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ORIGINAL ARTICLE

Maternal platelet-derived factors induce trophoblastic *LAIR2* expression to promote trophoblast invasion and inhibit platelet activation at the fetal-maternal interface

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Abstract

Background: During human placentation, extravillous trophoblasts (EVTs) arising from cell column trophoblasts (CCTs) invade the highly differentiated uterine mucosa, called decidua, where they erode blood vessels and replace vascular endothelial cells. Maternal platelets have been detected in intercellular gaps of CCTs, but their physiological role remains unclear.

Objectives: This study aimed to elucidate the impact of platelet-derived factors on trophoblasts that are exposed to maternal platelets through erosion of decidual blood vessels.

Methods: Trophoblast cell line ACH-3P spheroids were incubated either with platelet-derived factors or isolated platelets obtained from pregnant women and then subjected to RNA sequencing followed by validation using quantitative polymerase chain reaction, ELISA, and *in situ* padlock hybridization. Among the deregulated genes, leukocyte

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associated immunoglobulin-like receptor 2 (*LAIR2*) expression was confirmed in first trimester placenta and primary trophoblast organoids. The functional role of LAIR2 in trophoblast invasion and platelet activation was studied.

Results: Platelet-derived factors altered the transcriptional profile of ACH-3P spheroids, including deregulation of genes linked to embryonic development. Among them, LAIR2 was exclusively detected in CCTs and invaded EVTs of first trimester decidua. Histology showed extravasated maternal erythrocytes within interstitial gaps of highly invaded decidua samples, coinciding with LAIR2-positive EVTs. LAIR2 inhibited type 1 collagen-induced platelet activation and enhanced invasiveness of trophoblasts.

Conclusion: This study suggests that maternal platelet-derived factors affect the transcriptional profile of trophoblasts, including upregulation of *LAIR2*, which may be involved in fine-tuning the coagulation of maternal blood leaking from eroded decidual blood vessels and could increase the invasiveness of EVTs into the decidua through an autocrine mechanism.

KEYWORDS
first trimester, placenta, platelets, pregnancy

1 | INTRODUCTION

Human gestation is initiated by embryo implantation, which includes the anchoring of the blastocyst to the endometrial epithelium. While the inner cell mass of the blastocyst, the embryoblast, proceeds to develop into the embryo, amnion, and umbilical cord, the outer trophectoderm layer of the blastocyst provides the basis for the formation of the placenta by providing the source of specialized epithelial cells termed trophoblasts. Following implantation, the trophectoderm starts to proliferate and differentiate, fusing to a multinucleated syncytium, the so-called primitive syncytiotrophoblast (SCT). In this early stage, the primitive SCT possesses all necessary enzymes to penetrate the maternal endometrium, now referred to as the decidua [1,2]. Eventually, vacuoles start to grow in the primitive SCT, leading to the formation of lacunae, which are separated by SCT trabeculae and are precursors of the intervillous space [1]. These trabeculae then develop into primary villi, consisting of cytotrophoblasts (CTBs) as well as a layer of true SCT (that will form the surface of the placenta), before colonization of the extraembryonic mesoderm and development of placental blood vessels give rise to secondary villi and then tertiary villi, respectively. At the distal parts of the evolving villi, trophoblasts penetrate through the SCT and form trophoblast cell columns (CCTs), which eventually anchor the developing placenta to the decidua. Trophoblasts from distal areas of cell columns differentiate into an invading phenotype, the so-called extravillous trophoblast (EVT) [3]. This area has also been referred to as a transition zone, showing more intercellular space between the trophoblasts than in the proximal compact zone of cell columns, enabling a possible route for maternal blood plasma components to enter the early intervillous space [4]. There, EVTs are embedded in a self-secreted matrix of laminins, collagen type IV, vitronectin, heparan sulfate, and cellular fibronectins termed matrix-type fibrinoid [4,5]. Moreover, differentiation into the invasive phenotype is associated with an integrin switch and the expression of the bona fide EVT marker, major histocompatibility complex, class I, G (HLA-G). Failure in EVT lineage formation and differentiation can result in pregnancy complications such as preeclampsia, fetal growth restriction, and pregnancy loss [6]. EVTs invade the decidual stroma and its vessels, including uterine lymphatics, veins, and spiral arteries, forming trophoblast plugs within the latter to inhibit maternal blood cells from entering the early intervillous space. However, by the middle of the first trimester, cells in these trophoblast plugs become loosely cohesive, forming capillary sized channels [7]. Due to their small diameter of only 2 to 3 μm, platelets might be the first among the circulating maternal blood cells that can traverse these intercellular gaps. In fact, we and others have found platelets in between these trophoblast cell gaps, leading to the conclusion that maternal platelets might reach the intervillous space even before uteroplacental perfusion is fully established [8,9].

Several studies have reported a decrease in maternal platelet count during progression of pregnancy [10]. The decrease in mean platelet count gradually occurs from first trimester to second and third, with an overall decline of approximately 10% across gestation considered normal. This decline is a result of both dilution of platelet numbers by maternal blood plasma volume expansion and accelerated platelet sequestration and consumption in the utero-placental circulation [11–13]. It has been reported that in normal human pregnancy, maternal platelets can contribute to perivillous fibrin deposition, which can indirectly contribute to shaping of placental villi and intervillous space. However, excessive platelet activation at the fetal-maternal interface can lead to activation of inflammasomes in the

placental SCT, triggering a sterile inflammation of the placenta and potentially causing systemic inflammatory response in the mother. The question of whether maternal platelets are friend or foe of the human placenta remains unanswered [14].

Based on our recent observations showing maternal platelets in the intercellular space of CCTs, this study aimed to test the hypothesis that maternal platelets and their derived factors play a role in trophoblasts during the initial phase of placental development. For this purpose, platelets from pregnant women were cocultured with trophoblast spheroids and the latter subjected to molecular downstream analyses. Among the numerous genes involved in invasion and placentation processes, we identified leukocyte associated immunoglobulin-like receptor 2 (*LAIR2*) was deregulated in response to maternal platelet activation. This gene encodes a soluble receptor apparently highly expressed in distal parts of CCTs of anchoring villi during the first trimester and is associated with invasion capacities of EVTs.

2 | MATERIALS AND METHODS

2.1 | Platelet isolation and preparation of platelet releasate

This study was approved by the ethical committee of the Medical University of Graz (31-019 ex 18/19). Platelets were isolated as previously described [15,16]. The isolation protocol is provided in the Supplementary Material. Platelets were either directly used for coincubation with ACH-3P spheroids, aggregometry measurements, or further processed. For the generation of platelet releasate (PR), platelets were then incubated with one of the following agonists: 1 U/mL thrombin (Merck, Darmstadt KGaA), 10 μM ADP (2-methylthioadenosine diphosphate; Tocris), 1 μg/mL collagen type 1 (COL1A1; Chrono-Log Corporation), 50 μM thrombin receptor activator peptide 6 (TRAP6; HY-P0078, MedChemExpress) for 30 minutes at 37 °C. If not stated otherwise, PR used for experiments was activated with thrombin. For thrombin-induced PR preparation, thrombin was inactivated with 1.1 U/mL hirudin (Merck, Darmstadt KGaA) and platelets were centrifuged at 1962 × *g* for 15 minutes. The supernatant containing the released factors from activated platelets (PR), not platelets themselves, was collected, aliquoted, and stored at −80 °C for subsequent experiments. Characteristics of the study group are shown in Supplementary Table S1.

