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ORIGINAL ARTICLE

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Multiparity upregulates placental plasminogen and urokinase-type plasminogen activator

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Problem: Multiparity increased the number of trophoblast cells in decidua of both low and high fetal loss mouse models. However, they differ in fetal survival rate and maternal thymocyte subpopulations, suggesting that trophoblast invasiveness is not equivalent. Our aim was to explore the involved mechanism.

Method of study: We studied placentae from primiparous and multiparous females of low and high fetal loss models. We investigated invasiveness in vitro, expression of plasminogen, and its activators: tissue type (tPA)-urokinase type (uPA), and activity and expression of matrix metalloproteinases (MMP)-2 and MMP-9.

Results: Placental invasiveness is upregulated by multiparity, but lesser in the high fetal loss model. Multiparous animals showed elevated expression of plasminogen and uPA. However, the high fetal loss combination showed higher expression of a short and less active fragment of uPA (LMW-uPA). MMP-2, MMP-9, and tPA were unaffected.

Conclusion: uPA would participate in the increased multiparity-associated placental invasiveness.

KEYWORDS
mouse, multiparity, placenta, plasminogen, trophoblast invasion, uPA/PLAU

1 | INTRODUCTION

Fetally derived trophoblast cell invasion into the uterine wall is a characteristic of hemochorial placentation present in both humans and mice and is an essential process for a successful pregnancy. Invading trophoblast cells participate in a variety of physiological processes that are critical for the maintenance of pregnancy including communication with maternal immune cells, hormone and cytokine production, and angiogenesis and substitution of endothelial cells of maternal arterioles and of glandular epithelium.[1] The invasiveness of trophoblasts is maximal during the first stage of pregnancy. Then, it gradually reduces, and this fact might be related to the regulation of proteolytic enzymes/activator/inhibitor gene expression.[2] Loss of this tight regulation leads to gestational diseases: While a shallow vascular invasion is associated with the development of intrauterine growth restriction and preeclampsia,[3] an aggressive invasion to the uterine myometrium is observed in trophoblast cells of placenta accreta. The existence of associated factors such as previous Cesarean sections or placenta previa cannot be used as a predictor of this severe obstetric complication.[4] Up to the present, much effort has been focused on studying the regulation of trophoblast invasion during first-trimester pregnancies, but little attention has received the mechanisms operating at late gestation. Mouse models can bring a useful tool to investigate them. In this sense, we have been working with a physiological multiparity mouse model which can give a good approach to the knowledge of the factors that control this process in vivo.

The development of mouse placenta at mid-late gestation involves extensive tissue remodeling and cell invasion. From 14th day of gestation until its end at day 20-21, cells of the spongiotrophoblast lineage progressively increase the interstitial invasion and migrate into decidual tissue.[5] In previous works, we reported that placentae from mouse females at 18.5 days of their fourth gestation showed various layers of trophoblast cells (cytokeratin+, vimentin−) in decidua, whereas they constitute a single layer in placentae from primiparous females. This fact was observed in the CBA/J syngeneic combination and in allogeneic pregnancies from an abortion-prone model (CBA/J female mated

with DBA/2 males) and a non-abortion-prone one (CBA/J female mated with BALB/c males),[6-8] indicating that multiparity enhances trophoblast invasion into maternal tissue. These cells showed a high expression of M-CSF and G-CSF[7] and also of VEGF and of its receptor VEGF-R1 (Flt-1).[8] These evidences led us propose that VEGF could be involved in the regulation of the trophoblast invasion process in multiparity by means of activating proteases such as plasmin and matrix metalloproteases (MMPs).[9]

The fibrinolytic system comprises the inactive proenzyme plasminogen that can be converted to the active broad-spectrum proteinase plasmin by two physiological plasminogen activators (PAs): the tissue-type PA (tPA) and the urokinase-type PA (uPA/PLAU).[10] While tPA is mainly involved in the dissolution of fibrin clots, the primary role of uPA is considered to be the pericellular plasminogen activation, necessary for cell adhesion, migration, and tissue remodeling.[11,12] uPA activity is focalized to the cell surface by binding to its receptor uPAR/PLAUR, resulting in enhanced activation of cell bound plasminogen.[13] Plasmin, in turn, cleaves and converts tPA and uPA into two-chain proteases, which exhibit higher proteolytic activity, implying a positive feedback for the fibrinolytic cascade. Inhibition of the fibrinolytic system may occur either rapidly at the level of plasmin, mainly by alpha 2-antiplasmin or at the level of the PA, by specific plasminogen activator inhibitors (PAIs).

