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The Unique Expression and Function of miR-424 in Human Placental Trophoblasts[1]

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ABSTRACT

Placental hypoperfusion causes cellular hypoxia and is associated with fetal growth restriction and preeclampsia. In response to hypoxia, the repertoire of genes expressed in placental trophoblasts changes, which influences key cellular processes such as differentiation and fusion. Diverse miRNAs were recently found to modulate the cellular response to hypoxia. Here we show that miR-424, which was previously shown to be upregulated by hypoxia in nontrophoblastic cell types, is uniquely downregulated in primary human trophoblasts by hypoxia or chemicals known to hinder cell differentiation. We also identify FGFR1 as a direct target of miR-424 in human trophoblasts. This effect is unique to miR-424 and is not seen with other members of this miRNA family that are expressed in trophoblasts, such as miR-15 and miR-16. Our findings establish a unique role for miR-424 during differentiation of human trophoblasts.

FGFR1, hypoxia, microRNA, miR-424, trophoblasts

INTRODUCTION

Hypoperfusion, and the consequent cellular hypoxia, is one of the most common injuries to the placenta during human pregnancy [1, 2]. Hypoxia affects many molecular and cellular pathways, including the differentiation and function of placental trophoblasts. Thus, hypoxia may impact the maternal-fetal exchange interface, adversely influencing fetal growth.

As a part of our ongoing effort to better define the molecular mechanisms underlying trophoblast adaptation to injury, we recently focused on the expression of microRNAs (miRNAs) in placental trophoblasts exposed to hypoxia. MicroRNAs are small, noncoding RNAs that regulate gene expression at the posttranscriptional level through binding to partially complementary sequences in the 3′UTR of protein-coding mRNAs, causing translation inhibition and mRNA decay (review in [3]). MicroRNA pathways have emerged as important regulators of many cellular processes, and their aberrant expression has been associated with numerous pathological states [4–8]. High-throughput miRNA surveys have shown that placental trophoblasts express unique patterns of miRNAs [9–13]. The function of most of these trophoblastic miRNAs remains unknown, with only a few miRNAs associated with abnormal placentas derived from complicated pregnancies [14–17].

We recently identified a set of differentially regulated miRNAs in human primary trophoblasts that were exposed to hypoxic stress [18]. Among these miRNAs, we found that miR-424 is one of the only species that was downregulated in hypoxia. MicroR-424 is a mammalian-specific miRNA that is particularly abundant in placental trophoblasts [11, 12], and recent studies indicate that abundance is a critical determinant of the biological activity of miRNAs [19, 20]. MicroR-424 is known to regulate important cellular functions, including differentiation, proliferation, cell cycle, and angiogenesis [21–26]. Interestingly, it was recently shown that hypoxia upregulates the expression of miR-424 in endothelial cells [23]. Three other miRNA species—miR-15a, miR-15b, and miR-16—that share the same seed sequence with miR-424 are also highly expressed in placental trophoblasts. In this study, we investigated the expression of miR-424 and other related miRNAs in placental trophoblasts. We show that the reduced expression of miR-424 is not unique to hypoxia but is associated with hindered trophoblast differentiation. This effect was not observed in other miR-424 family members. We also show that miR-424 directly regulates the expression of FGFR1 in human trophoblasts through a discrete 3′UTR site. While miR-15/16 species are also expressed in trophoblasts and capable of targeting and silencing FGFR1, their expression level is not affected by hypoxia or the differentiation state of the cells.

MATERIALS AND METHODS

Cell Culture

Primary human trophoblasts (PHT) were prepared from normal term placentas using the trypsin-deoxyribonuclease-dispase/Percoll method as described by Kliman [27], with previously published modifications [28]. All placentas were obtained after term delivery using a protocol approved by the Institutional Review Board at the University of Pittsburgh.

Cultures were plated at a density of 350 000 cells/cm^2 and maintained in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Hyclone) and antibiotics at 37°C in a 5% carbon dioxide-air atmosphere. After 4 h, designed to allow cell attachment, the culture plates were allocated to either standard ($O_2 = 20\%$) or hypoxic ($O_2 < 1\%$) environments [18]. Differentiation was routinely monitored by medium human chorionic gonadotropin (hCG) levels using ELISA (DRG International), showing a characteristic increase in medium hCG as cytotrophoblasts differentiated into syncytiotrophoblasts, with attenuation of this process in hypoxic or undifferentiated cells [28, 29].

Immortalized, human, first-trimester, extravillous trophoblast cells (HTR8/SVneo), used as a convenient system for molecular assessment of miRNA-target interaction, were provided by C.H. Graham (Kingston, ON [30]) and were cultured in RPMI-1640 (Cellgro) supplemented with 5% bovine growth

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serum (HyClone) and antibiotics. JEG-3 human choriocarcinoma cells were maintained in DMEM containing 10% FBS and antibiotics as described [18]. Human umbilical vein endothelial cells (HUVEC; Lonza) were cultured in endothelial basal medium, phenol red-free (Lonza), supplemented with supplier-recommended concentrations of growth factors—human recombinant epidermal growth factor (hEGF), human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), ascorbic acid, hydrocortisone, and recombinant insulin-like growth factor (R3-IGF)—and 10% FBS (Lonza). Endothelial colony-forming cells (ECFC) were prepared as described in [31] and were maintained in endothelial basal medium, phenol red-free, supplemented with supplier-recommended concentrations of hEGF, hFGF, VEGF, ascorbic acid, hydrocortisone, and R3-IGF, with 10% FBS at 5×107 cells per well on collagen-coated six-well plates (BD Biosciences). HUVEC and ECFC were used at passage 3 and plated at a density of 48 000 cells per well in a six-well culture plate for incubations in a hypoxic chamber and at 96 000 cells per well in a six-well culture plate for incubations at standard oxygen concentration.