2.2 | Culture of trophoblast cell line ACH-3P and spheroid formation

The human first trimester trophoblast cell line ACH-3P [17] was kindly provided by Gernot Desoye (Department of Obstetrics and Gynecology, Medical University Graz, Austria). Cells were cultured as previously described [18]. Details are provided in the Supplementary Material. For spheroid formation, 2.5 × 10^4 cells were cultured per well of a Nunclon Sphera-treated 96-well, U-shaped-bottom microplate (Thermo Fisher Scientific). After 12 hours, cell spheroids were incubated with PR pooled from 10 independent pregnant donors (mean platelets/μL: 163 ± 44 × 10^3, mean gestational age: 270 ± 8 days) in a 1:2 dilution with culture medium. After culture, ≥8 spheroids were pooled and lysed with respective lysis buffer and vigorous up and down pipetting. Cell lysates were stored at −80 °C until further analysis. Four or more spheroids that underwent the same treatment were pooled and formalin-fixed and paraffin embedded (FFPE) according to standard procedure.

2.3 | LAIR2 silencing

ACH-3P cells were seeded in 24-well plates at a density of 1 × 10^5 cells per well and cultured for 24 hours. Cells were then transfected with a predesigned small interfering RNA (siRNA) targeting human *LAIR2* (5 pM, Silencer siRNA, Thermo Fisher Scientific) or nontargeting negative control siRNA (5 pM, Silencer siRNA, Thermo Fisher Scientific) using Lipofectamine as a transfection reagent in 1 ml Dulbecco's Modified Eagle Medium/F12 (1:1; Gibco, Life Technologies) supplemented only with 1% (v/v) L-glutamine (20 mM 100X; Gibco) for 24 hours.

2.4 | Viability and apoptosis determination for 3-dimensional (3D) cell culture

For viability and apoptosis determination, the commercially available CellTiter-Glo 3D Cell Viability Assay (Promega) and Caspase-Glo 3/7 (Promega) assays were performed according to the manufacturer's protocol. In brief, ACH-3P cells were seeded 24 hours prior to treatment as described previously to allow spheroid formation. PR in a 1:2 dilution was added directly to each well and incubated for 24 hours. The respective amount of culture medium was used as a vehicle control, whereas 10 μM staurosporine (Alexis Corporation) served as a control for apoptosis induction. After 24 hours of treatment, bioluminescence signals were measured using a CLARIOstar microplate reader (BMG Labtech). All values were blank adjusted (medium only). Cell viabilities were normalized to controls using GraphPad Prism.

2.5 | Coculture of ACH-3P spheroids with human platelets

ACH-3P cells were coincubated with isolated human platelets in a Nunclon Sphera-treated 96-well, U-shaped bottom microplate (Thermo Fisher Scientific). In each well, 2.5 × 10^4 cells were cultured and mixed with isolated human platelets (267 ± 44 × 10^3 platelets/μL) in an approximate 1:500 cell-to-platelet ratio, in the abovementioned ACH-3P media with or without 1 U/mL thrombin (Merck) for platelet activation. Controls were treated with the respective amount of the

ACH-3P culture medium without platelets or thrombin. After 24 hours, spheroids from each condition were pooled and either subjected to FFPE procedures or cell lysates were collected and stored at −80 °C until further analysis.

2.6 | RNA sequencing (RNA-seq) and bioinformatics analysis

Total RNA from matched PR-treated and control ACH-3P spheroids (5 per condition) was isolated with SV Total RNA Isolation System (Promega) according to the manufacturer's protocol. RNA samples were then sent to GENEWIZ from Azenta Life Sciences and subjected to RNA quantification using Qubit 4.0 Fluorometer (Life Technologies), and RNA integrity was checked with RNA Kit on Agilent 5300 Fragment Analyzer, RNA integrity number >9 (Agilent Technologies). Ribosomal RNA depletion was achieved using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat Cat. No. E6310). RNA-seq library preparation was performed using the NEBNext Ultra RNA Library Prep Kit for Illumina following the manufacturer's recommendations (New England Biolabs). Samples were sequenced on the Illumina NovaSeq 6000 instrument in a paired-end configuration, resulting in 2 × 250 bp read pairs.

RNA-seq analysis of the 10 samples was performed within Galaxy [19]. A description of the analysis is provided in the Supplementary Material. All raw RNA-seq files are provided through the National Center for Biotechnology Information (NCBI) Short Read Archive, accession number PRJNA1194512.

2.7 | Quantitative polymerase chain reaction (qPCR) analysis

Total RNA was isolated from pooled spheroid or organoid samples using the SV Total RNA Isolation System (Promega) according to the manufacturer's protocol. In addition, to spheroids and organoids, 100 µL of fresh culture medium supplemented with PR and 100 µL conditioned culture medium collected after cell culture were subjected to RNA isolation and subsequent qPCR analysis. A detailed description of the qPCR analysis is provided in the Supplementary Material. Primer sequences are shown in Supplementary Table S2.

2.8 | Analysis of single–nucleus RNA-sequencing data

Single–nucleus RNA-sequencing (snRNA-seq) human first trimester placental villi datasets from Nonn et al. [20] (n = 10 healthy tissues, n = 145,637 nuclei) were accessed from Zenodo (10.5281/zenodo.8159511) and used without additional preprocessing. Matrices were loaded into R (v4.1.2) and *LAIR2* expression visualized using Seurat (v4.1.0). Details about the analysis are provided in the Supplementary Material.

2.9 | *In situ* padlock probe analysis

The mRNA transcripts of *LAIR2* were visualized with *in situ* padlock probe hybridization as described previously [21,22]. Sequences for padlock probe design with the GenBank accession number NM_002288.6 (*LAIR2*) were obtained from NCBI. A detailed description of this method is provided in the Supplementary Material. Sequences of primers and padlock probes are shown in Supplementary Table S3.

2.10 | Primary trophoblast isolation and organoid formation

First trimester placental tissue was obtained from Auckland Medical Aid Centre, Auckland, New Zealand, after obtaining ethical approval from the Northern X Ethics Committee (NTX/12/06/057/AM012) and written informed consent. Primary mononuclear villous trophoblasts were isolated and cultured as organoids as previously described, with minor changes [23]. The detailed protocol is provided in the Supplementary Material. When most of the organoids reached a size of 200 to 500 µm, they were either stimulated to undergo differentiation toward the EVT lineage or maintained in trophoblast organoid media (TOM) as previously described [24]. To stimulate EVT differentiation, TOM was exchanged for extravillous trophoblast medium 1 (see Supplementary Material). Medium was changed every 2 to 3 days. After 7 days of culture in extravillous trophoblast medium 1 or respective TOM, half of the control organoids and half of the EVT-induced organoids were treated with PR pooled from 11 pregnant donors in a 1:2 dilution with the respective culture medium. The remaining organoids underwent medium change in their respective culture media. After 24 hours of incubation, all organoids from 2 wells of the same condition were pooled and cell lysates were collected and stored at −80 °C until further analysis. For FPPE processing, all organoids from 4 wells of the same condition were pooled.

2.11 | ELISA

LAIR2 levels were measured from pooled protein lysates of ACH-3P spheroids treated in presence or absence of PR using a LAIR2 ELISA kit (ELH-LAIR2, RayBiotech Life Inc), according to manufacturer's manual. Further description of this method is stated in the Supplementary Material.

2.12 | Immunohistochemistry

FFPE spheroids, organoids, or placenta tissue were cut into 5 µm sections and mounted on Superfrost Plus slides (Thermo Fisher Scientific). After standard deparaffinization steps, sections were subjected to antigen retrieval in Epitope Retrieval Solution pH 9.0 (Novocostra, Leica) or citrate buffer pH 6.0 2 times for 20 minutes

each at 150 W in a laboratory microwave (Miele). Immunohistochemistry was performed using primary antibodies specified in Supplementary Table S4 by applying the UltraVision Large Volume Detection System HRP Polymer Kit (Thermo Fisher Scientific) as previously described [25,26]. Hematoxylin was used as nuclei counterstain, and slides were mounted with Kaiser's Glycerin Gelatine (Merck) and a cover slip. Images were obtained with an Evident Olympus VS200 Slide Scanner (Evident Corporation).

2.13 | Immunofluorescence staining

For immunofluorescence double staining, 5 µm sections were mounted on Superfrost Plus slides (Thermo Fisher Scientific) and subjected to antigen retrieval as described above. The staining protocol is described in the Supplementary Material. Images were obtained with an Evident Olympus VS200 Slide Scanner or an Evident FV3000 Confocal Laser Scanning Microscope (Evident).

