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Research Article

NRF2 Serves a Critical Role in Regulation of Immune Checkpoint Proteins (ICPs) During Trophoblast Differentiation

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Abstract

Using cultured human trophoblast stem cells (hTSCs), mid-gestation human trophoblasts in primary culture, and gene-targeted mice, we tested the hypothesis that the multinucleated syncytiotrophoblast (SynT) serves a critical role in pregnancy maintenance through production of key immune modulators/checkpoint proteins (ICPs) under control of the O_2-regulated transcription factor, NRF2/NFE2L2. These ICPs potentially act at the maternal-fetal interface to protect the hemiallogeneic fetus from rejection by the maternal immune system. Using cultured hTSCs, we observed that several ICPs involved in the induction and maintenance of immune tolerance were markedly upregulated during differentiation of cytotrophoblasts (CytTs) to SynT. These included HMOX1, kynurenine receptor, aryl hydrocarbon receptor, PD-L1, and GDF15. Intriguingly, NRF2, C/EBPβ, and PPARγ were markedly induced when CytTs fused to form SynT in a 20% O_2 environment. Notably, when hTSCs were cultured in a hypoxic (2% O_2) environment, SynT fusion and the differentiation-associated induction of NRF2, C/EBPβ, aromatase (CYP19A1; SynT differentiation marker), and ICPs were blocked. NRF2 knockdown also prevented induction of aromatase, C/EBPβ and the previously mentioned ICPs. Chromatin immunoprecipitation-quantitative PCR revealed that temporal induction of the ICPs in hTSCs and mid-gestation human trophoblasts cultured in 20% O_2 was associated with increased binding of endogenous NRF2 to putative response elements within their promoters. Moreover, placentas of 12.5 days postcoitum mice with a global *Nrf2* knockout manifested decreased mRNA expression of C/ebpβ, Pparγ, Hmox1, aryl hydrocarbon receptor, and Nqo1, another direct downstream target of Nrf2, compared with wild-type mice. Collectively, these compelling findings suggest that O_2-regulated NRF2 serves as a key regulator of ICP expression during SynT differentiation.

Key Words: trophoblast, NRF2, C/EBPβ, immune modulators/checkpoint proteins (ICPs)

Abbreviations: AhR, aryl hydrocarbon receptor; BACH1, BTB domain and *cap "n" collar* homolog 1; CGB, chorionic gonadotropin subunit β; C/EBPβ, CCAAT-enhancer-binding protein β; ChIP, chromatin immunoprecipitation; Col, collagen; CyT, cytotrophoblast; dNK, decidual natural killer cell; EVT, extravillous trophoblast; GDF15, growth and differentiation factor 15; Hmox1, heme oxygenase I; hTSC, human trophoblast stem cell; ICP, immune modulators/checkpoint protein; IDO, indoleamine 2, 3-dioxygenase; Ig, immunoglobulin; KD, knockdown; NRF2, nuclear erythroid 2-related factor 2; PD-L1, programmed death-ligand 1; PPARγ, peroxisome proliferator activated receptor γ; PSG, pregnancy specific beta-1-glycoprotein; RT-qPCR, RT-quantitative PCR; SynT, syncytiotrophoblast; Treg, T regulatory cell; WT, wild-type

The placental syncytiotrophoblast (SynT) serves an essential role in pregnancy maintenance via transport of O_2 and nutrients, and synthesis of protein and steroid hormones (1). In this study, we have focused on the potential role of the SynT in sustaining pregnancy through production of immune modulators/checkpoint proteins (ICPs), to promote immune tolerance at the maternal-fetal interface/decidua and protect the fetus from rejection by the maternal immune system (2, 3). Using chromatin immunoprecipitation-sequencing, we recently found that genes manifesting increased binding of both Pol II and active histone marks during SynT differentiation were enriched for those involved in immune response and immune modulation (4).

Previously, we explored mechanisms that underlie human trophoblast differentiation and effects of O_2 tension by analyzing placenta-specific expression of the human aromatase (*hCYP19A1*) gene, which is controlled by tissue-specific promoters that lie upstream of tissue-specific first exons (5). The placenta-specific first exon of *hCYP19A1* (*exon I.1*), which encodes the unique 5′-untranslated region of its mRNA (5, 6), lies ~100 000 bp upstream of the translation initiation site in exon II. In studies using transfected cells and transgenic mice, we observed that as little as 246 bp immediately upstream of *exon 1.1* controls placenta-specific and temporal regulation of expression (7-9). Human trophoblast stem cells (hTSCs) and cytotrophoblasts (CytTs) do not express aromatase; however, when CytTs fuse to form SynT, aromatase is markedly induced (9, 10). By contrast, when CytTs are cultured in a hypoxic (2% O_2) environment, SynT

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differentiation and induction of *hCYP19A1* are prevented by increased binding of transcriptional repressors, MASH-2/ASCL2 (11) and USF1/2 (12), which are degraded upon SynT differentiation in 20% O_2 (13). This allows assembly of an enhanceosome comprising transcription factors Sp1 (9), estrogen receptor α (14), estrogen-related receptor γ (15) and glial cells missing 1 (16, 17), which serves a crucial role in placental development and SynT fusion (18, 19). Interestingly, this complex also contains the redox-regulated transcription factor, nuclear erythroid 2-related factor 2 (NRF2/NFE2L2), which is essential for O_2-mediated aromatase mRNA induction in cultured human trophoblasts (20).

Intriguingly, we discovered that NRF2 and coregulated transcription factors, CCAAT-enhancer-binding protein β (C/EBPβ) and peroxisome proliferator activated receptor γ (PPARγ), were markedly induced when CytTs fuse to form SynTs (20). NRF2 knockdown (KD) blocked the induction of *hCYP19A1*, as well as the upregulation of C/EBPβ and PPARγ (20). NRF2 deficiency has been implicated in preeclampsia (20-22), a hypertensive disorder of pregnancy associated with shallow implantation, placental hypoxia, and inflammation (23-26). In mice, *C/ebpα* and *C/ebpβ* are functionally redundant; targeted deletion of both genes caused arrest of embryonic development at embryonic day 10 (E10), due to defects in placental labyrinth formation (27). *Pparγ* null mouse embryos also die at E10, because of pronounced labyrinth defects (28), whereas *Pparγ* null mouse TSC fail to differentiate into SynT (29). Pparγ expression in mouse TSC was inhibited by hypoxia in a hypoxia-inducible factor-independent manner, suggesting the role of other redox-regulated factors (30), such as Nrf2. Mice with a global knockout of *Nrf2* were reported to manifest impaired fetal and placental growth, reduced labyrinth (31), increased placental inflammation, oxidative stress, and susceptibility to preterm labor induction (32).

NRF2, described as a "primary mediator of cellular adaptation to oxidative stress," is a *cap "n" collar*, bZIP transcription factor that interacts with small MAF proteins and binds to antioxidant response elements in promoters of antioxidant genes (eg, *HMOX1*, *MnSOD*) (33). NRF2 is regulated at different levels in response to changes in cellular redox state. At relatively low O_2 tension, NRF2 is repressed because of its covalent interaction in the cytoplasm with KEAP1, a cysteine-rich adaptor that facilitates NRF2 ubiquitination and degradation by the 26S proteasome (34). We observed that NRF2 was markedly upregulated during differentiation of human trophoblasts cultured in 20% O_2; however, NRF2 induction was prevented when CytT were cultured in a hypoxic environment (20).

In the present study, using hTSCs and human trophoblasts in primary culture, we observed that several ICPs involved in the induction and maintenance of immune tolerance were markedly upregulated during differentiation of CytT to SynT. These included: heme oxygenase I (HMOX1); the kynurenine receptor, aryl hydrocarbon receptor (AhR); programmed death-ligand 1 (PD-L1), and growth and differentiation factor 15 (GDF15), which we found to be secreted into the culture medium with SynT differentiation. Induction of these genes was dependent on expression and binding of NRF2. We also found that placentas of mice with a global deficiency in *Nrf2* were significantly smaller than wild-type (WT) at 10.5 and 12.5 days postcoitum and expressed decreased levels of C/ebpβ, Pparγ, Hmox1, and Ahr mRNA. The *Nrf2*-deficient fetuses also were significantly smaller in size. These and other findings have led us to hypothesize that O_2-regulated NRF2, and coregulated C/EBPβ and PPARγ, serve as key modulators of ICP expression during SynT differentiation. We posit that these ICPs are secreted into maternal blood within the intervillous space, or directly into the decidua, where they act on immune cells to maintain maternal tolerance to the hemiallogeneic fetus. We also propose that decreased placental expression of ICPs during late gestation contributes to a physiological "break" in maternal-fetal tolerance leading to labor at term, whereas their premature decline can lead to preterm parturition or preeclampsia.

