

Hypoxia-inducible factor 1 signaling drives placental aging and can provoke preterm labor

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Abstract

Most cases of preterm labor have unknown cause, and the burden of preterm birth is immense. Placental aging has been proposed to promote labor onset, but specific mechanisms remain elusive. We report findings stemming from unbiased transcriptomic analysis of mouse placenta, which revealed that hypoxia-inducible factor 1 (HIF-1) stabilization is a hallmark of advanced gestational timepoints, accompanied by mitochondrial dysregulation and cellular senescence; we detected similar effects in aging human placenta. In parallel in primary mouse trophoblasts and human choriocarcinoma cells, we modeled HIF-1 induction and demonstrated resultant mitochondrial dysfunction and cellular senescence. Transcriptomic analysis revealed that HIF-1 stabilization recapitulated gene signatures observed in aged placenta. Further, conditioned media from trophoblasts following HIF-1 induction promoted contractility in immortalized uterine myocytes, suggesting a mechanism by which the aging placenta may drive the transition from uterine quiescence to contractility at the onset of labor. Finally, pharmacological induction of HIF-1 via intraperitoneal administration of dimethyloxalyl glycine (DMOG) to pregnant mice caused preterm labor. These results provide clear evidence for placental aging in normal pregnancy, and demonstrate how HIF-1 signaling in late gestation may be a causal determinant of the mitochondrial dysfunction and senescence observed within the trophoblast as well as a trigger for uterine contraction.

eLife assessment

This **valuable** study provides insights into mechanisms of placental aging and its relationship to labor initiation. The authors provide **solid** evidence and have thoroughly investigated the molecular characteristics of normal placental aging using in vivo and in vitro model systems and human placental tissue analysis to corroborate their findings. This work contributes to existing work in placental aging and preterm birth and will be of interest to reproductive scientists.

Introduction

Preterm birth (birth prior to 37 completed weeks of gestation) is a massive global health burden: it leads all causes of death in neonates and children to the age of 5 worldwide¹, and survivors face a broad array of short- and long-term health challenges². Most preterm births in the United States are due to spontaneous onset of preterm labor, with unknown underlying cause³. Preterm labor has proven difficult to treat; there currently exist no highly effective interventions that prevent spontaneous preterm birth (SPTB)^{4, 5}. The dearth of effective treatments stems from our lack of understanding about the pathways regulating spontaneous onset of labor, both preterm and at term.

The placenta defines the maternal-fetal interface; it is capable of profoundly influencing both maternal and fetal physiology^{6–8}, and it is subject to remodeling in response to its local environment⁹. Placental disease is known to contribute to other disorders of pregnancy including preeclampsia^{10–12} and intrauterine growth restriction^{13, 14} and there is increasing interest in its potential role as a driver of preterm labor^{15–17}.

Recent studies have found that placentas from pregnancies that resulted in SPTB have unique metabolomic and transcriptomic signatures from term counterparts^{18–20}. The reported differences broadly implicate the stress response, inflammation, and various metabolic pathways. It has been challenging to narrow these findings or translate them for mechanistic relevance, given the lack of suitable culture systems that integrate experimental manipulation of placental cells with the uterine myocyte response. Furthermore, interpretation studies profiling human placentas from SPTB is sharply limited by the lack of gestational age-matched controls. Without these, the effects of gestational age cannot be distinguished from factors driving premature labor. The placenta encounters a dynamic local environment across its lifetime and faces evolving needs of the growing fetus, so the context of normal gestational age-related changes is vital for the correct interpretation of molecular characteristics distinguishing a SPTB placenta. Defining the molecular-level changes in the *healthy* placenta as it approaches the end of gestation, and their potential effects on the timing of labor onset would therefore address important knowledge gaps.

Here we report findings from unbiased transcriptomic analysis of healthy mouse placenta, which highlighted hypoxia-inducible factor 1 (HIF-1) signaling as a hallmark of advanced gestational timepoints, accompanied by mitochondrial dysfunction and cellular senescence. We detected some similar effects in human placentas, then modeled HIF-1 induction with two different stimuli and in two trophoblast cell models, demonstrating that mitochondrial dysfunction and cellular senescence arise secondary to HIF-1 stabilization. Whole transcriptome analysis revealed that upon HIF-1 stabilization, a trophoblast cell line acquires signatures that recapitulate the aged placenta. Finally, we show that conditioned media from these cells is sufficient to potentiate a contractile phenotype in uterine myocytes, implicating a mechanism by which the aging placenta may help drive the transition from uterine quiescence to contractility at the onset of labor. This mechanism is further reinforced by an *in vivo* model in which administration of the prolyl hydroxylase inhibitor dimethyloxalyl glycine (DMOG) to pregnant mice induces HIF-1 signaling in the placenta and causes SPTB.

Results

To illuminate gestational age-dependent transcriptional changes in across a healthy pregnancy, we collected whole mouse placentas at 48h intervals spanning embryonic day

13.5 to 17.5 (e13.5-e17.5) and quantified mRNA and protein targets that emerged from network analysis of an independently published microarray dataset from healthy mouse pregnancies. Upon searching Gene Expression Omnibus (GEO)²¹ datasets for “placenta AND transcriptome”, we were surprised that among 326 results, only nine included data from normal placenta across a series of gestational timepoints extending to late pregnancy^{22–28} (Figure 1). We applied weighted gene correlation network analysis (WGCNA) to a microarray study of mouse placenta²² (GEO accession GSE11224) spanning e8.5 to postnatal day 0 (p0) to assess the mRNA signature of the aging mouse placenta, using the dataset with the best temporal resolution across gestation. Distinct clusters emerged, each reflecting a group of genes whose expression changes across timepoints in a unified way (Figure 2A; accompanying statistics available at Mendeley Data, doi: 10.17632/g6vrw9jjn4.1). Genes in the ‘blue’ cluster showed increasingly positive correlation with advancing gestational timepoints; KEGG functional pathways significantly overrepresented among these genes include HIF-1 signaling, AMPK signaling, and cellular senescence. Genes in the ‘turquoise’ cluster showed increasingly negative correlation to advancing timepoints; KEGG functional pathways significantly overrepresented among these genes include the citric acid cycle, mitochondrial complex I biogenesis, and the mitotic cell cycle.

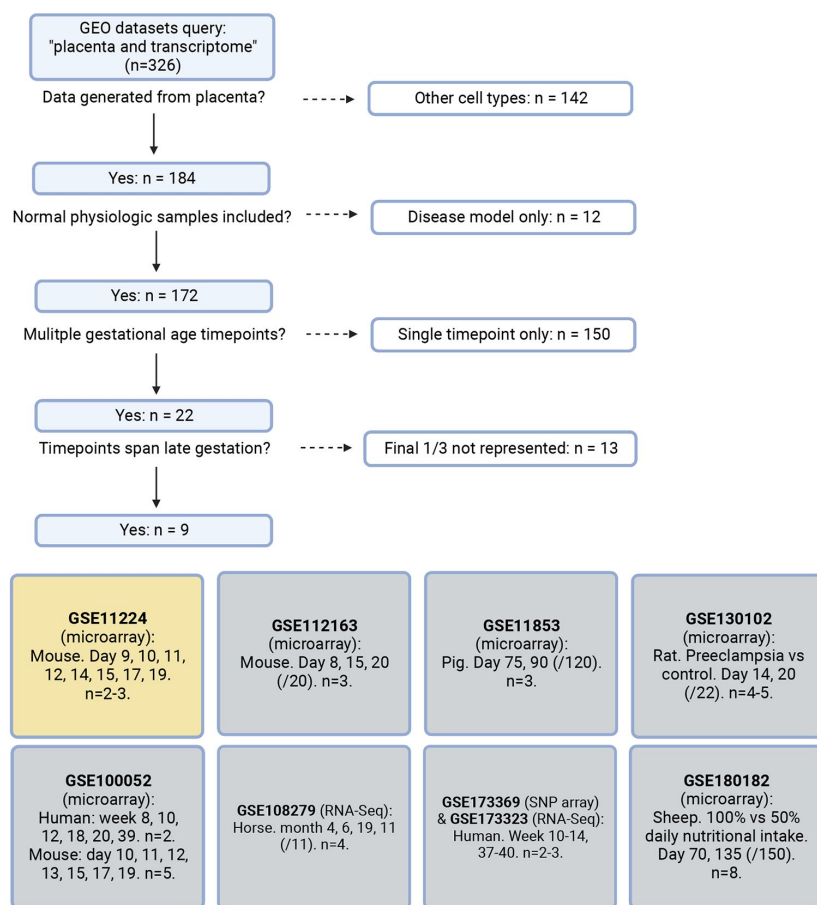


Figure 1.

Systematic search flow for placental transcriptomics datasets.

326 GEO datasets were identified by the search terms “placenta” and “transcriptome”; nine met criteria for containing placental transcriptomic data representing normal physiology at a range of gestational timepoints spanning through the final 1/3 of pregnancy. Dataset selected for further analysis in yellow.

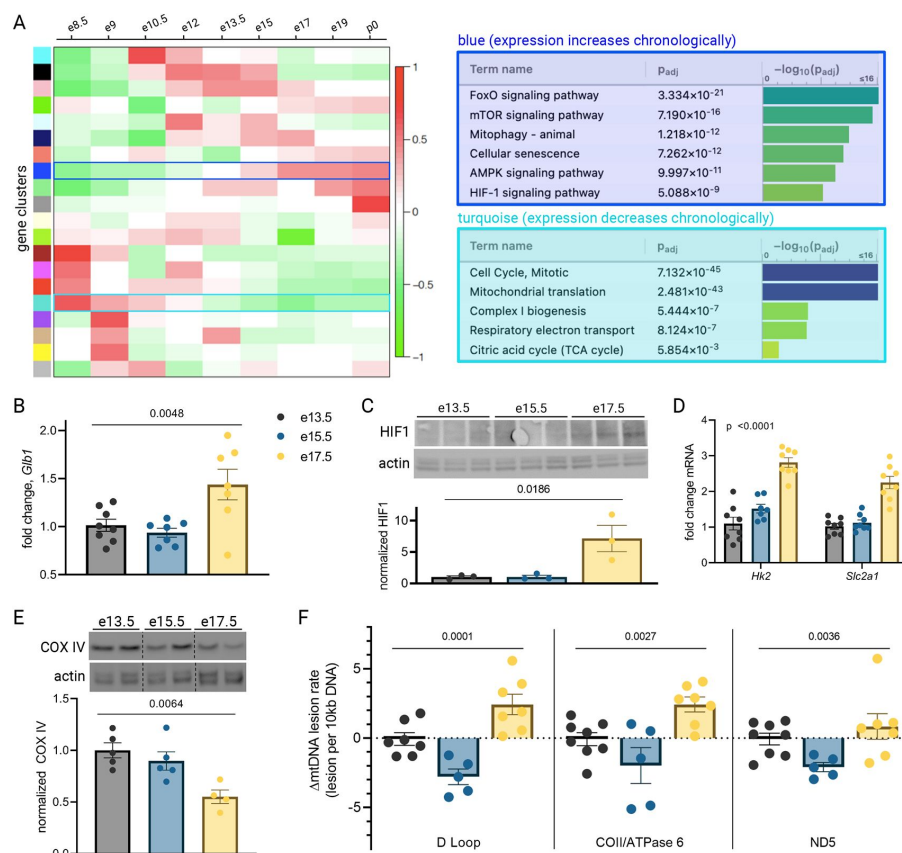


Figure 2.

Mouse placental aging is characterized by cellular senescence, HIF-1 signaling, and mitochondrial dysregulation.

WGCNA yielded 20 gene clusters. Functional pathways overrepresented in clusters found to increase (blue) and decrease (turquoise) across gestation highlight enhanced cellular senescence, increased HIF-1 signaling, and decreased mitochondrial synthesis and respiration late in pregnancy (A). mRNA expression of senescence marker *Glb1* peaks at e17.5 (B; one-way ANOVA $p=0.0048$). HIF-1 protein abundance is higher at e17.5 versus e13.5 and e15.5 (C; one-way ANOVA $p=0.019$), as is expression of HIF-1 targets *Hk2* and *Slc2a1* (D; two-way ANOVA $p<0.0001$ for

gestational age factor). (See [Figure 2 – Supplement 1](#) for analysis of gene expression changes across timepoints by placental sex.) Mitochondrial abundance, reflected by COX IV protein, decreases with gestational age (E, one-way ANOVA $p=0.0064$), and mitochondrial DNA lesion rate peaks at e17.5 in the regions of the D-loop (one-way ANOVA $p=0.0001$), COII/ATPase6 ($p=0.0027$), and ND5 ($p=0.036$) (F). B–F, Each data point represents a biological replicate (eg. RNA, protein, or DNA extracted from an individual placenta, in turn collected from one of 2–4 pregnant dams per group). Data normalized to mean at e13.5.