RNA Isolation, Reverse Transcription, and Real-Time Quantitative PCR

Total cellular RNA was purified using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. For mRNA analysis, reverse transcription (RT) was performed using High Capacity RNA-to-cDNA RMaster Mix (Applied Biosystems) in a 20-μl reaction mix at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. The RT product was used for real-time PCR as described previously [32]. Table 1 lists primer sequences used for the study. All primer sequences were BLAST-checked (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) for specificity. Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature. Control samples of H_2O were included in PCR reactions in each experiment. Samples were normalized to parallel reactions using primers specific for the trophoblast housekeeping gene YWHAZ [33]. The fold increase relative to control samples was determined by the 2-ΔΔCT method [34].

For miRNA analysis, RT and quantitative PCR (RT-qPCR) of duplicate samples was performed using the miScript PCR system (Qiagen) following the manufacturer's instructions. Primers (miScript) were used to detect expression of miR-15a, miR-15b, miR-16, miR-424, and RNU6B. Total RNA input was normalized using RNU6B RNA as an endogenous control. Dissociation curves and control samples were included, as described above.

Small RNA Sequencing

Small RNA of 18–28 nucleotides in length was gel-purified from 10 μg of total RNA isolated from PHT cells cultured in standard or hypoxic conditions for 48 h. Small RNA libraries were prepared and sequenced at the Genome Sequencing & Analysis Core Resource at Duke University using the Genome Analyzer II (Illumina). For analysis of the sequencing data, the FASTQ files were preprocessed to remove the adapter sequences. The trimmed reads, along with the trimmed quality scores, were aligned to human reference genome using the short-read aligner Bowtie [35]. Only perfect alignments were allowed. Ensembl annotation (http://uswest.ensembl.org/index.html) of all mRNA and noncoding RNAs were used to annotate the aligned reads. The annotated reads were summarized to derive each miRNA count. All miRNA counts from all libraries were further normalized using the quantile normalization method.

Plasmids, Mutagenesis, Transfection, and Luciferase Assay

MicroRNA expression vectors were engineered by cloning an approximately 500-bp fragment of genomic DNA that harbored the miRNA precursor along with its flanking sequences into a pcDNA3 vector (Invitrogen). For each miRNA, we designed an miRNA sensor construct by cloning a synthetic fragment that contained three perfectly matching miRNA-responsive elements (MRE) into psiCHECK2 (Promega). For 3′UTR of putative miRNA target genes, we PCR-amplified the relevant 3′UTR sequences using human genomic DNA as a template and inserted them into psiCHECK2 at the *Xho*I/*Not*I sites. Mutations in putative MREs were performed using a site-directed, ligase-independent mutagenesis [36] and included deletion of 10–20 nucleotides that included the MRE's seed sequences within the target 3′UTR. To reduce the risk of unintended mutations, each mutated insert was confirmed by sequencing and then subcloned back into a native psiCHECK2 vector that was not subject to mutagenesis.

Luciferase reporter constructs, along with the miRNA expression constructs, were cotransfected using polyethylenimine-mediated transfection [37]. HTR-8/SVneo cells, plated in 12-well plates, were transfected with 10 ng of reporter construct along with increasing concentrations of CMV-based vectors (pcDNA3) expressing relevant miRNAs. At 48 h after transfection, the cells were lysed with passive lysis buffer (Promega), and firefly and Renilla luciferase activities were measured consecutively with the Dual Luciferase Reporter system (Promega). Renilla luciferase activity was normalized to the firefly luciferase control. The miRCURY LNA microRNA Power Inhibitor, targeting miR-424, and a nontargeting control LNA oligonucleotide were obtained from Exiqon, Inc. HTR-8/SVneo cells were cotransfected with LNA inhibitors and a luciferase reporter plasmid using DharmaFECT Duo transfection reagent (Thermo Scientific). For transfection of PHT cells after 4–8 h, designed to allow PHT attachment to culture plates, cells were incubated with 25 nM and up to 100 nM of miRNA LNA inhibitor with DharmaFECT-1 transfection reagent in OPTI-MEM I (Invitrogen) for 24 h. Cotransfections of 25 nM antagomir with miRNA luciferase reporter construct were performed using DharmaFECT Duo transfection reagent in OPTI-MEM I for 24 h. After 24 h, the medium was changed to DMEM containing 10% FBS and antibiotics, and cells and media were harvested 48 h or 72 h after the start of transfection.

TABLE 1. List of primers used in this study with their sequences.

Name	Sequence
Mutagenic primers	
TM-FGFR1-F1	AGGTCCCTCAATAAAAATGCTTCATTTATCTATGGGCTG
M-FGFR1-F1	GCTTCATTTATCTATGGGCTG
TM-FGFR1-R1	ATTTTTATTGAGGGACCTAAACTGAAAATAGGTTTAGAA
M-FGFR1-R1	AAACTGAAAATAGGTTTAGAA
TM-FGFR1-F2	TATGTTTTCATTTCTGTAGGTTTCTGAGCTAGGGATTTTTTGG
M-FGFR1-F2	TTTCTGAGCTAGGGATTTTTTGG
TM-FGFR1-R2	CCTACAGAAATGAAAACATATTGAACTTTCTTTTGTATTTAGCAGTA
M-FGFR1-R2	TTGAACTTTCTTTTGTATTTAGCAGTA
TM-MAP2K1-F1	GCAGTGCATGTGAAGCATTGAAAATGAGCATCAGAGAGTGT
M-MAP2K1-F1	TGAAAATGAGCATCAGAGAGTGT
TM-MAP2K1-R1	ATGCTTCACATGCACTGCCTGTGAAGGATCTCACAAGGC
M-MAP2K1K1-F1	CTGTGAAGGATCTCACAAGGC
Primers for miRNA cloning	
M424-1F	GATCGGATCCGCAGCTCCTGGAAATCAAAT
M424-1R	GATCGGATCCCCCAGCCTAGCCAGGAATAC
M1516-1F	GATCGGATCCGGGCACAGAATGGACTTCAG
M1516-1R	GATCCTCGAGTTGATGGCATTCAATACAATTATTA
M1516-2F	GATCGGATCCAGAACGGCCTGCAGAGATAA
M1516-2R	GATCCTCGAGTGCTTAGGTAAATCAAACACCAA
Real-time PCR primers	
FGFR1-F	GTGACTTCCACAGCCAGATG
FGFR1-R	TTCATGGATGCACTGGAGTC

