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The expression and post-transcriptional regulation of FSTL1 transcripts in placental trophoblasts

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Abstract

Introduction—Follistatin-like-1 (FSTL1) is a widely expressed secreted protein with diverse but poorly understood functions. Originally described as a pro-inflammatory molecule, it has recently been reported to play a role in signaling pathways that regulate development and homeostasis. Distinctively, FSTL1 harbors within its 3'-UTR the sequence encoding microRNA-198 (miR-198), shown to be inversely regulated relative to FSTL1 expression and to exhibit opposite actions on cellular processes such as cell migration. We sought to investigate the expression of FSTL1 and to assess its interplay with miR-198 in human trophoblasts.

Methods—We used a combination of northern blot analyses, quantitative PCR, small RNA sequencing, western blot and immunohistochemistry to characterize FSTL1 and miR-198 expression in placental trophoblasts. We also used reporter assays to examine the post-transcriptional regulation of FSTL1 and assess its putative regulation by miR-198.

Results—We detected the expression of FSTL1 transcript in both the human extravillous trophoblast line HTR-8/SVneo and in primary term human villous trophoblasts. We also found that the expression of FSTL1 was largely restricted to extravillous trophoblasts. Hypoxia enhanced the expression of FSTL1 protein in cultured primary villous trophoblasts. Interestingly, we did not detect any evidence for expression or function of mature miR-198 in human trophoblasts.

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Discussion—Our data indicate that placental FSTL1 is expressed particularly in extravillous trophoblasts. We also found no evidence for placental expression of miR-198, or for its regulation of FSTL1, implying that the post-transcriptional regulation of FSTL1 by miR-198 is tissue specific.

Keywords

trophoblasts; hypoxia; FSTL1; miR-198

INTRODUCTION

Follistatin-like-1 (FSTL1) is a secreted glycoprotein with a follistatin domain and two non-functional calcium-binding motifs [1]. It was originally cloned as a TGF β 1 inducible factor [2] but subsequently shown to regulate diverse developmental pathways and tissue homeostasis. Ablation of the FSTL1 gene in the mouse results in several structural developmental defects and neonatal lethality due to respiratory failure, demonstrating its functional importance [3–5].

Despite its pleiotropic roles, the detailed molecular action of FSTL1 remains poorly understood. For example, FSTL1 suppresses BMP signaling, but the precise mechanism of its action has not been elucidated [4,6]. In addition, FSTL1 has been reported to exhibit both pro- and anti-inflammatory actions [7–13], with a specific anti-apoptotic and protective effect in cardiac or renal cell injury [14–16]. In addition, there are inconsistent results regarding the role of FSTL1 in cell migration and proliferation, with FSTL1 limiting cell migration in some systems [17–19] while enhancing cell proliferation and invasion in others [20,21]. FSTL1 is also upregulated during vascular development of the chick chorio-allantoic membrane, a structure that forms the placenta in mammals [22].

While some of the inconsistencies regarding the action of FSTL1 can be explained by tissue-specific function or by other experimental variables, results are also confounded by the fact that FSTL1 transcript encodes two distinct products: an mRNA for FSTL1 and a microRNA, miR-198, transcribed from the 3'-UTR of FSTL1. Interestingly, FSTL1 and miR-198 seem to have opposite functions, and are expressed in a mutually exclusive fashion, switched by environmental cues in a human skin organ culture system [21]. The expression profile of this bicistronic locus and, particularly, the relative expression level of the two transcripts have not been hitherto investigated outside this skin organ culture. Further, although FSTL1 lies at a crossroads of signaling pathways that are critical for placental function, its expression and regulation in the placenta have not been previously studied. In light of the information presented here, we tested the hypothesis that FSTL1 is expressed in human trophoblasts and regulated by miR-198. Although we found that FSTL1 is expressed in trophoblasts, with higher protein expression during hypoxic injury and prominent expression in extravillous trophoblasts (EVT) *in vivo*, we could not detect any evidence for the expression or function of miR-198 in trophoblasts. We detected several short RNA species that are strongly and exclusively induced in hypoxic primary human trophoblasts, suggesting complex transcriptional regulation and processing of the FSTL1 locus in trophoblasts.

