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Hypoxia enhances the expression of follistatin-like 3 in term human trophoblasts

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Abstract

Hypoxic injury hinders placental differentiation and alters trophoblast gene expression. We tested the hypothesis that the expression of follistatin-like 3 (FSTL3), a member of the follistatin family of proteins, is modulated by hypoxia in primary human trophoblast (PHT). Using immunofluorescence of human term placental villi we detected the expression of FSTL3 protein in placental villi, primarily in trophoblasts. We verified this finding in cultured term PHT cells. Basal expression of *FSTL3* transcript in cultured PHT cells, determined using quantitative PCR, was stable over the culture period. Importantly, when compared to culture in $\text{FiO}_2=20\%$ or $\text{FiO}_2=8\%$, PHT cells cultured in $\text{FiO}_2 < 1\%$ exhibited a 4–6 fold increase in *FSTL3* mRNA expression as early as 4 h in hypoxia. Whereas cellular FSTL3 protein was unchanged in hypoxia, we found that hypoxia increased the level of FSTL3 in the medium. Lastly, the exposure of PHT cells to either the hypoxia-mimetic cobalt chloride or the proline hydroxylase inhibitor dimethylxaloylglycine upregulated the expression of *FSTL3* transcript. Our data indicate that hypoxia enhances the expression of FSTL3 and its release from PHT cells. Our finding that hypoxia-mimetic agents enhance *FSTL3* expression implicates HIF1 α in this process.

1. Introduction

Unlike the physiological hypoxia that characterizes the implanting placenta early in human pregnancy [1–3], placental injury that is commonly linked to fetal growth restriction (FGR) is characterized by shallow invasion and increased resistance of the spiral arteries [4]. These abnormalities contribute to attenuated uteroplacental perfusion and reduced oxygen transport to the fetoplacental unit [5]. In addition to the well-characterized histological changes of injured trophoblasts [4,6], hypoxia influences the repertoire of trophoblast transcripts [7–9], with some of these effects attributed to the action of the transcription factor hypoxia inducible factor-1 α (HIF1 α).

Follistatin like 3 (FSTL3), also known as the product of *follistatin-related gene* (*FLRG*), is a 27 kDa glycoprotein, expressed from the human chromosome 19p13 region [10]. FSTL3 is a member of the follistatin family of proteins [11]. FSTL3 antagonizes the activity of members

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of the transforming growth factor beta (TGF β) family of signaling proteins. For example, FSTL3 is capable of binding activin, thereby hindering the ability of activin to bind its receptor [12–17]. This alters the influence of activin on diverse biological processes, including cellular growth and differentiation, immune responses, hematopoiesis, wound repair and response to injury [18–21]. FSTL3 is expressed in the cytoplasm and nucleus, and is also secreted from diverse cell types [14,16,22,23]. The expression of FSTL3 is high in the placental trophoblasts, and in villous endothelial cells [24]. It is also found in the amnion and chorion and extrauterine tissues including the testis, lung, kidney, pancreas and heart [10,25,26].

Using high-density oligonucleotide microarray screens, corroborated with RT-qPCR, we previously detected a higher expression of *FSTL3* mRNA in trophoblasts exposed to hypoxia, compared to trophoblasts cultured in standard conditions [7,27]. We and others also demonstrated enhanced placental *FSTL3* transcript in samples from pregnancies complicated by FGR vs. matched controls [7,28]. Because of these observations and in light of the influence of FSTL3 on activin we sought to examine the effect of hypoxia on the expression of FSTL3 mRNA and protein in term primary human trophoblasts (PHTs).

2. Methods

2.1. Immunofluorescence

The study was approved by the human research protection office of Washington University. Placentas were obtained within 20 min after term (37–41 weeks) uncomplicated singleton pregnancies and deliveries. Participants were delivered by either vaginal or abdominal delivery. Placental fragments (approximately 0.125 cm³) were sharply dissected at a site located at half the placental radius from the lateral placental margin, at the middle of the placental depth, 8–10 mm from the placental basal plate [29]. The biopsies were fixed for 24 h at room temperature in 10% neutral buffered formalin and embedded in paraffin. Sections (5 μ m) were deparaffinized in xylene and rehydrated in an ethanol gradient. For antigen retrieval, the sections were microwaved 11.5 min in 0.01 M NaCitrate, pH 6.0 and then cooled for 15 min to room temperature. The slides were washed, blocked with 10% Sea Block buffer (Pierce, Rockford, IL) in TBST solution (Tris buffered saline with 0.05% Tween 20) for 1 h in a humidity chamber, then incubated for 2 h in room temperature with goat polyclonal antibody against human FSTL3 (final 2 μ g/ml, Santa Cruz Biotechnology, Santa Cruz, CA). The slides washed twice and incubated for 2 h with Alexa Flour donkey anti-goat secondary antibody (final 10 μ g/ml, Invitrogen, Carlsbad, CA), washed with PBS and incubated with TO-PRO-3 iodide (642/661; Invitrogen, 1:200) in PBS for 10 min for nuclear staining. The slides were washed, covered and viewed using a confocal microscope (Nikon eclipse E800). For negative control we used non-immune serum instead of the primary antibody, and a secondary antibody as described. For immunofluorescence of primary PHTs, cultured cells were fixed with methanol, washed three times with PBS and stained as described above.