Correlation Studies

PHT cells from four placentas were cultured under standard and hypoxic conditions. Total RNA samples were extracted at time 0, 6, 12, 24, 48, and 72 h and hybridized to a microarray (Agilent SurePrint G3 Human GE 8x60K; Agilent). Expression of miR-424 was measured using Agilent microRNA microarray (Human miRNA Microarray Release 16.0, 8x60K). Data represent the average of log2 expression of the FGFR1 variants and miR-424 over the samples from four placentas. The Pearson correlation between the averaged log2 expression of FGFR1 variant 1 and the averaged log2 expression of miR424 is −0.7446, with a P value of 0.008591. The Pearson correlation between the averaged log2 expression of FGFR1 variant 6 and averaged log2 expression of miR424 is −0.7626, with a P value of 0.006341.

Western Immunoblotting

Cells were lysed in Cell Culture Lysis Reagent (Promega) supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific). Lysates were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes using standard procedures. Membranes were immunoblotted with a mouse monoclonal anti-FGFR1 antibody (MAB658; 0.5 mg/ml used at 1:500 dilution; R&D Systems) that recognizes most human FGFR1 isoforms. A goat anti-mouse conjugated with horseradish peroxidase (115-035-146; Jackson ImmunoResearch) was used as a secondary antibody. For normalization, the same membrane was immunoblotted with anti-actin antibody (MAB1501; EMD Millipore). The blots were washed and processed for chemiluminescence using SuperSignal West Dura (Thermo Scientific) and densitometrically quantified with VisionWorks LS software (version 6.6a; UVP BioImaging).

Statistics

All experiments were repeated at least three times. All data were analyzed using linear mixed-effect models, with the factor of interest as the fixed effect, and a single or nested random effect to represent the clustered structures. For each experiment, one or a few comparisons were preplanned to address a priori biological questions and tested by applying the Student t-test to the corresponding contrasts of the estimated coefficients of the fixed effects of the relevant linear mixed-effect models. No post hoc tests were performed. Significance level for each comparison was set at 0.05. For RT-qPCR data, the statistical analyses were done on the Ct values. For the microarray data, the robust multiarray average method [38], as employed in R-package AgiMicroRna [39], was used to obtain the summarized and normalized miRNA expression level. The statistical analyses of the miRNA and gene expression microarray data were done on the log2-transformed expression levels.

RESULTS

Expression of miR-424 in Hypoxic Human Trophoblasts

Previous analysis of miRNA profiles in PHT cultured in standard conditions ($O_2 = 20\%$) or hypoxic conditions ($O_2 < 1\%$) suggested reduced expression of miR-424 in response to 48 h of hypoxia [18]. Because the effect of hypoxia on miR-424 seemed inconsistent among various cell types [23], we analyzed the expression of trophoblastic miR-424 using deep sequencing of miRNAs in PHTs cultured in standard or hypoxic conditions and confirmed the reduced accumulation of miR-424 in hypoxic trophoblasts (Fig. 1A). To further detail miR-424 expression patterns, we used RT-qPCR to assess its expression over a period of 72 h in both standard conditions and hypoxia. FGFR1 is present as multiple splice variants that are not entirely characterized [40]. Therefore, primer pairs for FGFR1 were designed to anneal within common regions of the multiple transcripts of FGFR1. We found that miR-424 expression was relatively stable in PHTs during the first 24 h of culture; however, it was upregulated after 48 h of culture in standard conditions but not in hypoxia (Fig. 1B). To assess whether the effect of hypoxia was observed in other cell types, we examined miR-424 expression in other cells exposed to hypoxic conditions, including a choriocarcinoma cell line (JEG-3) and two models of endothelial cells: ECFC and HUVEC. Unlike PHT cells, these cells exhibited increased expression of miR-424 in hypoxia (Fig. 1C), consistent with a previous study reporting the induction of miR-424 by hypoxia in endothelial cells [23]. Thus, the relative reduction in miR-424 expression upon exposure to hypoxia seems to be a unique feature of placental PHTs.

We next sought to determine whether miRNA genes that are localized near miR-424 on chromosome X—genes that may constitute a unique polycistronic cluster (miR-503, mir-542-5p, mir-542-3p, mir-450a, mir-450b-5p, and mir-450b-3p) [21]—exhibit the same expression pattern as miR-424 in trophoblasts. Because some of these miRNA genes express two mature miRNAs, we report the results of the most abundant strand from each stem loop. We found that the expression pattern of miR-503 in hypoxia was similar to that of miR-424 (Fig. 1D), likely reflecting the fact that they are both derived from a unique common polycistronic precursor on the X-chromosome [21]. The other tested miRNAs were expressed at a markedly lower level and exhibited a pattern that was clearly distinct from that of miR-424 (Fig. 1D). The most distal species in this region, miR-450b, was below detection levels in PHT cells.

Expression of miR-424 During Trophoblast Differentiation

PHT cells isolated from term third-trimester placentas are mostly cytotrophoblasts, and they differentiate into syncytiotrophoblasts in vitro within 48–72 h in standard culture conditions. This differentiation is severely impaired when cells are cultured in low oxygen conditions [28, 41, 42]. To determine whether an altered differentiation state, even without hypoxia, reduces miR-424 expression, we measured the expression of miR-424 in primary trophoblasts grown in the presence of 1.5% dimethyl sulfoxide (DMSO), which is known to hinder trophoblast differentiation [43, 44]. A medium level of hCG in DMSO-exposed cells confirmed the attenuation of differentiation by DMSO (Fig. 2). We observed a substantial reduction of miR-424 expression in DMSO-exposed cells as compared to that of cells exposed to vehicle alone. No further decrease of miR-424 expression was observed when cells were exposed to hypoxia in addition to DMSO, supporting the conclusion that the reduction of miR-424 levels in hypoxic trophoblasts is to be attributed to abrogated differentiation of PHT cells in these cultures' conditions.