MATERIALS AND METHODS

Cell culture

All placentas ($n > 10$) were obtained after term delivery, using a protocol approved by the Institutional Review Board at the University of Pittsburgh. Primary human trophoblasts (PHT cells) were prepared from normal term placentas using the trypsin-deoxyribonuclease-Dispase/Percoll method as described by Kliman, with previously published modifications [23]. Cultures were plated at a density of 350,000 cells/cm² and maintained in standard or hypoxic growth conditions as we previously described [24,25]. In some of the cultures, PHT cells were exposed to 8-Bromo-cAMP (0.5 mM) or to DMSO (1.5%) as we previously described [26,27]. Immortalized human first trimester EVT cell line, HTR-8/SVneo, provided by Charles Graham, Queen's University, Kingston, Ontario [28], were cultured in RPMI 1640 (Mediatech, Manassas, VA), supplemented with 5% bovine growth serum (HyClone, Logan UT) and antibiotics. Other cell lines were cultured in standard conditions or in hypoxia, as indicated, using DMEM-based medium (MDA-MB, SKUT1, Caco2 and PANC1 lines), MEM-based medium (HepG2, Ishikawa, WISH, HeLa and JEG3 lines), RPMI-based medium (JAR line) and Ham's F-12K (BeWo line), supplemented by 10% bovine growth serum (HyClone) and antibiotics.

RNA detection using northern blot

For analysis of miRNA levels, total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform procedure of Chomczynski and Sacchi [29]. 20 µg of total RNA were denatured for 10 min at 65°C and subsequently resolved using 7 M urea/15% polyacrylamide gel electrophoresis (PAGE). The gel was stained using SYBR-gold (Invitrogen/Molecular Probes, Eugene, OR), and RNA was electro-transferred to nylon Hybond N+ membrane (GE Healthcare, Piscataway, NJ). Hybridization was performed overnight in 0.5 M Na₂HPO₄, pH 7.4, 7% SDS, 1 mM EDTA at 37°C, using DNA oligonucleotide probes, which were labeled with α-³²P]-dATP, using a StarFire labeling system (Integrated DNA Technologies, Coralville, IA). After washing, each membrane was exposed to film for 16–24 h. To assess FSTL1 expression, the RNA was resolved using MOPS-buffered 1.2% agarose gel containing formaldehyde, and then transferred to a nitrocellulose membrane by capillary blotting with 10× SSC. The membrane was hybridized overnight using a ³²P-labeled cDNA probe at 65°C in 0.5 M Na₂HPO₄, pH 7.4, 7% SDS, 1 mM EDTA.

Real-time quantitative PCR

RNA isolation and reverse transcription was performed as previously described. Briefly, total RNA was extracted from primary trophoblasts using TriReagent (MRC, Cincinnati, OH) according to the manufacturer's instructions, followed by purification on a silica spin column. RNA samples were exposed to DNaseI using a Turbo DNA-free Kit (Ambion). Reverse transcription (RT) was performed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA) in a 20 µl reaction mix at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. Reactions were run in duplicate using 384 well plates with 3 µL of cDNA per 10 µL of reaction mixture using SYBR Green PCR Master Mix (Applied Biosystems), and analyzed using an Applied Biosystems ViiA 7 Sequence

Detection System. Dissociation curves were run on all reactions, and samples were normalized to the expression of YWHAZ [30]. The Ct method was used to determine relative gene expression [31]. The primers used in this study are listed in table 1.

Small RNA sequencing

Small RNAs of 18 to 28 nucleotides in length were gel-purified from 10 µg of total RNA, isolated from PHT cells that were cultured in standard or hypoxic conditions for 48 h. Small RNA libraries were prepared and sequenced on a Illumina HiSeq-2000 platform. This was performed by Ocean Ridge Biosciences (Palm Beach Gardens, FL). The small RNA libraries were aligned to the NCBI37 human reference genome using Bowtie [32], then intersected with the mature miRNA sequenced annotated by miRBase [33] using BEDtools [34]. Assuming that the miRNA counts followed the Negative-Binomial distribution, we tested for differentially expressed transcripts using the algorithm implemented in the R package DESeq2, with the estimated relative size as the size factor for the DESeq2 algorithm [35]. The p-values of the Negative-Binomial tests were adjusted using the Benjamini and Hochberg's method, in order to control for false discovery rate [36].