2.2. Trophoblast isolation and culture

Term PHT cells were isolated and cultured as previously described [8,30,31]. After 4 h of culture in standard conditions, defined as 20% O₂/5% CO₂, non-adherent cells and syncytial fragments were removed by washing three times in PBS. Some plates were then cultured up to 48 h in an enclosed incubation chamber (Thermo Forma, Marietta, OH) that provided a defined hypoxic atmosphere of either <1% or 8% O₂, where stated (5% CO₂ and complemented by N₂, with added 10% H₂ to the <1% O₂), with continuous oxygen monitoring. The medium was replaced every 24 h. Where indicated, cultures were exposed to the hypoxia mimetic agent cobalt chloride (CoCl₂, Sigma, St. Louis, MO) or to the proline hydroxylase inhibitor dimethylxaloylglycine (DMOG, Cayman, Ann Arbor, MI) at the concentrations indicated.

2.3. RT-quantitative PCR (RT-qPCR)

RNA was purified using TriReagent (Sigma) according to the manufacturer's instructions, and cDNA synthesized using reverse transcription as we previously described [8]. PCR was performed in duplicate on 3 μ L RT product using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with the primer pairs listed below in a total reaction volume of 25 μ L, as described [8]. Reactions were analyzed using an Applied Biosystems Geneamp 7300 System. Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature. Fold change was computed relative to control using the $2^{-\Delta\Delta C_T}$ method [32,33]. Samples were normalized to *YWHA* expression, determined in parallel reactions. The primer sequences used included *FSTL3* (NM_005860) F (890–911)-GACACTCCTTAGAGCCCGGATT, and R (974–955) GCCTCCCTTCCTCAGTCCTT, *YWHAZ* (NM_145690) F (1133–1156)-ACTTTTGGTACATTGTGG CTTCAA, and R (1226–1207)-CCGCCAGGACAAACCAGTAT.

2.4. Western immunoblotting

PHT cells were rinsed with cold PBS and lysed with 150 μ L of lysis buffer with protease inhibitors, as we previously described [34]. The plates were scraped, and the lysate sonicated three times for 10 sec each, on ice. The lysates were centrifuged, and 50 μ g of protein/lane were separated on 12% SDS-PAGE at 100 V for 2 h. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) overnight at 4°C. Each blot was blocked using 10% Sea Block buffer (Pierce) in TBST for 2 h, then incubated overnight in 5% non-fat dry milk in TBST at 4°C with goat polyclonal antibody against human *FSTL3* (described above), or goat polyclonal anti-actin as a loading control (final 0.4 μ g/ml, Santa Cruz). Each blot was then washed and incubated for 2 h with horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz) at room temperature, then washed and processed for chemiluminescence using a Western Lightning ECL kit (Perkin Elmer, Boston, MA). For detection of *FSTL3* protein in the cultured medium we concentrated the medium by filtration using Centricon YM-10 Centrifugal Filters (MW cutoff 10 kD, Millipore). The medium was collected and centrifuged at 5,000 $\times$ g for 60 min. The concentrated sample was collected and added to a reducing SDS-PAGE loading buffer after adjustment for protein concentration in lysates from the plated cells. The samples were then electrophoresed and immunoblotted as described above. The results were densitometrically quantified using EpicheMI-3 software (UVP BioImaging System, Upland, CA).

2.5. Statistical analysis

Each experiment was repeated at least three times. Representative experiments are shown in the figures. Statistical analysis and determination of significance were performed based on all experiments. The fold change in mRNA expression was calculated using a geometric mean. Ordinal data were analyzed using the non-parametric Kruskal-Wallis analysis for multiple comparisons and a post-hoc Dunnett test or the Mann-Whitney test, using Primer of Biostatistics software (McGraw-Hill NY, NY). Significance was determined at $p < 0.05$.