Materials and Methods

Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. *Nfe2l2*^{tm1Ywk} (*NRF2*) knockout mice (stock number: 017009) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice at 6 to 8 weeks of age were time-mated overnight and pregnant mice were euthanized by isoflurane inhalation and cervical dislocation at 12.5 days postcoitum. After the fetuses were removed from their amniotic sacs together with their placentas, the decidual layers were removed; placentas were harvested and frozen in liquid nitrogen for subsequent RNA isolation. Placentas from fetuses of both sexes were used for this study.

Differentiation of hTSCs to SynT

hTSCs were generously provided by Drs. Hiroaki Okae and Takahiro Arima (Tohoku University Graduate School of Medicine, Sendai, Japan) and cultured as described (35). Briefly, a 100-mm culture dish or 6-well plate was precoated with 5 μg/mL Collagen (Col) IV (Corning, Corning, NY) for at least 1 hour at 37°C and washed twice with PBS. Stem cells were maintained in basal hTSC medium (DMEM/F12 supplemented with 0.1 mM 2-mercaptoethanol, 0.2% fetal bovine serum, 0.5% penicillin-streptomycin, 0.3% BSA, 1% ITS-X supplement, 1.5 μg/ml L-ascorbic acid, 50 ng/mL EGF, 2 μM CHIR99021, 0.5 μM A83-01, 1 μM SB431542, 0.8 mM VPA, and 5 μM Y27632) at 37°C in 5% CO_2. When cells reached 80% confluence, they were detached with TrypLE for 15 minutes at 37 °C and passaged to new Col IV-coated 100-mm plates at 1 to 2 × 10^5 cells per plate.

To promote SynT differentiation, hTSCs were seeded at a density of 1.5 × 10^5 cells per 6-well plate or 1 × 10^6 cells per 100-mm dish and cultured in SynT medium (DMEM/F12 supplemented with 0.1 mM 2-mercaptoethanol, 0.5% Penicillin-Streptomycin, 0.3% BSA, 1% ITS-X supplement, 2.5 μM Y27632, 2 μM forskolin, and 4% knockout serum replacement) at 20% O_2 in a standard CO_2 incubator, or at 2% O_2 in a HypOxystation (5% CO_2, 93% N_2). Fresh SynT medium was replaced on day 3 and the cells were harvested for RNA and protein analysis at 1, 3, and/or 5 days of culture. Before SynT differentiation, hTSCs were also harvested at (0 days), which is considered as CytT control.

Transient Transfection

RNAiMAX transfection reagent (Life Technologies) was used for NRF2 loss-of-function experiments according to the manufacturer's instructions. Briefly, 1.5 × 10^5 cells in 2 mL of basal hTSC culture medium were seeded onto Col IV precoated-6-well plate and transfected with On-TARGET*plus*

nontargeting control siRNA or siRNA against human *NFE2L2* (NRF2) (Dharmacon, Lafayette, CO) at a final concentration of 50 nM. After 24 hours, medium was replaced with fresh SynT differentiating medium and retransfected with the same concentration of siRNA. At 3 and 5 days posttransfection, cells were harvested for subsequent RNA analysis.

RT quantitative PCR

Total RNA was purified using QIAzol lysis reagent and a miRNeasy Mini Kit (Qiagen, Valencia, CA). The relative abundance of each transcript was deter1mined by RT-quantitative PCR (RT-qPCR) using SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA) or TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA). Mouse IL-1β TaqMan primer (Mm00434228_m1) and mouse 36B4 (*Rplp0*) TaqMan primer (Mm01974474-gH) were purchased from Thermo Fisher Scientific. All other primer sets used for RT-qPCR are listed in Table 1. The relative fold changes were calculated using the comparative cycle times method with 36B4 as an internal control on a Bio-Rad CFX384 real-time PCR detection system.

Western Blot Analysis

Nuclear and cytoplasmic extracts were prepared from hTSCs using a NE-PER extraction reagent kit (Pierce, Rockford, IL). Protein concentrations were determined by Pierce BCA Protein assay kit (Thermo Fisher Scientific) and equal amounts of protein (20-50 µg/lane) were loaded on a NuPAGE 4% to 12% Bis-Tris protein gel (Thermo Fisher Scientific). The following antibodies were used: rabbit anti-NRF2 antibody (ab62352, Abcam, Cambridge, UK) (36), goat anti-Keap1 antibody (ab166721, Abcam) (37), rabbit anti-C/EBPβ antibody (ab32358, Abcam) (38), mouse anti-PPARγ antibody (clone E-8; sc-7273, Santa Cruz Biotechnology) (39), rabbit anti-HMOX1 antibody (catalog no. 70081, Cell Signaling, Denver, MA) (40), rabbit anti-AhR antibody (catalog no. 83200, Cell Signaling) (41), rabbit anti-PD-L1 (catalog no. 13684, Cell Signaling) (42), and mouse anti-GDF15 antibody (clone G-5; sc-377195, Santa Cruz Biotechnology) (43). Anti-histone H3 antibody (catalog no. 17-168-1-AP; Proteintech, Rosemont, IL) (44) and anti-β-actin (ab8227, Abcam) (45) were used as loading controls for nuclear and cytoplasmic proteins, respectively. Horseradish peroxidase–conjugated anti-rabbit, anti-goat, and anti-mouse immunoglobulins (Igs) (Santa Cruz Biotechnology) (46-48) were used as secondary antibodies. The polyvinylidene fluoride membranes were incubated with enhanced SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) and exposed to X-ray film.

Human GDF15 ELISA

hTSCs were cultured at a density of 0.6×10^5 cells per 12-well plate in SynT differentiation medium in an atmosphere of 20% O_2 or 2% O_2; the supernatants were collected before (0 days) and after 5 days of culture. The levels of human GDF15 in the culture media were quantified using a human GDF15 Quantikine ELISA kit (DGD150, R&D Systems, Abingdon, UK) (49) according to the manufacturer's instructions.

Chromatin immunoprecipitation-qPCR

Cells were treated with 1% formaldehyde on a 100-mm culture plate to crosslink proteins and DNA; the reaction was

Table 1. Primer sequences used for RT-qPCR

Gene name	Forward (5′ to 3′)	Reverse (5′ to 3′)
Human		
36B4	TGCATCAGTACCCCATTCTATCA	AAGGTGTAATCCGTCTCCACAGA
NRF2	GTGGATCTGCCAACTACTCCC	TACAAACGGGAATGTCTGCG
Aromatase	ACGGAAGGTCCTGTGCTCG	GTATCGGGTTCAGCATTTCCA
CGB3	CGAGGTATAAAGCCAGGTACAC	CTCCTTGGATGCCCATGTC
PSG4	CAGAGGAGAACACACAAGCA	GGGCGGATTCCAGAAGTTTA
C/EBPβ	TTCATGCAACGCCTGGT	TCCGCCTCGTAGTAGAAGTT
PPARγ	GTCGTGTCTGTGGAGATAAA	ACCTGATGGCATTATGAGAC
Hmox1	GGTGAAGAAGAGGCCAAGACT	GCAGAATCTTGCACTTTGTTGCT
AhR	CATACCGAAGACCGAGCTGA	TCATTGCCAGAAAACCAGATGA
GDF15	ATGCACGCGCAGATCAA	ATGAGCACCATGGGATTGTAG
PD-L1	TCAATGCCCCATACAACAAA	GCTTGTCCAGATGACTTCG
IDO1	GGCTTTGCTCTGCCAAATCC	TTCTCAACTCTTTCTCGAAGCTG
Mouse		
36B4	CACTGGTCTAGGACCCGAGAAG	GGTGCCTCTGGAGATTTTCG
NRF2	CTCCGTGGAGTCTTCCATTTAC	GCACTATCTAGCTCCTCCATTTC
C/EBPβ	ATCGACTTCAGCCCCTACCT	TCACGTAACCGTAGTCGG
PPARγ	CAAGAATACCAAAGTGCGATCAA	GAGCTGGGTCTTTTCAGAATAATAAG
Hmox1	GTACACATCCAAGCCGAGAA	TGGTACAAGGAAGCCATCAC
AhR	GGCAGCTTATTCTGGGCTATAC	TGCCACTTTCTCCAGTCTTAATC
NQO1	GACAACGGTCCTTTCCAGAATA	CTCTGAATCGGCCAGAGAATG

Abbreviations: AhR, aryl hydrocarbon receptor; CGB, chorionic gonadotropin subunit β; C/EBPβ, CCAAT-enhancer-binding protein β; GDF15, growth and differentiation factor 15; Hmox, heme oxygenase I; IDO, indoleamine 2, 3-dioxygenase; NRF2, nuclear erythroid 2-related factor 2; PD-L1, programmed death-ligand 1; PPARγ, peroxisome proliferator activated receptor γ; PSG, pregnancy specific beta-1-glycoprotein.

stopped by adding glycine at a final concentration of 125 mM. After washing with cold PBS 2 to 3 times, cells were collected, and nuclear extracts were prepared with 1% SDS chromatin immunoprecipitation (ChIP) Lysis Buffer. The chromatin was sheared by sonication to produce DNA fragments; equivalent amounts of sheared DNA were used for immunoprecipitation with antibodies against NRF2 (Abcam) (36) or an equal amount of preimmune rabbit IgG (Millipore) (50). The same amount of crosslinked sheared DNA without antibody precipitation was processed in the same manner and served as an input control. Following immunoprecipitation, enriched DNA was purified and quantified by qPCR using the following primers: proximal *h (human) HMOX1* promoter, forward, 5′- GTCCCCTTGGGACTTGATGC-3′, reverse, 5′-GAGCCTGGGGTTGCTAAGTT-3′; proximal *hAhR* promoter,forward,5′-CCGTCTCTCAAACAGGTGAAG-3′,reverse, 5′- GGTAGCCATCTTCGTGCATAG-3′; proximal *hPD-L1* promoter, forward, 5′-ATCGGCGGAAGCTTTCAGTT-3′, reverse, 5′-TCTTCGAAACTCTTCCCGGTG-3′; proximal *hGDF15* promoter, forward, 5′-CCAGGAAGGGCAACCTTGAT-3′, reverse, 5′-CCTATGTGTCTGGCCCTGTG-3′. The qPCR was performed using SYBR Green PCR master mix (Applied Biosystems) on a Bio-Rad CFX384 real-time PCR detection system. Signals were normalized to input samples and expressed relative to that for control IgG. Samples from 3 independent ChIP experiments were analyzed for each treatment at each time point.