Targeting of MAP2K1 and FGFR1 by miR-424 in Trophoblasts

In our previous study, we reported FGFR1 as a potential target of miR-424 [18]. The TargetScan algorithm predicts three MREs within the 3′UTR of FGFR1. The two 5′-proximal sites overlap and were, therefore, considered as one site in subsequent experiments (Fig. 3A). We found that inactivation of the proximal conserved binding site (labeled 1 and 1′ in Fig. 3A) completely abolished the silencing by miR-424, while mutation in the 3′-end nonconserved element (site 2) had no effect on the reporter gene activity (Fig. 3B). These data confirmed the functional interaction between FGFR1 and miR-424 and indicated that the silencing of FGFR1 by miR-424 in HTR8/SVneo cells is entirely mediated by the conserved element. Interestingly, our data previously suggested that MAP2K1 might also be a target for miR-424. However, we noticed several sequence variations in the MAP2K1-3′UTR cDNA (Fig. 3C). Sequencing of additional MAP2K1 clones revealed two types of sequences, with one type corresponding to the true MAP2K1 and the other type showing a perfect match with MAP2K1P1, a pseudogene that is likely to have arisen by retrotransposition of a Map2k1 transcript [45]. Surprisingly, miR-424 had a weak yet consistent repressive

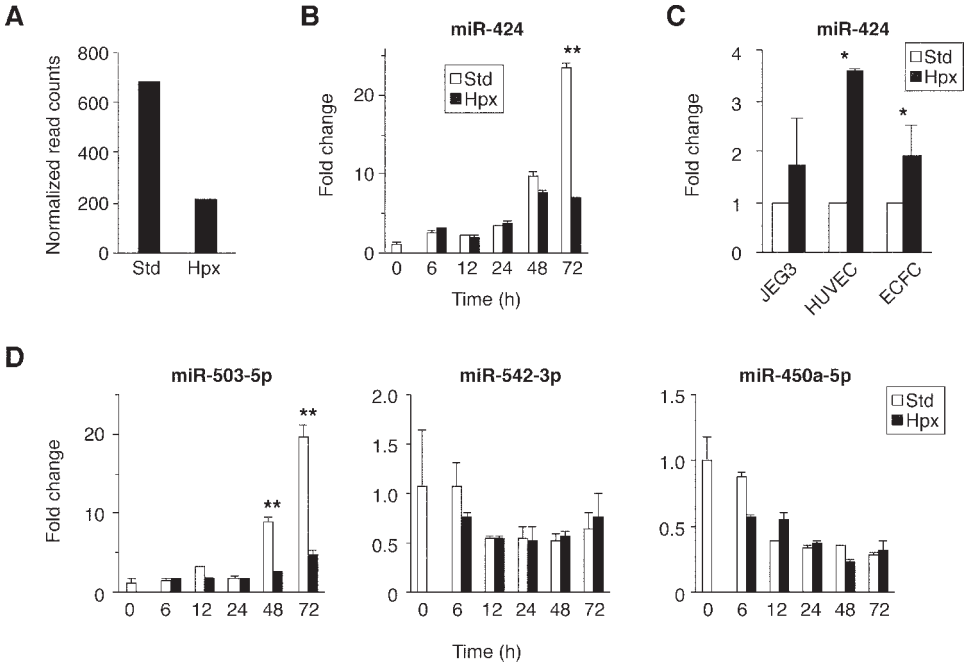

FIG. 1. MicroRNA expression in PHTs cultured in standard condition (Std) and hypoxia (Hpx). **A**) Expression level of miR-424-5p based on read counts from high-throughput sequencing (n = 1). Cells were cultured for 48 h in normoxia or hypoxia before RNA extraction. Two libraries of small RNAs were derived from these samples and deep-sequenced using an Illumina GAII analyzer. **B**) Time course of miR-424 expression in PHT cells cultured for 72 h in standard conditions compared to hypoxia and based on RT-qPCR. One representative of four experiments is shown. Error bars indicate the variability between two technical replicates. Data were normalized using U6 small nuclear RNA as an endogenous control. Statistical analysis was performed based on all independent experiments. $^{**}P < 0.01$ compared with the standard group. Note that miR-424 expression levels in standard conditions were also significantly ($P < 0.01$) elevated at 72 h compared to t = 0. **C**) Expression levels of miR-424 in JEG3, HUVEC, or ECFC cells. Data are mean ± SEM of three independent experiments, and each is measured in duplicate. Statistical analysis was performed on three independent experiments and is shown on a representative graph. $^{**}P < 0.01$ compared with standard conditions. **D**) Expression levels of the miR-424-clustered miRNAs in PHT cells cultured for 72 h in standard conditions compared to hypoxia. $^{*}P < 0.05$ compared with standard conditions.