See Figure 2 – Source Data 1. for uncropped blots.

To validate the dynamic pathway activity suggested by the WGCNA, we used qPCR to quantify mRNA expression of the senescence marker *Glb1*²⁹ in our mouse placentas, which mirrored the WGCNA finding that cellular senescence in the placenta peaks in the final days of gestation ([Figure 2B](#)). HIF-1 protein abundance was found to peak at e17.5 ([Figure 2C](#)) and HIF-1 targets *Hk2* and *Slc2a1* likewise confirmed increasing HIF-1 activation with advancing gestational age through e17.5 ([Figure 2D](#)). Of note, a modest but significant fetal sex-dependent difference in HIF-1 target expression (but not *Glb1*) was also observed across timepoints ([Figure 2 – Supplement 1](#)). To assess changes in mitochondrial abundance across gestation, we measured COX IV protein expression and found it declined progressively across mouse gestation ([Figure 2E](#)) as predicted by the WGCNA.

Having observed a pattern of declining mitochondrial abundance in the placenta with advancing gestational age, we next investigated the mitochondrial DNA (mtDNA) lesion rate. The mitochondrial genome is particularly vulnerable to reactive oxygen species (ROS) insults, and mtDNA damage participates in a vicious cycle with mitochondrial dysfunction and further ROS production; these effects are observed in a number of age-related diseases

in various tissues³⁰ and may drive age-associated loss of function³¹. We employed a semi-long run real-time qPCR approach³² to quantify relative mtDNA lesion rates in mouse placentas, normalized to e13.5. There was a measurable increase in the mtDNA lesion rate in the D-loop and COII/ATPase 6 regions of the mitochondrial genome at e17.5 (Figure 2F). Together, these results reflect a series of coordinated changes as gestation progresses — namely HIF-1 signaling induction, decreasing mitochondrial abundance, accumulating mtDNA damage, and escalating cellular senescence — confirming the patterns we discovered through reanalysis of published transcriptomic data.

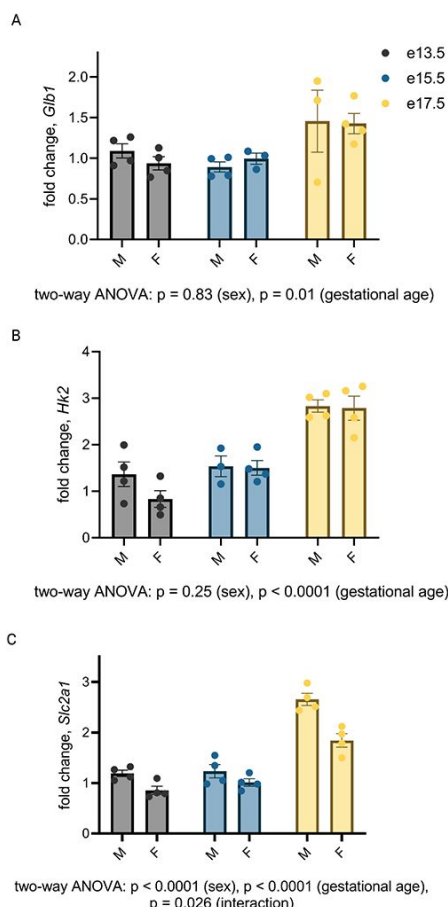


Figure 2 – Supplement 1.

Gestational age-dependent variability in expression of Hif-1 target *Slc2a1*, but not *Hk2*, is affected by placental sex.

Subgroup analysis did not reveal sex-dependent mRNA expression changes in *Glb1* (A) or *Hk2* (B) by qPCR, but did demonstrate consistently higher *Slc2a1* expression in male placentas at all gestational ages and an interaction between the effects of sex and gestational age on *Slc2a1* expression (C; two-way ANOVA $p < 0.0001$ for sex, $p < 0.0001$ for gestational age, and $p = 0.028$ for interaction). Each data point represents a biological replicate (RNA isolated from an individual placenta, collected from one of 2-4 dams per group). Data normalized to collective (M+F) mean at e13.5.

Figure 2 – Source Data 1. Uncropped, unedited blots from 2c (left) and 2e (right).

We next probed human placentas for the same gestational age-dependent changes. Studying normal human placenta at a range of gestational ages beyond the second trimester is challenging, as placental sampling is usually only possible after delivery, and most deliveries associated with healthy pregnancies occur at term. We therefore sought to capitalize on rare exceptions such as cases of placenta previa, vasa previa, or uterine dehiscence, where iatrogenic preterm delivery is indicated for reasons unrelated to placental health. With an objective to compare term versus preterm placentas, yet minimize confounding factors that accompany labor and disease states that could be expected to affect placental health, we designed a case-control study using the following exclusion criteria: onset of labor prior to delivery (spontaneous or induced); maternal history of hypertension, asthma, diabetes, or autoimmune disease; pregnancy complicated by gestational hypertension, gestational diabetes, preeclampsia, multiples, fetal anomalies, placenta accreta spectrum disorder, or smoking during pregnancy.

Placentas from 9 cesarean deliveries occurring before 35 weeks gestation and 11 cesarean deliveries occurring after 39 weeks gestation were studied (Table 1). Maternal

characteristics including race, nulliparity, and obesity were not statistically different across the early versus late groups; there was a small but statistically significant difference in maternal age at the time of delivery (31.9 ± 0.9 years versus 36.2 ± 1.0 for early gestation versus late, $p = 0.008$). The distribution of fetal sex was not different among groups.

Table 1.

Maternal and fetal characteristics.

Data summarized by mean $\pm$ SEM or n (%). p-values calculated via t-test (continuous variables) or Chi-square contingency table (categorical variables).

	<u>< 35 weeks</u>	<u>> 39 weeks</u>	<u>p value</u>
	n = 9	n = 11	
Gestational age at delivery (weeks)	34.0 $\pm$ 0.3	39.5 $\pm$ 0.1	< 0.0001
Maternal age (years)	31.9 $\pm$ 0.9	36.2 $\pm$ 1.0	0.008
Maternal BMI > 40	1 (11)	0 (0)	0.26
Maternal race			
White	7 (78)	9 (82)	0.13
Black	2 (22)	0 (0)	
Asian	0 (0)	2 (18)	
Primiparous	4 (44)	4 (36)	0.71
Female neonate	5 (55)	8 (73)	0.42
Indication for delivery	Placenta previa (3)	Scheduled repeat (7)	
	Vasa previa (4)	Breech presentation (3)	
	Thinned lower uterine segment (2)	Elective (1)	

qPCR revealed a trend toward increased mRNA expression of *GLB1* and HIF-1 targets *HK2* and *SLC2A1* in the > 39-week cohort (**Figure 3A**), consistent with the gestational age-dependent effect seen in mouse placenta. We also examined mitochondrial abundance in the two groups and found that mitochondrial RNA transcripts *ATP6* and *COX2* were significantly decreased (**Figure 3B**) and COX IV protein abundance was lower at the later timepoint (**Figure 3C**), mirroring the mouse findings. Of note, power calculation to reject the null hypothesis for difference between means for some of these measurements (with $\alpha=0.05$ and $\beta=0.2$) suggests a sample size of greater than 35 per group is required, assuming a similar effect size as seen in mouse data (eg. expecting *GLB1* fold change difference of 40% across groups), and greater variability than seen for mouse data (eg. expected standard deviation of *GLB1* fold change equal to 0.6, versus 0.3 in mice). Practical constraints, especially given our strict exclusion criteria, make a study of this size unfeasible; nonetheless, we have included analysis of 20 human placentas here in recognition of the vital importance of translating mouse findings to human biology, even preliminarily. The data should be interpreted in the context of these statistical realities.

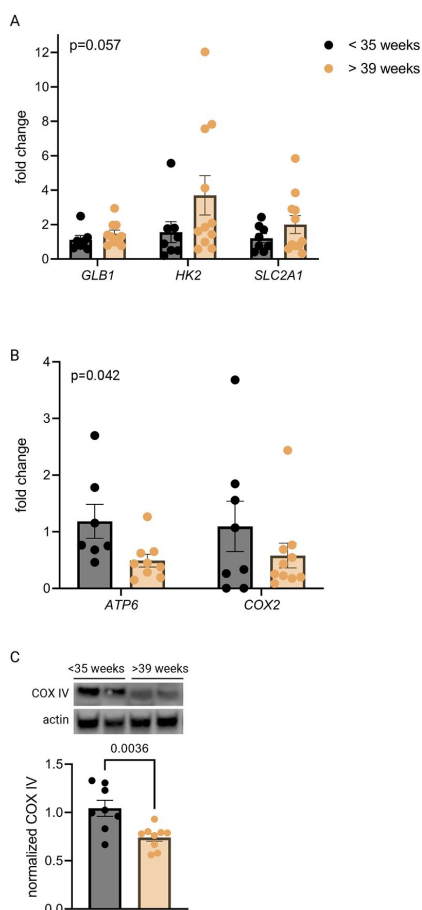


Figure 3.

Senescence, HIF-1 signaling, and decreased mitochondrial abundance characterize late-gestation human placenta.

mRNA expression of senescence marker *GLB1* and HIF-1 targets *HK2* and *SLC2A1* trends higher in placentas from > 39-week cohort vs < 35-week cohort (A; 2-way ANOVA gestational age factor $p=0.057$). Mitochondrial abundance, reflected by mitochondrial genes *ATP6* and *COX2* (B; two-way ANOVA gestational age factor $p=0.042$) and COX IV protein (C; $p=0.0036$) decreases with advancing gestational age. Each data point represents a biological replicate (RNA or protein isolated from an individual placenta). Data normalized to mean in < 35 week group. See Figure 3 – Source Data 1 for uncropped blots.

Figure 3 – Source Data 1. Uncropped, unedited blots from Figure 3.

The co-appearance of cellular senescence, HIF-1 signaling, and mitochondrial dysregulation in the placenta as it approaches the end of gestation led us to hypothesize that in aging placental cells, HIF-1 induction could be upstream of mitochondrial dysregulation and cellular senescence, as is seen in other systems in emerging aging research^{33–35}. To test this hypothesis, we established a pharmacological model of HIF-1 induction in primary mouse trophoblasts using cobalt chloride, a prolyl hydroxylase inhibitor that stabilizes HIF-1 α ^{36, 37} and has been widely used to model hypoxia. After six hours of CoCl_2 exposure, we confirmed HIF-1 protein accumulation in cultured trophoblasts (Figure 4A). After 48 hours of CoCl_2 exposure, mouse trophoblasts exhibit decreased mitochondrial abundance, by *Cox2* mRNA expression (Figure 4B) and Cox IV protein abundance (Figure 4C), and an increase in senescence-associated beta galactosidase (SA- β Gal), encoded by *Glb1* (Figure 4D) and detected as a blue stain in an X-gal assay for senescence (Figure 4E). These findings suggest that HIF-1 stabilization induces subsequent mitochondrial dysfunction and senescence in trophoblasts.

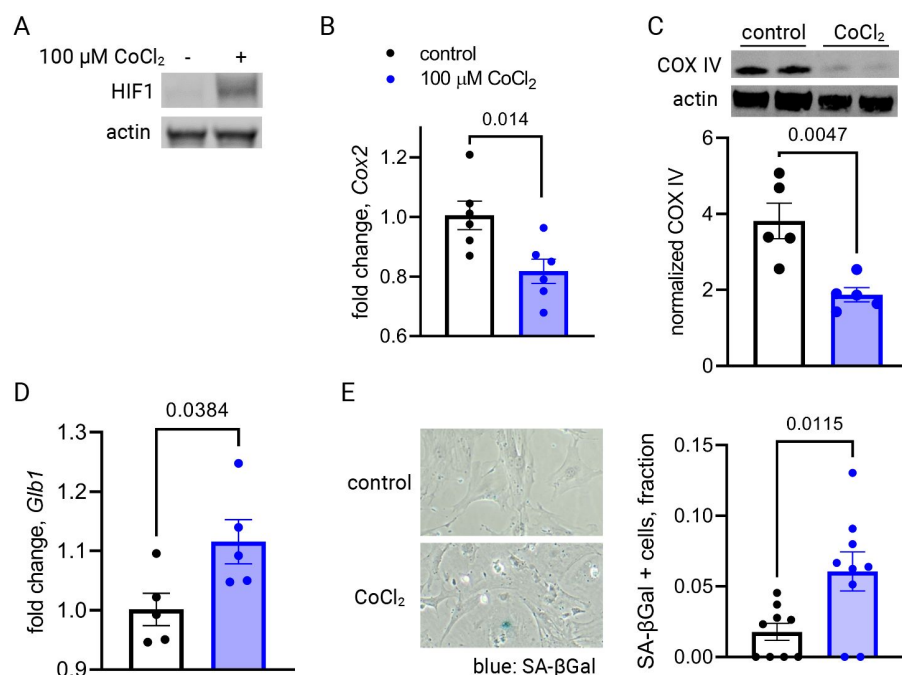


Figure 4.