Morphological Analysis of Trophoblast Stem Cell Fusion

The hTSCs were seeded onto Col VI-coated Nunc Lab-Tek II Chamber slides (Thermo Fisher Scientific) in SynT differentiation medium for 5 days and fixed with 4% paraformaldehyde for 20 minutes at room temperature. After 3 washes with PBS, cells were blocked with 3% BSA in PBS and incubated with a primary anti-plakoglobin antibody (1:250, clone 15; BD Biosciences, San Jose, CA) (20, 51, 52) overnight at 4°C. Then, the cells were washed with PBS 3 times and incubated in Alexa Fluor 488-conjugated secondary antibodies (1:500, Life Technologies) (53) for 1 hour at room temperature. The chamber slides were then washed 3 times with PBS and the nuclei were stained with 4′,6-diamidino-2-phenylindole (1:2000; Sigma-Aldrich, St. Louis, MO), and mounted using Mounting Medium (Fluoroshield; Sigma-Aldrich). Images were captured using a Zeiss confocal microscope at ×400 magnification (Carl Zeiss, Oberkochen, Germany).

Statistical Analyses

Data are expressed as the mean ± standard error of the mean. Differences between groups were analyzed by 1-way ANOVA followed by a Tukey post hoc test using GraphPad Prism 7.03. Statistical significance was set as P values of < 0.05, and each experiment was performed at least 3 times.

Results

Hypoxia Impairs SynT Differentiation and Syncytialization of hTSCs

Changes in placental oxygen tension caused by spiral artery remodeling starting after 8 to 10 weeks of gestation contribute to trophoblast differentiation. During the first trimester, blood flow is low and the placenta is relatively hypoxic (1%-2% O_2) (54), promoting CytT proliferation. The oxygen gradient within the placenta during the second trimester is dramatically increased as the development of maternal spiral arterioles occurs, resulting in formation of an oxygen-rich environment to support SynT differentiation (54, 55). On the other hand, defects in these processes result in placental hypoxia, which is believed to be an underlying cause of preeclampsia. Because trophoblast differentiation is an oxygen-dependent process, we investigated the effects of low oxygen tension (2% O_2, ~14 mm Hg) on expression of SynT specific markers, and on trophoblast fusion. When hTSCs were cultured in medium containing forskolin to promote SynT differentiation in 20% O_2 (simulates O_2 milieu of trophoblasts in direct contact with maternal arterial blood, which is estimated to be 90 to 100 mm Hg (54)), SynT-specific markers, including aromatase (CYP19A1), chorionic gonadotropin subunit β (CGB) 3, and pregnancy specific beta-1-glycoprotein (PSG) 4, were significantly induced during SynT differentiation, whereas hypoxic conditions (2% O_2) prevented SynT-specific gene expression (Fig. 1A-C). The marked induction of these placenta-specific genes supports the authentication of these cells as human trophoblasts, as characterized in detail by Okae et al (35).

To define the underlying mechanisms whereby hypoxia prevents SynT differentiation, hTSCs were cultured in a 20% or 2% O_2 environment for up to 5 days and the cells were immunostained with antibodies to plakoglobin (green). Plakoglobin, also known as γ-catenin, is a cytoplasmic component of desmosomes and adherens junctions, which facilitates cell-cell adhesion by linking desmosomal cadherins to the cytoskeleton (56). Nuclei were visualized by staining with 4′,6-diamidino-2-phenylindole (blue). The cells cultured in 20% O_2 exhibited clear syncytium formation, whereas those cultured in 2% oxygen manifested a significantly reduced percentage of fused cells as well as a decreased size of syncytia compared to cells cultured at 20% O_2 (Fig. 1D). Thus, hypoxia inhibited the induction of SynT-specific gene expression and cell fusion.

Hypoxia Prevents Upregulation of NRF2, C/EBPβ, and Immune Modulatory Genes/*ICPs* on SynT Differentiation

We previously discovered that the redox-sensitive transcription factor NRF2 mediates the induction of miR-1246, one of the most highly upregulated miRNAs during SynT differentiation of human trophoblasts in primary culture (20). Transcription factors C/EBPβ and PPARγ have been identified as downstream regulators of NRF2 transactivation; NRF2 was found to directly bind to AREs in the *C/EBPβ* promoter region during the early stages of adipocyte differentiation (57). Thus, to identify the effects of hypoxia on transcription factors important for SynT differentiation, we analyzed expression of NRF2, C/EBPβ, and PPARγ in hTSCs cultured in 20% or 2% O_2 for 5 days. We observed that NRF2 and C/EBPβ were dramatically upregulated at both mRNA and protein levels upon culture in 20% O_2; this induction was significantly reduced in cells cultured in 2% O_2 (Fig. 2A, B, D). Protein expression levels of Keap1, which binds and targets NRF2 for proteasomal degradation, were significantly downregulated during SynT differentiation of hTSCs cultured in a 20% O_2 environment (Fig. 2D). By contrast, Keap1 protein levels were unaffected in hTSCs cultured in 2% O_2 compared with undifferentiated CytT (Fig. 2D). Because BACH1 (BTB domain and *cap "n" collar* homolog 1) is another known transcriptional repressor of NRF2, which competes with NRF2 for binding to antioxidant-response elements

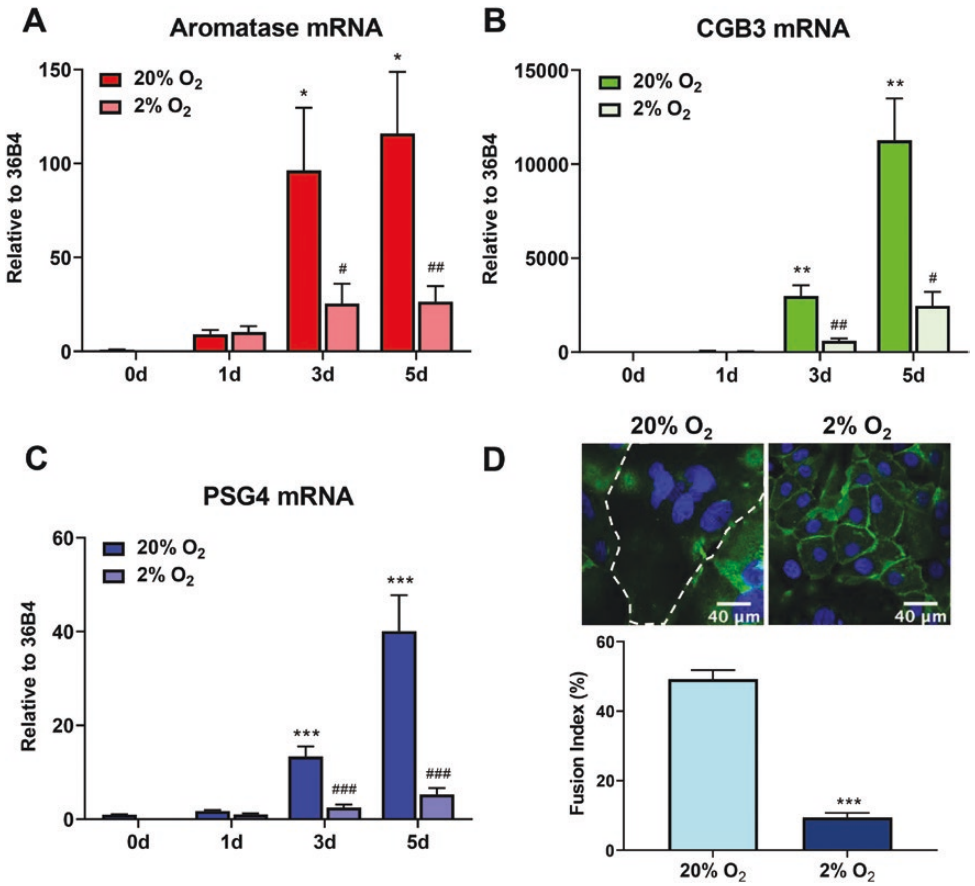

Figure 1. Hypoxia inhibits cell fusion and mRNA expression of SynT-specific markers in cultured hTSCs. hTSCs were cultured in medium containing 2 μM forskolin to promote SynT differentiation. The cells were cultured in 20% O_2 or 2% O_2 for 1, 3, or 5 days. (A-C) Hypoxia inhibited the temporal induction of SynT differentiation markers (aromatase, CGB3, and PSG4), compared with cells cultured in 20% O_2. (D) Plakoglobin immunostaining (green) of hTSCs after 5 days of culture in 20% O_2 or 2% O_2. Nuclei were stained with DAPI (blue). Shown is a representative confocal fluorescence image (magnification: 400×). ImageJ was used to quantify fused cells in 10 fields in each condition. Fusion index = (N – S)/T×100%. N refers to the number of nuclei in the syncytia, S refers to the number of syncytia, and T refers to the total number of nuclei. Scale bar: 40 μm. Values are the mean ± SEM (n ≥ 3); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs 0 d; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs 20% O_2.