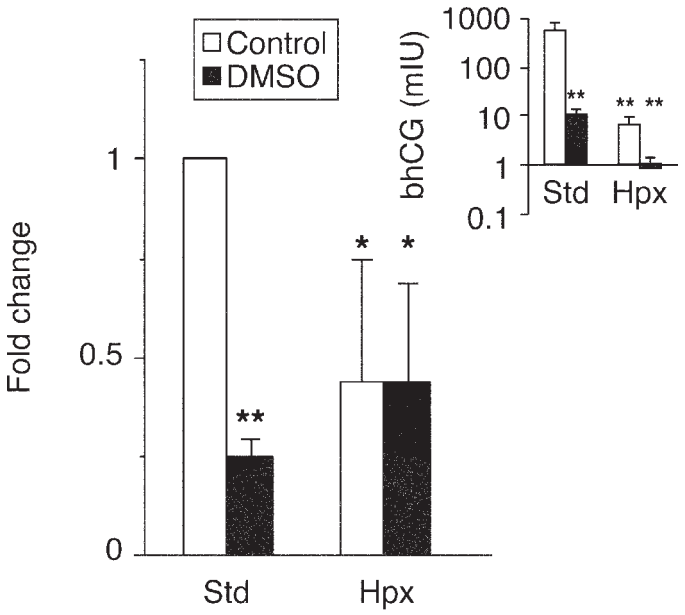

FIG. 2. Trophoblast differentiation enhances the expression of miR-424. RT-qPCR analysis of miR-424 expression in PHT cells cultured for 48 h in standard conditions (Std) or hypoxia (Hpx) and in the absence or presence of DMSO. Data were normalized using U6 small nuclear RNA as an endogenous control. Inset: the concentration of hCG released in the medium, measured by ELISA as described in *Materials and Methods*. Data represent the geometric mean of three independent experiments, each performed in duplicate and plotted on a logarithmic scale. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control in the standard conditions group.

effect on the true MAP2K1 sequence in HTR8/SVneo cells (Fig. 3D, left panel). In contrast, whereas MAP2K1P1 was repressed by miR-424 at a level comparable to other validated targets (Fig. 3D, right panel), a mutation of the putative miR-424 element on MAP2K1P1 did not abrogate the repressive effect (Fig. 3D), suggesting that a further downstream perfect seed match element may mediate the silencing of our MAP2K1P1 reporter.

Because expressed pseudogenes have the potential to act as miRNA decoys [46], we investigated the expression status of MAP2K1P1 in PHT cells. Using RT-qPCR, we detected only the expression of MAP2K1 but not the pseudogene transcripts, indicating that the pseudogene MAP2K1P1 is not expressed in these cells. To further confirm that miR-424 can target FGFR1, we used a locked nucleic acid-based miR-424 inhibitor to repress endogenous miR-424 levels in the human trophoblast cell line HTR8/SVneo. As shown in Figure 4, luciferase activity from an FGFR1 luciferase reporter plasmid was enhanced proportionally to the amount of transfected miR-424 inhibitor, with no effect by the control (scrambled) oligonucleotide.

Lastly, we tested whether members of the miR15/16 family, which share a seed element with miR-424, can also regulate the FGFR1 reporter. Although miR-15 and miR-16 silenced FGFR1 luciferase reporter similarly to miR-424, and this effect was also primarily mediated by the conserved proximal element of FGFR1 3′UTR (Fig. 5A), we found that the expression of miR-15/16 species, while abundant in trophoblasts, is not altered by hypoxia (Fig. 5B). We noted a modest increase in luciferase activity of FGFR-Δ2 mutant when co-transfected with miR-15b/16.2 (Fig. 5A, right panel). This