Short-term HIF-1 stabilization in primary mouse trophoblasts leads to decreased mitochondrial abundance and cellular senescence.

HIF-1 is detected in cultured trophoblasts exposed to CoCl_2 (A). After 48 hours of CoCl_2 exposure, trophoblasts exhibit decreased mitochondrial abundance reflected by *Cox2* mRNA expression levels (B; $p=0.014$) and COX IV protein levels (C; $p=0.0047$). Senescence marker *Glib1* is increased (D; $p=0.038$) and SA- β Gal accumulation is noted by X-gal assay (E; $p=0.012$). Each data

point represents a technical replicate (eg. protein, RNA, or β -Gal measured from an individual well of cells grown in treated or control condition). Data normalized to mean of control treatment group. See Figure 4 – Source Data 1 for uncropped blots.

Figure 4 – Source Data 1. Uncropped, unedited blots from 4a (left) and 4c (right)

Primary trophoblasts undergo spontaneous syncytialization in culture, a phenomenon that limits the duration of study and may also confound the interpretation of experimental changes in key metabolic factors³⁸. We therefore also modeled HIF-1 activation in JAR cells, a trophoblast cell line that does not undergo syncytialization³⁹. Consistent with our results in primary cells, we found that HIF-1 is stabilized in CoCl_2 -treated JAR cells (Figure 5A), and after 6 days of exposure, mitochondrial DNA and protein abundance declined (Figure 5B-C). We further defined the time course of mitochondrial downregulation: by 72 hours the effect began to appear (Figure 5 – Supplement 1). Furthermore, at the 6-day timepoint we found that CoCl_2 exposure leads to accumulation of mitochondrial ROS, as measured by mtSOX, a fluorescent mt-superoxide indicator dye (Figure 5D), and impairs mitochondrial polarization, as measured by tetramethylrhodamine ethyl ester (TMRE) staining, a cell-permeant fluorescent dye that accumulates in polarized mitochondria (Figure 5E). Additionally, we observed pronounced signs of cellular senescence: morphological hallmarks (cellular swelling) and β -galactosidase overexpression (Figure 5F); growth arrest which persists for days after removal of the HIF-1 stabilizing compound (Figure 5G); and a senescence-associated secretory phenotype (SASP, Figure 5H) reflected by increases in mRNA expression of the genes encoding VEGF, TNF α and IL-1 α and a decrease in mRNA expression of the gene encoding anti-inflammatory cytokine IL-10.

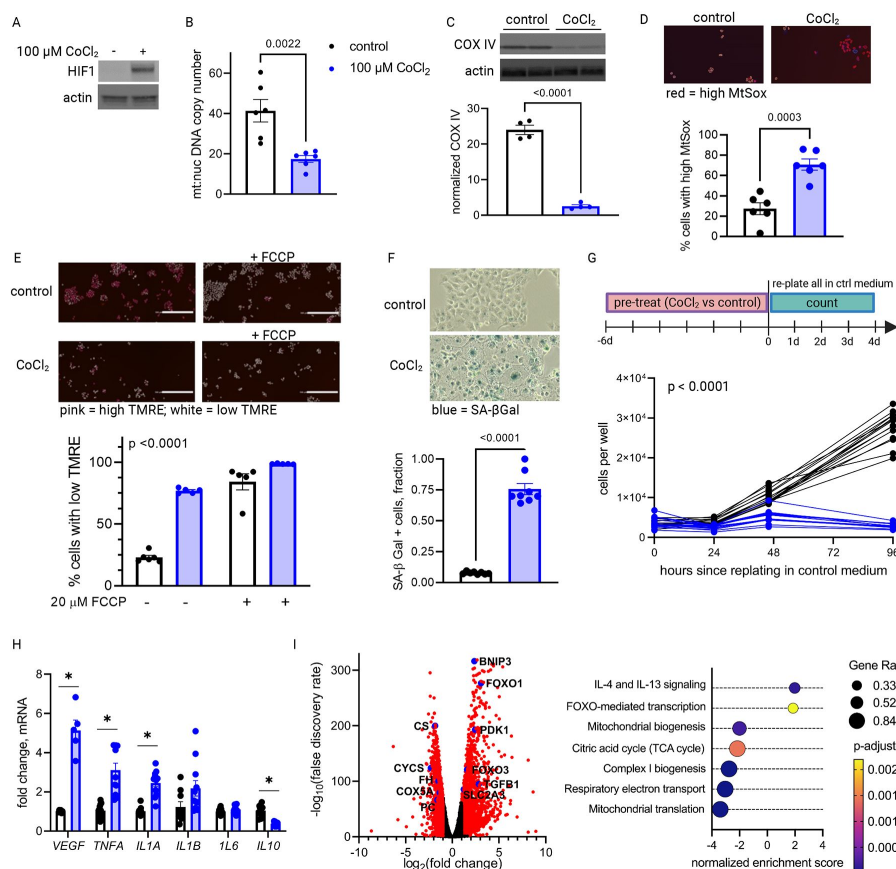


Figure 5.

Long-term HIF-1 stabilization in JAR cells leads to mitochondrial dysfunction, cellular senescence, and metabolic reprogramming.

HIF-1 is stabilized at 6 day timepoint of CoCl₂ exposure (A). After 6 days, mitochondrial abundance is decreased as reflected by a drop in the mitochondrial:nuclear DNA copy number (B) and a decrease in COX IV protein (C). (See [Figure 5 – Supplement 1](#) for timecourse of declining mitochondrial abundance.) Cells also exhibit augmented signs of mitochondrial dysfunction via MtSox (D; p=0.0003) and TMRE staining (E; two-way ANOVA CoCl₂ factor p<0.0001). SA- β Gal staining reflects a high proportion of senescent cells (F; p<0.0001) and growth arrest is confirmed by cell counting following a 6-day pre-treatment with CoCl₂ (G; two-way ANOVA p<0.0001 for interaction of CoCl₂ factor with time). (See [Figure 5 – Supplement 2](#) for assessment of cell death by propidium iodide staining.) mRNA expression of SASP candidates *VEGF*, *TNFA*, *IL1A*, and *IL10* is altered after CoCl₂ exposure (H; *, adjusted p<0.01). RNA Seq revealed upregulation of 2188 and downregulation of 1389 genes (I; genes with | log₂(FC) | > 1 and -log(FDR)>2 indicated in red) after CoCl₂ treatment, with geneset enrichment analysis revealing several pathways significantly dysregulated after CoCl₂ treatment recapitulating changes seen in transcriptomic analysis of late versus early gestation mouse placenta. Scale marker = 200 μ m. FCCP= carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, an ionophore uncoupler of oxidative phosphorylation which depolarizes mt membrane potential. See [Figure 5 – Supplement 3](#) for assessment of effects of HIF-1 stabilization in JAR cells using DMOG. Each data point represents a technical replicate (measurement from an independent well of cells grown in treatment vs control condition). Data normalized to mean of control group. See Fig 5 Source Data 1 for uncropped blots.

We conducted additional studies to confirm that cell death was not a primary contributor to the lack of proliferation. Propidium iodide staining with quantitative fluorescence cytometry indicated that CoCl₂ treatment only increased cell death by 0-3% ([Figure 5 – Supplement 2](#)). Importantly, cells remained adherent and continued to acidify culture medium beyond 14 days of CoCl₂ exposure, providing confidence that HIF-1 stabilization induces a phenotype characterized by predominantly viable cells that are no longer proliferating, namely cellular senescence. Finally, to assess whether the effects we observed were attributable to HIF-1 stabilization and not an off-target effect of CoCl₂, we also evaluated an alternative prolyl hydroxylase inhibitor, dimethylxalylglycine (DMOG)⁴⁰, and found similar effects on HIF-1, mitochondrial abundance and cellular senescence ([Figure 5 – Supplement 3](#)).

To determine if other features of the aged placenta phenotype were recapitulated in this model, we performed whole transcriptome analysis via RNA-Seq. Differential expression ($\log_2$ fold change > 1 ; FDR < 0.05) was found for 2188 upregulated and 1389 downregulated genes (Figure 5I). Gene set enrichment analysis revealed that chemical hypoxia led to upregulation of inflammatory signaling, and downregulation of the TCA cycle, mitochondrial biogenesis, and respiratory electron transport. These data therefore recapitulated metabolic patterns that emerged from time course transcriptomic analysis of intact placentas (Figure 2A).

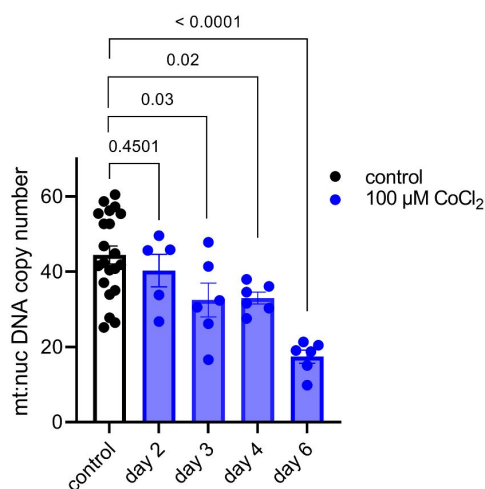


Figure 5 – Supplement 1.

The mitochondrial effects of HIF-1 stabilization in JAR cells begin to appear on day 3 following CoCl₂ exposure. Each data point represents a technical replicate (independent well of cells grown in control vs treatment condition).

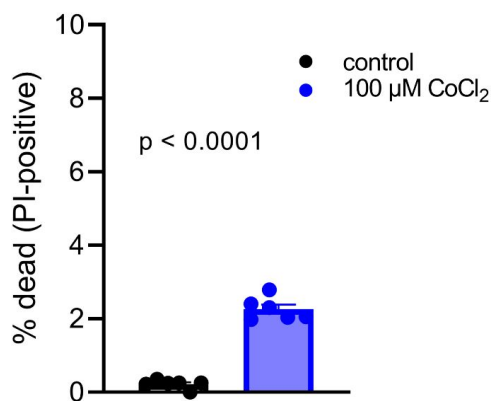


Figure 5 – Supplement 2.

Increased number of JAR cells stain with propidium iodide, but the absolute number remains low following 6 days of CoCl₂ treatment. Each data point represents a technical replicate (independent well of cells grown in control vs treatment condition).

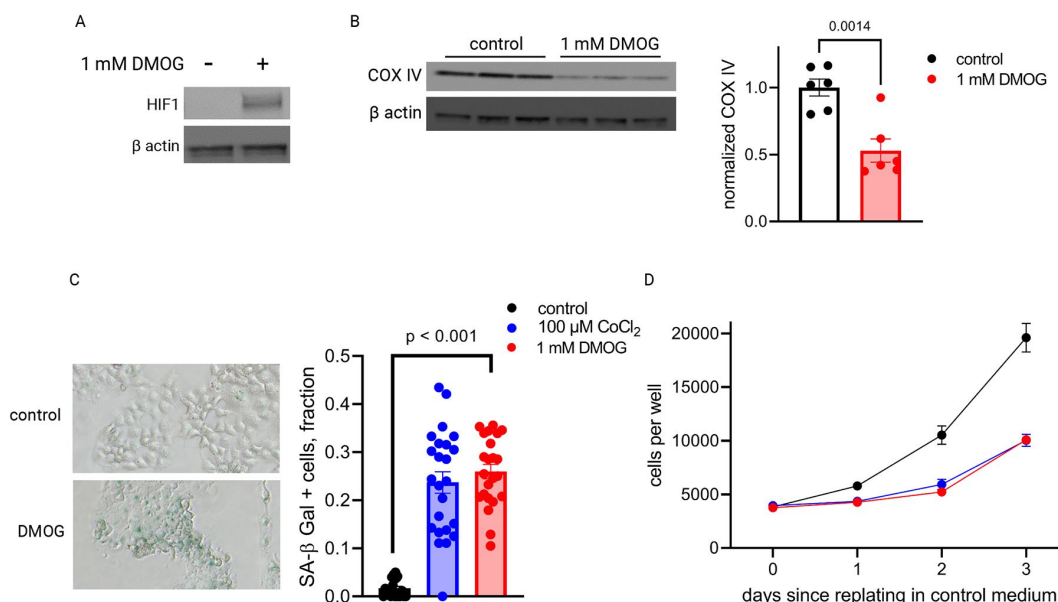


Figure 5 – Supplement 3.