3. Results

We initially determined the expression of *FSTL3* protein in placental villi derived from term uncomplicated pregnancies. Using immunofluorescence we detected *FSTL3* primarily in the villous trophoblast bilayer (Fig. 1), with a lower level of expression within the villous core. To evaluate the effect of culture on *FSTL3* expression we first used RT-qPCR to measure basal *FSTL3* mRNA expression in PHT cells cultured in standard conditions (FiO₂ 20%). As shown in Fig. 2A, *FSTL3* mRNA was not significantly changed during 48 h in culture. To assess the influence of hypoxia on the expression of trophoblast *FSTL3* transcript we exposed cultured

PHT cells to hypoxia *in vitro*, and used RT-qPCR to measure *FSTL3* expression. Because human villous trophoblasts beyond the first trimester of normal pregnancy are likely exposed to an FiO_2 near 8% [2] we compared *FSTL3* mRNA in cells cultured in $\text{FiO}_2 < 1\%$ to expression in $\text{FiO}_2 = 8\%$, as well as standard culture conditions ($\text{FiO}_2 = 20\%$). We have previously characterized trophoblast gene expression in our hypoxic conditions, demonstrating a reduction in hCG and transferrin expression and an increase in erythropoietin and erythropoietin receptor expression (Biron-Shental *et al*, *in press*). As shown in Fig. 2B, there was no difference in *FSTL3* mRNA level between PHT cells cultured in $\text{FiO}_2 = 8\%$ or 20% for 48 h. In contrast, we observed a marked increase in *FSTL3* levels in cells cultured in $\text{FiO}_2 < 1\%$. This effect was time-dependent, reaching a maximum after 8 h in hypoxia (Fig. 2C).

We used western immunoblotting to determine if the increase in *FSTL3* transcript in hypoxic PHT cells translated to a higher level of cellular FSTL3 protein. Interestingly, we found that the expression level of cellular FSTL3 was unchanged in our culture conditions (Fig 3A), with some of the cultures exhibiting a non-significant decline in FSTL3 levels after 48 h. We expanded these results using immunofluorescence, demonstrating that intracellular localization of FSTL3 was also unchanged in standard and hypoxic PHTs (Fig. 3B). Because FSTL3 is a secreted protein [14,23,35] we examined FSTL3 levels in the culture medium and found that unlike intracellular FSTL3 protein expression, the level of FSTL3 in medium of PHT cells cultured in $\text{FiO}_2 < 1\%$ was significantly higher than its level in medium from cells cultured in standard conditions (Fig. 3A, lower panel).

Finally, because the influence of hypoxia on cells is frequently mediated by HIF1 α we tested for the effect of the HIF1 α -stabilizing, hypoxia-mimetics CoCl_2 and DMOG [36–38] on *FSTL3* mRNA. As shown in Fig. 4, we found that both hypoxia-mimetics increased *FSTL3* mRNA expression. This effect of CoCl_2 and DMOG was recapitulated using the human placental line JEG3 (not shown).

4. Discussion

FSTL3 has been previously detected in the murine and human placenta [7,14,24,25,28]. Here we expanded these observations, and showed that FSTL3 protein is expressed primarily in the trophoblast layer of term human placental villi. The expression of FSTL3 mRNA and protein was constant when PHT cells differentiate *in vitro* from cytotrophoblasts to predominantly syncytiotrophoblasts [31,34]. Importantly, hypoxia ($\text{pO}_2 < 1\%$) upregulated *FSTL3* mRNA in PHT cells. Whereas the expression of cellular FSTL3 protein was stable, the level of FSTL3 in the culture medium was increased by hypoxia. Our data, along with information on FSTL3 secretion [14,39], suggest that while differentiation does not alter the expression of FSTL3, hypoxia enhances the secretion of FSTL3 from trophoblasts.

Although our study was not designed to detail the mechanisms underlying the regulation of *FSTL3* gene by hypoxia, our data support a role for HIF1 α in this process. The transcription factor HIF1 α regulates the expression of many genes in response to hypoxia. CoCl_2 antagonizes the action of iron in converting the stable HIF1 α to a polyubiquitinated form, and DMOG non-selectively inhibits prolyl-hydroxylases that are needed for this process, rendering HIF1 α transcriptionally active in the absence of hypoxia [36–38]. Exposure of PHT cells to either CoCl_2 or DMOG recapitulates the effect of hypoxia on *FSTL3* gene expression, and therefore invokes HIF1 α in this response. Consistent with these findings, our analysis reveals that human FSTL3 5'-sequences, identified from cosmid R33586 on chromosome 19 (accession No. AC004156) [35], harbor at least two (–388 and –746 from the start site) putative HIF1 α binding elements [40]. Our understanding of the function of these promoter sites in regulation of *FSTL3* gene by hypoxia requires additional studies.