2.14 | Hematoxylin and eosin staining

After FFPE processing, tissue blocks were cut into 5 µm sections and stained with hematoxylin and eosin as previously described [18]. A brief description can be found in the Supplementary Material. Images were obtained with an Evident Olympus VS200 Slide Scanner (Evident Corporation).

2.15 | Platelet aggregometry

Aggregometry of isolated platelets from pregnant women was assessed using impedance measurements with Chrono-log Model 700 Aggregometer (Chrono-Log Corporation). For activation, either TRAP6 (HY-P0078, MedChemExpress) at a final concentration of 50 µM or Col1 (Chrono-Log Corporation) at a final concentration of 1 µg/mL was added to the platelet suspension. To measure the influence of LAIR2 on platelet activation, agonists were preincubated with or without 1 µg/mL recombinant human (rh)LAIR2 protein (ab182705, abcam) for 30 minutes at ambient temperature before measurements. Further details on the methodology can be found in the Supplementary Material.

2.16 | Invasion assay

To measure the invasion ability of the ACH-3P cells through an extracellular matrix (ECM), a QCM 96-Well Cell Invasion Assay (ECM555, Merck) was performed according to manufacturer's protocol. Cells were seeded with or without 1 µg/mL rhLAIR2 protein (ab182705, abcam). Additionally, we used cells that underwent siRNA-mediated knockdown of *LAIR2* expression prior to this assay. Samples were measured with a CLARIOStar fluorescence plate reader (BMG LABTECH GmbH). Further description of the assay is provided in the Supplementary Material.

2.17 | Statistical analysis

Data were analyzed using GraphPad Prism 10.1.2 and are presented as mean with SD. Standard normality was tested by performing the Shapiro–Wilk test, and significance was tested with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to compare groups with one another. Student's *t*-test was used when only 2 groups were compared.

3 | RESULTS

3.1 | Platelet-derived factors modify the trophoblast gene expression profile

To determine the effect of maternal platelet-derived factors on the gene expression profile of trophoblasts, blood was collected from pregnant women just prior to cesarean section, and platelets were isolated and activated with thrombin. The resulting supernatant was collected and used as PR fraction for subsequent experiments. Trophoblast spheroids, generated from the first trimester trophoblast cell line ACH-3P, were cultured with and without PR and subjected to RNA-seq (Figure 1A). Moreover, the impact of platelet-derived factors on the viability and late apoptosis of ACH-3P spheroids was investigated. While the cell viability was significantly enhanced with PR compared to untreated spheroids (Figure 1B), caspase 3-dependent apoptosis was not induced by this treatment (Figure 1C). RNA-seq and subsequent bioinformatics analysis showed that the presence of PR altered the gene expression profile of the trophoblast spheroids, including 65 significantly up and 13 significantly downregulated genes (Figure 1D, Supplementary Table S5). From this set of deregulated genes, we proceeded to identify those that have been previously discussed in the context of placental development and/or platelet function, such as integrins (*ITGA2*, $\log_2$ fold change (FC) = 1.31; *ITGA3*, $\log_2$FC = 0.86), metalloproteinase (*MMP15*, $\log_2$FC = 0.97), and NOD-like receptor proteins (*NLRP2*, $\log_2$FC = 0.61; *NLRP7*, $\log_2$FC = 0.59) (Figure 1E), and confirmed their deregulation in response to PR via qPCR (Figure 1F). Since platelets have been reported to carry very small amounts of mRNA and exhibit high levels of integrins, culture medium supplemented with PR was subjected to qPCR analysis for *ITGA2*, which, however, showed no signal, ruling out possible data falsification by traces of platelet RNA. Further gene set enrichment analysis revealed several significant gene ontology terms, including but not limited to terms associated with cell migration and adhesion as well as tissue, cellular, and vascular development (Supplementary Figure S1, Supplementary Table S6).