in promoters of target genes (58), we measured BACH1 expression at 20% O_2 or 2% O_2 during SynT differentiation. Unexpectedly, our findings showed that BACH1 mRNA was induced at 20% O_2 and was significantly inhibited at 2% O_2, whereas BACH1 nuclear protein was barely detectable and not changed at 2% O_2 (data not shown). Thus, reduction of NRF2 protein under hypoxic conditions may be regulated, at least in part, by Keap1 induction and binding. On the other hand, we found that mRNA expression of PPARγ did not change significantly during SynT differentiation in 20% O_2, or in response to culture in a 2% O_2 environment (Fig. 2C), whereas its protein levels were modestly, but significantly, reduced in 2% O_2 (Fig. 2G, I), suggesting that hypoxia-mediated factors may contribute to regulation of PPARγ protein stability. Indeed, it has been suggested that the stability of PPARγ is downregulated by CUL4B, which is directly activated in response to hypoxia, specifically via hypoxia-inducible factor-1α (59, 60).

We previously observed that NRF2 mediated the induction C/EBPβ during differentiation of mid-gestation human trophoblasts in primary culture (20). Moreover, NRF2 has multiple direct or indirect downstream targets in various cell types comprising ICPs, including HMOX1, AhR, PD-L1, and GDF15 (58, 61-63). Thus, we next determined the effects of culture of hTSCs for up to 5 days in 2% vs 20% O_2 on mRNA and protein expression of ICPs. We observed a marked temporal induction of HMOX1, AhR, PD-L1, and GDF15 mRNA (Fig. 3A-D) and protein (Fig. 3E-I) in cells cultured in a 20% O_2 environment for up to 5 days, but these increases were significantly inhibited in cells cultured in 2% O_2 after 3 and 5 days of culture (Fig. 3). Importantly, hypoxia resulted in ~75% and 85% reduction of AhR and GDF15 protein compared with cells cultured in 20% O_2, respectively, indicating that hypoxia has a stronger effect on protein expression than mRNA expression of ICPs (Fig. 3E, G, and I). This suggests that hypoxia-mediated factors may also contribute to the regulation of their protein expression along with differentiation-induced transcription factors, NRF2, and C/EBPβ.

Importantly, mature GDF15 is secreted following intracellular cleavage, and serum GDF15 levels are higher during pregnancy than in the nonpregnant state (64). This leads to the question of whether GDF15 levels in the culture medium may also increase during SynT differentiation. Intriguingly, GDF15 levels in culture medium collected at 0 day were 0.6 ng/mL and were markedly increased to 16 ng/mL after 5 days of SynT differentiation in 20% O_2, whereas its secretion was significantly reduced in cells cultured in 2% O_2 (Fig. 3J), consistent with changes in intracellular GDF15 levels measured by immunoblotting (Fig. 3E, I). This suggests that

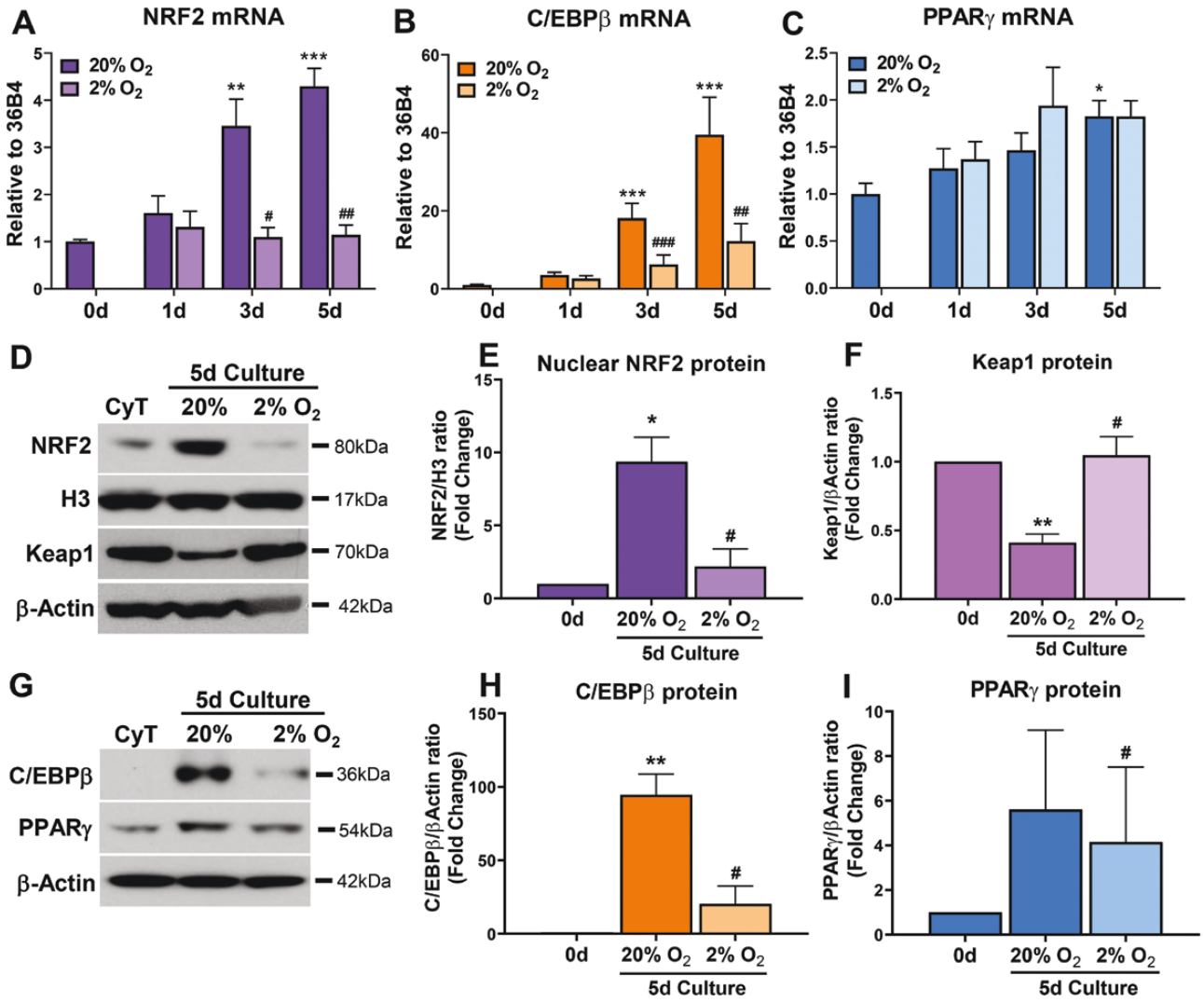

Figure 2. Hypoxia inhibits expression of transcription factors NRF2 and C/EBPβ in cultured hTSCs. hTSCs cultured in SynT differentiation medium containing forskolin in 20% O_2 or 2% O_2 for 1, 3, or 5 days were harvested for analysis by RT-qPCR and western blotting. mRNA (A) and nuclear protein levels (D, E) of NRF2 increased markedly during culture of hTSCs in 20% O_2 and were inhibited in response to culture in a hypoxic (2% O_2) environment after 3 and 5 days of culture. (A, D, E). Keap1 protein expression was reduced during SynT differentiation after 5 days of culture in 20% oxygen and remained increased at levels similar to those of undifferentiated CytT after 5 days of culture in 2% O_2 (D, F). Expression of C/EBPβ at both mRNA (B) and protein (G, H) levels was markedly increased after 3 and 5 days of culture in SynT differentiation medium in 20% O_2 and was inhibited in response to culture in 2% O_2. Shown are representative images of immunoblots (D, G) and quantifications of the scans of blots (E-I). Values are the means ±SEM of data from 3 to 5 independent experiments; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs 0 d; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs 20% O_2 at 5 days.