Immunohistochemistry (IHC)

Immunohistochemical staining was performed on 5 µm paraffin-embedded sections of human 4% paraformaldehyde-fixed placentas using an automated immunostainer (Leica Bond-Max IHC stainer, San Diego, CA). FSTL1 IHC was performed using a goat polyclonal antibody to FSTL1 (1:30, R&D Systems, Minneapolis, MN). Cytokeratin-7 staining was performed using a pre-diluted monoclonal antibody (CK7, clone OV-TL-12/30) from DAKO (DAKO Corporation, Carpinteria, CA). Placental lactogen (hPL) staining was performed using a pre-diluted rabbit polyclonal antibody (Biogenex, San Ramon, CA, USA). CD34 IHC was performed using a prediluted antibody (CD34, clone QBEnd/10, 1 mg/L, Leica Biosystems Inc., Buffalo Grove, IL). Bond Ready-to-Use Negative Control (Leica), negative (Rabbit) and negative (Mouse) were used as control. The Bond Refine Polymer detection system was used for visualization. IHC of CD68 and HLA-G was performed manually. For detection of CD68, we used an anti-mouse IgG biotin conjugated secondary antibody (1:200, Boehringer Mannheim, Indianapolis, IN).

Immunohistochemistry of HLA-G was performed on 4% paraformaldehyde fixed frozen sections using a mouse monoclonal antibody (20 µg/ml, MEM-G/9, Abcam, Cambridge, MA, ab7758) and an Anti-mouse IgG Biotin conjugated secondary antibody (1:200, Boehringer Mannheim) as a secondary antibody. The sections were developed with diaminobenzidine (DAB Chromogen/Substrate Kit [High Contrast], ScyTek, Logan UT).

Western blot

The cells were lysed in Cell Culture Lysis Reagent (Promega), supplemented with protease inhibitors (Halt Protease Inhibitor cocktail, Thermo Scientific, Waltham MA). Lysates were separated on SDS-PAGE and transferred to PVDF membranes, using standard procedures [37]. Membranes were immunoblotted with goat Anti-FSTL1 antibody (0.5 µg/ml, ab11805, Abcam) followed by anti-goat HRP secondary antibody (0.8 mg/ml, Jackson ImmunoResearch, West Grove, PA). For normalization, the membrane was immunoblotted with anti-actin antibody (1:30,000, clone C4, 50-174-848, Thermo Scientific). Signals were

visualized using SuperSignal West Dura (Thermo Scientific) and densitometrically quantified with VisionWorks LS software (UVP BioImaging, Upland, CA).

Plasmids, transfection, and luciferase assay

Expression vectors for miR-198 were constructed by PCR amplification from an FSTL1 clone (Ref SC115714, Origene, Rockville, MD), and fragments of several sizes were cloned into a pcDNA3 vector (Invitrogen, Carlsbad, CA). A miR-198 sensor construct was generated by cloning a synthetic fragment that contained three perfectly matching miRNA responsive elements (MRE) into psiCHECK2, downstream of luciferase (Promega, Madison, WI). Cells, plated in 12-well plates, were transfected using polyethylenimine-mediated transfection [38] with 10 ng of reporter construct along with increasing concentrations of CMV-based pcDNA3vector expressing relevant miRNAs. FSTL1-3'UTR sub-regions were amplified by PCR and cloned into XhoI/NotI sites of psiCHECK2 vector. Deletion of the miR-198 sequence was performed using the Q5 site-directed mutagenesis kit (New England Biolabs). After verification of the mutation, the mutated FSTL1-3'UTR insert was sub-cloned back into the original psiCHECK2 vector to ensure that no unintended mutations were inserted in the plasmid backbone. The primer pairs used in this study are detailed in Table 1. Luciferase activity was measured 48 h after transfection using the Dual-Glo Luciferase Assay System (Promega). Renilla luciferase activity was normalized to firefly luciferase activity. For expression of miR-198 mimic, cells were transfected with 100 nM double-stranded miR-198 mimic (miRIDIAN, Dharmacon, Lafayette, CO), using DharmaFect Duo transfection reagent in Opti-MEM for 24 h.