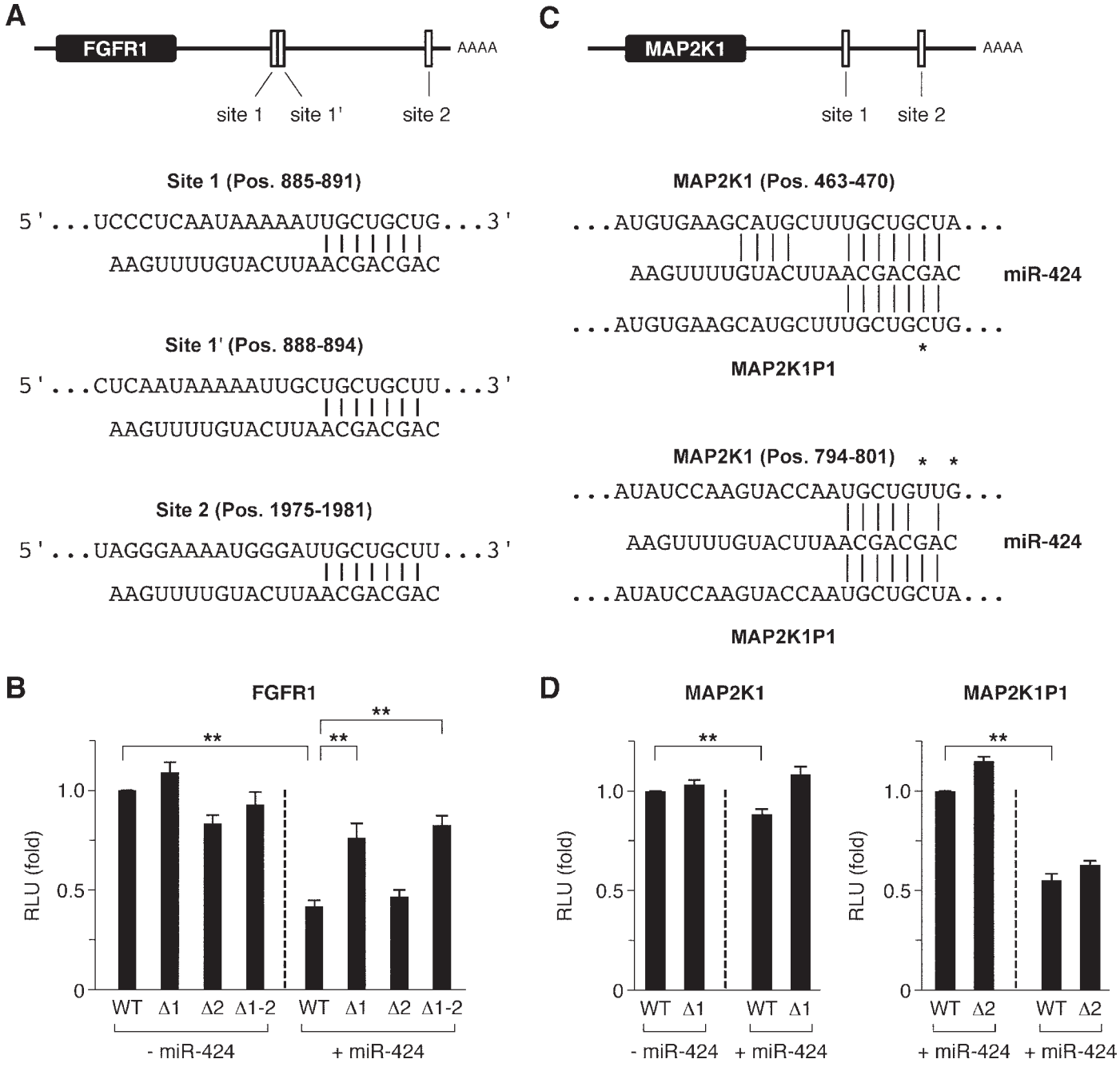

FIG. 3. MicroR-424 targets a discrete 3′UTR sequence in FGFR1 and MAP2K1 genes. **A**) Schematic representation of the predicted miR-424 MREs within the 3′UTR of the FGFR1 mRNA. Alignments between the miR-424 binding sites and miR-424 are shown. **B**) Deletion analysis of FGFR1 3′UTR. HTR8/SVneo cells were transfected with the wild-type FGFR1-3′UTR reporter (WT) or with a promoter that harbors a mutation in each of the two miR-424 binding sites, alone (Δ1 or Δ2) or in combination (Δ1,2). Each reporter was transfected along with an empty vector (pcDNA3) or a vector expressing miR-424. **C**) Schematic representation of a conserved miR-424 MRE within MAP2K1 (site 1) and of a putative miR-424 8-mer site (site 2) in the sequence of MAP2K1P1. Alignments between miR-424 and MAP2K1 and MAP2K1P1 are shown. Asterisks indicate the diverging nucleotides in the seed region between the sequences of MAP2K1 and MAP2K1P1. **D**) Luciferase reporter assay using MAP2K1-3′UTR and MAP2K1P1 reporter that harbors a mutation in each of putative binding sites (Δ1 or Δ2) or wild-type (WT) MRE. Relative luciferase unit (RLU) activity from each reporter, normalized to firefly luciferase activity, was determined 48 h later. Experiments using MAP2K1 (left panel) were performed seven times (n = 7), and statistical significance was determined using a linear mixed-effects model with the experiment batch as the random effect. Using this method, MAP2K1 WT was repressed in the presence of transfected miR-424 by about 17.45%, with $P = 3.28 \times 10^{-14}$ (**). Data derived from the MAP2K1P1 construct are geometric means of three independent experiments performed in duplicate. Statistical significance was determined by the Student *t*-test (SEM). $^{*}P < 0.01$.

effect was modest, yet may represent a weak synergistic action of miR-15/16 activity on that site. Together, these data suggest that miR-424 or the miR15/16 family has the ability to modulate the expression of FGFR1 in PHT cells, but only miR-424 controls FGFR1 expression in altered differentiation or in hypoxia.

FGFR1 Expression Is Increased in Hypoxic Trophoblasts

Having confirmed the functional interaction between FGFR1 reporter and miR-424 in a trophoblast cell line, we sought to interrogate this interaction in PHT cells. As predicted, both hypoxia and exposure to DMSO increased in

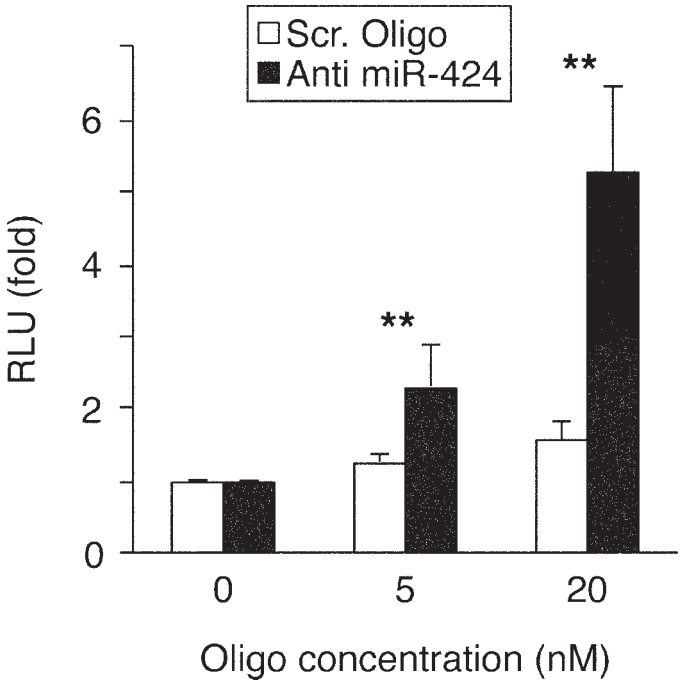

FIG. 4. Inhibition of miR-424 derepresses FGFR1 expression. Luciferase assay of HTR8/SVneo cells cotransfected with FGFR1-luciferase reporter and either anti-miR-424 or a scrambled-sequence oligonucleotide as control. RLU activity from each reporter construct, normalized to firefly luciferase activity, was determined 48 h later. Data are geometric means of three independent experiments performed in duplicate. $^{**}P < 0.01$ compared with the control group (scrambled [Scr.] oligo).

the accumulation of FGFR1 transcripts (Fig. 6A). Similarly, we observed a twofold increase of FGFR1 protein in PHT cells exposed to hypoxia or DMSO (Fig. 6B). To further document the inverse relationship between miR-424 and FGFR1 in trophoblasts, we performed an analysis of the correlation between expression levels of miR-15/16/424 and several isoforms of FGFR1. Using microarray data from experiments in which we analyzed four sets of PHTs cultured in standard conditions or hypoxia across different time points (0, 12, 24, 48, and 72 h), we found a statistically strong negative correlation between FGFR1 transcripts and miR-424, but not miR-15/16 (Fig. 7).