SynT-derived secreted form of GDF15 may not only have an impact on the immediate microenvironment, but also travel through the maternal blood and send a signal to different tissues/organs.

NRF2 Mediates the Induction of SynT-specific Genes, Transcription Factors, and ICPs in hTSCs

To investigate the role of the redox-sensitive transcription factor NRF2 in the expression of the ICPs induced during SynT differentiation, hTSCs were transiently transfected with small interfering RNA against *Nrf2* or nontargeting control for 3 and 5 days. First, we confirmed that transfection of the hTSCs with NRF2 siRNA achieved an 80% KD of endogenous NRF2 mRNA (Fig. 4A) and confirmed previously reported knockdown efficiency of NRF2 siRNA by immunoblotting in primary trophoblasts (20). NRF2 KD blocked SynT differentiation-mediated induction of C/EBPβ (Fig. 4B) as well as SynT-specific genes, including aromatase and CGB3 (Fig. 4D and E). We also observed an induction of PPARγ during SynT differentiation of hTSCs, but NRF2 knockdown only modestly and insignificantly downregulated its expression (Fig. 4C), indicating that inhibition of NRF2 alone was insufficient to downregulate PPARγ mRNA expression. Thus, it is likely that NRF2 together with upstream regulators may act to induce PPARγ expression. NRF2 KD caused a statistically significant reduction in HMOX1, AhR, PD-L1, and GDF15 (Fig. 4F-I). The NRF2 KD-mediated reduction in HMOX1, AhR, and PD-L1 was also evident in human trophoblasts in primary culture isolated from mid-gestation placenta (Supplemental Fig. 1) (65). Interestingly, during SynT differentiation of mid-gestation human trophoblasts in primary culture, we also observed induction of indoleamine 2, 3-dioxygenase (IDO) 1 (Supplemental Fig. 1) (65), which has been reported to be important for maintaining immune tolerance at the maternal-fetal interface (66). The induction of IDO1 in primary human trophoblasts was blocked by NRF2

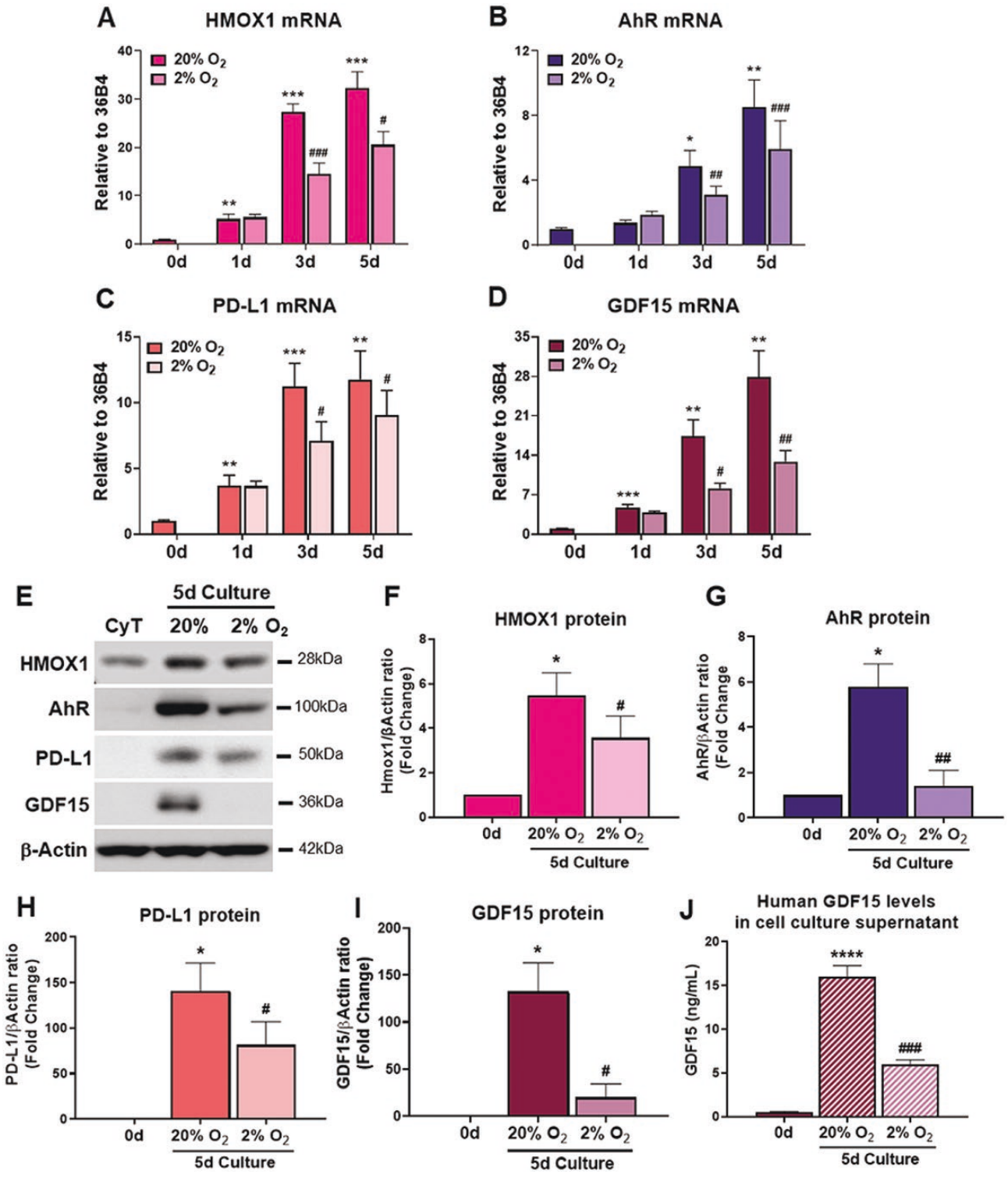

Figure 3. Expression of ICPs was increased during hTSC differentiation to SynT and was inhibited by hypoxia. hTSCs were cultured for up to 5 days in SynT differentiation medium in 20% or 2% O_2 and analyzed for HMOX1, AhR, PD-L1, and GDF15 mRNA (A-D) and protein (E-I) expression. (A-D) mRNA expression of the ICPs increased significantly during SynT differentiation in 20% O_2 and was inhibited by culture in 2% O_2. (E) Representative immunoblots of HMOX1, AhR, PD-L1, and GDF15 protein levels in hTSCs before (CytT) and after culture for 5 days in 20% or 2% O_2. (F-I) Quantifications of scans of immunoblots from at least 3 independent experiments. (J) Consistent with GDF15 protein levels in cell lysates, the secreted form of GDF15, measured by human GDF15 ELISA of the culture medium, was increased during SynT differentiation in 20% O_2, but inhibited by culture in 2% O_2. Values are the means ± SEM of data from ≥ 3 independent experiments; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs 0 d (CytT); $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs 20% O_2.

KD (Supplemental Fig. 1) (65). By contrast, IDO1 was not detectable in hTSCs during SynT differentiation in culture and was decreased during SynT differentiation of primary trophoblasts isolated from term placenta (data not shown). These findings indicate that hTSCs derived from first-trimester human placenta have the ability to recapitulate most, but not all of the biological processes observed during differentiation of mid-gestation human trophoblasts in primary culture.