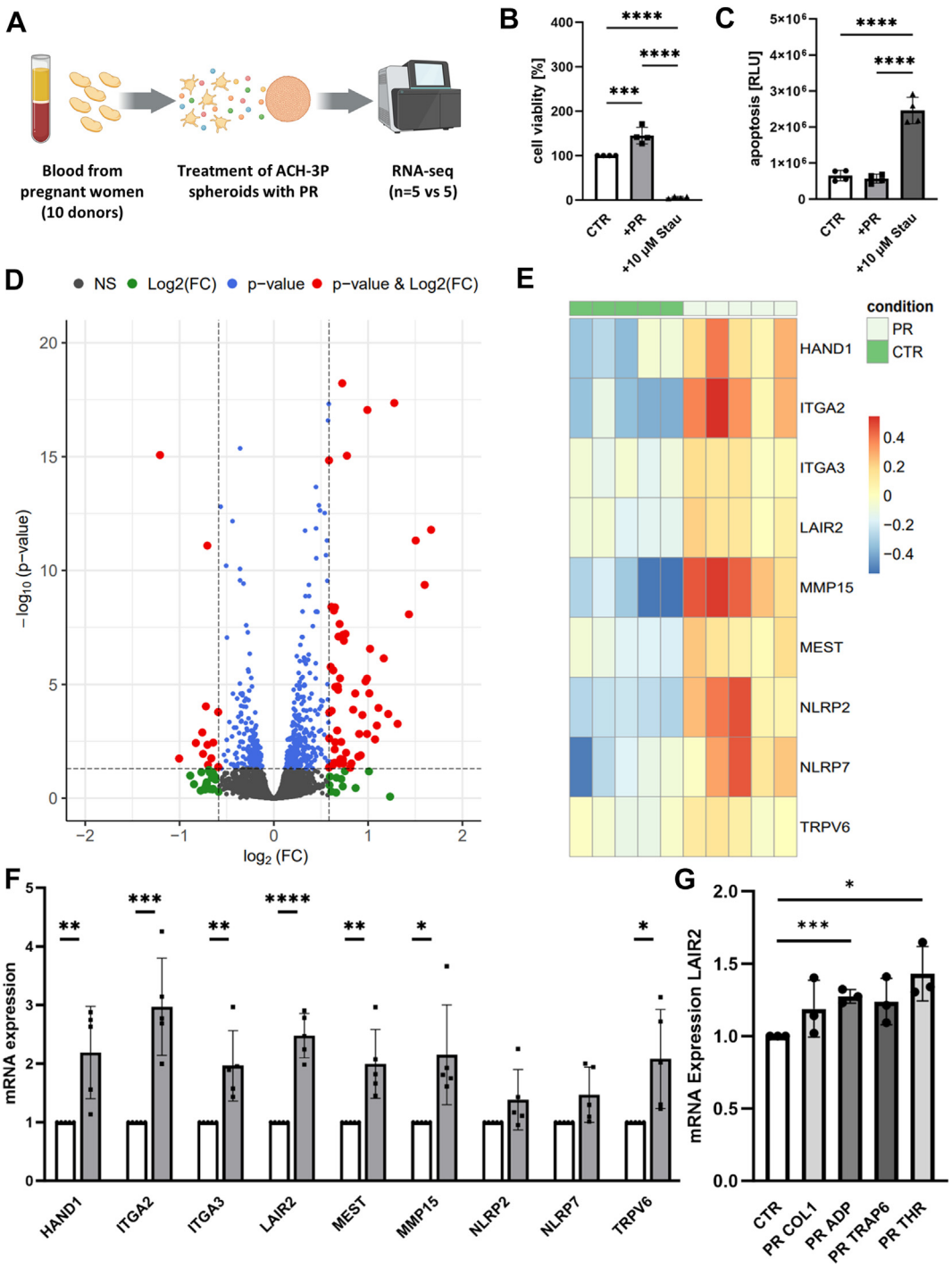

FIGURE 1 Platelet-derived factors modulate transcriptome of trophoblasts. Platelets were isolated from whole blood from pregnant women (n = 10) and activated with thrombin. ACH-3P spheroids were treated with supernatant from activated platelets (platelet releasate fraction, PR) and analyzed by RNA-seq (A). PR enhanced the cell viability of the spheroids (B) but did not affect apoptosis (C) in comparison to control spheroids (CTR). Staurosporine (Stau, 10 µM, 24 hours) was applied to induce apoptosis. RNA-seq data revealed a significant upregulation of 65 genes and a significant downregulation of 13 genes in spheroids upon treatment with PR (D). Gene expression patterns (based on relative rlog values) of genes involved in embryonic or placental development (E). Deregulation of gene expression was confirmed by qPCR (F). *LAIR2* expression was analyzed in ACH-3P monolayer cultures that were incubated with either collagen I-, ADP-, TRAP6- or thrombin-induced PR, which was prepared from 4 different donors (G). Data are presented as mean ± SD. FC, fold change; HAND1, heart and neural crest derivatives expressed 1; ITGA, integrin subunit alpha; LAIR2, leukocyte associated immunoglobulin-like receptor 2; MEST, mesoderm specific transcript; MMP15, matrix metalloproteinase 15; NLRP, NOD-like receptor protein; NS, not significant; qPCR, quantitative polymerase chain reaction; RNA-seq, RNA-sequencing; TRPV6, transient receptor potential cation channel subfamily V member 6. $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .0002$, $^{****}P \leq 0.0001$. Parts of the figure were created with BioRender.com.

3.2 | LAIR2 is highly expressed in invading trophoblasts of the first trimester placenta

From the obtained RNA-seq data set, we identified *LAIR2* (log_2FC = 1.60) as a gene of interest (Figure 1E) potentially linking platelet function and placental development. It is worth mentioning that, besides thrombin-induced PR, COL1A1-, ADP-, and TRAP6-induced PR, although less pronounced, also elicited the same trend of increased *LAIR2* expression in ACH-3P cells grown in monolayers, with only ADP-induced PR reaching statistical significance (Figure 1G). Previous studies have implicated the involvement of LAIR2 in the invasion capacity of EVTs and in uterine spiral artery remodeling, as well as collagen-binding and platelet activation [27–29]. Analysis of our recent snRNA-seq data from human first trimester placenta samples [20] revealed strong expression of *LAIR2* in the CCTs (Figure 2A, B). Immunohistochemical staining was consistent with these findings, showing LAIR2-positive cells throughout cell columns of anchoring villi (Figure 2C). Of note, the platelets themselves were negative for LAIR2 staining (Supplementary Figure S2). To validate snRNA-seq and immunostaining, we performed mRNA-based padlock probe *in situ* hybridization in combination with immunofluorescence staining, which confirmed *LAIR2* expression in trophoblasts positively stained for EVT marker HLA-G in first trimester villous tissue (Figure 2D, E). To further validate *LAIR2* expression in early placental development, we isolated primary trophoblasts from first trimester placental tissue and cultured them as organoids. Interestingly, immunofluorescence double staining confirmed that neither HLA-G (Figure 2F) nor LAIR2 (Figure 2G) was expressed in trophoblast organoids, while organoids in which EVT differentiation was induced showed strong HLA-G expression (Figure 2H) and a trend toward increased *LAIR2* expression (Figure 2I, J). This trend was even more pronounced when organoids in which EVT differentiation was induced were incubated with PR (Figure 2J). However, no statistical significance was reached, likely due to substantial interdonor variations in the primary cells used.

3.3 | Platelets and their released factors induce trophoblastic *LAIR2* expression

To more accurately investigate the influence of maternal platelet activation on trophoblastic *LAIR2* mRNA expression, an *in situ* padlock probe approach was performed on ACH-3P spheroids in the absence and presence of PR from pregnant women. It is worth mentioning that in both settings, cells of the superficial layers of spheroids generally showed more overall, as well as more intensive, signals than the middle layers and the inner core (Figure 3A, D). Although control spheroids showed more evenly distributed signals altogether, *LAIR2* signals clearly accumulated more in PR-treated spheroids (Figure 3E) compared with controls (Figure 3B). *LAIR2* expression was calculated relative to that of actin β (*ACTB*, Figure 3C, F). In line with RNA-seq data, PR-treated spheroids showed an increase in normalized *LAIR2* signals compared to control spheroids (Figure 3G). ELISA analysis of cell protein lysates from ACH-3P spheroids were consistent with the mRNA expression and showed significantly upregulated LAIR2 in spheroids exposed to PR compared to controls (Figure 3H). In addition, ACH-3P cells were cultured to form spheroids either in absence of platelets or presence of nonactivated (ie, resting) and activated platelets. While resting platelets appeared to have no impact on *LAIR2* mRNA expression, thrombin-activated platelets significantly induced *LAIR2* expression in coculture (Figure 3I). Immunohistochemical staining of all 3 groups of spheroids revealed the distinct cavity (Figure 3J), as previously described [18], while platelets, visualized with platelet marker CD42b, accumulated not only in the cavity but also in between trophoblast layers in both resting (Figure 3K) and activated states (Figure 3L).

3.4 | LAIR2 promotes trophoblast invasion and inhibits COL1A1-induced platelet activation

Next, we stained human first trimester decidua samples for HLA-G, confirming substantial invasion of EVTs in decidua basalis samples (Figure 4A), while the decidua parietalis—the noninvaded region of the pregnant uterine mucosa—was, as expected, negative for HLA-G staining (Figure 4B). HLA-G staining revealed erosion of decidual blood vessels (arrowhead in Figure 4A), accompanied by extravasation of maternal blood cells into the decidual stroma. The extravasation was visualized by hematoxylin and eosin staining of consecutive sections, showing erythrocytes in adjacent perivascular areas (Figure 4C), and confirmed by immunostaining for endothelial marker CD31, which also revealed erythrocytes in perivascular areas (Figure 4E). Since leakage of blood into the decidual stroma can be expected to coagulate in presence of ECM components, we stained for COL1A1, a highly thrombogenic ECM protein. In the decidua parietalis, COL1A1 was most prominent in subepithelial areas of the zona compacta (the uppermost densely packed layer, rich in decidual stroma cells and ECM) (Figure 4H), gradually decreasing toward the zona spongiosa (layer showing dilated and differentiated uterine glands), in which it was prevailing in the tunica adventitia of the spiral arteries. COL1A1 was also present in the decidua basalis (Figure 4G), where we observed EVT-eroded blood vessels and extravasated blood. Congruent regions of the decidua basalis positive for HLA-G were also positive for LAIR2 (Figure 4I), whereas the noninvaded decidua parietalis was negative (Figure 4J), cumulatively suggesting that within decidual tissue, only invading EVTs express *LAIR2* at the fetal-maternal interface.