DISCUSSION

Placental trophoblasts express common, as well as unique, patterns of miRNAs. Although not restricted to trophoblasts, miR-424 is highly abundant in the placenta, as are with several members of the miR-16 family of miRNAs. In this study, we found that the expression of miR-424 directly correlates with the differentiation of trophoblasts. The upregulation of miR-424 as cells differentiate in vitro is completely abolished when differentiation into syncytiotrophoblasts is abrogated by hypoxia or exposure to DMSO. In addition, we found that downregulation of miR-424 in cells exposed to hypoxia is specific to primary trophoblasts and was not observed in a trophoblast cell line (JEG-3) or in either of two models of endothelial cells (HUVEC and ECFC), in which miR-424 was upregulated, as shown by us and others [23]. Interestingly, we recently reported that miR-424 was upregulated in the plasma of pregnant women with fetal growth restriction, compared to

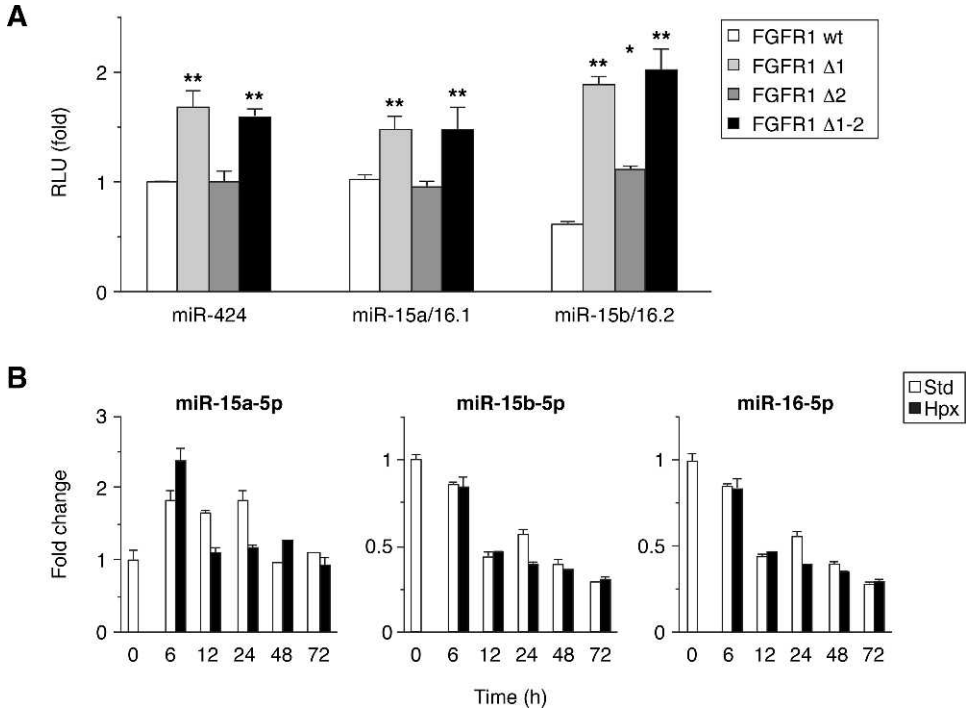

FIG. 5. Silencing of FGFR1 by members of the miR-15/16 family. **A**) Activity of wild-type and mutant FGFR1-3′UTR reporter genes in response to expression of members of the miR-15/16 family of miRNAs. HTR8/SVneo cells were transfected with FGFR1 reporters along with an miR-424 or miR-15/16 expression vector. RLU activity from each reporter construct, normalized to firefly luciferase activity, was determined 48 h later. Data are geometric means of three independent experiments performed in duplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group for each miRNA expression vector (FGFR1 wt). **B**) Expression levels of some members of the miR-424 family in PHT cells cultured for 72 h in standard conditions (Std) compared to hypoxia (Hpx). One representative of four experiments is shown, with error bars indicating the variability between two technical replicates. Data were normalized using U6 small nuclear RNA as an endogenous control.

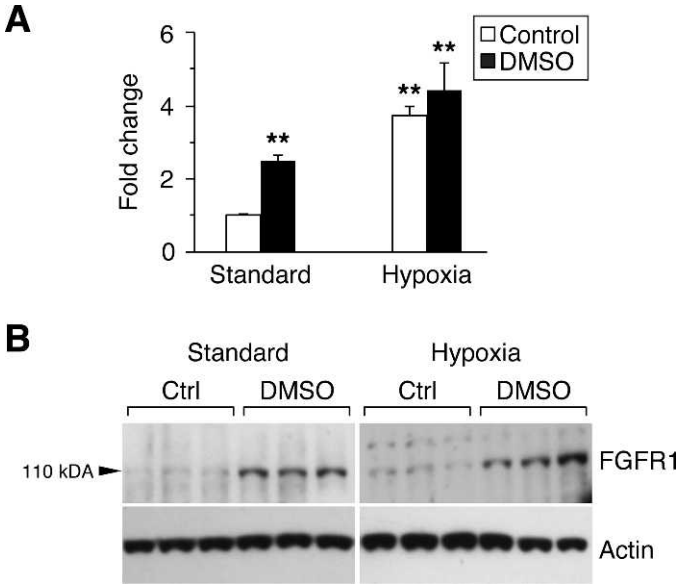

FIG. 6. The effect of the differentiation state on the expression of FGFR1 in PHT cells. **A**) RT-qPCR analysis of FGFR1 expression in PHT cells cultured in hypoxia and exposed to DMSO for 72 h. Data represent the geometric mean of three independent experiments performed in duplicate. **B**) Impaired trophoblast differentiation by hypoxia or DMSO enhances FGFR1 expression. Western blot analysis of FGFR1 in three sets of PHT cells that were cultured for 72 h in standard conditions, in hypoxia in complete medium (Ctrl), or in complete medium containing 1.5% of DMSO. $^{**}P < 0.01$ compared with the control group in standard conditions.