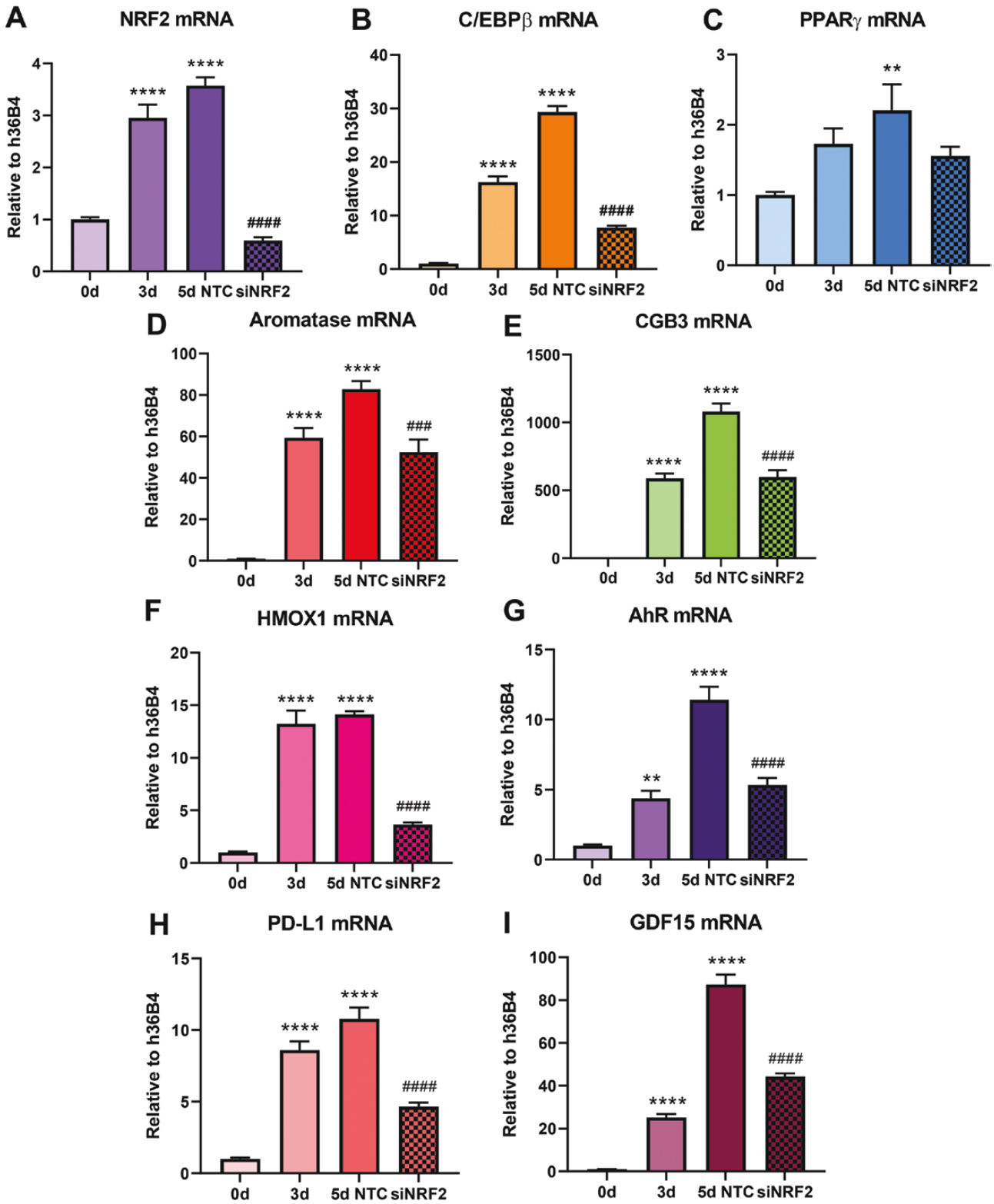

Figure 4. NRF2 knockdown (KD) inhibits induction of C/EBPβ, aromatase, CGB3, and ICPs during hTSC differentiation. Inhibition of NRF2 by transfection of NRF2 siRNA (A) inhibited mRNA expression of C/EBPβ (B), SynT differentiation markers aromatase and CGB3 (D, E), and ICPs (F-I). Values are the means ± SEM of data from at least 3 independent experiments (n ≥ 3); $^{*}P < 0.05$, $^{***}P < 0.001$ vs 0 day; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs transfection of nontargeting control.

Collectively, our findings indicate that NRF2 is an important mediator of immune tolerance during trophoblast differentiation. Further, this may contribute to immune suppression at the maternal-fetal interface.

NRF2 Binds Directly to the Promotors of *ICPs* in hTSCs During SynT Differentiation

Next, to determine whether NRF2 regulates the previously mentioned ICPs by direct binding to their promoters during

SynT differentiation, we searched 1 kb upstream of transcription start sites of the each of the ICPs for putative NRF2-binding site using TFBIND (https://tfbind.hgc.jp/) (67) and/or PROMO algorithm (http://alggen.lsi.upc.es/) (68) and performed ChIP-qPCR using hTSCs (Fig. 5) and primary trophoblasts freshly isolated from mid-gestation human placenta (Supplemental Fig. 2) (65). We found that temporal induction of the ICPs, HMOX1, AhR, PD-L1, and GDF15 in hTSCs and primary trophoblasts from mid-gestation human placenta was associated with increased binding of endogenous NRF2 to their promoters during SynT differentiation (Fig. 5A-D and Supplemental Fig. 2) (65). In addition, the induction of *IDO1* in primary human trophoblasts from mid-gestation human placentas was associated with increased binding of NRF2 to its promoter (Supplemental Fig. 2) (65). Taken together, our findings suggest that NRF2 serves a role as a master regulator in the maintenance of immune privilege during pregnancy by transcriptional regulation of ICPs.

Global *Nrf2* KO Mice Display an Inhibition of Selective ICPs and an Increase in IL-1β During Mid-gestation

Because it has been reported that global NRF2 deletion in mice resulted in significantly reduced fetal weight and labyrinth/placental volume (31), we next evaluated the expression of ICPs in placentas of WT vs *Nrf2* KO mice at E12.5, which is considered to be mid-gestation in the mouse. Importantly, mid-gestation is a critical period for successful pregnancy because it has been shown that genes involved in immune tolerance, angiogenesis, and cell differentiation/mobility are upregulated in mid-gestation placentas, compared with those at later stages of pregnancy (69). RNA was prepared from at least 12 placentas of *Nrf2* KO mice and from 8 gestation-matched placentas of WT mice at e12.5. We confirmed that NRF2 mRNA expression was completely ablated in placentas of *Nrf2* KO mice (Fig. 6A). We previously observed that NRF2 KD inhibited differentiation-associated expression of C/EBPβ and PPARγ in mid-gestation human trophoblasts in primary culture (20). C/EBPβ and PPARγ have been reported to function as key transcription factors in mouse placental development (27, 28). In the present study, we observed that *Nrf2* KO resulted in a significant inhibition of C/EBPβ and PPARγ mRNA expression at E12.5 (Fig. 6B). Furthermore, we found that HMOX1, AhR, and NQO1 mRNA were significantly reduced in *Nrf2* KO placentas compared with WT (Fig. 6C). Notably, with *Nrf2* KO, the pro-inflammatory cytokine IL-1β was up-regulated in mouse placentas at E12.5 (Fig. 6D). By contrast, TNFα and IL-6 were not changed and CXCL15, the human homolog of IL-8, was not detectable (data not shown). These findings suggest that *Nrf2* KO may upregulate ROS and oxidative stress that, in turn, activates the inflammasome and increases IL-1β production in placenta. Taken together, NRF2 may play an important immunomodulatory role during early placental development by inducing ICPs and reducing inflammatory signaling in the form of IL-1β.

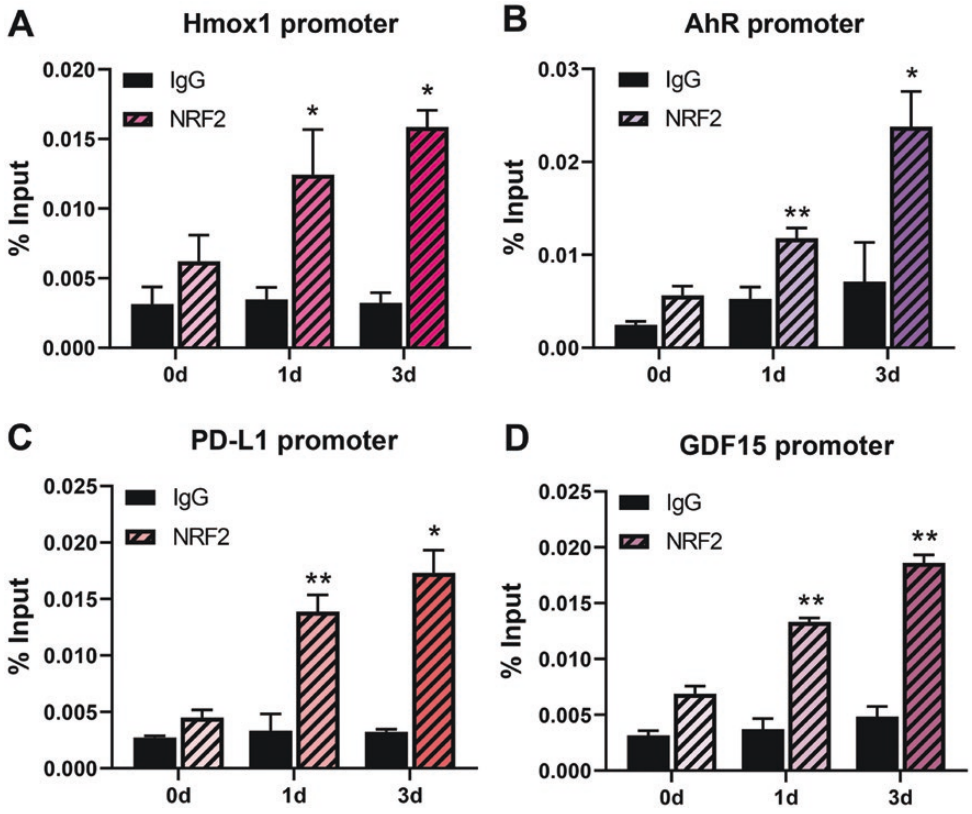

Figure 5. Binding of endogenous NRF2 to promoters of *ICPs* in hTSCs increases during SynT differentiation. ChIP-qPCR was used to analyze binding of endogenous NRF2 to promoters of genes encoding HMOX1 (A), AhR (B), PD-L1 (C), and GDF15 (D) before (0 days) and after 1 and 3 days of culture in SynT differentiation medium. Nonimmune IgG was used as a nonspecific control. Values are shown as percentage input. Values are the means ± SEM of data from at least 3 independent experiments (n ≥ 3); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs NRF2 binding at 0 hours.