Immunofluorescence staining showed colocalization of HLA-G (green) and LAIR2 (red), confirming the presence of LAIR2 in invaded EVTs in the decidua basalis (Figure 5A–D). Since LAIR2 is a secreted decoy receptor for collagen and collagen is present in the decidua, we next tested whether LAIR2 plays a role in the EVT invasion process. For this purpose, we performed siRNA-mediated silencing of *LAIR2*, which resulted in a 73% knockdown of *LAIR2* expression compared to control (Figure 5E). The subsequent invasion behavior of ACH-3P cells was studied using an ECM invasion assay. The results showed that cell invasion was significantly increased

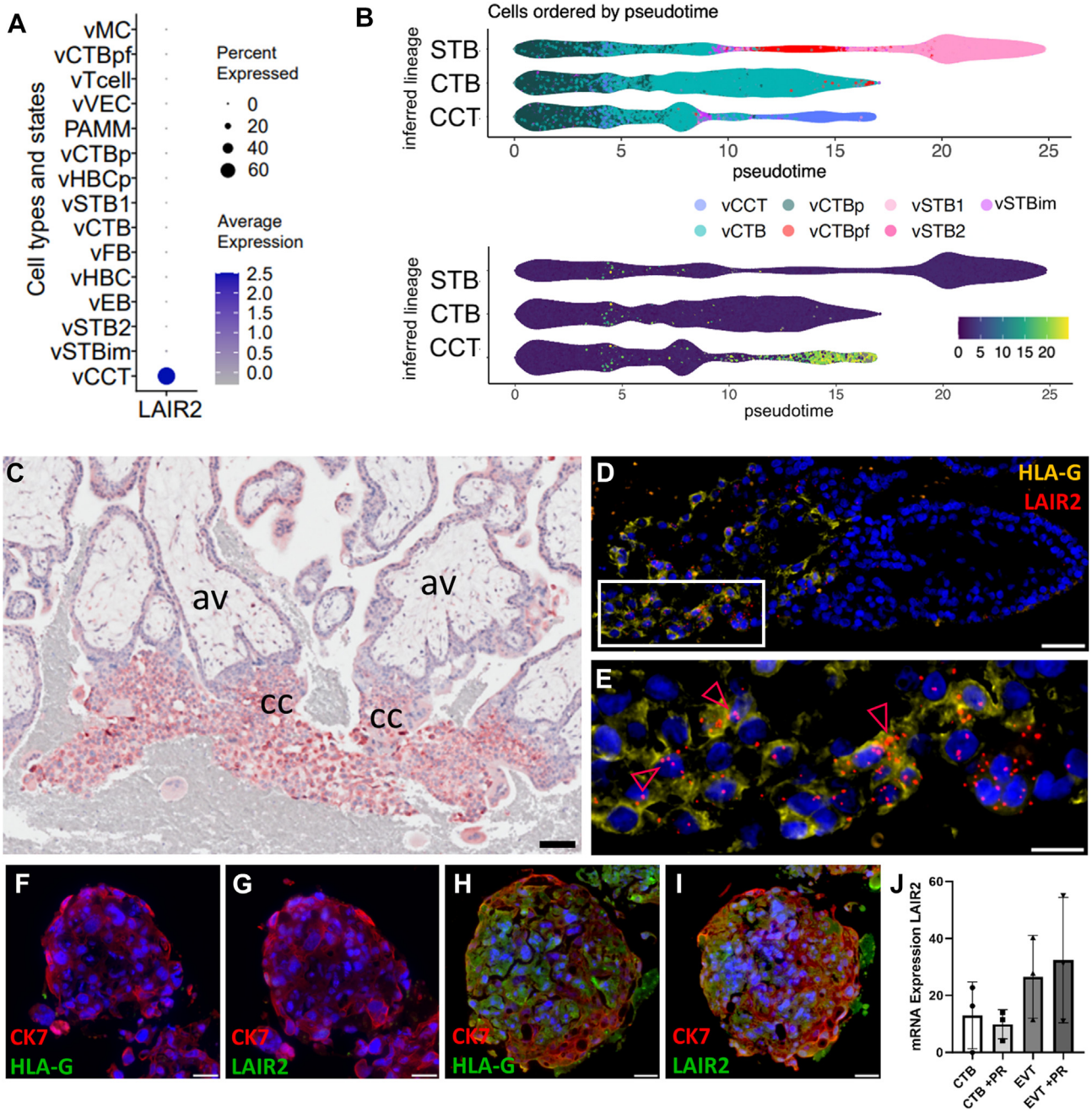

FIGURE 2 Expression of *LAIR2* in human placenta samples. Analysis of snRNA-seq data showed that *LAIR2* is predominantly expressed in the cell column trophoblast (vCCT) population of human villous first trimester placenta; abbreviations for other villus-associated cell populations: myocyte (vMC); prefusion cytotrophoblast (vCTPpf); vascular endothelial cell (vVEC); placenta-associated maternal monocyte/macrophage (PAMM); proliferating cytotrophoblast (vCTBp); proliferating Hofbauer cell (vHBCp); syncytiotrophoblast 1 (vSTB1) and 2 (vSTB2); cytotrophoblast (vCTB); fibroblast (vFB); Hofbauer cell (vHBC); erythroblast (vEB); immature syncytiotrophoblast (vSTBim); (A). Scatter plot of modeled pseudotime values separated by inferred lineage. Each dot represents a nucleus, colored by its annotated cell type or state membership (B). Immunohistochemistry confirmed LAIR2 expression in distal parts of cell columns (cc) of anchoring villi (av) (C). *In situ* padlock probe hybridization approach showing strong LAIR2 mRNA signals (arrowheads) in HLA-G positive (yellow) cells of villous tissue (D and E, enlargement of the boxed region in D). Double immunofluorescence of adjacent section of trophoblast organoids for CK7 (F–I, red) with either HLA-G or LAIR2 (both green) showed that undifferentiated primary trophoblast organoids were negative for HLA-G (F) and LAIR2 (G), while organoids induced to undergo differentiation toward the EVT phenotype showed strong expression of HLA-G (H) and LAIR2 (I). First trimester trophoblast organoids that underwent EVT differentiation showed increased LAIR2 mRNA expression, which is slightly increased in presence of PR (J). Data in J are presented as mean ± SD from 3 different experiments. Scale bar in C represents 100 μm. Scale bars in D and F–I represent 50 μm. Scale bar in E represents 20 μm. CCT, cell column trophoblast; CTB, cytotrophoblast; EVT, extravillous trophoblast; HLA-G, major histocompatibility complex, class I, G; LAIR2, leukocyte associated immunoglobulin-like receptor 2; PR, platelet releasate fraction; snRNA-seq, single–nucleus RNA-sequencing; STB, syncytiotrophoblast.