DMOG stabilizes HIF-1 in JAR cells (A) and induces similar effects as CoCl₂ on COX IV protein (B), SA-βGal expression (C), and cell growth (D) after 4 days. Each data point represents a technical replicate (measurement from an independent well of cells grown in treatment vs control condition). See Figure 5 – Source Data 1 for uncropped blots.

Figure 5 – Source Data 1. Uncropped, unedited blots from 5a (left) and 5c (right)

The onset of labor is accompanied by a transformation of the uterus from quiescence into a distinct physiologic state in which it generates powerful, coordinated contractions. This phenotypic change is characterized by upregulation of a cadre of contraction associated proteins (CAPs), notably: cyclooxygenase-2 (COX-2, encoded by *PTGS2*), prostaglandin F_{2α} receptor (encoded by *PTGFR*), interleukin-6 (IL-6), and connexin 43 (Cx43, encoded by *GJA1*). Phenotypic switching can be modeled in primary and immortalized uterine myocytes upon stimulation with inflammatory mediators such as IL-1β, PGF_{2α}, and thrombin^{41–43}.

To test whether primary placental metabolic disruptions could crosstalk with uterine myocytes to trigger the contractile phenotype, we collected conditioned media from our JAR cell model of HIF-1-driven senescence and measured the effect on expression of CAPs in uterine myocytes (hTERT-HM cells). We observed a robust and specific effect: co-stimulation of uterine myocytes with IL-1β plus conditioned media from JAR cells treated with CoCl₂ (but not from untreated JAR cells) potentiated the induction of *PTGS2*, *GJA1*, *PTGFR*, and *IL6* mRNA expression (Figure 6A–D). We next employed a collagen lattice assay previously described for assessing contractility of myometrial cells *in vitro*^{41, 44}, and demonstrated that myocyte contraction is augmented upon exposure to conditioned media from JAR cells treated with CoCl₂ but not from untreated JAR cells (Figure 6E). These transcriptional and functional results collectively demonstrate that uterine myocytes are responsive to the secretome of JAR cells driven to senescence via HIF-1 induction.

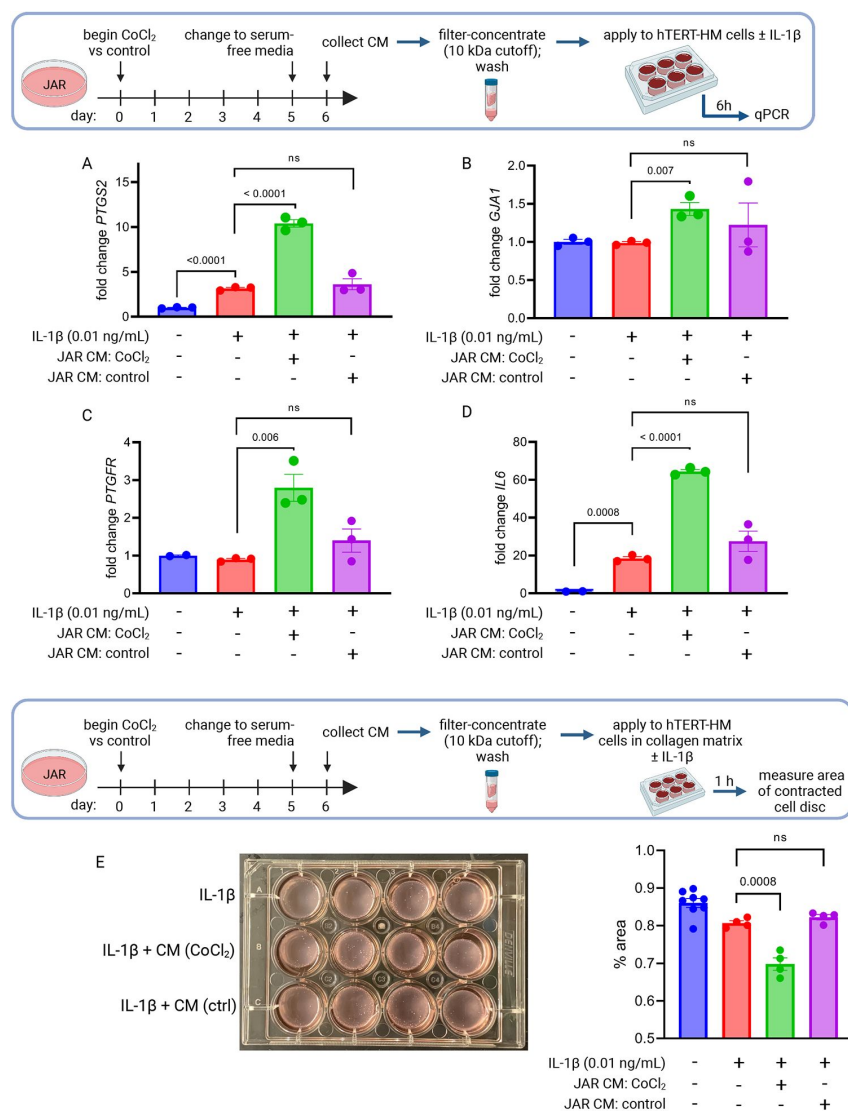


Figure 6.

Conditioned media (CM) from JAR cells following HIF-1 stabilization induces expression of contractile-associated proteins and augments contraction in immortalized human uterine myocytes.

hTERT-HM mRNA expression of *PTGS2* (A), *GJA1* (B), *PTGFR* (C), and *IL6* (D) was induced by CM from JAR cells following CoCl₂ treatment (but not in control conditions), potentiating the effect of stimulation of myocytes by exogenous IL-1β. Data normalized to mean of null treatment group. Percent well area occupied by hTERT-HM cells embedded in collagen matrix is significantly smaller after stimulation with IL-1β plus JAR CM from CoCl₂ condition, reflecting greater degree of hTERT-HM cellular contraction (E). Each data point represents a technical replicate (measurement from an independent well of cells grown in treatment vs control condition).

To test whether HIF-1 induction drives labor onset *in vivo*, we administered DMOG intraperitoneally to pregnant mice on gestational day e16.5. Analysis of placentas recovered 12 hours following injection indicated that HIF-1 protein is stabilized in placenta following maternal DMOG injection (Figure 7A), and transcription of HIF-1 target genes *Hk2* and *Slc2a1* was significantly increased (Figure 7B). Following injection of DMOG, gestational length was significantly shortened compared to injection of vehicle alone (Figure 7C-D).

Discussion

The subject of whether the healthy placenta undergoes aging (with accompanying dysfunction) within its 40-week lifespan has been of great interest and debate for many years. Age-related functional decline is a near-universal phenomenon affecting many tissues, but the underlying biochemical driving factors vary widely depending on the context of cell type and local stressors. Mechanisms well described in other tissues are rooted in genomic instability, mitochondrial dysfunction and oxidative stress, nutrient deprivation and metabolic insufficiency, or loss of proteostasis⁴⁵. While characterization of the placenta as an aging organ appeared in the literature beginning nearly fifty years ago^{46, 47}, a counter-

argument has been offered that there is no logical reason for the placenta to undergo accelerated aging relative to the fetus, since both share the same genes and environment⁴⁸. A third viewpoint proposes that aging in the placenta is indicative of a disease state—not normal progression of healthy gestation—one usually reflective of maladaptive responses to oxidative stress^{49–53}. Finally, recent reports have noted prominent signs of aging in another fetally derived tissue, the chorioamniotic membranes, in healthy pregnancies⁵⁴, and have linked aging of the fetal membranes to the onset of labor^{55, 56}. The results presented here span mouse and human placentas to provide evidence for placental aging in normal pregnancy. Mechanistic dissection of this phenomenon further demonstrates how hypoxia in late gestation may be both a causal determinant of mitochondrial dysfunction and senescence observed within the trophoblast as well as a trigger to induce uterine contraction.

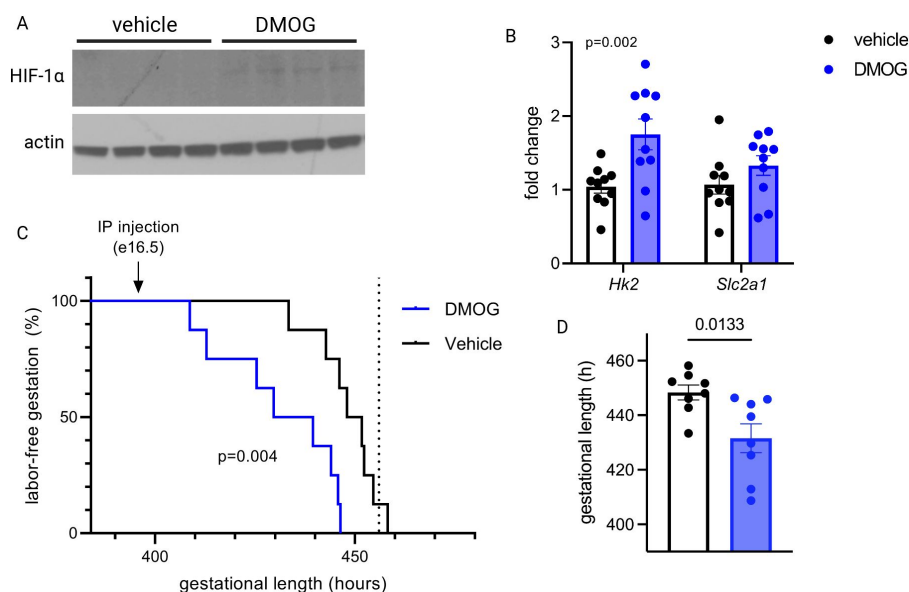


Figure 7.

Maternal DMOG injection on e16.5 stabilizes placental HIF-1 and induces preterm labor.

HIF-1α protein is detected in placental lysates 12 hours following DMOG injection but not vehicle (A). mRNA expression of HIF-1 targets *Hk2* and *Slc2a1* is upregulated following DMOG injection ($p = 0.002$ for DMOG vs vehicle, two-way ANOVA) (B). Gestational length is significantly shortened following DMOG injection versus vehicle (C-D). Each data point represents a biological replicate (in A

and B, each measurement from an individual placenta collected from one of 2 pregnant dams). Data normalized to mean of vehicle group. See Figure 7 – Source Data 1 for uncropped blots.

Figure 7 – Source Data 1. Uncropped, unedited blots from Figure 7.

To our knowledge, this is the first report to propose that placental aging in healthy pregnancy is characterized by induction of HIF-1 signaling, accompanied by downstream effects including mitochondrial dysfunction and cellular senescence. These findings have implications for the role of the placenta in signaling that promotes the onset of labor, particularly given our demonstration that upon HIF-1 stabilization, trophoblasts can induce inflammatory changes in uterine myocytes and potentiate their contractility, and our *in vivo* demonstration that administration of DMOG stabilizes placental HIF-1 and leads to preterm labor in mice.

Important next steps prompted by this work include determining the specific components of the secretome of HIF-1-activated trophoblasts which are responsible for inducing myocyte transformation, anticipating overlap with findings of prior studies delineating paracrine signals that mediate labor onset^{57–60}. Additionally, having established that HIF-1 signaling is upstream of mitochondrial dysfunction and cellular senescence in late-gestation placenta in normal pregnancies, future studies can investigate whether factors that modulate this

pathway could increase or decrease vulnerability to preterm labor. In studies targeted to uterine decidua, a link between cellular senescence and labor onset has previously been established, with evidence implicating phospho-Akt and mTORC1 signaling upstream of prostaglandin synthesis as a key mechanism in this model^{61, 62}. It is possible that senescence signaling from the placenta and decidua converge, and labor is provoked upon these convergent endpoint signals surpassing a threshold, regardless of their origin within the gestational compartment. Future studies using targeted manipulation of senescence and metabolism in these compartments individually will help clarify their specific contributions to labor onset.

Our results suggest that placental HIF-1 becomes stabilized late in gestation, and this could be secondary to the onset of hypoxia sensing as oxygen demand outstrips supply in the fetoplacental unit. However, placental hypoxia has been difficult to examine *in vivo*. Despite evolving approaches for measurement of placental oxygenation, findings have varied widely and adequate spatiotemporal resolution has so far been challenging⁶³. However, it is apparent that due to arteriovenous shunting and high metabolic extraction rates, the placenta experiences hypoxia throughout pregnancy with a reported partial pressure of oxygen 30-50 mm Hg in the intervillous space, so perhaps a relevant question is what prevents HIF-1 stabilization and its downstream effects early in gestation. It is possible that the gestational-age-dependent HIF-1 induction we observed reflects not a change in oxygen availability, but rather a change in other HIF-regulatory factors such as nicotinamide adenine dinucleotide (NAD⁺) depletion, as has been demonstrated to occur in muscle and is accompanied by metabolic reprogramming and mitochondrial decline⁶⁴. Importantly, replenishment of NAD⁺ was shown to restore markers of mitochondrial function in these aged cells, suggesting that mitochondrial effects of HIF-1-driven aging are reversible in some systems. Separately, a recent study which compared the miR-nomes of 1st-versus 3rd-trimester human placenta highlighted differential expression of miRNAs with functional links to silencing of hypoxia response and cellular senescence pathways in the first trimester⁶⁵. Future investigations into the impact of placental HIF-1 regulation will require a more precise understanding of placental oxygenation *in vivo* and a sophisticated approach to model these factors in placental explants or other cellular systems.

