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A distant trophoblast-specific enhancer controls HLA-G expression at the maternal–fetal interface

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HLA-G, a nonclassical HLA molecule uniquely expressed in the placenta, is a central component of fetuses-induced immune tolerance during pregnancy. The tissue-specific expression of HLA-G, however, remains poorly understood. Here, systematic interrogation of the HLA-G locus using massively parallel reporter assay (MPRA) uncovered a previously unidentified cis-regulatory element 12 kb upstream of HLA-G with enhancer activity, Enhancer L. Strikingly, clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas9-mediated deletion of this enhancer resulted in ablation of HLA-G expression in JEG3 cells and in primary human trophoblasts isolated from placenta. RNA-seq analysis demonstrated that Enhancer L specifically controls HLA-G expression. Moreover, DNase-seq and chromatin conformation capture (3C) defined Enhancer L as a cell type-specific enhancer that loops into the HLA-G promoter. Interestingly, MPRA-based saturation mutagenesis of Enhancer L identified motifs for transcription factors of the CEBP and GATA families essential for placentaation. These factors associate with Enhancer L and regulate HLA-G expression. Our findings identify long-range chromatin looping mediated by core trophoblast transcription factors as the mechanism controlling tissue-specific HLA-G expression at the maternal–fetal interface. More broadly, these results establish the combination of MPRA and CRISPR/Cas9 deletion as a powerful strategy to investigate human immune gene regulation.

During pregnancy, a semiallogeneic fetus expressing paternaly derived antigens is nurtured for months without suffering rejection by the maternal immune system. This state of immune tolerance is established at a precise anatomical location, the placenta, a transient organ consisting of fetal trophoblasts and a specialized uterine mucosa, the decidua. During implantation, HLA-G+ extravillous trophoblasts (EVTs) invade the maternal tissue, defining the boundary between mother and fetus. HLA-G, a nonclassical nonpolymorphic major histocompatibility complex (MHC) class I molecule, is uniquely expressed by EVT tissue (5, 4), where it plays a central role in inducing immune tolerance. Several inhibitory receptors present on natural killer (NK) cells, the most abundant immune cell type at the maternal–fetal interface, and on myeloid cells, have been shown to bind to HLA-G (5–7). An HLA-G cycle between decidual NK cells and EVT provides for both NK cell tolerance and antiviral immunity (8–10). Importantly, HLA-G is sufficient to inhibit NK cell cytotoxicity (11) and required to protect trophoblasts against NK cell-induced lysis (12). Several pregnancy-related disorders, including miscarriage, recurrent fetal loss, and preeclampsia, have been associated with polymorphisms resulting in reduced HLA-G expression levels (13, 14). Intriguingly, HLA-G expression has also been detected in tumor lesions, where it may facilitate immune evasion (15, 16). However, despite substantial effort, the mechanism by which the EVT-specific expression of HLA-G is obtained has remained elusive for more than two decades (13, 17, 18).

Tissue-specific gene expression is primarily regulated at the level of transcription by distant cis-regulatory elements—enhancers (19, 20). Traditionally, enhancer discovery has relied on examining features predictive of enhancer activity, such as chromatin accessibility, DNA and chromatin covalent modifications, and sequence conservation between species (21). This approach has been successfully used to gain important insights into immune gene regulation, such as the discovery of enhancers controlling the expression of murine Foxp3, a transcription factor governing the commitment and stability of regulatory T cells (22).

However, substantial differences in regulatory sequences between species limit the ability to derive conclusions from model organisms regarding human gene regulation. In particular, the MHC locus differs significantly between mouse and humans (23), and HLA-G lacks a clear ortholog in mice.

In this study, we used an unbiased high-throughput approach, massively parallel reporter assay (MPRA) (24), to interrogate a human immune gene regulation: pregnancy | immune tolerance | MPRA | CRISPR/Cas9

Significance

Successful pregnancy poses an immunological paradox, as the mother’s immune system does not reject a fetus, even though it is a partially foreign tissue. Fetal extravillous trophoblasts (EVTs) deeply invade the uterus and interact with maternal immune cells without facing rejection. The nonclassical major histocompatibility complex (MHC) molecule HLA-G is essential for immune tolerance induction in pregnancy, yet the mechanism by which EVT s uniquely express HLA-G remains unknown.

Using high-throughput cis-regulatory element dissection and genome editing tools, we discovered a remote enhancer essential for HLA-G expression in human EVT s, describing the basis for its selective expression at the maternal–fetal interface. These findings provide insight into immune tolerance induction during pregnancy and may yield new therapeutic targets for pregnancy-related disorders.