During pregnancy, the conversion of plasminogen to plasmin creates a powerful proteolytic system that participates in the trophoblast invasion by remodeling the extracellular matrix and activating growth factors and other proteases. In particular, plasmin can activate the zymogen forms of specific decidual MMPs such as gelatinases (gelatinase A: MMP-2 and gelatinase B: MMP-9), regarded as key enzymes in the invasion process.[14] Furthermore, plasmin can release and activate sequestered growth factors including vascular endothelial growth factor (VEGF) which in turn induces the expression of collagenase, PAs, urokinase receptor (uPAR/PLAUR), and also PAIs, amplifying the invasive process.[9] The expression of components of the uPA system (uPA, uPAR, and PAIs) has been demonstrated in mouse[15] and in human[16] at early and term gestation.[17] However, no report has been published investigating the expression of plasminogen in placenta.

Despite histological similarities between placentae from multiparous females of both allogeneic combinations, further evidences suggested that trophoblast invasiveness may not be equivalent. First, multiparous females from the low fetal loss model and in the control syngeneic combinations showed an increase in the number of live fetuses in comparison with primiparous age-matched females. This effect was not seen in the high fetal loss model, which in addition showed an increase in the abortion rate.[18] Second, allogeneic pregnancies induce quantitative variations in maternal thymocyte subpopulations, but its effects differ according to the parity status of the female and the male component of the cross-breeding (BALB/c or DBA/2).[18]

To explore the mechanism involved in the increased trophoblast invasion induced by multiparity in the low and high fetal loss model, in the present work we investigated the following: (i) the in vitro placental invasiveness, (ii) the expression of plasminogen, tPA, and uPA, and (iii) the expression and activity of gelatinases MMP-2 and MMP-9. We employed placentae from first and fourth pregnancies of each mouse combination. As in previous studies, the influence of mother's age was also analyzed. The obtained results show that (i) placental invasiveness is upregulated by four previous gestations even though lesser in the high fetal loss model, (ii) the higher expression of plasminogen and uPA found in placentae from multiparous females might explain such differences, and (iii) interestingly, multiparous placentae from the high fetal loss combination express a higher proportion of the less active low molecular weight-uPA (LMW-uPA).

2 | MATERIALS AND METHODS

2.1 | Animals

Two-month-old mice were purchased from Comisión Nacional de Energía Atómica (Ezeiza, Buenos Aires, Argentina) and were housed in our institutional facilities. CBA/J female mice mated with DBA/2 (high fetal loss model), BALB/c (low fetal loss model), or CBA/J males were randomized into three groups (each one, n=5-6): primiparous young (3.0±0.5 months old), primiparous old (8.5±0.5 months old), and multiparous old (8.5±0.5 months old at their fourth pregnancy). The observation of a vaginal plug was considered as 12 hours post-conception. All mice were killed on the 18.5th day of gestation, and the placentae were removed and divided for further analyses. Some of them were immediately processed for their culture, others were fixed for Immunohistochemistry (IHC) studies, and the rest were stored at −70°C for Western blot (WB) analyses. For invasion assays, all placentas from one pregnant mice were pooled for each experiment (n=5 mice/group). This study was approved by our local institutional animal care (CICUAL, Universidad de Buenos Aires, Argentina) which is in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2 | Placental culture supernatants

Placentae were mechanically disrupted and cultured during 48 hours at 37°C and 5% CO_2 at 76±4 mg/mL in RPMI 1640 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2 g/L $NaHCO_3$, 2 mmol/L L-glutamine, 1 mmol/L pyruvate, 20 μg/mL penicillin, 20 μg/mL streptomycin (all Sigma-Aldrich, St. Louis, MO, USA), and 10% fetal bovine serum (FBS, v/v; Natocor, Córdoba, Argentina). Culture supernatants were collected and stored at −20°C.