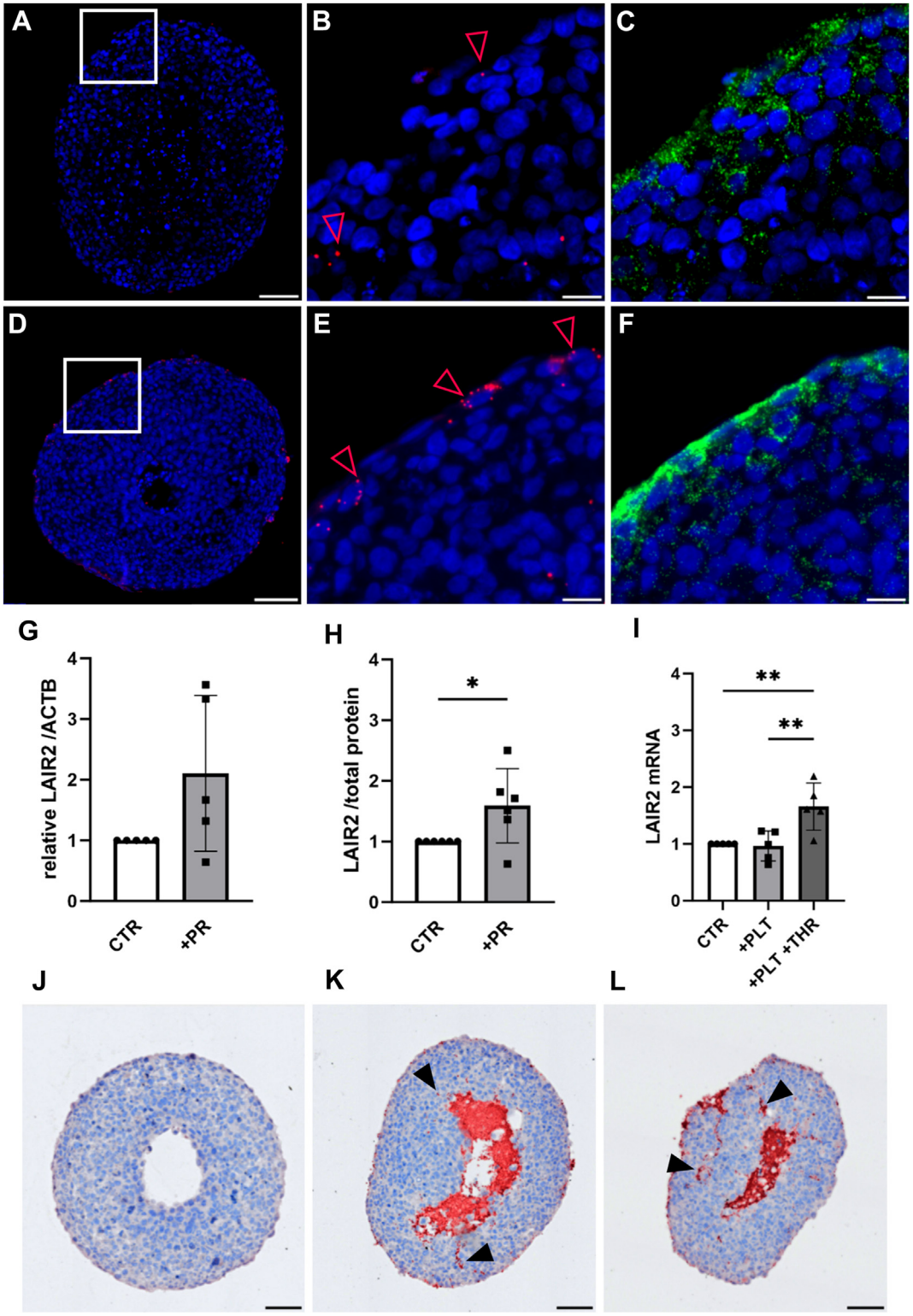

FIGURE 3 Platelets and their derived factors induce *LAIR2* expression of trophoblast spheroids. Padlock probe-based *in situ* hybridization was performed on ACH-3P spheroids incubated in absence (A–C) or presence (D–F) of platelet releasate fraction (PR). Results showed only moderate expression of *LAIR2* in control ACH-3P spheroids (B, arrowheads), while spheroids incubated with PR showed strong signals mostly in the superficial cell layers (E, arrowheads). Expression of actin β (*ACTB*) was used as an internal reference, shown in green (C and F). Software-based analysis of signals in the superficial layers of the spheroids confirmed higher expression of *LAIR2* in PR-treated spheroids (G). ELISA measurements of protein lysates of the spheroids showed significantly increased LAIR2 (H). Coincubation of spheroids with resting platelets (+PLT) showed no effect, whereas activation of the platelets with thrombin (+PLT +THR) significantly increased *LAIR2* expression (I). Spheroids formed cavities in their core (J). Upon coincubation of spheroids with resting (K) or activated platelets (L), platelets can be found—irrespective of their activation status—in the cavities as well as in the intercellular space of trophoblasts (arrowheads). Scale bars in A, D, and J–L represent 100 μm. Scale bars in all other images represent 20 μm. Data are presented as mean ± SD from 5 (A–G and I) or 6 (H) different experiments. CTR, control spheroids; LAIR2, leukocyte associated immunoglobulin-like receptor 2. $^{*}P \leq .05$, $^{**}P \leq .01$.

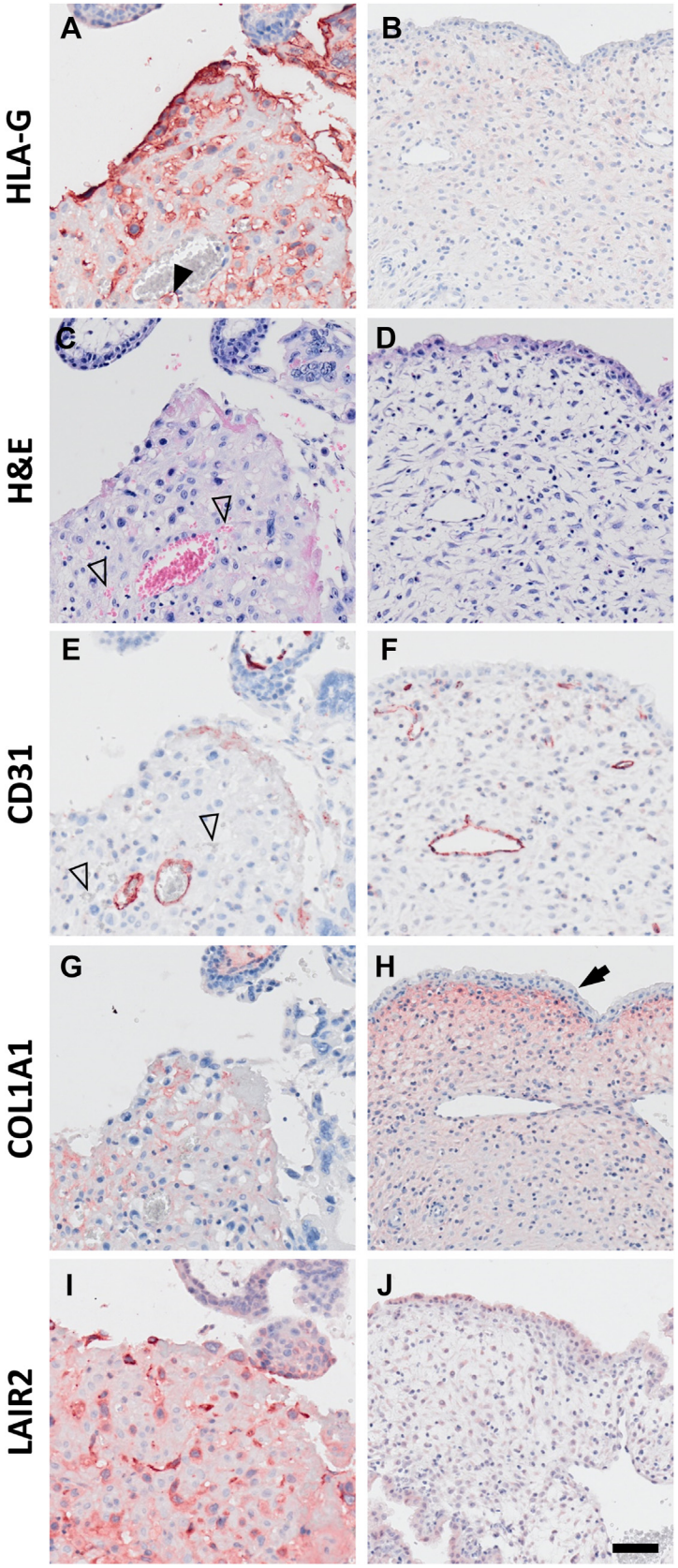

FIGURE 4 LAIR2-positive extravillous trophoblasts (EVTs) erode decidual blood vessels, leading to extravasation of maternal blood. Decidua basalis (left column) and corresponding decidua parietalis (right column) tissue sections were stained for major histocompatibility complex, class I, G (HLA-G) (A and B) and showed invasion of EVTs (arrowhead) into decidual blood vessels. Hematoxylin and eosin (H&E) staining (C and D) of consecutive sections revealed extravasated maternal erythrocytes in the invaded decidua basalis regions (C, arrowheads). This extravasation of erythrocytes (arrowheads) was confirmed by CD31 staining of the decidua basalis (E) and parietalis (F). Collagen I (COL1A1) (G and H) was detected in both decidua basalis (G) and parietalis, the latter of which showed prominent COL1A1 content underneath the uterine epithelium (H, arrow). LAIR2 staining (I and J) revealed positive cells only in the decidua basalis. Representative images were acquired from a decidua at gestational age 7+0. Scale bar represents 50 μm. LAIR2, leukocyte associated immunoglobulin like receptor 2.