Statistics

Real-time PCR expression data of FSTL1 and NDRG1 was analyzed using two way ANOVA analysis after normalization to YWHA expression. Differences in relative luciferase activity of the constructs tested in this study were analyzed using two-way ANOVA on the log2 transformed hRuc.U/hluc ratio, followed by the post hoc Dunnett's test. The data represent the means $\pm$ SD from at least three independent experiments. A p value < 0.05 was considered statistically significant.

RESULTS

The expression of FSTL1 in human trophoblasts

We used northern blot to examine the expression FSTL1 mRNA in PHT cells and in several trophoblast-derived cell lines. FSTL1 mRNA was detected in several non-trophoblastic cell lines (Fig. 1A). Among trophoblasts, FSTL1 was detected only in the EVT line HTR-8/SVneo and in PHT cells, but not in other villous trophoblast tumor-derived lines (JEG3, BeWo, JAR, Fig. 1A). To confirm these findings and better characterize the expression of FSTL1 protein in various sub-populations of placental trophoblasts, we used IHC, performed using adjacent sections of a normal term human placenta. We detected relatively high expression of FSTL1 protein in regions enriched for EVTs (Fig. 1B). Images of the basal plate and areas of villous fibrin (Fig. 1B and Suppl. fig. 1) were immunopositive for FSTL1 at nearly all of the large polygonal EVTs. We confirmed cell lineage identity using immunostaining of serial sections for HLA-G (EVTs), CD68 (uterine macrophages and

Hofbauer cells), CK7 (epithelial cells of the uterus and placenta), hPL (villous trophoblast and a subset of EVT), and CD34 (vascular endothelial cells). We noted relatively weaker FSTL1 immunostaining in the syncytiotrophoblast layer of chorionic villi. When viewing sections of villous tissue (especially at low power), we identified FSTL1 immunopositive patches, which were largely adjacent to areas of perivillous fibrin. These data defined the expression of FSTL1 in human trophoblasts, indicating prominent expression in EVTs.

The expression of FSTL1 in hypoxic trophoblasts

Several studies have found a protective effect of FSTL1 against tissue injury [16,21,39–41]. We therefore sought to examine the effect of hypoxia, a common type of placental injury, on FSTL1 expression in PHT cells. RT-qPCR analysis of FSTL1 expression showed an insignificant change in FSTL1 mRNA levels in PHT cells exposed to hypoxia (Fig. 2A). In contrast, the mRNA for NDRG1, a hypoxia-induced gene that attenuates hypoxic injury in trophoblasts (and used here as a control, 42) was markedly upregulated. We confirmed our PCR results using northern blot of PHT cells and several other cell lines (Suppl. fig. 2), confirming that hypoxia had an insignificant effect on FSTL1 expression. Interestingly, using western blot analysis we found that hypoxia strongly upregulated FSTL1 protein expression in PHT cells (Fig. 2B). This increase in protein expression in hypoxia was unrelated to the differentiation state of PHT cells, because it was unaffected in conditions that promote or hinder differentiation (Fig. 2B) [26,27]. A time-course analysis of FSTL1 protein expression showed a relatively slow increase in FSTL1 in hypoxia, with a major accumulation occurring only after 48 h of exposure to hypoxia (Fig. 2C). Together, these data show that hypoxia enhances the expression of FSTL1 protein in trophoblasts and suggest that this effect may be attributed to increased translational efficiency or FSTL1 protein stability.