The shift in metabolic phenotype away from oxidative respiration in late pregnancy as demonstrated by our transcriptomics analysis is consistent with earlier findings from respirometry studies in mouse placenta⁶⁶, where rates of oxidative phosphorylation were noted to decline from e14 to e19, particularly in the labyrinthine zone. It is still unclear if this shift reflects changing substrate availability, or oxygen content, or perhaps the metabolic needs of the developing fetus. A better understanding of the dynamic metabolism of the placenta across gestation will be crucial for developing strategies for optimizing placental health, with major implications for both maternal and fetal outcomes. For example, placental metabolomics studies with a gestational time series could help corroborate the metabolic implications interpreted from our transcriptomic data. And while our study stemmed from analysis of the whole-organ placental transcriptome, single-cell transcriptomics across advancing gestational age in healthy placentas would offer critical cell-type localization of specific metabolic events noted to occur late in pregnancy.

Our data have implications for other adverse pregnancy outcomes beyond preterm birth, including intrauterine growth restriction and preeclampsia. Two important recent studies have examined separate mouse models with constitutively active placental HIF-1 and found that when induced from the beginning of gestation, placental HIF-1 signaling drives abnormal placentogenesis and impaired spiral artery remodeling, leading to placental hypoperfusion, fetal growth restriction, and a preeclampsia-like clinical syndrome^{67, 68}. In the context of these earlier results, our study highlights the critical gestational age-dependence of HIF-1 effects, having demonstrated that in a normally developed mouse

placenta, induction of HIF-1 signaling during the final days of gestation leads to labor onset. Timing may be key to understanding how two distinct pregnancy complications – preterm labor and preeclampsia – appear to emerge separately from overlapping pathophysiologic mechanisms^{69–73}.

In summary, we report a molecular characterization of placental aging phenomena occurring in normal pregnancies, stemming from induction of HIF-1 signaling and downstream mitochondrial dysfunction and cellular senescence. These findings establish aging in the placenta as a feature of normal gestation; identify HIF-1 signaling as an upstream trigger leading to mitochondrial dysfunction and senescence in the placenta; demonstrate that the secretome of senescent trophoblasts is sufficient to potentiate uterine myocyte transformation and contractility; and establish that HIF-1 induction *in vivo* can induce preterm labor. These findings may have important implications for illuminating the factors that determine gestational length both in health and disease.

Data Availability

Statistics accompanying WGCNA (Figure 2) are accessible through Mendeley Data, doi: 10.17632/g6vrw9jjn4.1. (<https://data.mendeley.com/datasets/g6vrw9jjn4/1>)

The RNA Seq dataset has been deposited in NCBI's Gene Expression Omnibus²¹ and is accessible through GEO Series accession number GSE199278. (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE199278>)

Additional source data (original images for all blots) available in attached files.

Methods

Mouse placenta collection

Wild-type C57BL/6 mice (Jackson Laboratory #000664) were fed a chow diet and housed at 20°C in a 12-hour light/12-hour dark cycle. Nulliparous females (< 6 months of age) were housed for a single dark cycle (midnight = gestational day 0) with a stud male. On gestational day e13.5–17.5, placentas were isolated via laparotomy from pregnant females anesthetized via surgical-plane isoflurane. Placentas were quartered, immersion-rinsed in dH₂O and blotted dry, then snap frozen in liquid nitrogen and stored at -80°C prior to use.

Mouse trophoblast isolation

Protocol described in full in Pennington *et al.*⁷⁴. On gestational day e15.5, placentas were collected via laparotomy from pregnant wild-type C57BL/6 female mice anesthetized with surgical-plane isoflurane. After dissection, placentas were quartered, immersion-rinsed in dH₂O, then placed in ice-cold digestion buffer: DMEM plus HEPES 20 mM, collagenase 1mg/mL, and DNase I 4000U/mL. Placentas were digested for 20–40 minutes at 37°C with trituration and examined under a microscope until optimal digestion achieved.

Digested cells were passed through a 70 micron strainer, washed in DMEM, then separated using a Percoll gradient. Trophoblast fraction was washed once more then plated in complete medium: DMEM with HEPES 20 mM, FBS 10%, and supplemented with sodium

pyruvate, pen-strep-glutamine, non-essential amino acids, and gentamicin. Cells were grown at 37°C in 95% ambient air with 5% CO₂.

Mouse DMOG injection and measurement of gestational length

On gestational day e16.5, dams received an intraperitoneal injection of DMOG (Selleck Chemicals) 7.5 mg in 0.3 mL sterile saline (approximately 250 mg/kg) versus vehicle alone. Video recordings were used to measure gestational length, defined as interval from midnight of timed mating period until birth of the first pup.

Human placental specimens

Placenta samples were collected at the time of cesarean delivery, within 20 minutes of delivery of the placenta. Placental tissue was sampled from approximately 1cm deep to the maternal surface after dissecting away membranes. Cotyledons from all 4 quadrants were collected and minced together prior to immersion rinse in dH₂O and storage of separate aliquots by snap freezing versus immersion in RNA Later. All samples were stored at – 80°C prior to use.

Cell culture

All cells were grown at 37°C in 95% ambient air with 5% CO₂. JAR choriocarcinoma cells (ATCC HTB-144; authenticated by STR profiling and confirmed negative for mycoplasma contamination) were grown in RPMI 1640 media (4.5 g/L glucose) supplemented with 10% FBS. Adherent JAR cells were treated with HIF1-stabilizing agents dissolved in 1X PBS (pH 7.4): cobalt chloride hexahydrate (100 µM CoCl₂, Millipore Sigma) and dimethyloxalylglycine, N-(methoxyoxoacetyl)-glycine methyl ester (1 mM DMOG, Millipore Sigma). JAR cells were treated with 100 µM CoCl₂ or 1 mM DMOG in culture media as indicated prior to endpoint assays.

Preparation of conditioned media: On day 5 of JAR cell treatment with CoCl₂ (versus control condition), media exchange was performed to apply serum-free media for both conditions. 24 hours later, conditioned media samples were collected and applied to 10 kDa molecular weight cutoff filters (Amicon Ultra), with centrifugation for 20 minutes at 4000 x g, 4°C. Filters were washed with one volume of hTERT-HM base medium (see below), with repeat centrifugation. Concentrated proteins retained in suspension above the filter were collected and stored at – 80°C.

hTERT-HM cells (generously supplied by Dr. Jennifer Condon, Wayne State; STR reference profile not available) were grown in DMEM/F12 medium (Gibco) with 10% FBS and Antibiotic-Antimycotic (1X, Gibco). Cells were stimulated by addition of recombinant human IL-1β (R&D Systems) and/or filter-concentrated JAR cell conditioned media as indicated, 6 hours prior to collection of cellular RNA.

RT – qPCR

Total RNA was reverse transcribed to cDNA with the Superscript III reverse transcriptase (Invitrogen) system with random hexamer primers (Invitrogen) according to manufacturer's instructions. cDNA was amplified via real-time quantitative PCR with TaqMan Fast Advanced Master Mix (Applied Biosystems) or SYBR Green PCR Master mix (Qiagen) in a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). In mouse samples, target gene expression was normalized to endogenous levels of housekeeping gene, β-actin. In human samples,

target gene expression was normalized to endogenous levels of housekeeping gene, YWHAZ (TaqMan) or β -actin (SYBR). Mouse and human primers are listed in Table 2.

Table 2.

Primers

Target	Sequence	Platform
<i>Glb1</i>	Mm00515342_m1	TaqMan
<i>Hk2</i>	Mm00443385_m1	TaqMan
<i>Slc2a1</i>	Mm00441473_m1	TaqMan
D loop (long, 801 bp amplicon)	F: CGTACATTAACTATTTTCCCAAG R: GAGTTTGGTTCACGGAACAT	SYBR
COII/ATPase 6 (long, 855 bp amplicon)	F: TTGGTCTACAAGACGCCACA R: ATTTTGGTGAAGGTGCCAGT	SYBR
Nd5 (long, 930 bp amplicon)	F: CGCCTACTCCTCAGTTAGCC R: ATGGTGA CTAGTGCCAGGT	SYBR
Nd2/Nd1 (long, 832 bp amplicon)	F: GGATGAGCCTCAAAC TCCAA R: ATGATGGCAAGGGTGATAGG	SYBR
D loop (short)	F: TGA CTATCCCCTTCCCCATT R: TTGTTGGTTTCACGGAGGAT	SYBR
COII/ATPase 6 (short)	F: TCTCCCCTCTCTACGCATT C R: CGGTTAATACGGGGTTGTTG	SYBR
Nd5 (short)	F: GGCCTCACATCATCACTCCT R: GCTGTGGATCCGTTCTAGT	SYBR
Nd2/Nd1 (short)	F: GGATGAGCCTCAAAC TCCAA R: GGCTCGTAAAGCTCCGAATA	SYBR
<i>Actb</i>	Mm00607939_s1	TaqMan

Human

Target	Sequence	Platform
<i>HK2</i>	Hs00606086_m1	TaqMan
<i>SLC2A1</i>	Hs00892681_m1	TaqMan
<i>IL1A</i>	Hs00174092_m1	TaqMan
<i>IL1B</i>	Hs01555410_m1	TaqMan
<i>TNFA</i>	Hs00174128_m1	TaqMan
<i>IL6</i>	Hs00174131_m1	TaqMan
<i>IL10</i>	Hs00961622_m1	TaqMan
<i>MT-ATP6</i>	Hs02596862_g1	TaqMan
<i>MT-COX2</i>	Hs02596865_g1	TaqMan
<i>ND1</i>	F: CCATAAAACCCGCCACACT	SYBR
	R: GAGCGATGGTGAGAGCTAAGGT	
<i>18S</i>	F: CGCAGCTAGGAATAATGGAATAGG R: CATGGCCTCAGTTCCGAAA	SYBR
<i>GJA1</i>	Hs.PT.58.38338544	SYBR
<i>PTGS2</i>	Hs.PT.58.77266	SYBR
<i>ACTB</i>	Hs.PT.39a.22214847	SYBR
<i>YWHAZ</i>	Hs01122445_g1	TaqMan

Collagen lattice contraction assay

As previously described^{41, 44}, hTERT-HM cells were suspended in a collagen gel matrix (Cellmatrix collagen type I-A, Fisher Scientific, prepared with MEM, NaOH and HEPES) then plated in 12-well dishes and grown in hTERT-HM medium. When cells reached approximately 60% confluence, gel matrix was detached from plate and experimental treatments were applied in fresh cell culture medium. After 1 hour incubation, a photograph was taken of each well for manual measurement of percent well area occupied by collagen disc (using imageJ), reflecting cellular contraction.

mtDNA damage assay

Mitochondrial DNA damage was assayed from total DNA via a semi-long run real-time PCR approach, as described elsewhere³². Briefly, separate qPCR reactions were assayed to compare a long versus short amplicon representing each genomic region. A lesion rate per 10 kb of mtDNA was calculated as:

$$\text{lesion rate} = \frac{10000 \text{ (bp)}}{\text{size of long fragment (bp)}} * (1 - FC_{\text{long-short}}),$$

with $FC_{\text{long-short}} = 2^{-\Delta\Delta Ct}$ calculated in the usual method.

Relative mtDNA copy number

qPCR assays for nuclear and mitochondrial genes were performed using total DNA as a template. Relative mitochondrial DNA was calculated as: follows:

$$\text{mt:nuc DNA copy number} = 2 * 2^{\Delta Ct}, \text{ where } \Delta Ct = Ct_{\text{nuclear gene}} - Ct_{\text{mitochondrial gene}}.$$

Immunoblots

Protein extraction was performed on cell pellets using RIPA lysis buffer, or on snap-frozen tissue using RIPA lysis buffer and a bead homogenizer. Protein lysates were fractionated using NuPage Bis-Tris polyacrylamide gels (20µg total protein per well) and transferred to PVDF membranes. Antibodies are listed in [Table 3](#).

Table 3.

Antibodies

Antibody	Species	Working Concentration	Source
CoxIV	Mouse IgG mAb	1:1,000	Cell Signaling Technology, #11967S
β -Actin-HRP conjugate	Rabbit IgG mAb	1:2,000	Cell Signaling Technology, # 5125S
HIF1	Rabbit	1:1000	Cell Signaling Technology, #14179S

SA- β -Galactosidase Assay

Adherent cells were fixed and stained for SA- β Gal via a Senescence Detection kit (Abcam, ab65351) per manufacturer's instructions. Representative images were used to calculate the fraction of SA- β Gal-positive cells, scored by an observer blinded to treatment condition.