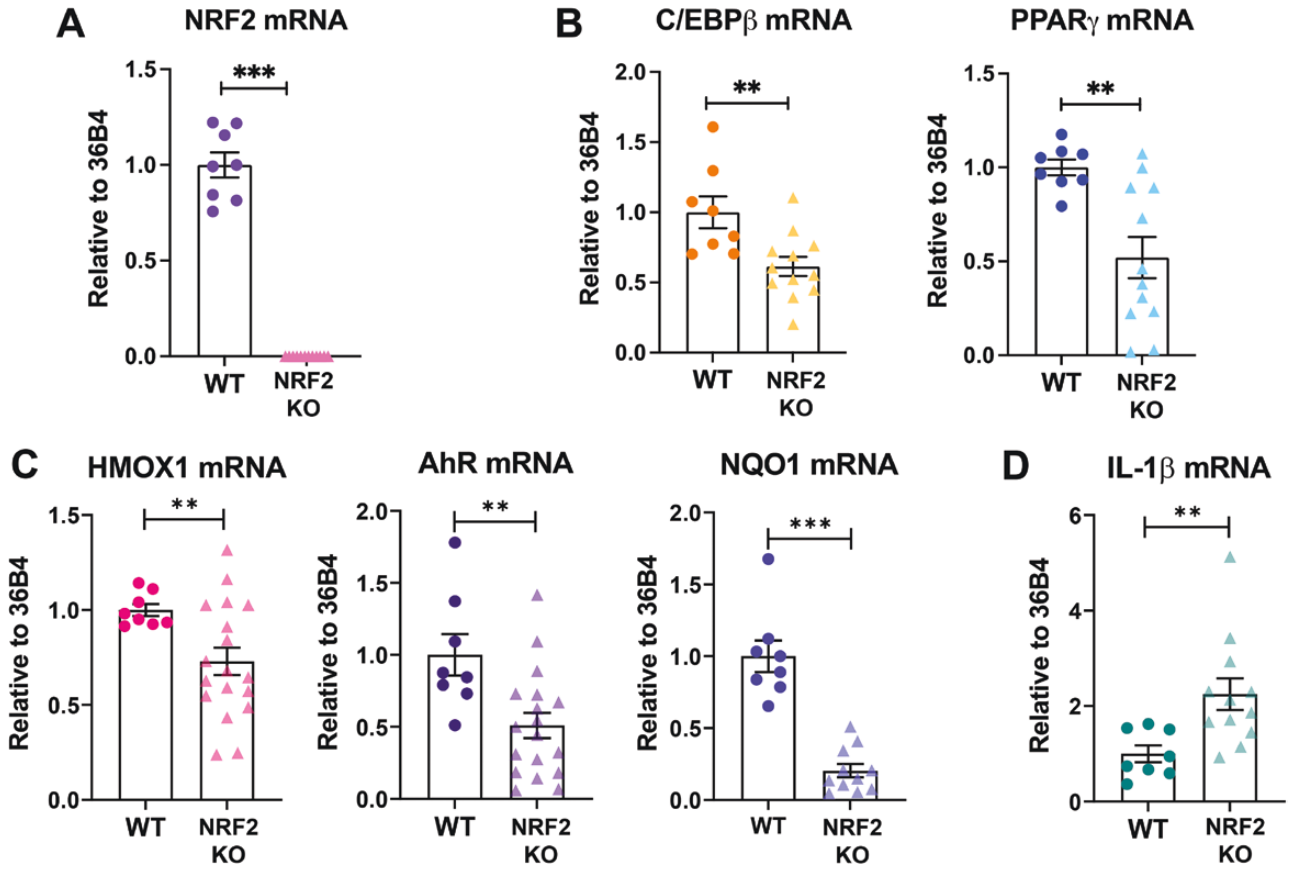

Figure 6. Placental immune modulator gene expression is significantly decreased, whereas proinflammatory IL-1β is increased in *Nrf2* global KO mice at E12.5. (A) Abrogated mRNA expression of *Nrf2* in placentas of *Nrf2* KO mice was confirmed by qRT-PCR. (B, C) Placentas of *Nrf2* KO mice at E12.5 displayed a significant decrease in C/EBPβ, PPARγ, HMOX1, AhR, and another direct downstream target of *Nrf2*, Nqo1. (D) IL-1β mRNA was significantly increased in placentas of *Nrf2* KO mice, compared with wild-type (WT). (n ≥ 8 for WT; n ≥ 12 for *Nrf2* KO.) $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs WT.

Discussion

In this study, we have demonstrated that the redox-regulated transcription factor, NRF2, which is markedly induced during human SynT differentiation, via upregulation of C/EBPβ (20), serves an important regulatory role in the increased expression of ICPs, including HMOX1, AhR, PD-L1, and GDF15 (summarized in Fig. 7). These factors, which have been reported to be important inducers of an immunosuppressive/tolerogenic phenotype of immune cells (70-75), were found in the present study to be downstream targets of NRF2 (Figs. 4 and 5, Supplemental Figs. 1 and 2) (65). Based on the potential role of NRF2 as a redox-sensitive transcription factor, we observed that, compared with cells cultured in 20% O_2, hypoxia (2% O_2) caused a reduction in nuclear NRF2 protein levels, whereas cytoplasmic levels of its negative regulator, KEAP1, increased and were similar to those of undifferentiated CytT (Fig. 2). Moreover, we found reduced levels of C/EBPβ and the previously mentioned ICPs in cells cultured in 2% vs 20% O_2 (Figs. 2 and 3), and in response to NRF2 knockdown (Fig. 4). This suggests an important role of NRF2 in trophoblast expression of multiple ICPs, which may affect immune tolerance at the maternal-fetal interface.

Single-cell transcriptome analysis of human trophoblast, decidual, and immune cell types revealed a large number of ligand-receptor pair interactions between SynT and decidual cells, suggesting that the decidua is the focus of signaling from the SynT (76). Immune tolerance is thought to be maintained throughout most of pregnancy in mice and humans by decidual immunosuppressive cells, including regulatory T cells (Tregs) (77), decidual natural killer cells (dNK) (78) and alternatively activated (M2) macrophages (Mϕ) (79). dNK cells comprise ~70% of decidual immune cells during early pregnancy and are critical for trophoblast invasion and vascular remodeling (80, 81). These cells may act with immature dendritic cells to promote expansion of Tregs and to protect the fetus from maternal rejection (82). Moreover, dNK cells express the antimicrobial peptide Granulysin, which selectively targets bacterial infection without killing trophoblasts and can be transported to extravillous trophoblasts (EVTs) by nanotubes for host defense against intracellular bacterial infection (83). Importantly, dNK cells decline before labor at term. Notably, ~25% of the immune cells in decidua during early to mid-pregnancy are Mϕ, which predominately express an M2/immunosuppressive phenotype (84). However, in mice, Mϕ that invade the decidua near term manifest an inflammatory M1 phenotype (85).

The placenta is a unique and transient organ consisting of trophoblasts, which are the earliest differentiated cells derived from the blastocyst, specialized to deliver essential nutrients for adequate fetal growth. There has been a critical need to develop in vitro culture models that recapitulate differentiation of trophoblast lineages that arise throughout gestation. Over the past 2 decades, we have studied trophoblast differentiation

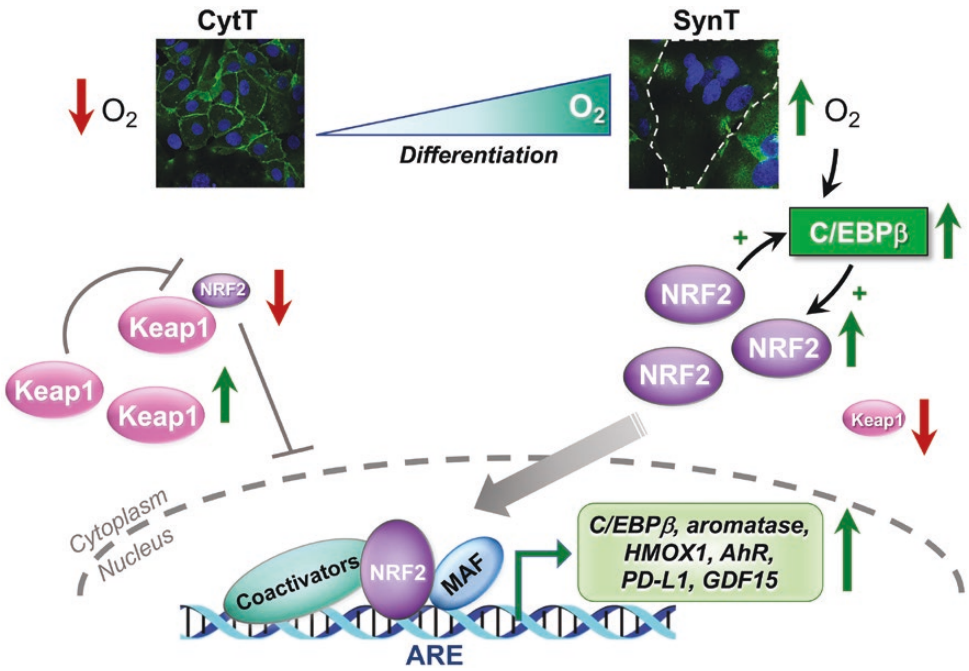

Figure 7. Increased O_2 tension promotes SynT differentiation with induction of the redox-sensitive transcription factor, NRF2, which induces expression of *C/EBPβ*, aromatase/*CYP19a1* and *ICPs* through binding to their promoters. In this manner, NRF2 and downstream *ICP* target genes serve a critical role in the maintenance of immune tolerance at the maternal-fetal interface.

