117–6128 DOI: 10.1128/MCB.23.17.6117-6128.2003.
- (9) Kumar, P.; Mendelson, C. R. Estrogen-related receptor  $\gamma$  (ERR $\gamma$ ) mediates oxygen-dependent induction of aromatase (CYP19) gene expression during human trophoblast differentiation. *Mol. Endocrinol.* **2011**, *25* (9), 1513–1526 DOI: 10.1210/me.2011-1012.
- (10) Bruder, E. D.; Nagler, A. K.; Raff, H. Oxygen-dependence of ACTH-stimulated aldosterone and corticosterone synthesis in the rat adrenal cortex: Developmental aspects. *J. Endocrinol.* **2002**, *172* (3), 595–604 DOI: 10.1677/joe.0.1720595.
- (11) Raff, H.; Bruder, E. D. Steroidogenesis in human aldosterone-secreting adenomas and adrenal hyperplasias: Effects of hypoxia *in vitro*. *Am. J. Physiol. Endocrinol. Metab.* **2006**, *290* (1), E199–E203 DOI: 10.1152/ajpendo.00337.2005.
- (12) Shang, E. H.; Yu, R. M. K.; Wu, R. S. S. Hypoxia affects sex differentiation and development, leading to a male-dominated population in zebrafish (*Danio rerio*). *Environ. Sci. Technol.* **2006**, *40* (9), 3118–3122 DOI: 10.1021/es0522579.
- (13) Yu, R. M. K.; Chu, D. L. H.; Tan, T. F.; Li, V. W. T.; Chan, A. K. Y.; Giesy, J. P.; Cheng, S. H.; Wu, R. S. S.; Kong, R. Y. C. Leptin-mediated modulation of steroidogenic gene expression in hypoxic zebrafish embryos: Implications for the disruption of sex steroids. *Environ. Sci. Technol.* **2012**, *46* (16), 9112–9119 DOI: 10.1021/es301758c.
- (14) Samarajeewa, N. U.; Yang, F.; Docanto, M. M.; Sakurai, M.; McNamara, K. M.; Sasano, H.; Fox, S. B.; Simpson, E. R.; Brown, K. A. HIF-1 $\alpha$  stimulates aromatase expression driven by prostaglandin E2 in breast adipose stroma. *Breast Cancer Res.* **2013**, *15* (2), R30 DOI: 10.1186/bcr3410.
- (15) Wang, G. L.; Jiang, B. H.; Rue, E. A.; Semenza, G. L. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O<sub>2</sub> tension. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92* (12), 5510–5514 DOI: 10.1073/pnas.92.12.5510.
- (16) Weidemann, A.; Johnson, R. S. Biology of HIF-1 $\alpha$ . *Cell Death Differ.* **2008**, *15* (4), 621–627 DOI: 10.1038/cdd.2008.12.
- (17) Kumar, A.; Rani, L.; Dhole, B. Role of oxygen in the regulation of Leydig tumor derived MA-10 cell steroid production: The effect of cobalt chloride. *Syst. Biol. Reprod. Med.* **2014**, *60* (2), 112–118 DOI: 10.3109/19396368.2013.861034.
- (18) Lewis, B. P.; Burge, C. B.; Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **2005**, *120* (1), 15–20 DOI: 10.1016/j.cell.2004.12.035.
- (19) Sirotkin, A. V.; Ovcharenko, D.; Grossmann, R.; Lauková, M.; Mlynček, M. Identification of microRNAs controlling human ovarian cell steroidogenesis via a genome-scale screen. *J. Cell. Physiol.* **2009**, *219* (2), 415–420 DOI: 10.1002/jcp.21689.
- (20) Hollert, H.; Giesy, J. The OECD validation program of the H295R steroidogenesis assay for the identification of *in vitro* inhibitors and inducers of testosterone and estradiol production. Phase 2: Inter-laboratory pre-validation studies (8pp). *Environ. Sci. Pollut. Res. Int.* **2007**, *14* (1), 23–30 DOI: 10.1065/espr2007.03.402.