2.3 | Placental homogenates

Placentae were mechanically minced, homogenized in lysis buffer (1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin, all Sigma-Aldrich; in 10 mmol/L Tris pH 8.0), sonicated in ice, and centrifuged at 10 000 *g* for 10 minutes at 4°C. The protein content was determined using Bio-Rad Protein Assay® (Bio-Rad Laboratories, Hercules, CA, USA), employing BSA (ICN Biochemicals, Irvine, CA, USA) as standard.

2.4 | Trophoblast cell isolation

Trophoblast cells were cultured according to a previously described method[19] with modifications.[20] Briefly, placentae were isolated in sterile conditions and washed in RPMI1640 with 20 μg/mL penicillin and 20 μg/mL streptomycin. The placentae were then incubated in dissociation medium (wash medium containing 1 mg/mL collagenase, 20 mg/mL DNase, both Sigma-Aldrich) for 1 hour at 37°C, with gentle pipetting to separate cells. Cells were washed to remove dissociation medium, filtered to remove undigested tissue, and separated on an isotonic 40% Percoll gradient. The trophoblast cell layer was collected and washed with RPMI medium. The purity of trophoblast cells (identified as cytokeratin 7 positive) was around 90% (assessed by FACS analysis, as reported by Hanna et al.[21] and Lin et al.[22]).

2.5 | Matrigel invasion assay

Inserts with 8.0-μm pore polyethylene terephthalate membranes (BD Biosciences, San Jose, CA, USA) were placed in a 24-well plate (BD Biosciences). Diluted 1:3 Matrigel (BD Biosciences) in FBS-free RPMI medium was added to the upper chamber and incubated at 37°C for gelling. Trophoblast cells were added to upper wells (5×10^4 in RPMI-0.1% FBS v/v), while 500 μL of RPMI 5% FBS (v/v) medium was added to the lower well as chemo-attractant. Plates were incubated at 37°C and 5% CO_2 for 24 hours, and then, non-invaded cells were scraped off. Migrated cells were fixed with 1% paraformaldehyde (v/v). Membranes were cut, and invasive cells were stained with 10% DAPI (v/v; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and mounted on a microscope slide. Each sample was assayed by duplicate. The number of invasive cells in a sample was expressed as the mean number and standard deviation of ten 400× microscopic fields in two membranes counted in a fluorescent microscope (OLYMPUS BX51 with a camera Q color3; Olympus Corporation, Center Valley, PA, USA).

2.6 | Western blot and dot blot analysis

Placental lysates were solubilized in sample buffer (1% SDS v/v in 125 mmol/L Tris pH 6.8) with or without 300 mmol/L β-mercaptoethanol (2-ME). Protein samples were subjected to electrophoresis and blotted onto nitrocellulose membranes (Hybond-ECL; Amersham, GE Healthcare, Little Chalfont, UK). For dot blot (DB), different amounts of placental homogenates were seeded directly on the membranes and dried. Blots were blocked with 5% of not fat milk (w/v) in TBST (50 mmol/L Tris-HCl, 150 mmol/L NaCl pH 7.5, 5% Tween-20 v/v) and then incubated with the corresponding primary antibody: rabbit antiplasminogen, goat anti-tPA, rabbit anti-uPA (H-140) which detects all forms of uPA, and goat anti-β-actin (all, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-MMP-9, and rabbit anti-MMP-2 (Calbiochem, Merck Millipore, Darmstadt, Germany). After washing, blots were incubated with the respective secondary antibody: anti-rabbit IgG-HRP (ZyMax; Invitrogen, Thermo Fisher Scientific) and anti-goat IgG-HRP (Santa Cruz Biotechnology), developed with ECL Advance Western Blotting Detection Kit (Amersham, GE Healthcare) and exposed to autoradiography films (AGFA CP-BU NEW; Agfa Healthcare, Mortsel, Belgium). Bands were quantified employing ImageJ 1.42q (National Institutes of Health, Bethesda, MD, USA). β-Actin was employed as loading control. The protein molecular weight was estimated by comparison with Full-Range Rainbow Molecular Weight Marker (Amersham, GE Healthcare).