Live cell staining

Adherent cells were stained using the TMRE-Mitochondrial Membrane Potential Assay Kit (Abcam, ab113852) per manufacturer's instructions. Select wells were pretreated with 20 μ M FCCP, a decoupling agent, as a positive control for 20 minutes before TMRE and Hoechst counterstain was applied. Live JAR cells were analyzed by the Celigo Image Cytometer (Nexcelom BioScience) for fluorescence quantification and gating. Adherent live JAR cells were stained with MitoSOX™ Red Mitochondrial Superoxide Indicator kit (Invitrogen) and analyzed using the cytometer in a similar manner.

Cell counting

JAR cells were cultured for 6 days $\pm$ cobalt chloride exposure prior to cell counting. On day 6, cells from each condition were replated in a 96-well plate in CoCl₂-free media at a density of 5×10^3 cells per well. Celigo Image Cytometer was used for automated cell counting (total cells per well) at 24-hour intervals.

RNA-Seq

Following 6 days of treatment with CoCl₂ 100 μ M versus control media, total RNA was isolated from JAR cells as described above. Extracted RNA (300ng) was treated with NEBNext rRNA Depletion Kit v2 (E7400X) and cDNA was generated using random hexamer priming and Maxima H Minus Reverse Transcriptase. cDNA was converted into double stranded cDNA using NEBNext mRNA Second Strand Synthesis Module (E6111L) and sequencing libraries were generated by tagmentation using Nextera XT DNA Library Preparation Kit (Illumina FC-131) with 12 cycles of PCR amplification. Sequencing libraries were analyzed by

Qubit and Agilent Bioanalyzer, pooled at a final concentration of 1.2pM, and sequenced on an Illumina Nextseq500 instrument 36x8x36 read structure.

Transcriptomics Analysis

Sequencing reads were demultiplexed and trimmed for adapters using bcl2fastq (v2.20.0). Secondary adapter trimming, NextSeq/Poly(G) tail trimming, and read filtering was performed using fastp (v0.20.0)⁷⁵; low quality reads and reads shorter than 18nt after trimming were removed from the read pool. Salmon (v1.1.0)⁷⁶ was used to simultaneously map and quantify reads to transcripts in the GENCODE 33 genome annotation of the GRCh38/hg38 human assembly. Salmon was run using full selective alignment, with sequence-specific and fragment GC-bias correction turned on (`--seqBias` and `--gcBias` options, respectively). Transcript abundances were collated and summarized to gene abundances using the tximport package for R⁷⁷. Normalization and differential expression analysis were performed using edgeR^{78, 79}. For differential gene expression analysis, genes were considered significant if they passed a fold change (FC) cutoff of $\log_2\text{FC} > 1$ and a false discovery rate (FDR) cutoff of $\text{FDR} < 0.05$. Functional enrichment analyses were performed using g:Profiler⁸⁰. WGCNA⁸¹ was performed after removing genes with low expression as previously described⁸².

Statistics

Student's two-tailed unpaired t-test, ordinary one-way, and two-way ANOVA statistical tests were applied as indicated to compare biological replicates in each experiment. Data were excluded as outliers using the interquartile range method (lower limit = 1st quartile – 1.5 x IQR; upper limit = 3rd quartile + 1.5 x IQR).

Study Approval

All animal experiments were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (protocol #008-2022). Human placental specimens and data were biobanked and accessed under protocols approved by the Beth Israel Deaconess Medical Center Institutional Review Board, and written informed consent was obtained before subject participation (protocols #2008P000061, 2020P000997, 2021P000897).

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The authors declare no competing interests.

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Reviewer #1 (Public Review):

Ciampa et al. investigated the role of the hypoxia-inducible factor 1 (HIF-1) pathway in placental aging. They performed transcriptomic analysis of prior data of placental gene expression over serial timepoints throughout gestation in a mouse model and identified increased expression of senescence and HIF-1 pathways and decreased expression of cell cycle and mitochondrial transcripts with advancing gestational age. These findings were confirmed by RT-PCR, Western blot, and mitochondrial assessment from mouse placental tissues from late gestation time points. Studies of human placental samples at similar late gestational ages showed similar trends in increased HIF-1 targets and decreased mitochondrial abundance with increasing gestation, but were not significantly significant due to the limited availability of uncomplicated preterm placenta samples. The authors demonstrated that stabilization of HIF-1 in vitro using primary trophoblasts and choriocarcinoma cell lines recapitulated the gene and mitochondrial dysfunction seen in the placental tissues and were consistent with senescence. Interestingly, cell-conditioned media from HIF-1 stabilized placenta cell lines induced myometrial cell contractions in vitro and correspondingly, induction of HIF-1 in pregnant mice was associated with preterm labor in vivo. These data support the role of the HIF-1 pathway in the process of placental senescence with increasing gestational age and highlight this pathway as a potentially important contributor to gestational length and a potential target for therapeutics to reduce preterm birth.

Overall, the conclusions of this study are mostly well supported by the data. The concept of placental aging has been controversial, with several prior studies with conflicting viewpoints on whether placental aging occurs at all, is a normal process during gestation, or rather only a pathologic phenomenon in abnormal pregnancies. This has been rather difficult to study given the difficulty of obtaining serial placental samples in late gestation. The authors used both a mouse model of serial placental sampling and human placental samples obtained at preterm, but non-pathologic deliveries, which is an impressive accomplishment as it provides insight into a previously poorly understood timepoint of pregnancy. The data clearly demonstrate changes in the HIF-1 pathway and cellular senescence at increasing gestational ages in the third trimester, which is consistent with the process of aging in other tissues.

Weaknesses of this study are that although the authors attribute alterations in HIF-1 pathways in advanced gestation to hypoxia, there are no experiments directly assessing whether the changes in HIF-1 pathways are due to hypoxia in either in vitro or in vivo experiments. HIF-1 has both oxygen-dependent and oxygen-independent regulation, so it is unclear which pathways contribute to placental HIF-1 activity during late gestation, especially since the third-trimester placenta is exposed to significantly higher oxygen levels compared to the early pregnancy environment. Additionally, the placenta is in close proximity to the maternal decidua, which consists of immune and stromal cells, which are also significantly affected by HIF-1. Although the in vitro experimental data in this study demonstrate that HIF-1 induction leads to a placenta senescence phenotype, it is unclear whether the in vivo treatment with HIF-1 induction acts directly on the placenta or rather on uterine myometrium or decidua, which could also contribute to the initiation of preterm labor.

Reviewer #2 (Public Review):

The authors sought to characterize normal placental aging to better understand how the molecular and cellular events that trigger the labor process. An understanding of these mechanisms would not only provide insight into term labor, but also potential triggers of preterm labor, a common pregnancy complication with no effective intervention. Using bulk transcriptomic analysis of mouse and human placenta at different gestational timepoints, the authors determined that stabilization of HIF-1 signaling accompanied by mitochondrial dysfunction and cellular senescence are molecular signatures of term placenta. They also used in vitro trophoblast (choriocarcinoma) and a uterine myocyte culture system to further validate their findings. Lastly, using chemically induced HIF-1 induction in vivo in mice, the authors showed that stabilization of HIF-1 protein in the placenta reduced the gestational length significantly.

The major strength of this study is the use of multiple model systems to address the question at hand. The consistency of findings between mouse and human placenta, and the validation of mechanisms in vitro and in vivo modeling are strong support for their conclusions. The rationale for studying the term placentas to understand the abnormal process of preterm birth is clearly explained. Although the idea that hypoxic stress and placental senescence are triggers for labor is not novel, the comprehensiveness of the approach and idea to study the normal aging process are appreciated.

There are some areas of the manuscript that lack clarity and weaknesses in the methodology worth noting. The rationale for focusing on senescence and HIF-1 is not clearly given that other pathways were more significantly altered in the WGCNA analysis. The placental gene expression data were from bulk transcriptomic analyses, yet the authors do not explicitly discuss the limitations of this approach. Although the reader can assume that the authors attribute the mRNA signature of aging to trophoblasts - of which, there are different types - clarification regarding their interpretation of the data and the relevant cell types would strengthen the paper. Additionally, while the inclusion of human placenta data is a major strength, the differences between mouse and human placental structure and cell types make highlighting the specific cells of interest even more important; although there are correlations between mouse and human placenta, there are also many differences, and the comparison is further limited when considering the whole placenta rather than specific cell populations.

Additional details regarding methods and the reasons for choosing certain readouts are needed. Trophoblasts are sensitive to oxygen tension which varies according to gestational age, and it is unclear if this variable was taken into consideration in this study. Many of the cellular processes examined are well characterized in the literature yet the rationale for choosing certain markers is unclear (e.g., Glb1 for senescence; the transcripts selected as representative of the senescence-associated secretory phenotype; mtDNA lesion rate).

Overall, the findings presented are a valuable contribution to the field. The authors provide a thoughtful discussion that places their findings in the context of current literature and poses interesting questions for future pursuit. Their efforts to be comprehensive in the characterization of placental aging is a major strength; few placental studies attempt to integrate mouse and human data to this extent, and the validation and presentation of a potential mechanism by which fetal trophoblasts signal to maternal uterine myocytes are exciting. Nevertheless, a clear discussion of the methodologic limitations of the study would strengthen the manuscript.

Reviewer #3 (Public Review):

In this study, Ciampa and colleagues demonstrate that HIF-1 α activity is increased with gestation in humans and mice placentas and use several in vitro models to indicate that HIF activation in trophoblasts may release factors (yet to be identified) which promote myometrial contraction. Previous studies have linked placental factors to the preparation of the myometrium for labour (e.g. prostaglandins), but HIF-1 α has not been implicated.

Weaknesses and concerns:

1. The author's rebuttal state that placentas undergo subclinical cellular aging as they reach term. Although several future studies are described to test functional deficits at the cellular level, the current manuscript does not provide convincing evidence of cellular aging. The only evidence of cellular senescence provided in both human and mouse data is the mRNA expression of a single gene associated with senescence.
2. The authors have not responded to the concern regarding CoCl₂ mediating differentiation. The paragraph from a ref states that JAR cells do not respond as well as BeWOs to forskolin. However, this does not mean that JAR cells do not differentiate. This point is particularly pertinent as a quick search of their RNA-seq data shows upregulation of STB genes following CoCl₂ treatment including ERVs (ERVFRD1, ERVV-1, ERVV-2, ERV3-1), CYP19A1 and OVOL1 just to name a few. If the authors' conclusion is that CoCl₂ treatment did not alter trophoblast differentiation, the authors should provide additional data showing this. For example, cell fusion assays showing E-cadherin/desmoplakin staining and nuclear localization within stained boundaries.
3. The authors acknowledge the possibility of extraplacental effects of DMOG in the initiation of labour in their model, no additional evidence has been provided to support placental effects of their model. The authors also argue that although PMID 30808919 (which specifically overexpressed HIF-1 α in the placenta) did not show changes in birth length, they propose that this may be due to constitutive HIF1 α expression at the beginning of pregnancy. This argument is invalid since placental maldevelopment is consistently linked with several pregnancy complications including spontaneous preterm birth. If anything, perturbations in the beginning of pregnancy are more likely to lead to worse outcomes than those at the end of pregnancy.
4. Regarding induction of syncytialisation, please provide additional evidence that the cells have/have not syncytialised.
5. Lack of cohesion between experimental models. Please provide evidence that DMOG mediates similar effects on SA- β gal activity as CoCl₂ in JARs.
6. Evidence of senescence and mitochondrial abundance could be strengthened by providing additional markers. E.g. only GLB1 mRNA expression is provided as evidence of senescence, and COX IV protein for mitochondrial abundance in mouse and human placentas. This point has not been addressed. Please provide at least one additional marker of senescence and mitochondrial abundance.

Author Response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

[...] Overall, the conclusions of this study are mostly well supported by the data. The concept of placental aging has been controversial, with several prior studies with conflicting viewpoints on whether placental aging occurs at all, is a normal process during gestation, or rather only a pathologic phenomenon in abnormal pregnancies. This has been rather difficult to study given the difficulty of obtaining serial placental samples in late gestation. The authors used both a mouse model of serial placental sampling and human placental samples obtained at preterm, but non-pathologic deliveries, which is an impressive accomplishment as it provides insight into a previously poorly understood timepoint of pregnancy. The data clearly demonstrate changes in the HIF-1 pathway and cellular senescence at increasing gestational ages in the third trimester, which is consistent with the process of aging in other tissues.