- (21) Hilscherova, K.; Jones, P. D.; Gracia, T.; Newsted, J. L.; Zhang, X.; Sanderson, J. T.; Yu, R. M. K.; Wu, R. S. S.; Giesy, J. P. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. *Toxicol. Sci.* **2004**, *81* (1), 78–89 DOI: 10.1093/toxsci/kfh191.
- (22) Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29* (9), e45 DOI: 10.1093/nar/29.9.e45.
- (23) Nusrin, S.; Tong, S. K. H.; Chaturvedi, G.; Wu, R. S. S.; Giesy, J. P.; Kong, R. Y. C. Regulation of CYP11B1 and CYP11B2 steroidogenic genes by hypoxia-inducible miR-10b in H295R cells. *Mar. Pollut. Bull.* **2014**, *85* (2), 344–351 DOI: 10.1016/j.marpolbul.2014.04.002.
- (24) Hecker, M.; Newsted, J. L.; Murphy, M. B.; Higley, E. B.; Jones, P. D.; Wu, R.; Giesy, J. P. Human adrenocarcinoma (H295R) cells for rapid *in vitro* determination of effects on steroidogenesis: Hormone production. *Toxicol. Appl. Pharmacol.* **2006**, *217* (1), 114–124 DOI: 10.1016/j.taap.2006.07.007.
- (25) Ivan, M.; Kondo, K.; Yang, H.; Kim, W.; Valiando, J.; Ohh, M.; Salic, A.; Asara, J. M.; Lane, W. S.; Kaelin, W. G. HIF $\alpha$  targeted for VHL-mediated destruction by proline hydroxylation: Implications for O<sub>2</sub> sensing. *Science* **2001**, *292* (5516), 464–468 DOI: 10.1126/science.1059817.
- (26) Masson, N.; Willam, C.; Maxwell, P. H.; Pugh, C. W.; Ratcliffe, P. J. Independent function of two destruction domains in hypoxia-inducible factor- $\alpha$  chains activated by prolyl hydroxylation. *EMBO J.* **2001**, *20* (18), 5197–5206 DOI: 10.1093/emboj/20.18.5197.
- (27) Chaturvedi, G. *Hypoxia Inducible Factors and Associated MicroRNAs in Regulation of Steroidogenesis in the H295R Human Adrenocortical Carcinoma Cells*. Ph.D. Dissertation, City University of Hong Kong, Hong Kong, 2011.
- (28) Moreau, F.; Mittre, H.; Benhaim, A.; Bois, C.; Bertherat, J.; Carreau, S.; Reznik, Y. Aromatase expression in the normal human adult adrenal and in adrenocortical tumors: Biochemical, immunohistochemical, and molecular studies. *Eur. J. Endocrinol.* **2009**, *160* (1), 93–99 DOI: 10.1530/EJE-08-0215.
- (29) Laderoute, K. R.; Calaoagan, J. M.; Gustafson-Brown, C.; Knapp, A. M.; Li, G. C.; Mendonca, H. L.; Ryan, H. E.; Wang, Z.; Johnson, R. S. The response of c-jun/AP-1 to chronic hypoxia is hypoxia-inducible factor 1 $\alpha$  dependent. *Mol. Cell. Biol.* **2002**, *22* (8), 2515–2523 DOI: 10.1128/MCB.22.8.2515-2523.2002.
- (30) Ghosh, S.; Wu, Y.; Li, R.; Hu, Y. Jun proteins modulate the ovary-specific promoter of aromatase gene in ovarian granulosa cells via a cAMP-responsive element. *Oncogene* **2005**, *24* (13), 2236–2246 DOI: 10.1038/sj.onc.1208415.
- (31) Zhang, X.; Yu, R. M. K.; Jones, P. D.; Lam, G. K. W.; Newsted, J. L.; Gracia, T.; Hecker, M.; Hilscherova, K.; Sanderson, T.; Wu, R. S. S.; Giesy, J. P. Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. *Environ. Sci. Technol.* **2005**, *39* (8), 2777–2785 DOI: 10.1021/es048679k.