2.7 | Zymography assay

Assessment of gelatin-degrading enzymes from placenta was performed by electrophoretic zymography. Placental homogenates or culture supernatants were subjected to 10% SDS-PAGE containing 2 mg/mL gelatin (Merck Millipore). The enzyme activity was restored by removal SDS by shaking with 2.5% Triton X-100 (v/v). Gels were incubated 24 hours at 37°C with Substrate Buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, 2 μmol/L $ZnCl_2$, and 0.02% NaN_3 w/v) and then stained with 0.05% Coomassie Brilliant Blue G-250 (w/v) (Bio-Rad, Hercules, CA, USA) in methanol: acetic acid: water (25:10:65). After removing the dye, proteolytic activity was visualized as clear zones of proteolysis which were measured with ImageJ 1.42q (National Institutes of Health). The protein molecular weight was estimated as described for WB.

2.8 | Immunohistochemistry

First, each placental section was stained with hematoxylin and eosin in order to check that there was no evidence of infection or any histological alteration. IHC was performed in paraffin-fixed placental sections. Briefly, paraffin was removed by consecutive washes of xylene, absolute ethanol, and 96% ethanol. After washing with PBS, endogenous peroxidase activity was blocked with 1% hydrogen peroxide (v/v) in methanol for 30 minutes. Tissue sections were incubated with a normal serum from the same host of the detection antibody used in each case (rabbit or goat) for 30 minutes at 37°C and stained overnight with the respective primary antibody (or not immune polyclonal IgG of the same origin, as control) at 4°C. After washings with PBS, samples were incubated with a biotinylated antibody for 30 minutes at room temperature and then were washed again. Color was developed using Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and by adding diaminobenzidine substrate kit (Vector Laboratories). The sections were counterstained with hematoxylin (Biopur, Rosario, Argentina) and mounted employing Canadax® (Biopur).

The primary antibodies used were rabbit antiplasminogen, rabbit anti-uPA (H-140), and goat anti-tPA (all, Santa Cruz Biotechnology), and the secondary ones were anti-rabbit IgG Biotin-conjugated (Zymed Laboratories, Thermo Fisher Scientific, Waltham, MA, USA) or anti-goat IgG Biotin-conjugated (Vector Laboratories).

2.9 | Statistical analysis

Results were expressed as mean±SD for each group. Repeated-measurement analysis of variance (ANOVA) and Newman-Keuls test were used to assess differences between means employing the