miR-198 is not expressed in trophoblasts

The eleventh exon of FSTL1 encodes the 3'UTR of FSTL1 and also contains sequences encoding miR-198, which may regulate the expression of FSTL1 [21]. We therefore performed northern blot analysis of miR-198 in PHT cells and in several cell lines, cultured in standard or hypoxic conditions. Despite the relatively high expression level of the FSTL1 transcript in PHT cells, we could not detect a signal corresponding to the size of a mature 22-nucleotide-long miRNA in these cells. As control, we detected a faint miR-198 signal in 293T and SKUT1 cells (Fig. 3). Similarly, we did not detect miR-198 in HTR-8/SVneo cells, which express a high level of FSTL1 (Fig. 1A). We also did not detect any evidence for miR-198 expression in PHT cells by RT-qPCR or RNA sequencing (data not shown). We attempted to overexpress miR-198 in PHT cells, using a transfected expression vector containing segments of FSTL1 3'UTR of different sizes that span the miR-198 sequences. While this strategy has been used in our laboratory to overexpress several miRNA species [24,43], none of these approaches resulted in expression of the mature miR-198 transcript, suggesting that the miR-198 transcript is not processed into a mature miRNA in trophoblasts. Intriguingly, our northern blot experiments using the miR-198 probe revealed a unique pattern of hybridization signals in PHT cells, consisting of several signal bands with estimated sizes of ~17, ~60, ~70, and ~120 nucleotides, which were upregulated in cells cultured in hypoxic conditions (Fig. 3). This pattern was consistently observed in primary

trophoblasts, but not in other cell types, ruling out a non-specific degradation of the FSTL1 transcript.

Post-transcriptional regulation of the FSTL1 transcript

Several miRNA databases, including TargetScan, specified the presence of putative binding sites for miR-198 within the FSTL1 3'UTR. To analyze the possibility that FSTL1 might be regulated by its own passenger miRNA, we transfected a miR-198 mimic, along with an FSTL1-Luc reporter that harbors the miR-198 MRE or a positive control reporter (sensor) containing three artificial binding elements for miR-198 downstream from luciferase in a psiCHECK2 plasmid. As shown in Fig. 4, the miR-198 mimic had a potent repressive effect on the miRNA sensor control in HTR-8/SVneo cells, but virtually no effect on the FSTL1-Luc construct, suggesting that FSTL1 is not a direct target of miR-198 in these cells.

In addition to miRNA target sites, the 3'UTR of FSTL1 contains at least seven prototypic AU-rich elements (AREs), consisting of AUUUA pentamers, which are present in many unstable mRNAs. These elements are scattered throughout the FSTL1 3'UTR, suggesting that they play a role in regulation of FSTL1 expression. To examine this possibility, we designed a series of luciferase-based reporters harboring sub-regions of the FSTL1-3'UTR that may play a role in the post-transcriptional regulation of FSTL1 (Fig. 5). Because our northern blot analyses of FSTL1 and miR-198 showed a cell-specific expression pattern (Fig. 1A), we performed these transfection assays in HTR-8/SVneo cells and in PHT cells (Fig. 5). Interestingly, the expression pattern of each construct was not uniform among the cell types, likely reflecting the interaction of these domains with cell type-specific factors. For example, in HTR-8/SVneo cells the complete FSTL1 3'UTR decreased activity of the luciferase reporter by less than 15%, while it reduced the activity to almost 60% of the basal activity in PHT cells. Analysis of the different 3'UTR fragments suggests the existence of sequence elements with variable effects on the luciferase activity. Deletion of increasingly larger segments starting from the distal part of the 3'UTR reduced the luciferase activity. In contrast, deletion of some of the proximal AREs led to increased luciferase activity, suggesting that the 5' moiety of the region accelerates transcript stability or translation in PHT cells.

We also made constructs in which we deleted the 20 nucleotides corresponding to the sequence encoding miR-198. Since the FSTL1 transcript gives rise either to the FSTL1 mRNA or can be processed to miR-198 [21] its deletion could potentially drive higher expression of the FSTL1. Surprisingly, removal of the miR-198 sequence in the context of the intact 3'UTR resulted in a sharp decrease of the luciferase activity in both PHT and HTR-8/SVneo cells. Deletions of miR-198 sequence in the context of partial segments of the 3'UTR had a variable effect, which was dependent upon the cell type and the remaining 3'UTR moieties, suggesting a complex interaction between the miR-198 sequence and different 3'UTR elements.