Key words: CRISPR/Cas9, enhancer, HLA-G, JEG3, placenta, trophoblasts

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27-kb region spanning the HLA-G locus for functional activation of transcription. Our results uncover a private enhancer, which controls the tissue-specific expression of HLA-G at the maternal-fetal interface, and provide a relevant methodology to dissect human immune gene regulation without prior sequence knowledge.

Results

Identification of a Trophoblast-Specific Enhancer 12 kb Upstream of HLA-G. To systematically interrogate the HLA-G locus for active cis-regulatory elements, we set up a MPRA screen (24). For this purpose, 12,000 partially overlapping 121-bp-long elements (tiles) spanning 27 kb of the HLA-G locus were synthesized, coupled to unique DNA tags, and cloned into plasmids containing an invariant promoter and a firefly luciferase reporter gene. For greater confidence, two different promoters were used in parallel libraries, a strong promoter (SV40P) and a minimal TATA box synthetic promoter (minP). The resulting libraries were cotransfected into JEG3 cells, an HLA-G+ chorioncarcinoma cell line commonly used in EVT studies (25). To measure the relative enhancer activity of each tested element, we performed high-throughput sequencing and quantified the relative abundance of each element’s tag reads in mRNA isolated from the transfected cells and in the pooled libraries. Enhancer activity was calculated as the median (cDNA count divided by the DNA count) of tags representing a tile, divided by the median ratio for all tags in a library. Nonlinear candidates were defined as any tile where enhancer activity measurements were >1 and P values were <0.05 for both biologically replicates of each library transfaction.

Our unbiased MPRA screen yielded several enhancer candidates upstream of HLA-G (Fig. 1A). The four most confident hits, indicated in Fig. 1A and B, were then carried on for further analysis using classical luciferase reporter gene assays. The most confident candidate, located 12 kb upstream of the HLA-G gene, was the only tile with enhancer activity greater than 2 with both promoters tested, displaying the highest enhancer activity with minP (8.4) and second highest enhancer activity with SV40P (12.4) overall. This region specifically enhanced firefly luciferase activity upstream of the minimal promoter by 20-fold in HLA-G+ JEG3 cells (Fig. 1C). We named this previously unidentified putative regulatory element Enhancer L, for being a long-range enhancer discovered with our unbiased enhancer screen. Importantly, Enhancer L was not active in HEK293T cells, an HLA-G−negative control cell line (Fig. 1D). Moreover, this cell type-specific activity pattern was maintained even when Enhancer L was cloned in an inverted orientation (Fig. 1D), a classical hallmark of an enhancer element (19). Of note, candidate numbers 3 and 4 from our MPRA screen, located near or even partially overlapping with Enhancer L, respectively, displayed negligible activity in JEG3 cells (Fig. 1C). Altogether, these observations suggest that Enhancer L corresponds to a narrowly defined regulatory region in the HLA-G locus that may confer tissue-specific HLA-G expression to trophoblasts.

Enhancer L Is Essential for HLA-G Expression in JEG3 Cells. Next, we sought to investigate whether Enhancer L modulates endogenous HLA-G expression. To directly target Enhancer L in JEG3 cells, we used a clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas9 dual-guide approach (26, 27) by targeting two guide RNAs (gRNAs) to sites linking Enhancer L (Fig. 2A). We used a Streptococcus pyogenes Cas9 linked via a self-cleaving 2A peptide to a green fluorescent protein (GFP) to facilitate identification of Cas9-expressing cells. GFP+ cells were sorted and plated at clonal density, and the emerging single-cell–derived colonies were transferred 10 d postplating into 96-well plates. PCR analysis of CRISPR/Cas9 targeted single-cell–derived clones was used to identify homozygous Enhancer L KO clones (Fig. 2B). We observed a clonal targeting efficiency of 29.5%, with homozygous deletions occurring at a frequency of 8.7%. Four independent Enhancer L-null clones and three WT clones were selected for further characterization. As expected, Sanger sequencing demonstrated excision of the DNA between the predicted Cas9 cleavage sites (three bases 5′ of the PAM sequence), with three out of four clones having the same exact deletion of 154 bp (Fig. 2C).