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We thank Reviewer #1 for the thoughtful analysis offered here. We agree that our study has not determined whether placental HIF-1 activation occurring during late gestation is due to oxygen-dependent or oxygen-independent regulation; both possibilities are outlined in paragraph 3 of the Discussion. We used a pharmacological approach in our experiments characterizing the effects of HIF-1 stabilization in trophoblasts because it allows superior command of experimental conditions, but in future studies using hypoxic growth conditions we will determine whether oxygen sensing is a critical component of the aging effects on mitochondrial abundance, metabolism, and cellular senescence in the placenta.

Reviewer #1 also appropriately highlights the possibility that extra-placental effects of DMOG may contribute to the initiation of preterm labor in our mouse model. Future studies making use of mice with placenta-specific transgenes will allow clarification of the specific contributions of placental HIF-1 signaling to labor onset.

Reviewer #2 (Public Review):

[...] The major strength of this study is the use of multiple model systems to address the question at hand. The consistency of findings between mouse and human placenta, and the validation of mechanisms in vitro and in vivo modeling are strong support for their conclusions. The rationale for studying the term placentas to understand the abnormal process of preterm birth is clearly explained. Although the idea that hypoxic stress and placental senescence are triggers for labor is not novel, the comprehensiveness of the approach and idea to study the normal aging process are appreciated.

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Overall, the findings presented are a valuable contribution to the field. The authors provide a thoughtful discussion that places their findings in the context of current literature and poses interesting questions for future pursuit. Their efforts to be comprehensive in the characterization of placental aging is a major strength; few placental studies attempt to integrate mouse and human data to this extent, and the validation and presentation of a potential mechanism by which fetal trophoblasts signal to maternal uterine myocytes are exciting.

Nevertheless, a clear discussion of the methodologic limitations of the study would strengthen the manuscript.

We thank Reviewer #2 for careful consideration of our data and for the valuable feedback.

We chose to focus on HIF-1 signaling, mitochondrial function and abundance, and cellular senescence among the pathways that emerged from WGCNA based on our testable hypothesis that these three phenomena could be linked, with HIF-1 upstream of mitochondrial changes and cellular senescence (noted in Lines 166-169 with references to studies on aging establishing this connection in other systems). The other pathways not studied here (FOXO, AMPK, mTOR signaling) are important stress-response mediators which likely play additional key roles in the biology we have begun to describe; extensive future studies are warranted to explore this fully.

While we focused on establishing new mechanistic insights for aging in the placenta as a whole, localization of the effects described here to specific placental cell populations will be important to clarify in future studies, as is proposed in the Discussion (lines 316-319, which has been updated for emphasis). To our knowledge, no single-cell transcriptomics studies of

the placenta have been published to date describing gene expression changes across advancing gestational age in healthy pregnancies, and the quantitative value of immunolocalization studies of candidate proteins in isolation would be limited.

We do not dispute the limitations of mouse placenta as an imperfect model for the human organ; we have provided parallel data from human specimens wherever possible. We agree that this will continue to be critical in future studies, especially those aiming to achieve cell-type localization of these signaling pathways.

As mentioned in the response to Reviewer #1, we utilized pharmacological HIF-1 induction in our experimental models rather than manipulation of oxygen tension but acknowledge the value of follow-up studies utilizing hypoxic growth conditions in the Discussion.

SA-b-Gal activity is a key biomarker of cellular senescence, and this is most commonly assessed histochemically. Unfortunately, detecting b-galactosidase enzyme activity was not possible in the biobanked human specimens we accessed in this study (not collected/stored in a suitable format for histochemical processing), which is why we instead quantified expression of the lysosomal enzyme b-D-galactosidase, encoded by *GLB1*, the gene responsible for SA-b-Gal activity (Lee BY *et al.* Senescence-associated β -galactosidase is lysosomal β -galactosidase. *Aging Cell* 2006 – cited in line 106). A host of other senescence markers exists, but their appearance in senescent cells depends on the cell type and underlying drivers of the senescent phenotype (reference #45), with SA-b-Gal activity among the most universal. Similarly, the specific SASP components depend on cell type and senescence stimulus; we selected the markers in Figure 5H based on their previously established roles as mediators of placental signaling. As noted in the text (lines 120-121 with references to the relevant literature), mtDNA damage has previously been implicated as a driver of age-related loss-of-function in other tissues, which led us to explore whether mtDNA damage accompanies the other signs of mitochondrial dysfunction and dysregulation that were emerging in our data.

Reviewer #3 (Public Review):

In this study, Ciampa and colleagues demonstrate that HIF-1 α activity is increased with gestation in humans and mice placentas and use several in vitro models to indicate that HIF activation in trophoblasts may release factors (yet to be identified) which promote myometrial contraction. Previous studies have linked placental factors to the preparation of the myometrium for labour (e.g. prostaglandins), but HIF-1 α has not been implicated. Due to several issues regarding the experimental design, the results do not currently support the conclusions.

Major concerns:

- 1. The hypothesis states that placental aging promotes parturition via HIF-1 α activation, the study does not provide any evidence of an aged placenta. Aging is considered a progressive and irreversible loss of functional capacity, inability to maintain homeostasis, and decreased ability to repair the damage. The placenta retains all these abilities throughout pregnancy [PMID: 9462184], and there's no evidence that the placenta functionally declines between 35-39 weeks, otherwise, it wouldn't be able to support fetal development. However, there is evidence of a functional decline in post-term placentas (i.e. >40 weeks in humans) but the authors compare preterm placentas with E17.5 mice placentas or 39-week human placentas, both these gestational periods are prior to the onset of parturition in most pregnancies (human = 40wkGA, mice=E18.5).*

We thank Reviewer #3 for careful consideration of our manuscript and the thoughtful feedback.

Our stance that the placenta ages across its normal lifespan is based on the appearance of cellular senescence as an emerging pathway in latter gestational timepoints in the WGCNA, with subsequent validation of cellular senescence markers accumulating in placental samples from the advanced gestational age cohort. Although functional deficits stemming from the appearance of cellular senescence late in pregnancy may not be appreciable at the whole-system level until post-dates, we propose that the subclinical cellular aging that we have detected even before labor onset may be relevant in the setting of a “second hit” stressor — eg, impaired ability to maintain homeostasis, repair damage.

Future studies will examine functional deficits at the cellular level in response to HIF-1 stabilization (eg. Seahorse assay) and in early- versus late-gestational age primary cells. We hypothesize such studies will reveal impaired resistance to metabolic stressors in the senescent phenotype. Further, there will be value in exploring the impact of senolytics in restoring function to aged tissue.

In both mouse and human, our selection of placentas that had not yet been exposed to spontaneous labor was deliberate, in order to avoid confounding from the inflammatory effects of labor and delivery itself (due to large swings in perfusion pressure and local ischemia-reperfusion events).

1. While the authors provide evidence that HIF-1 α activity increases in both the human and mice placenta as gestation progresses, the mechanistic link between placental HIF-1 α and parturition is not strongly supported. For example, most of the evidence is based on in vitro studies showing that conditioned media from trophoblasts treated with CoCl₂ increased the contraction of myometrial cells. The specific factor responsible was not identified but the authors allude to pro- inflammatory factors such as cytokines. It was therefore interesting to note that the conditioned media had undergone a filtration step that removes all substances >10kDa, which includes the majority of cytokines and hormones.

We appreciate the opportunity to clarify that in the filtration step, we *retained the >10 kDa fraction*, allowing us to clear CoCl₂ itself among other <10kDa molecules. A 10kDa cutoff was chosen to allow for retention of cytokines including those previously implicated as signals that can promote contractility in uterine myocytes. As mentioned in the discussion, future studies will aim to identify specific factors within the secretome that are necessary and sufficient to induce the contractile changes.

1. An alternative explanation is that CoCl₂ treatment-induced trophoblast differentiation and the effects on myometrial contraction may be related to differences in secreted factors produced by cytotrophoblasts versus syncytiotrophoblast. Although JAR cells do not spontaneously differentiate, they can be induced to syncytialise upon cAMP stimulation. Ref 39 the authors cite shows this. Indeed, the morphology of the cells in Fig5F that were exposed to CoCl₂ indicates that they may be syncytialised. Syncytialised trophoblasts also express markers of senescence including increased SA- β -gal activity and reductions in mitochondrial activity.

The following is taken from Reference 39, final paragraph:

For instance, among the tested cell lines the choriocarcinoma cell line BeWo is best suited for studies on syncy8al fusion. However, ACH-3P, JAR and Jeg-3 cells react to forskolin treatment with elevated levels of hCG they do not form syncy8a73 and are therefore poor models for syncy8aliza8on over a period of 7

days.

1. The in vivo experiment showing reduced gestation length in pregnant mice receiving DMOG injection is interesting. However, we cannot exclude the effects of DMOG on non-placental tissues (both maternal and fetal) or the non-specific effects of DMOG (i.e. HIF-1α independent). Furthermore, previous studies using a more direct approach to alter HIF-1α activity in the placenta using trophoblast-specific overexpression of HIF-1α in mice do not lead to changes in gestation length [PMID: 30808910].

As stated in the response to Reviewer #1, we acknowledge the possibility that extra-placental effects of DMOG may contribute to the initiation of preterm labor in our mouse model. Future studies making use of mice with placenta-specific transgenes will allow clarification of the specific contributions of placental HIF-1 signaling to labor onset.

Regarding PMID 30808919, as noted in our Discussion (lines 326-335), an important distinction is that the referenced paper studied effects of trophoblast-specific expression of a constitutively active HIF1 from the beginning of pregnancy, and their findings highlight markedly abnormal placental development in that context. By contrast, we describe effects of HIF-1 stabilization late in pregnancy in a normally developed placenta.

1. Lack of appropriate experimental models. E.g. JAR choriocarcinomas are not an ideal model of the human trophoblast as they are malignant. Much better models are available e.g. primary human trophoblasts from term placentas or human trophoblast stem cells from first-trimester placentas. Similarly, the mouse model is also not specific as discussed above.

We agree with the Reviewer that the JAR cell line has important differences from human trophoblasts, nonetheless as stated in the Results section (Lines 181-184) they were used in order to model long-term exposure to HIF-1 induction without underlying syncytialization confounding the findings, as would be the case with primary cells.

1. Lack of cohesion between the different experimental models. E.g. CoCl₂ was used to induce hypoxia/HIF1α in mouse TBs, but DMOG was used in vivo in mice. SA-β Gal staining was carried out in cells but not in mouse or human tissues.

We used two distinct prolyl hydroxylase inhibitors (CoCl₂ and DMOG) in our *in vitro* studies (Figures 4, 5, and 5 Supplement) to demonstrate reproducibility across models and to help attribute the effects to HIF-1 stabilization rather than off-target effects. DMOG was chosen for the *in vivo* studies because of its prior use in mice.

As mentioned in response to reviewer 2, detecting b-galactosidase enzyme activity was not possible in the biobanked human specimens we accessed in this study (not collected/stored in a suitable format for histochemical processing), which is why we instead quantified expression of the lysosomal enzyme b-D- galactosidase, encoded by *GLB1*, the gene responsible for SA-b-Gal activity (Lee BY *et al.* Senescence-associated β-galactosidase is lysosomal β-galactosidase. *Aging Cell* 2006 – cited in line 106).

1. Evidence of senescence and mitochondrial abundance could be strengthened by providing additional markers. E.g. only GLB1 mRNA expression is provided as evidence of senescence, and COX IV protein for mitochondrial abundance in mouse and human placentas.

As mentioned in response to Reviewer 2, the appearance of other senescence markers depends on the cell type and underlying drivers of the senescent phenotype (reference #45), with SA-b-Gal activity among the most universal. Future studies will further probe which markers accompany cellular senescence in aging placenta to define the senescence phenotype in this setting.

1. Given that the main goal of this study was to investigate the role of hypoxia, hypoxia (i.e. low oxygen) was never directly induced and the results were based on chemical inducers of HIF-1α which have multiple off-target effects.

As mentioned in response to Reviewer 1, we agree that our study has not determined whether placental HIF-1 activation occurring during late gestation is due to oxygen-dependent or oxygen-independent regulation; both possibilities are outlined in paragraph 3 of the Discussion. We used a pharmacological approach in our foundational experiments characterizing the effects of HIF-1 stabilization in trophoblasts because it allows superior command of experimental conditions, but in future studies using hypoxic growth conditions we will determine whether oxygen sensing is a critical component of the aging effects on mitochondrial abundance, metabolism, and cellular senescence in the placenta. We are encouraged by the consistency of the senescence phenotype in JAR cells following administration of two distinct prolyl hydroxylase inhibitors, CoCl₂ and DMOG, suggesting that the effects seen are more likely attributable to HIF-1 stabilization (versus off-target effects).