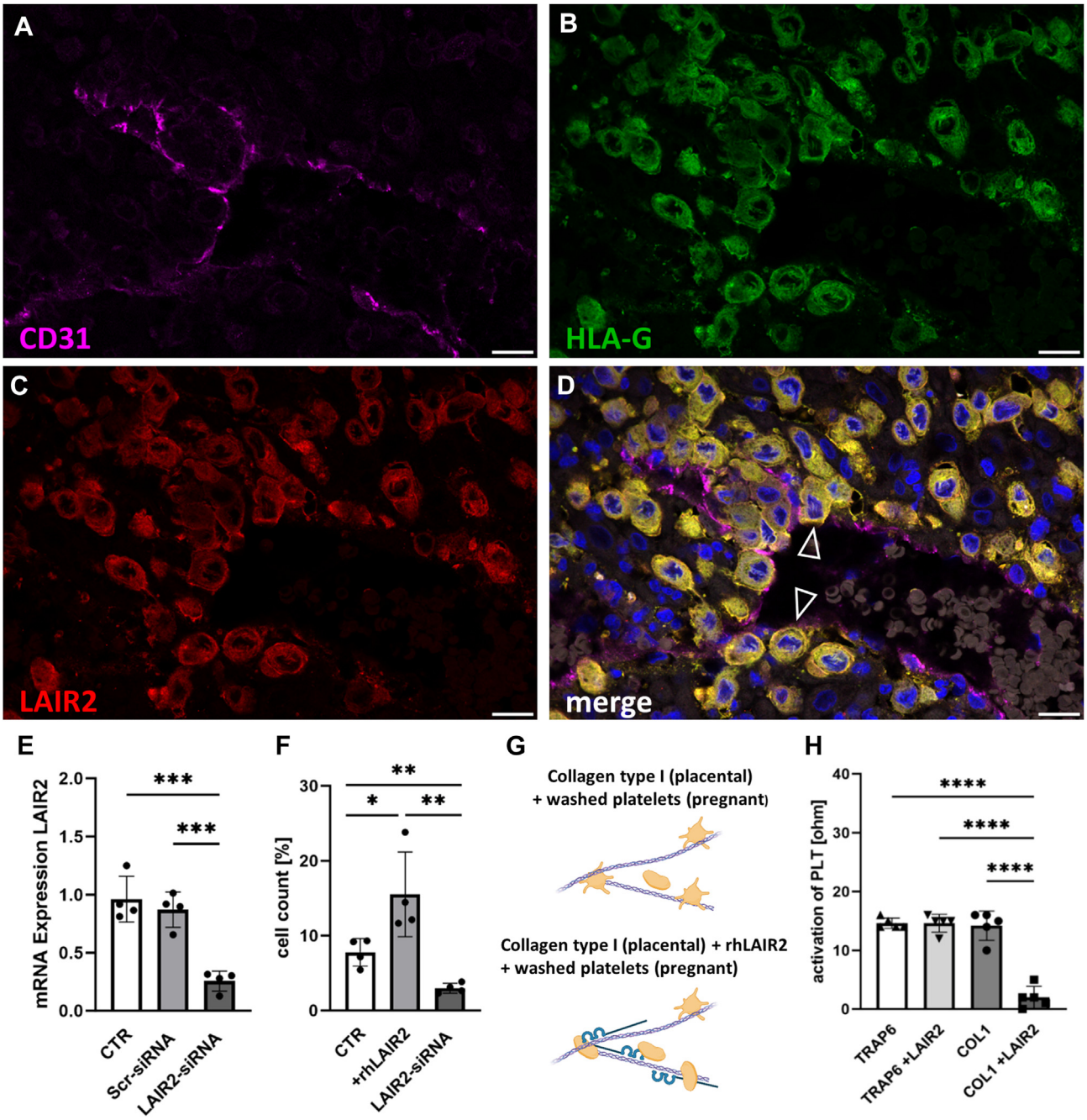

FIGURE 5 LAIR2 promotes trophoblast invasion and inhibits maternal platelet activation. Triple immunofluorescence for endothelial marker CD31 (A), HLA-G (B) and LAIR2 (C), confirmed LAIR2 expression in extravillous trophoblasts (arrowheads) that invaded in a decidual blood vessel and partly replaced the endothelial lining (D, merge). LAIR2 mRNA expression in control ACH-3P cells (CTR) was compared with that in cells treated with either scrambled siRNA (Scr-siRNA) or LAIR2-siRNA (E). Invasion of ACH-3P cells was determined by an extracellular matrix invasion assay, which showed increased invasion in the presence of rhLAIR2 and decreased invasion after siRNA-mediated LAIR2 silencing (F). To test the effect of LAIR2 on collagen-induced platelet activation, platelets from pregnant women were stimulated with either collagen type I (COL1) or TRAP6, which were either preincubated with or without rhLAIR2 (G). Subsequent aggregometry showed that preincubation with LAIR2 inhibited platelet activation induced by COL1 but not TRAP6 (H). Scale bar represents 20 μm. Data are presented as mean ± SD from 4 (E and F) and 5 (H) different experiments. CTR, control spheroids; HLA-G, major histocompatibility complex, class I, G; LAIR2, leukocyte associated immunoglobulin-like receptor 2; PLT, platelets; rhLAIR2, recombinant human LAIR2; siRNA, small interfering RNA; TRAP6, thrombin receptor activator peptide 6. $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .0002$, $^{****}P \leq .0001$. Parts of the figure were created with BioRender.com.

nearly 2-fold, in the presence of rhLAIR2 protein, whereas it was reduced by 2.6-fold after *LAIR2* silencing, compared with the control (Figure 5F). Besides affecting invasion, LAIR2 could play a crucial role in regions where maternal blood leaks into perivascular ECM by binding and sequestering collagen, thereby possibly affecting activation of leaking maternal platelets. Aggregometry measurements using

platelet samples from pregnant women revealed that both TRAP6, a synthetic peptide that mimics the action of thrombin, as well as COL1A1 strongly activated maternal platelets. However, when both agonists were preincubated with rhLAIR2 protein, activation via collagen was almost completely blocked, while there was no interference whatsoever with TRAP6 (Figure 5G, H).

4 | DISCUSSION

Our study suggests that activation of maternal platelets and release of their cargo affect the transcriptional profile of invasive trophoblasts at the maternal-fetal interface. Bioinformatics analysis of the trophoblast transcriptome shows that cells shift toward an expression pattern associated with migration and adhesion, thereby contributing to tissue and vascular development in response to platelet-derived factors. Among the deregulated genes, *LAIR2*—a gene expressed exclusively in the extravillous lineage among trophoblast populations—is upregulated by platelet-derived factors. Importantly, bioinformatics pseudotime analysis of a snRNA-seq dataset using first trimester placenta mapped *LAIR2* expression to the CCT trajectory. This finding is substantiated not only *in situ* on mRNA and protein levels, but also *in vitro*, when primary trophoblast organoids are triggered to undergo differentiation toward the EVT phenotype. The fact that the presence of resting platelets within trophoblast spheroids did not induce *LAIR2* expression, whereas thrombin-activated platelets did, suggests that it is not the platelets themselves, but rather platelet-derived factors that play the regulatory role. In this context, we propose a novel 3D concept to study the interaction of human platelets with trophoblasts *in vitro*, mimicking the presence of platelets within the intercellular space of distal CCTs in first trimester of pregnancy [9,30]. Evidence from our previous transmission electron microscopy investigations suggests that platelets are activated in this location, further substantiating the assumption that the release of platelet-derived factors could partly influence cell column trophoblast fate and in particular induce *LAIR2* expression in this trophoblast lineage [9]. One of these factors could be platelet-derived transforming growth factor β, which has recently been suggested as a crucial driver in the differentiation of placental EVTs into decidual EVTs [31]. As expected, our data show that platelet-derived factors do not induce caspase-dependent apoptosis in trophoblast spheroids, but, in fact, increased their viability significantly. While this finding may not be surprising, given the established role of platelet-rich plasma in promoting cell viability and its increasing use as an alternative to fetal bovine serum in cell culture experiments, it nonetheless represents a noteworthy observation [32].