Strikingly, deletion of Enhancer L resulted in complete ablation of HLA-G expression, as determined by flow cytometry (Fig. 2D) and quantitative real-time PCR (qRT-PCR) (Fig. 2E). Surveying the whole genome for chromatin accessibility using genome-wide DNase-seq revealed that Enhancer L is located within a DNase I hypersensitivity site (DHS) in JEG3 cells (Fig. 2C), supporting the hypothesis that Enhancer L is indeed an active regulatory element in its endogenous chromatin context.

Deletion of Enhancer L in JEG3 Cells Uniquely Ablates HLA-G Expression. Following our observation that Enhancer L is required for HLA-G expression, we then asked whether Enhancer L acts specifically on HLA-G. Previous studies have identified enhancers that affect multiple genes spanning regions of hundreds of kilobases (28, 29). To investigate whether Enhancer L also regulates other genes in the HLA locus or elsewhere on chromosome 6, we sequenced polyA+ mRNA from three Enhancer L KO JEG3 clones, as well as three WT clones and two independent samples of the parental JEG3 cell line as controls. RNA-seq confirmed that HLA-G is completely ablated across all KO clones (Fig. S1A), and that it is the only such gene within 2 Mb of Enhancer L (Fig. S1B), suggesting that HLA-G is the only direct cis target of Enhancer L. Looking beyond chromosome 6, transcriptome-wide analysis revealed statistically
Fig. 2. Enhancer L is required for HLA-G expression in the JEG3 trophoblast cell line. (A) Dual-CRISPR guide strategy to delete Enhancer L. Arrows represent the primers used for PCR screening. (B) PCR screening of CRISPR/Cas9-targeted JEG3 single-cell-derived clones. Green*, wild type; yellow*, heterozygote; red*, null clone. (C) Sanger sequencing of four independent homozygous Enhancer L KO clones and three independent WT clones resulting from CRISPR/Cas9 targeting of Enhancer L (black box) in JEG3 cells using a dual-CRISPR guide RNA approach. Binding sites for the gRNAs targeting Enhancer L are underlined and shaded. PAM motifs are italicized in green type. Enhancer L is part of a DNase I hypersensitive site (DHS) in JEG3 cells, as determined by genome-wide DNase-seq. EL, Enhancer L. (D) Combined FACS histogram demonstrating complete ablation of HLA-G surface expression in Enhancer L KO JEG3 clones. (E) HLA-G transcript levels of Enhancer L KO clones, with JEG3 cells and HEK293T cells as controls. Gene expression normalized to GAPDH expression. Error bars represent SEM of replicates of a representative experiment (n = 2).

Significant differences in the expression of 321 genes using Cuffdiff [false-discovery rate (FDR) < 0.05]. To rule out the possibility that these changes were caused by CRISPR/Cas9-induced off-target effects, we performed in silico off-target analyses of our Enhancer L gRNAs using the CRISPR design tool at crispr.mit.edu (30). The top 50 predicted off-target sites yielded maximum scores of 3.3 for gRNA 1, and 0.9 for gRNA 2 (out of 100), suggesting that the observed global changes in gene expression are not likely to be a result of off-target cleavage at these sites. Gene set enrichment analysis (GSEA) of the most differentially expressed genes revealed statistically significant enrichment (FDR < 0.05) for six gene sets, all of which are related to steroid hormone biosynthesis and G-protein-coupled receptor signaling, processes expected to play a role in trophoblast physiology. Pairwise comparison of all three experimental groups (WT, KO, parental JEG3), however, revealed that, despite the observed transcriptome-wide changes in gene expression, HLA-G was by far the most down-regulated gene upon Enhancer L deletion at the whole-transcriptome level (Fig. SIC), indicating that Enhancer L uniquely modulates HLA-G expression.

Enhancer L Is Required for HLA-G Expression in Primary EVTs. To confirm the role of Enhancer L in primary human trophoblasts, we obtained villi from first-trimester human placental tissue and purified HLA-G+ EVTs by flow cytometry (31). Cas9-2A-GFP and gRNAs targeting Enhancer L were successfully codelivered into primary EVTs using lentiviral particles, as assessed by GFP expression (Fig. S4). As expected, Enhancer L deletion resulted in a significant decrease in HLA-G mRNA levels [74.12 ± 13.61% (SEM); n = 3] (Fig. 3B).