DISCUSSION

Our data indicate that FSTL1 is highly expressed in human trophoblasts, particularly in EVTs. We also found a marked divergence between the expression of FSTL1 mRNA and

protein. For example, whereas the expression of FSTL1 protein is low in villous trophoblasts, FSTL1 mRNA is strongly expressed in these cells. Similarly, hypoxia had a marked effect on FSTL1 protein expression in PHT cells, but not on mRNA levels. These observations imply that post-transcriptional processes, including translation, stability and degradation, may be crucial for controlling FSTL1 protein output, as recently shown for other proteins [44–46]. AREs are known to regulate mRNA stability, and are often present in the 3'UTR of cytokine mRNAs, where they mediate the rapid mRNA elimination of these potent regulatory molecules (review in 47). Although the deletions of a number of AREs had an impact on the reporter constructs, we could not attribute a clear role to a specific motif in our luciferase reporter assay. The effects were dependent on the range of the deletion and the relative position of the deleted fragment, implying a functional interaction between different elements. Notably, some of the deletions also resulted in PHT-specific effects. Together, these data suggest the existence of complex, cell-specific mechanisms that regulate FSTL1 expression, likely involving the interplay between different sub-regions of FSTL1 3'UTR.

Although the FSTL1 transcript harbors the miR-198 sequence within its 3'UTR, and FSTL1 transcript is relatively abundant in trophoblasts, we could not detect mature miR-198 in either PHT or HTR-8/SVneo. Intriguingly, in our initial experiments we detected a signal for miR-198 using a microarray analysis and *in situ* hybridization (data not shown). In contrast, by more definitive and sensitive technologies, including qPCR and northern blot, we could not detect miR-198 expression in trophoblasts. These discrepancies prompted us to perform RNAseq experiments, where we sequenced several small RNA libraries derived from PHT cells and confirmed the absence of any miR-198 sequence reads. On the other hand, using RNAseq we detected the presence of RNA fragments that partially overlap with the sequence of miR-198 (as also shown in Fig. 3), and likely explain the detection signal observed in some of our early, hybridization based technologies. We also noted that deletion of the miR-198 sequence from the FSTL1 3'UTR resulted in a reduction of the reporter's luciferase activity, indicating a positive influence of this motif on gene expression. Together, these results suggest that miR-198 sequence is not processed as a miR in trophoblasts, but perhaps mediates the formation of a secondary structure that could regulate FSTL1 expression. This might be markedly different from epidermal cells, where a rapid alternate strong anti-correlated expression pattern ("seesaw") between FSTL1 and its passenger miR-198 was described [21]. In these cells, miR-198 is normally abundantly expressed and FSTL1 is barely detectable. During injury, the anti-migratory miR-198 is switched-off and the expression of the pro-migratory FSTL1 is turned on to allow the migration of keratinocytes and the re-epithelialization of the damaged epidermis [21]. In the epidermis, binding of the RNA-binding protein KSRP drives the expression of miR-198, while reduced levels of KSRP cause switching to FSTL1 expression. Curiously, the protein expression database (The Human Protein Atlas, 48) indicates that trophoblasts express a high level of KSRP, suggesting that trophoblasts specifically suppress miR-198 biogenesis.

Our northern blot analyses also revealed the presence of signal bands that were uniquely detected in PHT cells and were absent in other cells tested. The accumulation of these short transcripts was remarkably induced when PHT cells were exposed to hypoxic culture

conditions. To date, the nature of these PHT- and hypoxia-specific RNA species remains unknown. A more thorough analysis of deep transcriptome sequencing data may reveal the nature of these small RNA species.

The finding that EVTs constitutively express a high level of FSTL1 is particularly interesting in the context of invasiveness, which is one of the distinctive features of this subpopulation of trophoblasts. A role for FSTL1 in promoting cell migration and invasion has been reported in several studies. For example, FSTL1 promotes cell proliferation in cartilage cells during development, and also enhances cell migration and metastasis in bone tumor cells [49]. As noted earlier, the pro-migratory capabilities of FSTL1 are evident in the more physiological context of wound healing, where FSTL1 stimulates migration of keratinocytes to promote wound closure [21]. In contrast, a recent study indicated that exogenous FSTL1 had rather an inhibitory effect on colon cancer cell migration [50], suggesting that the effect of FSTL1 on cell proliferation and migration might be context-dependent. Functional experiments targeting the intracellular levels of FSTL1 will be essential in order to decipher the effect of FSTL1 on EVT invasion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- FSTL1 is expressed in the human placenta, mainly in extravillous trophoblasts.
- Basal FSTL1 protein expression is low in villous trophoblasts, but is increased in hypoxic conditions.
- FSTL1 expression in trophoblasts is regulated by post-transcriptional mechanisms.
- There is no evidence of expression of the miR-198 passenger miRNA in trophoblasts, or for its effect on FSTL1 expression.