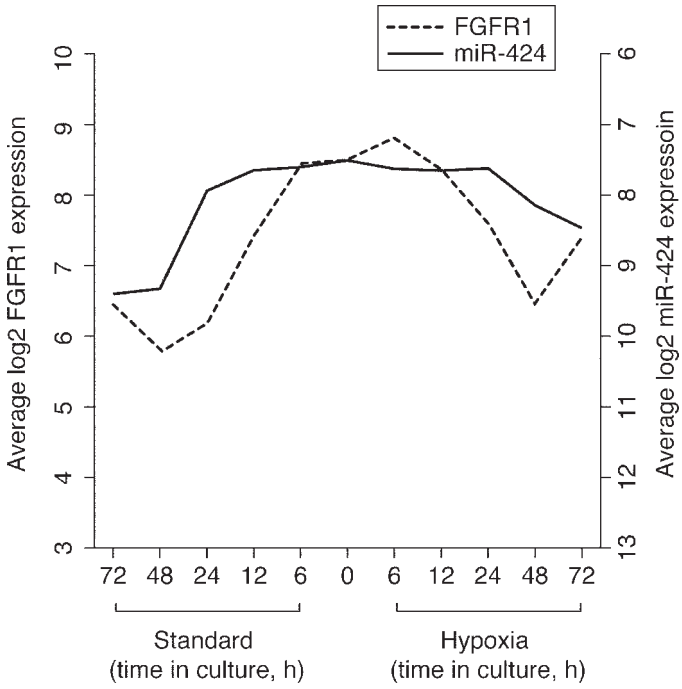

FIG. 7. Inverse correlation between FGFR1 mRNA and miR-424 in PHT cells. FGFR1 (most abundant transcript) or miR-424 was detected using microarray, and log2-transformed expression was plotted against the log2-transformed expression of miR-424 (note the inverted axes, designed to illustrate the expression pattern). Data represent the average of log2 expression of the FGFR1 variants and miR-424 for samples derived from four placentas. The Pearson correlation between the averaged log2 expression of FGFR1 and the averaged log2 expression of miR424 is -0.7446 ($P < 0.01$).

controls [16]. This observation was unexpected, because fetal growth restriction is commonly associated with placental insufficiency and cellular hypoxia. However, our past observation could be explained by changes in maternal endothelial cells. It will be interesting to dissect the molecular mechanisms underlying the difference in miRNA response to hypoxia in PHT and endothelial cells.

We also found that the pattern of miR-424 expression in trophoblasts is specific to miR-424 and its clustered partner miR-503. Analysis of several miRNAs located in close genomic proximity, including miR-542 and members of the miR-450 family, did not reveal significant changes when cells were exposed to hypoxia. Similarly, miRNAs that share the same seed sequence of the miR-424 family, including miR-16 and miR-15, were unchanged by hypoxia, while miR-195 and miR-497 were almost undetected in trophoblasts.

We defined the molecular interaction of miR-424 with 3′UTR sites within the FGFR1 gene, showing that only the conserved binding site proximal to the stop codon mediates the silencing activity of miR-424. The distal, less conserved site did not play a role in miR-424 action. Interestingly, the proximal site also mediated the suppressive activity of miR-16 and miR-15. Two recent studies have also reported the targeting of FGFR1 by miR-424 [47, 48], further confirming FGFR1 as a target of miR-424. Expression of FGF receptors, including FGFR1, has been confirmed in the human placenta, where it is postulated to play a role in villous development [49, 50].

FGFR1 has a complex genomic organization and has been implicated throughout development in many signaling pathways controlling cellular proliferation, differentiation, survival, and angiogenesis [51]. Deregulated FGF signaling is also common in many types of cancers, and FGFR1 amplification has also been reported in breast cancers [52], oral squamous carcinoma [53], ovarian cancer [54], bladder cancer [55], and rhabdomyosarcoma [56]. To date we have not been able to silence FGFR1 expression in PHTs, likely reflecting suboptimal silencing efficiency in PHTs. Additionally, FGFR1 is highly regulated posttranscriptionally, resulting in many splicing variants [57], and the precise genomic context of the miR-424 binding elements(s) remains to be validated. In the mouse, deletion of FGFR1 results in embryonic lethality around Day 7.5 to 9.5 (E7.5 to E9.5), preventing a detailed analysis of its role in the placenta [58, 59]. In humans, inactivating mutations in the FGFR1 gene are associated with the Kallmann syndrome, a rare genetic disorder characterized by hypogonadism, infertility, and anosmia [60]. Kim et al. [48] recently showed that miR-424 and miR-503 regulate FGF signaling in the lungs by targeting FGFR1 and the ligand FGF2. We also tested the 3′UTR of FGF2 in our system and observed a repression of the luciferase activity in cells cotransfected with miR-424 (data not shown). Together, these observations support the relevance of miR-424 to FGF signaling, which probably plays a role in placental development and adaptation to stress.

Lastly, we noted a weak effect of miR-424 on MAP2K1 reporter, with a more potent effect on the pseudogene MAP2K1P1. It is unclear whether this low repression effect results from suboptimal experimental conditions or is a feature of the pair MAP2K1-miR-424 in trophoblasts. It was recently shown that MAP2K1 is a target of miR-424 in senile hemangioma [61] and that MAP2K1 can be silenced by miR-497, which harbors the same seed sequence as miR-424 [62]. Altogether, these findings strongly suggest that MAP2K1 is

probably a genuine target of this family of miRNA, yet is unlikely to be relevant to trophoblastic miR-424.

An interesting finding that will need further investigation is the potential interplay between miR-424 and other members of the family, particularly miR-15 and miR-16. We showed that these miRNAs have the ability to repress an FGFR1 reporter. These miRNAs are normally abundant in trophoblasts, but their levels did not change in the conditions tested; therefore, it is unlikely that they affect FGFR1 during the process of differentiation or response to hypoxia. However, we cannot rule out the possibility that, in other circumstances, miR-15/16 could play a role in the regulation of FGFR1.

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