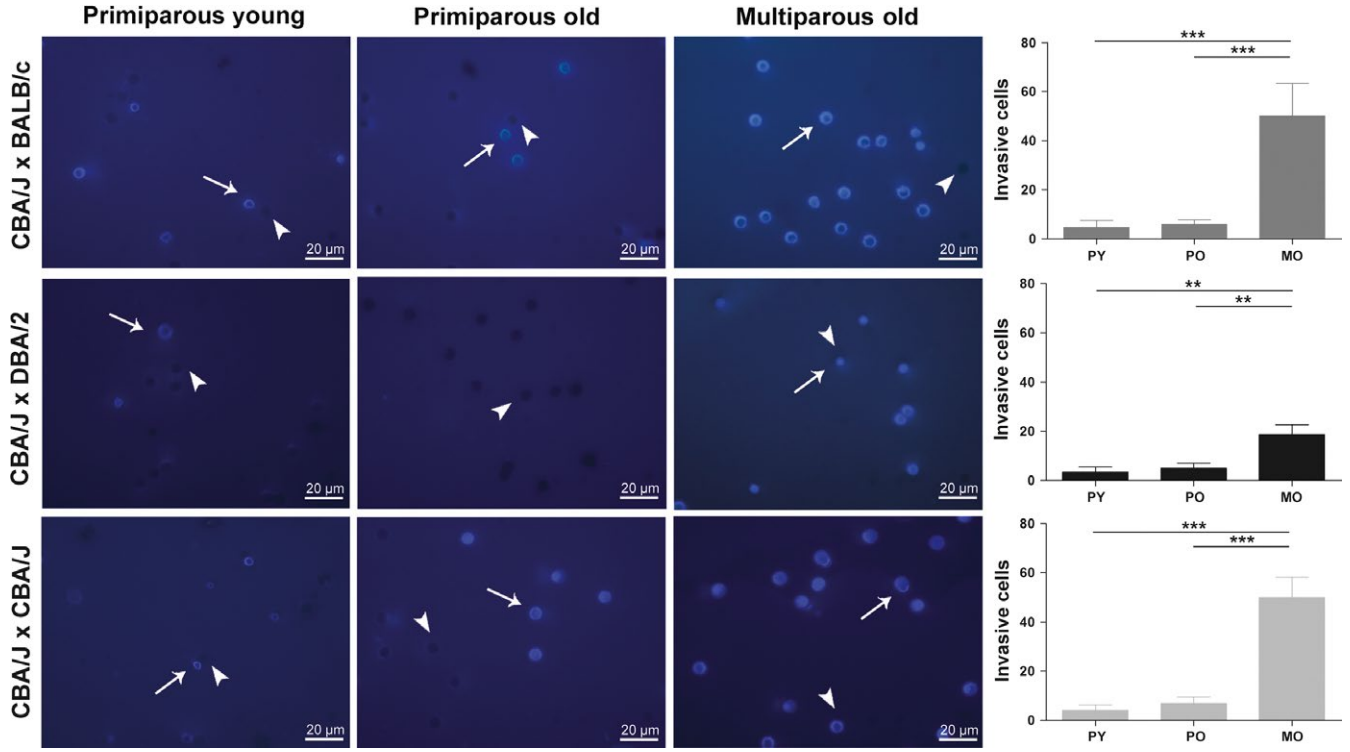


FIGURE 1 Invasive capacity of placental cells. Placentae were obtained from primiparous young (PY), primiparous old (PO), and multiparous old (MO) CBA/J females mated with BALB/c (CBA/J×BALB/c, low fetal resorption model) or DBA/2 males (CBA/J×DBA/2, abortion-prone model). Each group n=5. Trophoblast cells were assed to migrate through the Matrigel™. Representative photographs of DAPI-stained cells from each group are shown. Arrows indicate invasive cells, and arrowheads indicate membrane pores. On the right, statistical analysis is shown. Mean and standard deviation of cell counted in 10 fields at 400× were determined (Newman-Keuls test, $^{**}P<.01$; $^{***}P<.001$)

GraphPad Prism software (http://www.graphpad.com). Differences at $P<.05$ were statistically significant.

3 | RESULTS

3.1 | Placental invasive capacity is upregulated by previous gestations and can be related to gestational success

The placental invasive behavior of each mating group was compared by Matrigel migration assays. Placentae obtained from young and old primiparous females of all combinations showed a similar number of invading cells. In contrast, placentae obtained from multiparous females significantly increased the number of invasive cells with respect to young or age-matched primiparous females of the same cross-breeding. However, the invasiveness of trophoblast suspensions from CBA/J×CBA/J and CBA/J×BALB/c multiparous groups was around 8.5 times greater than their respective primiparous combinations, while in the case of the high fetal loss model, the invasiveness of multiparous placentae exceeded only almost four times greater than that of primiparous ones (Figure 1).

These results indicate that the placental invasiveness does not depend on maternal age or on the mating histocompatibility differences. Interestingly, the placental invasive capacity is upregulated by four previous gestations, mainly in low fetal loss combinations.