Fig. 1. The expression of FSTL1 in human trophoblasts, cell lines, and human placenta
 (A) Northern blot analysis of FSTL1 expression in PHT cells (cultured for 48 h) and in diverse trophoblastic and non-trophoblastic cell lines. After transfer, the blot was hybridized with ³²P-labeled FSTL1. Cells of trophoblastic origin are labeled in blue. rRNA was used as a loading control. The blot represents three independent experiments. (B) The expression of FSTL1 in the term human placenta, detected by IHC. Placental bed (1st and 2nd rows), villous sections (3rd and 4th rows), and villous fibrin (5th and 6th rows) were analyzed by IHC, using antibodies against proteins that mark of placental cell types, including HLA-G

(primarily EVTs) and CD68 (uterine macrophages and Hofbauer cells). Hematoxylin and eosin (H&E) staining is shown on the left panel and a control (mouse IgG as primary antibody) is shown on the right. Higher magnification views (40×) of the boxed regions are shown in the panels below the low magnification views (10×). The scale bar for the 10× images is 50 microns, and for the 40× images is 25 microns. The panels represent multiple images, obtained using at least two different placentas.

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Fig. 2. The expression of FSTL1 in PHT cells, exposed to hypoxia

(A) PHT cells were cultured in standard ($O_2=20\%$) or hypoxic ($O_2 < 1\%$) conditions for 48 h. FSTL1 mRNA expression was analyzed by RT-qPCR as described in material and methods. Expression of NDRG1, a hypoxia-inducible gene is shown as a control. The graph represents cells that were isolated from different placentas ($n=5$). The difference in FSTL1 expression in cell cultured in standard or hypoxic conditions was insignificant ($p=0.22$), and significant for NDRG1 ($p < 0.0001$). (B) Western blot analysis of FSTL1 expression in PHT cells, cultured for a total of 72 h either in standard conditions, in the presence of 1.5%

DMSO, in hypoxia, or in the presence of 0.5 mM 8-Br-cAMP. 40 µg of a protein lysate were analyzed using an FSTL1 antibody, as described in Methods. Actin was used as a loading control. (C) A time-course analysis of FSTL1 expression in PHT cells exposed to hypoxic conditions. Cells were seeded and cultured in standard conditions for 4 h (T=0) then transferred into the hypoxic chamber. Cells were exposed to hypoxia for 6 h to 72 h and harvested at the indicated times. Band intensity was quantified using UVP VisionWorksLS, normalized to the corresponding actin level, and expressed as fold change relative to the intensity at time 0. Note that the additional faint band below the FSTL1 has been previously noted in one of the first FSTL1-related publications [51].

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Fig. 3. The expression of miR-198 containing 3'UTR fragments in PHT cells and in cell lines, exposed to hypoxia

Northern blot of total RNA from cells exposed to standard conditions (N) or hypoxia (H), as defined in Fig. 1. Three different cell lines and PHT cells isolated from different placentas (n=3) were analyzed. Total RNA (30 µg/sample) was analyzed as described in Methods. rRNA was used as a loading control.

Fig. 4. The effect of miR-198 on an FSTL1-3'UTR reporter

A schematic representation of the FSTL1 3'UTR mRNA, which harbors the miR-198 sequence and two predicted miR-198 binding sites. Alignments between the miR-198 binding sites and miR-198 are shown. A sensor construct harboring three copies of sites perfectly complementary to miR-198, FSTL1-3'UTR reporter construct, or an empty luciferase control vector (10 ng each) were transfected into HTR-8/SVneo cells along with 10 nM of miR-198 mimic. Relative luciferase unit (RLU) activity was determined as described in Methods. The data represent the geometric means $\pm$ SD of three independent experiments, performed in duplicate, and shown as fold change compared to the vector alone, with scrambled oligonucleotide. * $p < 0.01$ vs. control (scr. oligo).