using primary trophoblasts freshly isolated from mid-gestation human placenta (4, 13, 20, 51). Others have used either first trimester (86) or term placental cells, immortalized cell lines established from first trimester extravillous trophoblast (ie, HTR-8/SVneo) (87) or choriocarcinoma cells (ie, BeWo and JEG-3) derived from metastatic cancer cells (88, 89). However, these cell lines have limitations because they cannot mimic proliferative villous cytotrophoblasts that are capable of differentiation to either EVT or SynT. Okae et al recently reported the derivation of hTSCs from first trimester human placental CytT (35). These cells are maintained in a proliferative state and can be differentiated into the 2 main trophoblast lineages, SynT or EVT (35). Our findings in the present study provide further evidence that these hTSCs offer a robust and biologically relevant system for identifying genes, pathways, and mechanisms underlying the control of *ICP* gene expression during human SynT differentiation. Using this system, we observed that upon culture of hTSCs under conditions that lead to SynT differentiation, ~50% of the cells underwent cell fusion/syncytialization with marked induction of SynT markers (aromatase, CGB3, and PSG4), which is similar to what we observed using primary trophoblasts isolated from mid-gestation human placenta (51). Overall, our findings indicate that hTSCs serve as a relevant culture model for studies of gene regulation and function of human trophoblasts.

The increase in oxygen availability following enlargement of the placental spiral arteries after mid-gestation has a critical impact on trophoblast differentiation and function. As placental blood flow markedly increases, the increased O_2 tension causes induction of trophoblast fusion and SynT differentiation (54). By contrast, the low ambient oxygen accessibility during early gestation promotes CytT proliferation (28) and prevents cell fusion and differentiation (29, 30) (Fig. 7). As mentioned, NRF2 plays a central transcriptional role in the response of cells to changes in redox state (90). Indeed, NRF2 deficiency in women has been implicated in preeclampsia, which is characterized by abnormal placentation with impaired vascularization and hypoxia (91). Accordingly, NRF2 KO mice manifested impaired fetal and placental growth, reduced labyrinth volume (31), and a marked reduction in expression of the labyrinth-specific gene Distal-Less Homeobox 3 (92). *Nrf2* KO mice also manifested increased placental expression of the proinflammatory cytokine IL-1β (Fig. 6D) and an enhanced susceptibility to preterm birth induction by intrauterine injection of lipopolysaccharide (32). Using cultured hTSCs, we observed that the hypoxia-mediated reduction of NRF2 led to inhibition of cell fusion/syncytialization and the repression of genes encoding ICPs, which protect the fetus from maternal rejection.

The multinucleated SynT covering the surface of chorionic villi is bathed in maternal blood and thus can interact with maternal and fetal immune cells within the local environment. Emerging evidence suggests that the SynT may contribute to immune tolerance at the maternal-fetal interface via secretion of ICPs to protect the hemiallogeneic fetus from rejection by the maternal immune system (70). We observed that differentiation of hTSCs (Figs. 4 and 7) and freshly isolated CytT from mid-gestation human placenta to SynT (20), resulted in marked induction of NRF2, C/EBPβ, and PPARγ. Moreover, C/EBPβ and PPARγ have been found to act as signaling partners of NRF2 and are implicated in the development of an anti-inflammatory M2 Mϕ phenotype (93, 94). Further, C/EBPβ was reported to be important for B-cell development, whereas C/EBPβ-deficient mice were highly susceptible to *Listeria monocytogenes* infection (95, 96). Notably, the PPARγ agonist, rosiglitazone, induced M2 Mϕ polarization in circulating blood mononuclear cells (93) and reduced the rate of lipopolysaccharide-induced preterm birth and decidual M1 Mϕ activation in pregnant mice (85). Notably,

we observed that PPARγ expression was not significantly affected by NRF2 KD or by culture of hTSCs under hypoxic conditions. On the other hand, we found that Pparγ was significantly decreased in *Nrf2* KO mouse placentas at E12.5 compared with WT, suggesting that multiple cell types within the placenta may play a cooperative role in the regulation of PPARγ.

SynTs are a major source of placenta-derived microvesicles, and exosomes, which play a key role in immune tolerance by delivery of bioactive signaling molecules and immunomodulatory factors from the fetus to the mother (97). Specifically, placental exosomes were found to contain ICPs, such as PD-L1, TGF-β, and TRAIL (98). Intriguingly, PD-L1 is one of several known direct downstream targets of NRF2 (62). PD-L1 is one of the most studied ICPs; PD-1/PD-L1 signaling downregulates the function and proliferation of effector T cells and induces differentiation and immunosuppressive capacity of Tregs (71). We discovered that PD-L1 was highly expressed in hTSCs upon SynT differentiation in 20% O_2 (Fig. 3), and its expression was directly mediated by NRF2 binding to the *PD-L1* promoter in hTSCs (Fig. 5) and in mid-gestation human SynTs in primary culture (Supplemental Figure S2) (65). Furthermore, PD-L1 was significantly downregulated when hTSCs were cultured in a hypoxic environment (Fig. 3). Interestingly, PD-L1 mRNA was unchanged in global *Nrf2* KO mouse placentas at E12.5 compared with WT; however, PDL-1 was found to be significantly reduced in *Nrf2* KO placentas at E10.5 (data not shown). This suggests that the Nrf2-PD-L1 axis may be more important at earlier stages of pregnancy in mice.

HMOX1 and AhR are other direct downstream targets of NRF2 involved in immune modulation (58, 61). HMOX1 was observed to regulate placental vascular development and to play a key role in immune homeostasis in mice by maintaining a tolerogenic dendritic cell environment (70). Carbon monoxide produced by the enzymatic action of HMOX1 has been found to increase the expansion and differentiation of myeloid cells, which are important for maintenance of tissue homeostasis and immune tolerance (72). In addition, *AhR* null mice exhibited reduced fertility because of abnormal placental development and impaired generation of Tregs (73). We found that HMOX1 and AhR were induced during SynT differentiation of mid-gestation human trophoblasts in primary culture (Supplemental Figure S1) (65) and in hTSCs during SynT differentiation (Fig. 3); this induction was directly mediated by NRF2 (Supplemental Figure S2, Figs. 4 and 5) (65), suggesting a conserved role of NRF2 in the regulation of immunomodulatory factors in humans and mice.

GDF15, a TGFβ superfamily member, also has been proposed to promote immunosuppression by inducing differentiation of tolerogenic DCs and Tregs (74, 75). GDF15 is most highly expressed in the placenta, compared with other normal tissues, and its deficiency is associated with miscarriage and preeclampsia (99, 100). We found that during SynT differentiation, GDF15 was inhibited by hypoxia, as well as by NRF2 knockdown (Figs. 3 and 4). Importantly, we observed that GDF15 protein was secreted into the culture medium bathing hTSCs; its levels increased > 25-fold during SynT differentiation and were markedly inhibited in cells cultured in a hypoxic environment (Fig. 3J). Thus, hypoxia-mediated reduction in NRF2 likely results in inhibition of immunosuppressive GDF15, together with other ICPs, HMOX1, AhR, and PD-L1.

Importantly, these ICPs also are expressed in a variety of tumors and invading leukocytes, where they function to promote immune evasion, tumor metastasis, and progression (101). In fact, among a wide range of tumor types, GDF15 was found to be the most highly overexpressed soluble factor (102). Thus, NRF2 likely serves a key role in the regulation of immune tolerance to enhance both tumor progression (103) and placental development (31). These findings suggest that a variety of cancers have usurped the role of NRF2 to protect themselves from rejection by the host's immune system.

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Author Contributions

Y.-T.K. and S.M. contributed to the studies using isolation and culture of human primary trophoblasts (Supplemental figures) (65); K.H. carried out all other experiments; K.H. and C.R.M. contributed to the conceptual design, interpreted the data, and wrote the manuscript. All authors have reviewed and commented on the manuscript.

Disclosure Summary

The authors have no conflicts of interest to disclose.

Data Availability

Original data generated and analyzed during this study are included in this published article or in the data repositories listed in References.

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