Reviewer #1 (Recommendations For The Authors):

This is a very interesting and well-written study that supports the concept of placental aging using a combination of a mouse model, in vitro cell lines, and human placental samples.

Overall this is an important contribution to our current understanding of placental biology highlighting the role of the HIF-1 pathway and merits publication.

This study would be strengthened by the following addition:

- As stated in the Public Review, the authors attribute HIF-1 induction at increased gestation to hypoxia, however, this has not been demonstrated experimentally and HIF-1 has both O₂-dependent and independent regulation. The authors could perform an in vitro culture of primary placental cells or JAR cells under hypoxic conditions and assess the HIF-1 pathway/mitochondria activity to provide support for a hypoxia-dependent mechanism.

We thank Reviewer #1 for the thoughtful analysis offered here. We agree that our study has not determined whether placental HIF-1 activation occurring during late gestation is due to oxygen-dependent or oxygen-independent regulation; both possibilities are outlined in paragraph 3 of the Discussion. We used a pharmacological approach to characterize effects of HIF-1 stabilization in trophoblasts because it allows superior command of experimental conditions, but in future studies using hypoxic growth conditions we will determine whether oxygen sensing is a critical component of the aging effects on mitochondrial abundance, metabolism, and cellular senescence in the placenta.

Reviewer #2 (Recommendations For The Authors):

Major comments:

1. The rationale for the pursuit of HIF-1 and cellular senescence after initial WGCNA was weakly supported, though this avenue led to interesting and impactful results. The text could provide a stronger rationale for pursuing these pathways as opposed to the top-upregulated and downregulated pathways, perhaps by emphasizing previously published work in the field.

We thank Reviewer #2 for careful consideration of our data and for the valuable feedback.

We chose to focus on HIF-1 signaling, mitochondrial function and abundance, and cellular senescence among the pathways that emerged from WGCNA based on our testable hypothesis that these three phenomena could be linked, with HIF-1 upstream of

mitochondrial changes and cellular senescence (noted in Lines 166-169 with references to studies establishing this connection in other systems). The other pathways not studied here (FOXO, AMPK, mTOR signaling) are important stress-response mediators which likely play additional key roles in the biology we have begun to describe; extensive future studies are warranted to explore this fully.

1. Validation of the gene expression data with placental histology and immunolocalization of proteins of interest would bolster the study by identifying the relevant cell types and showing changes in protein levels over time. Additionally, single-cell RNA-seq data from mouse and human placenta are available. Exploration of these published datasets would also be interesting.

While we focused on establishing new mechanistic insights for aging in the placenta as a whole, localization of the effects described here to specific placental cell populations will be important to clarify in future studies, as is proposed in the Discussion (lines 316-319, which has been updated for emphasis). To our knowledge, no single-cell transcriptomics studies of the placenta have been published to date describing gene expression across advancing gestational age timepoints, and the value of single timepoint “snapshots” that exist in the literature is limited for the purpose of validating the aging mechanisms we have proposed here.

3. In Figure 2, all of the data have a gestational age-dependent trend except for Fig 2F where the mtDNA lesion rate drops at e15.5. What is the authors' interpretation of these results?

A testable hypothesis to explain this result is that as mtDNA damage begins to accumulate, cells are initially able to respond via mitophagy, removing those mitochondria with damaged DNA (e15.5), until that response is overwhelmed, allowing the detectable mtDNA lesion rate to spike at e17.5.

1. In paragraph three of the results, the authors conclude that there is an accumulation of ROS stress, yet there is no direct measurement of ROS. Measuring ROS directly in this setting would strengthen this conclusion (similar to what is done in Figure 5E).

We interpreted the accumulation of mtDNA damage as a marker of ROS stress, but the Reviewer correctly points out that we did not measure ROS directly in this model. We have updated the language (line 126) to be more accurate.

1. There is a discrepancy in the length of CoCl₂ treatment in primary trophoblasts vs. JAR cells (48 hours vs. 6 days). Treatment with DMOG in JAR cells also differed (4 days). Do the authors have any evidence that longer vs. shorter stabilization of HIF-1 has secondary effects in these cells that could affect the results of the study?

We preliminarily explored the timecourse of the effects of HIF-1 stabilization in JAR cells, as shown in Fig 5 – Supp 1, and also found that the decline in mt abundance precedes the appearance of senescence markers (data not shown). JAR cells are a much better model for exploring effects of chronic exposure to HIF-1 stabilization because they do not syncytialize as primary trophoblasts do. We limited our studies in primary cells for this reason to a 48h-timepoint, because the effects of syncytialization would confound longer studies. With the aim of simply validating our CoCl₂ findings with a separate prolyl hydroxylase inhibitor, we picked an intermediate timepoint for convenience. The reviewer correctly pinpoints the value of future studies that further dissect the kinetics of these phenomena, which could also potentially identify at which phases the effects are reversible.

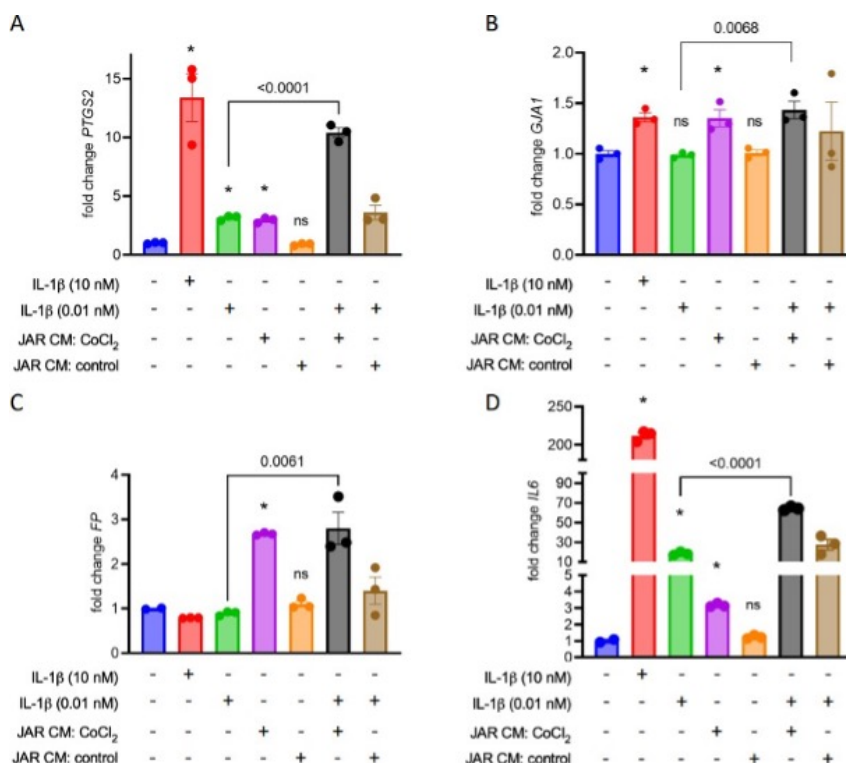
1. The authors evaluated mitochondrial effects in a time course experiment (Figure 5 Supplement 1) and found that the effects of HIF-1 stabilization emerged after three days of treatment, but no such experiment was conducted to determine the timing of senescence with SA- β Gal. It would be interesting to correlate the mitochondrial effects and onset of senescence caused by HIF-1 stabilization.

In future studies we will continue to explore the relative dynamics of HIF1 stabilization vs mitochondrial effects and senescence. In doing so it will be important to explore other markers of senescence; while SAbGal is the most universal senescence marker, others (such as p16 or p21 induction), if present, may lend themselves to more precise quantification and a clearer definition of senescence “start time”.

7. IL-1 β is used in experiments testing the effect of JAR-conditioned media on uterine myocytes. The conclusion of this experiment is that conditioned media from JAR cells treated with CoCl₂, but not from untreated JAR cells, results in myocyte contraction (Figure 6E) and expression of contraction-associated genes (Figure 6A-D). Although the figure shows that IL-1 β + conditioned media increases expression of these genes compared to IL-1 β alone, an added control condition where conditioned media is used in the absence of IL-1 β would underscore this conclusion and show whether the components in the conditioned media are sufficient to induce gene expression and contraction. There is also no justification for the 10 kDa cutoff in this experiment.

We did test whether conditioned media could induce contractile changes in myocytes in the absence of IL-1b co-stimulation, and indeed found that the CoCl₂-stimulated conditioned media does elicit this effect on its own. We eliminated these conditions from the published figure in an aim to limit its complexity, but present them here (*, p < 0.05 vs no treatment):

Author response image 1.



The filtration step was implemented to concentrate the conditioned media prior to applying it to the myocytes. A 10kDa cutoff was chosen to ensure retention of most cytokines, especially

those previously implicated in contractile switching of uterine myocytes (eg. IL1b, IL1a, TNFa each approximately 18 kDa, IL6 approximately 21 kDa). The filtration and wash steps also ensured clearance of CoCl₂ out of the conditioned media before it was applied to myocytes.

1. Figure 7 shows the use of DMOG in vivo to stabilize HIF-1, which induces preterm labor. Is there a way to inhibit HIF-1 signaling downstream to show that preterm labor in vivo is specifically due to HIF-1 stabilization and not an off-target effect of DMOG? Rescue experiments either in vitro or in DMOG-treated mice using HIF-1s inhibitors would be very compelling although we recognize these may not be feasible. Regardless, a comment on the translational impact of this study and the potential of targeting the HIF pathway to treat or prevent SPTB should be considered.

There is considerable research into HIF inhibitors as cancer therapeutics (and FDA approval of a HIF2a inhibitor, belzutifan, for von Hippel Lindau disease). Future studies into the ability of HIF-1 inhibitors to rescue preterm labor are certainly of interest, though translational potential may be limited by systemic toxicity unless a targeted placenta-specific delivery system can be achieved. Genetic approaches using placenta-specific knockout might also be useful, particularly if conditional knockout can be achieved to limit the effects on HIF-1 signaling to late pregnancy, after placental development is complete.

9. The effect of JAR-conditioned media on uterine myocytes is very interesting. The authors might consider additional discussion of what the putative mediators are and what is suggested in the preterm birth literature (e.g., Sheller-Miller, PMID: 30679631). Assessment of other SASP factors in using ELISA, e.g., would strengthen the study, or at least a rationale for the genes evaluated.

We agree that follow-up studies should be done to identify which components of the secretome are key for mediating the contractile effect in myocytes, as noted in the Discussion (Lines 271-273), now updated for emphasis and with the suggested references.

Additional minor comments:

1. For Figure 1A, without reading the figure legend it is unclear that the vertical color graph represents different gene clusters; consider labeling the y-axis with 'Gene clusters.' Also, blue and turquoise clusters could be labeled as "upregulated" or "downregulated" for simplicity and clarity.

Updated, thank you for the suggestions.

1. For mRNA expression wherever relevant, state in the figure legends and main text the method used (i.e., qPCR) and what the reference timepoint and normalization strategy was. For instance, in Figure 2 (and supplement 1), we were of the impression that the e15.5 and e17.5 values were normalized to e13.5.

Updated, thank you for the suggestions.

1. For Figure 5, can the authors explain in the main text what is Mtsox and how is it a marker for mitochondrial depolarization? In 5E, it would be helpful to mention what is TMRE and FCCP are and how it measures mitochondrial ROS.

Updated, thank you for the suggestions.

1. Figure 5 Supplement 2 and Figure 5 Supplement 3 appear to be missing labels indicating black vs. blue vs. red datasets.

Updated, thank you for the suggestion.

1. Figure 7c, what is the *n* in each group?

Gestational length data in Figures 7c and 7d each reflect the same *n*=8 mice per group.

1. Minor edits are needed for inconsistent use of terms (*pre-term* vs. *preterm*, for example) and grammar.

Updated, thank you for the suggestion.

Suggested additions to the Methods section to improve reproducibility:

1. Include more detail re: cell culture conditions, including % oxygen.

Updated, thank you.

1. Collagen lattice contraction assay - include details on how measurements of collagen discs were performed. Was this automated?

Updated, thank you.

1. Immunoblots. Details, such as the amount of protein loaded, gel composition, protein extraction method, etc., would be helpful.

Updated, thank you.

Reviewer #3 (Recommendations For The Authors):

Minor comments:

1. It is unclear why 2-way ANOVA was performed in figure 3 when there are only 2 groups under comparison: <35 wks vs >39 wks

As in Figure 2D, multiple genes are analyzed together in Figure 3A using 2-way ANOVA with the two factors being 1) gestational age and 2) individual gene targets (GLB1, HK2, GLUT1). This approach allows us to define the combined effect of gestational age on expression level of all of the genes whose expression is increasing.

1. Scale bars missing in some figures - Fig4E, Fig 5D, 5F, Fig5 - Suppl 3C.

Scale bars were not captured with the original images; we regret this omission.