Loss of HLA-G surface expression as a result of lentiviral CRISPR/Cas9-mediated ablation of Enhancer L was first evaluated in JEG3 cells, which divide rapidly in culture. We observed complete loss of HLA-G surface expression 1 wk posttransduction in a large percentage of transduced cells [61.9 ± 1.93% (SEM); n = 3] (Fig. S2A). Detecting changes in HLA-G surface expression in primary EVTs, however, is hampered by the unusually long half-life of HLA-G protein on the cell membrane (32), and the fact that primary EVTs can only be cultured ex vivo for a short period (<5 d). Despite these technical limitations, we were able to detect a significant reduction in HLA-G surface expression 5 d after targeting Enhancer L in primary EVTs [60.71 ± 10.68% (SEM); n = 3] (Fig. 3 C and D, and Fig. S2B). Successful genomic deletion of Enhancer L was confirmed by PCR sequencing (Fig. S2 C and D). Our results demonstrate that Enhancer L is indeed necessary for HLA-G expression in primary human EVTs.

Enhancer L Is a Distant Regulatory Element That Loops into the HLA-G Proximal Promoter. Next, we aimed to characterize the mechanism by which Enhancer L activates HLA-G expression at a distance. The current model of long-range gene regulation posulates that remote cis-regulatory elements come into close proximity to the promoters of the genes they regulate via chromatin looping (33). To test for the involvement of looping in Enhancer L–HLA-G promoter long-range communication, we carried out chromatin conformation capture (3C) assays in JEG3 and HLA-G-negative HEK293T cells (Fig. S3 A and C, and Materials and Methods). We detected a looping interaction between Enhancer L and the classical promoter of HLA-G specifically in JEG3 cells (Fig. S3B), confirming the nature of the resulting hybrid DNA molecule consisting of Enhancer L and the proximal promoter by sequencing (Fig. S3D). Of note, this looping interaction was absent in

Fig. 3. Enhancer L is necessary for HLA-G expression in primary extravillous trophoblasts (EVTs). (A) Transduction of first-trimester HLA-G+ EVT with lentiviral Cas9 and Enhancer L gRNAs, assessed based on GFP expression. BF, bright-field (40x magnification). (B) Reduction of HLA-G expression at the mRNA level following Enhancer L deletion. Bars represent average ± SEM of three independent experiments. Gene expression normalized to GAPDH expression. *P < 0.05, paired Student’s t test. EL, Enhancer L. (C) Enhancer L deletion leads to significant reduction in HLA-G surface expression in primary EVTs, as assessed by FACS. One representative experiment is shown (n = 3). (D) Significant reduction in HLA-G surface expression upon Enhancer L deletion in primary EVTs [mean fluorescence intensity (MFI)]. Bars represent average ± SEM of three independent experiments. *P < 0.05, paired Student’s t test.
HEK293T cells (Fig. S3B), in agreement with the lack of Enhancer L activity in these cells (Fig. 1D).

**MPRA-Based Scanning Mutagenesis Reveals Motifs Controlling Enhancer L Activity.** Having established Enhancer L as a bona fide enhancer upon the HLA-G locus, we sought to identify the transcriptional regulators that mediate its action. To our surprise, truncation of Enhancer L invariably led to loss of enhancer activity in firefly luciferase reporter gene assays, suggesting multiple active motifs spread across its length (Fig. S4A). To fine map the active regulatory motifs responsible for Enhancer L activity, we carried out an MPRA-based scanning mutagenesis at the single-base pair resolution (24). In brief, we generated a total of 12,000 Enhancer L variants, representing all possible single substitutions, as well as small insertions or deletions at all positions. To reduce experimental noise, each variant was coupled to 16 tags on average, for a total of 200,000 distinct variant–tag combinations. As before, this complex library was cotransfected into JEG3 cells, followed by RNA harvesting and sequencing analysis. This fine mapping of Enhancer L led to the identification of five putative regulatory motifs, consistent across both promoters tested (SV40P and minP) (Fig. 4A and Fig. S4B). Reporter gene assays with truncated versions of Enhancer L lacking each one of these motifs (M1 through M5) showed that each one of them is essential for optimal Enhancer L activity in JEG3 cells (Fig. S4C). Subsequent in silico analysis using the TRANSFAC database (34) predicted binding of CEBP and GATA family transcription factors within these five motifs (Fig. 4A and Fig. S4B).