At the present time the role of FSTL3 in response to placental hypoxia remains unknown. Others have found that brain *FSTL3* expression increases after penetrating injury [20], and that FSTL3 is expressed during wound healing and hematopoiesis [21]. FSTL3-deficient mice do not exhibit an abnormal phenotype during embryonic development, but have not been tested under stress conditions. These mice exhibit pancreatic β -cell hyperplasia, insulin sensitivity without an increase in muscle mass yet with hepatic steatosis, increased hepatic gluconeogenesis and decreased visceral fat [41]. Male mice with targeted gonadal overexpression of human *FSTL3* exhibit smaller testes with reduced sperm counts, degenerating seminiferous tubules and reduced fertility, while females have fewer preantral and antral follicles and a greater incidence of follicular atresia. These abnormalities were associated with reduced body weight [42].

Mechanistically, FSTL3 is known to bind activin. This binding antagonizes the biological activity of activin [14–17,19]. TGF β and activin, in turn, up-regulate the activity of *FSTL3* promoter, acting mainly via the binding of SMAD3/4 to *FSTL3* promoter [19,35]. Together, these signaling pathways complete a feedback loop that controls activin action. Although studies using gene ablation in mice did not uncover a significant role for activin in placental function [43], activin and its receptors are expressed in trophoblasts, and activin expression is enhanced in placentas from pregnancies complicated by preeclampsia, and reduced in placentas exposed to hypoxia [44]. The biological activity of placental FSTL3 may also be related to other proteins that interact with FSTL3, including myostatin, ADAM12 and fibronectin [22, 45,46]. These interactions may shed light on the function of FSTL3 in hypoxic trophoblasts, and on the potential paracrine effect of FSTL3 on non-trophoblastic villous cells, such as fibroblasts, macrophages or endothelial cells.

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Figure 1.

FSTL3 expression in placental villi. Formalin-fixed sections of normal term human placentas were stained for FSTL3 using immunofluorescence. (A) Negative control using non-immune serum instead of the primary antibody and a secondary antibody. (B) FSTL3 staining, with inset demonstrating trophoblast staining (arrows). The results were repeated in samples from three different placentas. Blue: nuclei; Green: FSTL3. Magnification $\times 200$, bar = 30 μM .

Figure 2.

The effect of hypoxia on the expression of *FSTL3* mRNA in PHT, analyzed using RT-qPCR. (A) The expression of *FSTL3* in standard conditions. Cells were cultured for 48 h in FiO₂=20%, and the basal expression of *FSTL3* was analyzed (n=6). (B) The influence of hypoxia (FiO₂=8% or FiO₂ ≤1%) on *FSTL3* expression. (C) A time course for the effect of hypoxia (FiO₂ ≤1%) on *FSTL3* mRNA expression in PHT. The graphs represent data reproduced in five experiments (* denotes p <0.05).

Figure 3.

Cellular and culture medium levels of FSTL3 protein in standard and hypoxic conditions. (A) Western immunoblotting depicts the expression of FSTL3 in standard conditions and hypoxia ($\text{FiO}_2 \leq 1\%$, a representative experiment from $n=4$). Medium samples were collected from trophoblasts that were cultured in serum free conditions and concentrated by filter centrifugation as described in methods. The graph represents the signal density for FSTL3 in the concentrated medium, with white bars representing standard conditions and black bars representing hypoxia (densitometry of a representative experiment, $n=4$). (B) Immunofluorescence of FSTL3 in PHT cells obtained from PHTs cultured for 24 h in standard

conditions or hypoxia ($O_2 \leq 1\%$, magnification $\times 600$, $n=3$). Blue: nuclei; Green: FSTL3, bar=10 μM .

Figure 4.

The effect of hypoxia-mimetic chemicals on *FSTL3* mRNA expression in cultured PHT cells. Trophoblasts were exposed to CoCl₂ (0.2 μ M) or DMOG (0.25, 0.5 or 1 μ M) at the time points indicated. RNA was isolated and quantified using RT-qPCR (* denotes p < 0.05 compared to control, n=3).