From the distal ends of the CCTs, invasive EVTs start to invade into the decidua basalis stroma, where they erode maternal blood vessels and replace vascular endothelial cells. Intriguingly, our histological survey revealed maternal erythrocytes within interstitial gaps of highly invaded decidua samples, suggesting extravasation of maternal blood in these areas. Leakage of blood cells usually occurs due to increased vascular permeability or compromised integrity of the vessel wall and has been described for pathological scenarios at both macroscopic and microscopic levels, including tissue trauma and within tissues characterized by leaky or immature blood vessels, such as in large atherosclerotic plaques and tumors [33]. The presence of collagen, primarily produced by decidual stromal cells, as well as EVT-derived fibronectin and laminin [34], can be expected to quickly activate leaking platelets and trigger coagulation of extravasated maternal blood. Coagulation and deposition of fibrin may contribute to the initial steps of development of the Nitabuch stria, a layer of fibrinoid located deeper in the basal plate embedding maternal and fetal cells [5]. LAIR2 is a soluble collagen receptor that can modulate immune responses by interacting with collagen. It acts as a decoy receptor, preventing the binding of LAIR1 to collagen, which can influence immune cell behavior and tumor microenvironments [35]. In the context of tumor research, *LAIR2* expression is associated with immune infiltration and can serve as a biomarker for T cell exhaustion in the tumor microenvironment [36]. Based on knowledge from tumor research, it is thus tempting to speculate about a modulating role of EVT-derived LAIR2 in the immune interactions of maternal immune cells with invading trophoblasts. However, another role of LAIR2 may be fine-tuning the coagulation of leaked maternal blood in perivascular regions of the invaded decidua (Figure 6), thereby contributing to fibrinoid deposition and the development of the Nitabuch stria. In particular, we propose that EVT invasion into decidual blood vessels and replacement of their endothelial lining is associated with maternal blood leakage into the perivascular regions of the decidual stroma, where coagulation is likely to occur in the presence of ECM components. Initial activation of platelets and the release of their cargo promotes trophoblastic *LAIR2* expression, which is subsequently secreted into the surrounding intercellular space. There, LAIR2 functions as a decoy receptor by binding to COL1A1, facilitating further trophoblast invasion while simultaneously preventing excessive maternal platelet activation.

Our results align with previous studies showing strong *LAIR2* expression mainly in CCTs as well as EVTs of first trimester placenta [27,37]. We and others have shown that LAIR2 contributes to increased invasiveness of ACH-3P cells, implicating that an upregulation of this gene might also enhance trophoblast invasiveness in an autocrine manner, potentially positively impacting placental development and function [28]. Even slightly abnormal changes in the molecular profile of trophoblasts undergoing differentiation toward the EVT lineage are proposed factors responsible for overinvasion, leading to pathologies such as abnormal invasive placenta [38]. Of note, previous publications have even discussed a potential correlation between diminished *LAIR2* expression in placental tissue and the development of preeclampsia. More specifically, it has been proposed that the downregulation of *LAIR2* expression may lead to impaired physiologic spiral artery conversion. A difference in expression levels of LAIR2 in placental tissue between women who developed preeclampsia and women who remained healthy throughout gestation

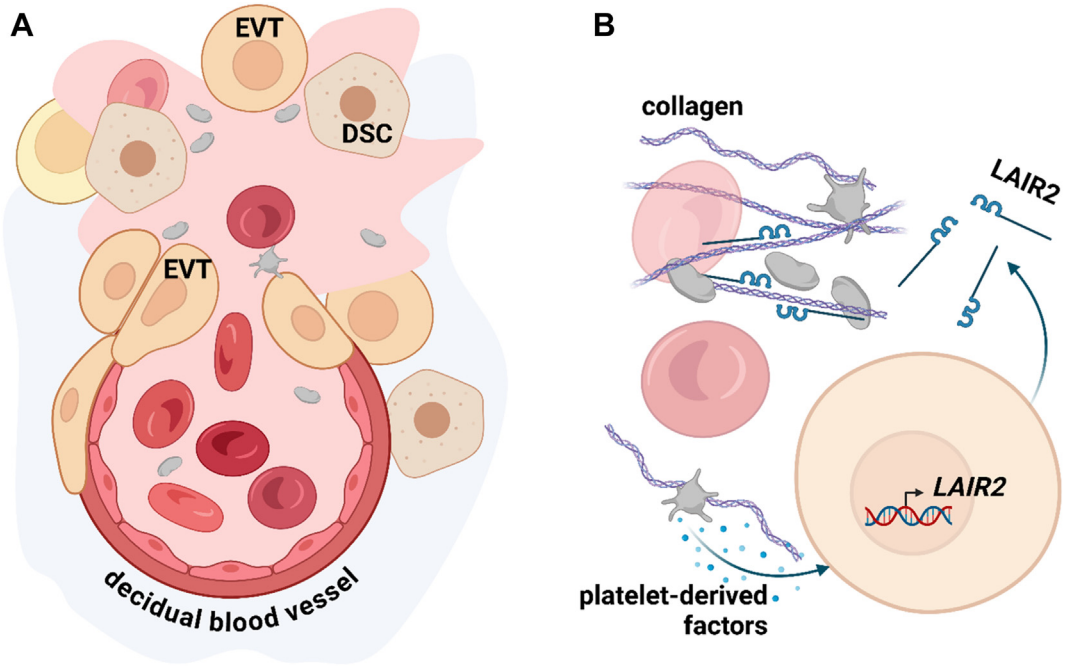

FIGURE 6 Proposed concept for LAIR2 in the maternal-fetal interface. Invaded extravillous trophoblasts (EVT) erode decidual blood vessels and partly replace the endothelial lining. Erosion of blood vessels is accompanied by leakage of maternal blood into perivascular regions of the decidual stroma (decidual stroma cells [DSC]) and can be expected to coagulate in presence of extracellular matrix components (A). Initial platelet activation and the release of their cargo induce trophoblastic LAIR2 expression, which is then secreted into the adjacent intercellular space. There, it acts as a decoy receptor by binding to collagen I, subsequently promoting further trophoblast invasion while also inhibiting excessive activation of maternal platelets (B). LAIR2, leukocyte associated immunoglobulin-like receptor 2. Figure was created with BioRender.com.

was detected by chorionic villus biopsy and has been suggested as a biomarker for preeclampsia risk prediction [27,37,39].

Bioinformatics analysis of the RNA-seq data revealed significant gene ontology terms, such as regulation of cell development and embryonic morphogenesis. Heart and neural crest derivatives expressed 1 (*HAND1*), found to be deregulated in response to platelet-derived factors, might be an important player in these processes. Here, the trophoblast cell line ACH-3P was used for functional coculture experiments with maternal platelets. This may be considered a limitation, since expression of *HAND1*, a key regulator of murine trophoblast development, is present in choriocarcinoma cells and the human trophectoderm, whereas it is absent from first trimester placenta [40]. However, the use of platelets obtained from pregnant individuals and the novel 3D coculture with trophoblasts to mimic the exposure of extravasated maternal platelets to EVTs, as described in the intercellular space of CCTs or EVT-eroded decidual blood vessels, can be considered as a strength of the current study.

In conclusion, this study suggests that maternal platelet-derived factors regulate the transcriptional profile of trophoblasts. This includes regulation of genes involved in placental development, including *LAIR2*, a gene exclusively expressed in the invasive trophoblast linage. Trophoblast-derived LAIR2 may be involved in the fine-tuning of coagulation of leaking maternal blood in the decidua and the invasiveness of EVTs into it through an autocrine manner.

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AUTHOR CONTRIBUTIONS

F.L. and M.G. conceived and designed the study, analyzed data, and drafted the manuscript. F.L., D.F., J.G., N.K., K.U., L.N., C.D., D.V., J.C.K., A.L.H., and D.S.V. performed experiments and analyzed data. A.E.H., D.K., J.F., G.M., B.R., G.C., F.H., S.W., J.P., and J.L.J. provided acquisition, analysis, and interpretation of data and performed statistical analysis. All authors revised and approved the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the authors used ChatGPT to enhance clarity and refine complex sentence structures. After using

this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2025.03.020.