Fig. 5. Elements of the FSTL1-3'UTR regulate reporter gene activity

The central panel depicts the constructs used in the study, in which a Renilla luciferase cassette driven by the SV40 promoter (orange box) is cloned upstream of the indicated portion of the FSTL1 3'UTR in psiCHECK2 vector. Also shown are the positions of the AU-Rich Elements (AREs, vertical blue bars) and the location of the pre-miR-198 stem loop. The empty psiCHECK2 plasmid (uppermost construct) was used as control. The right and left panels show the results of the luciferase reporter assay. Each construct was transfected in HTR-8/SVneo or PHT cells. Luciferase activity was determined 48 h after transfection. Relative luciferase unit (RLU) activity from the control reporter construct was set at 1. Renilla luciferase activity was normalized to firefly luciferase, to control for transfection efficiency and cell viability. The data represent the means $\pm$ SD from three independent experiments. The construct with the complete 3'UTR was compared to the control (empty vector) but all the FSTL1 3'UTR truncations were compared the construct containing the full-length 3'UTR. * denotes $p < 0.01$, ** denotes $p < 0.001$, vs. control (empty vector), performed using ANOVA with post-hoc Dunnett's test.

Table 1

List and sequences of primers used in this study

Primer	Direction	Sequence
FSTL1- 1	forward	GATCCTCGAGCAGTCGCCAAATCACCAGTA
	reverse	GATGCGGCCGCTCGCTAAGGGGCTCAAAGTA
FSTL1- 2	forward	GATCCTCGAGCAGTCGCCAAATCACCAGTA
	reverse	GATGCGGCCGCGAAGAGACAGGGCCAACTCA
FSTL1- 3	forward	GATCCTCGAGCAGTCGCCAAATCACCAGTA
	reverse	GATGCGGCCGCGAGAAGAAGGAAAAATCACAGGAA
FSTL1- 4	forward	GATCCTCGAGCAGTCGCCAAATCACCAGTA
	reverse	GATGCGGCCGCTCACAGTTGCCTGTGCATT
FSTL1- 5	forward	GATCCTCGAGCAGTCGCCAAATCACCAGTA
	reverse	GATGCGGCCGCCACAGAAGCTGCAGAACTCAA
FSTL1- 6	forward	GATCCTCGAG GAGCCCCTTAGCGACCTAAC
	reverse	GATGCGGCCGCGGGGAAAGGAAACCA ATAA
FSTL1- 7	forward	GATCCTCGAGGAGCTGGTAAGAATGCTACTAACC
	reverse	GATGCGGCCGCGGGGAAAGGAAACCA ATAA
FSTL1- 8	forward	GATCCTCGAGTTAACCTCCGGCTGGAAATA
	reverse	GATGCGGCCGCGGGGAAAGGAAACCA ATAA
FSTL1- 9	forward	GATCCTCGAGACCCTGCAACATTGAACTCC
	reverse	GATGCGGCCGCGGGGAAAGGAAACCA ATAA
FSTL1- 198	forward	CCTGTGATTTTTCCTTCTCT
	reverse	CAATGATCAGAACCACAGAATTTATAAC
<i>Real-time PCR primers</i>		
FSTL1	forward	TCTGTGCCAATGTGTTTTGTGG
	reverse	TGAGGTAGGTCTTGCCATTACTG
NDRG1	forward	CAGTTTGGGCTGAAAAGCAT
	reverse	CTCCACCATCTCAGGGTTGT
YWHAZ	forward	CTGAACTCCCCAGAGAAAGC
	reverse	CCGATGTCCACAATGTCAAG
Renilla Luc [1]	forward	AACGCGCCTCTTCTTATTT
	reverse	GTCTGGTATAATACACCGCG

[1] Roberts AP, Doidge R, Tarr AW, Jopling CL. The P body protein LSM1 contributes to stimulation of hepatitis C virus translation, but not replication, by microRNA-122. *Nucleic Acids Res* 2014;42:1257–69.