**CEBP and GATA Factors Regulate Trophoblast-Specific HLA-G Expression.** Motif sequence analysis alone does not allow discrimination between different members of transcription factor families. We reasoned that the transcription factors controlling HLA-G expression via Enhancer L must be highly expressed specifically in HLA-G+ trophoblasts. Microarray analysis of primary cells isolated from human placental tissue, and JEG3 cells (31), revealed that CEBPA, CEBPB, GATA2, and GATA3 are the most highly expressed genes within their respective transcription factor families (Fig. 4B). Our whole-transcriptome RNA-seq analysis (Fig. 4C) confirmed high expression levels of CEBPB, GATA2, and GATA3 in JEG3 cells. In addition, a survey of publicly available gene expression profiles (BioGPS) revealed that these three transcription factors are highly coexpressed in human placenta, and also more restricted in expression to this tissue than any other CEBP or GATA transcription factor family member. Importantly, CEBPB, GATA2, and GATA3 have been implicated in murine placental development and trophoblast-specific gene regulation (35–37), making them strong candidates for transcriptional regulators of HLA-G expression in human trophoblasts.

To test our prediction, we sought to determine whether CEBPB, GATA2, and GATA3 bind to Enhancer L. Indeed, chromatin immunoprecipitation (ChIP) using validated ChIP-grade antibodies, followed by qPCR analysis (ChIP-qPCR), revealed a 40-fold enrichment for CEBPB on Enhancer L (Fig. 4D). Similarly, a significant enrichment for GATA2 and GATA3 (fivefold) was detected on Enhancer L (Fig. 4E), indicating that, in JEG3 cells, endogenous CEBPB, GATA2, and GATA3 associate with Enhancer L. In addition, all three factors were found to bind to the proximal promoter of HLA-G (Fig. 4D and E), providing further evidence for the existence of a chromatin loop between Enhancer L and the core promoter of HLA-G, possibly established by GATA2 and GATA3 (38, 39). Of note, Pol II associated with both Enhancer L and the HLA-G core promoter (Fig. S4D), suggesting that active transcription is involved in the formation of this long-range chromatin loop. Consistent with a role in HLA-G transcriptional activation, transient overexpression of CEBPB, GATA2, and GATA3 individually in JEG3 cells led to an up to eightfold increase in HLA-G expression, indicating that these three factors are transcriptional activators of HLA-G expression (Fig. 4F). Taken together, our data support a model where CEBPB and GATA2/3 mediate long-range chromatin interactions between Enhancer L and the classical promoter of HLA-G (Fig. 4G), driving HLA-G expression specifically in EVTs at the maternal–fetal interface.

**Discussion**

Genome-wide association studies (GWAS) have uncovered an astonishing number of disease-associated noncoding loci (40), posing a challenge to functionally validate and characterize putative regulatory elements. MPRA represents an unbiased high-throughput method for de novo discovery and validation of cis-regulatory elements.
regions. In this study, the most confident candidate from our MPRA screen, located 12 kb upstream of HLA-G, was found to be active specifically in the HLA-G* JEG3 choriocarcinoma cell line (Fig. 1), suggesting that it may be involved in tissue-specific HLA-G transcriptional regulation. Indeed, CRISPR/Cas9 genome editing revealed that this previously unidentified enhancer, Enhancer L, is essential for trophoblast expression of HLA-G (Figs. 2 and 3, and Figs. S1 and S2).

Previous studies established that MHC gene expression is mainly controlled at the level of a conserved proximal promoter. Upon interaction with a transcriptional activator—CITA for class II and NLRC5 for class I genes—a multiprotein transcription factor complex is assembled, forming the MHC enhanceosome (17, 41, 42). Even though the enhanceosome is essential for basal and induced expression of MHC class I genes, its relevance in trophoblasts is uncertain: EVTs do not express NLRC5 or CITA (31) and the HLA-G proximal promoter harbors several non-functional motifs (18), suggesting that tissue-specific HLA-G expression is mediated by a distinct mechanism. Although several studies have described cis-regulatory regions involved in HLA-G transcriptional regulation (13, 43, 44), the present study is the first (to our knowledge) to report a noncoding sequence, Enhancer L, absolutely required for the tissue-specific expression of HLA-G in trophoblasts.

Interestingly, Enhancer L is contained within a long terminal repeat (LTR) sequence, LTR7 (45), associated with a human endogenous retroviral element (ERV), ERV1, as indicated in Fig. S5A. LTR sequences have been co-opted by mammalian genomes as regulatory elements, especially in the placenta (46). Well-known examples include the placenta-specific promoter of CYP19 (47) and MER20, regulatory sequences found upstream of progesterone-responsive genes essential for decidualization (48). Enhancer L sequence is unique in the human genome and well conserved across apes and Old World monkeys, yet absent in New World monkeys (Fig. S5 A and B), where HLA-G appears to be a classical MHC molecule (49–51). Intriguingly, the orangutan genome, the only ape genome containing a functional HLA-G promoter (X2 and Y cis-elements matching those in the HLA-A promoter, Fig. S5C), does not harbor the Enhancer L sequence. In addition, similar to New World monkeys, the orangutan HLA-G ortholog is a polymorphic MHC molecule. Perhaps in orangutans, because they are predominantly monogamous and thus less exposed to allogeenic fetuses (49), HLA-G functions as a classical antigen-presenting molecule. The observation that Enhancer L is only found in genomes that lack a functional HLA-G classical promoter raises the possibility that a retroviral element was co-opted during evolution to function in trophoblast-specific tolerogenic MHC expression.