- (32) Hebert, C.; Norris, K.; Scheper, M. A.; Nikitakis, N.; Sauk, J. J. High mobility group A2 is a target for miRNA-98 in head and neck squamous cell carcinoma. *Mol. Cancer* **2007**, *6*, 5 DOI: 10.1186/1476-4598-6-5.
- (33) Shen, G.; Li, X.; Jia, Y. F.; Piazza, G. A.; Xi, Y. Hypoxia-regulated microRNAs in human cancer. *Acta Pharmacol. Sin.* **2013**, *34* (3), 336–341 DOI: 10.1038/aps.2012.195.
- (34) Ting, H. J.; Messing, J.; Yasmin-Karim, S.; Lee, Y. F. Identification of microRNA-98 as a therapeutic target inhibiting prostate cancer growth and a biomarker induced by vitamin D. *J. Biol. Chem.* **2013**, *288* (1), 1–9 DOI: 10.1074/jbc.M112.395947.
- (35) Oszolák, F.; Poling, L. L.; Wang, Z.; Liu, H.; Liu, X. S.; Roeder, R. G.; Zhang, X.; Song, J. S.; Fisher, D. E. Chromatin structure analyses identify miRNA promoters. *Genes Dev.* **2008**, *22* (22), 3172–3183 DOI: 10.1101/gad.1706508.
- (36) Marson, A.; Levine, S. S.; Cole, M. F.; Frampton, G. M.; Brambrink, T.; Johnstone, S.; Guenther, M. G.; Johnston, W. K.; Wernig, M.; Newman, J.; Calabrese, J. M.; Dennis, L. M.; Volkert, T. L.; Gupta, S.; Love, J.; Hannett, N.; Sharp, P. A.; Bartel, D. P.; Jaenisch, R.; Young, R. A. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell* **2008**, *134* (3), 521–533 DOI: 10.1016/j.cell.2008.07.020.
- (37) Iorio, M. V.; Visone, R.; Di Leva, G.; Donati, V.; Petrocca, F.; Casalini, P.; Taccioli, C.; Volinia, S.; Liu, C. G.; Alder, H.; Calin, G. A.; Ménard, S.; Croce, C. M. MicroRNA signatures in human ovarian cancer. *Cancer Res.* **2007**, *67* (18), 8699–8707 DOI: 10.1158/0008-5472.CAN-07-1936.
- (38) Gu, Y.; Sun, J.; Groome, L. J.; Wang, Y. Differential miRNA expression profiles between the first and third trimester human placentas. *Am. J. Physiol. Endocrinol. Metab.* **2013**, *304* (8), E836–843 DOI: 10.1152/ajpendo.00660.2012.
- (39) Basini, G.; Bianco, F.; Grasselli, F.; Tirelli, M.; Bussolati, S.; Tamanini, C. The effects of reduced oxygen tension on swine granulosa cell. *Regul. Pept.* **2004**, *120* (1–3), 69–75 DOI: 10.1016/j.regpep.2004.02.013.

(40) Ma, T.; Yang, S. T.; Kniss, D. A. Oxygen tension influences proliferation and differentiation in a tissue-engineered model of placental trophoblast-like cells. *Tissue Eng.* **2001**, *7* (5), 495–506 DOI: 10.1089/107632701753213129.

(41) Bhat-Nakshatri, P.; Wang, G.; Collins, N. R.; Thomson, M. J.; Geistlinger, T. R.; Carroll, J. S.; Brown, M.; Hammond, S.; Srour, E. F.; Liu, Y.; Nakshatri, H. Estradiol-regulated microRNAs control estradiol response in breast cancer cells. *Nucleic Acids Res.* **2009**, *37* (14), 4850–4861 DOI: 10.1093/nar/gkp500.

(42) Cohen, A.; Shmoish, M.; Levi, L.; Cheruti, U.; Levavi-Sivan, B.; Lubzens, E. Alterations in micro-ribonucleic acid expression profiles reveal a novel pathway for estrogen regulation. *Endocrinology* **2008**, *149* (4), 1687–1696 DOI: 10.1210/en.2007-0969.