Previous literature suggests that differential expression of transcription factors plays a role in cell-type-specific HLA-G transcription. The identity of such factors, however, has remained elusive (52, 53). In our study, MPRA-based saturation mutagenesis allowed us to fine map the regulatory elements responsible for Enhancer L activity, ultimately pointing toward CEBP and GATA factors as candidates for transcriptional activators of HLA-G expression in trophoblasts (Fig. 4). Indeed, ChIP and transient transfection studies revealed that CEBP, GATA2, and GATA3 associate with Enhancer L (Fig. 4 D and E) and are positive regulators of HLA-G expression (Fig. 4F).

3C revealed that Enhancer L loops across a 12-kb distance into the classical promoter of HLA-G (Fig. S3). Consistent with this long-range chromatin interaction, genome-wide DNase-seq demonstrated that Enhancer L is part of a DHS specifically in HLA-G* JEG3 cells (Fig. 2C and Fig. S6). Publicly available ChIP-seq data indicates CTCF binding flanking Enhancer L and the HLA-G coding sequence (Fig. S6). This CTCF binding pattern suggests the existence of an insulated chromatin domain (54) for HLA-G transcriptional regulation, corroborated by our observation that Enhancer L deletion does not significantly alter the expression of any gene other than HLA-G on chromosome 6 (Fig. S1). Interestingly, a long-range chromat interaction mediated by the insulator CTCF has been described in the MHC class II locus (55). Our data suggest that the looping interaction between Enhancer L and the promoter of HLA-G is mediated by GATA2/3, possibly in association with CEBP (38, 39, 56).

In conclusion, we have demonstrated that trophoblast HLA-G expression is contingent upon the activity of a remote enhancer, Enhancer L. Our data are consistent with a model where CEBP and GATA2/3 associate with Enhancer L, are recruited to the core promoter of HLA-G via chromatin looping, and up-regulate HLA-G expression (Fig. 4G). These findings establish chromatin looping mediated by lineage-specific transcription factors as a mechanism governing tissue-specific immune gene expression at the maternal–fetal interface. Future studies further dissecting the transcriptional regulation of HLA-G will not only shed light on immune privilege during pregnancy, but may also enable us to specifically control HLA-G expression to induce tolerance in transplantation therapies.

Materials and Methods

All of the human tissue used for this research was deidentified, discarded clinical material. The Committee on the Use of Human Subjects [the Harvard institutional review board (IRB)] determined that this use of all of this human material is exempt from the requirements of IRB review.

Cell Culture. JEG3 and HEK293T cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% (vol/vol) FBS, Gultaxam, and penicillin–streptomycin. Transfections were carried out using FuGENE 6 (Promega) according to the manufacturer’s instructions and analyzed 48 h posttransfection.

Flow Cytometry. Cells were harvested, blocked in 4% (vol/vol) FBS for 30 min, stained with HLA-G PE (clone MEMG9; Abcam) in 1% FBS for 1 h, washed thrice, and re suspeded in 1% FBS. Cells were cultured using either a FACScalibur or an LSRII instrument (BD Biosciences) and analyzed with FlowJo (Tree Star) software.

qRT-PCR Analysis. Total RNA was isolated using TRizol (Life Technologies), according to manufacturer’s instructions. A total of 1,500 ng of RNA was used for qDNA synthesis with the qScript CDNA SuperMix (Quanta Biosciences). A total of 30 ng of CDNA was used per qRT-PCR, performed using SYBR Green (Life Technologies) on a Viia7 system real-time PCR system (Life Technologies). Target gene expression levels were normalized to GAPDH. Primer pairs used are listed in Table S1.

details of molecular biology, MPRA, luciferase reporter gene assay, genome-wide DNase-seq, 3C, CRISPR/Cas9 genome editing, transcription-wise RNA-seq, first-trimester primary EVT isolation and transduction, and ChIP (ChIP-qPCR) experiments are given in SI Materials and Methods.

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