3.2 | Parity status, male genotype, and pregnancy success do not modify placental MMP-2 and MMP-9 expression and activity

Western blot analysis of placental homogenates revealed a band at 72 kDa compatible with MMP-2 pro-form, while the presence of MMP-9 appeared as a high molecular complex of 190 kDa (Figure 2A) which after treatment with 2-ME was reduced to 120 kDa (not shown). Gelatinase activity was detected by zymography in placental culture supernatants (Figure 2B) and tissue homogenates (not shown). In both samples, the pro-forms of both MMPs were detected in agreement with previous reports.[23] Nevertheless, the comparison of the expression and activity of both gelatinases among the studied groups did not show differences. Similar results were obtained with the syngeneic cross-breeding. These results indicate that MMP-2 and MMP-9 are not responsible for the increased placental invasiveness observed in multiparous females.

3.3 | Plasminogen expression is upregulated in multiparous females from normal and abortion-prone gestations

The expression of plasminogen was firstly analyzed by IHC in placental tissues. Results showed that plasminogen is preferentially expressed in the spongy zone in primiparous females regardless of

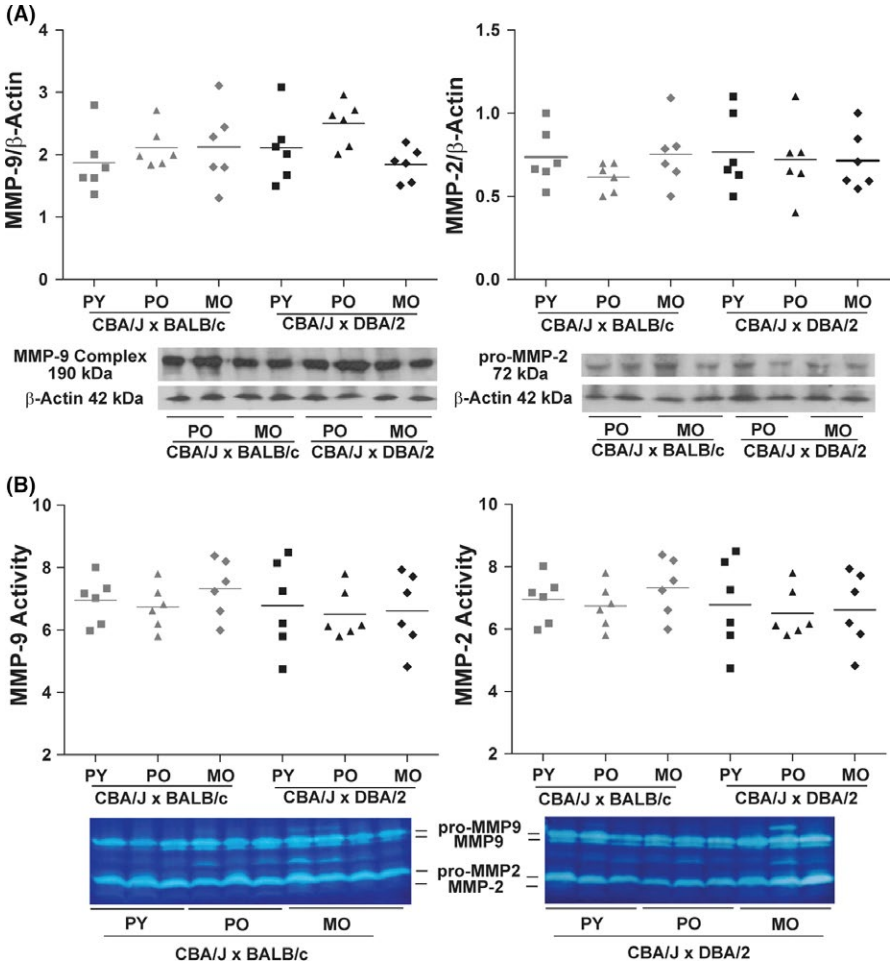


FIGURE 2 Study of MMP-9 and MMP-2 gelatinases. Placentae were obtained from primiparous young (PY), primiparous old (PO), and multiparous old (MO) CBA/J females mated with BALB/c (CBA/J×BALB/c, low fetal resorption model) or DBA/2 males (CBA/J×DBA/2, abortion-prone model). Each group n=6. (A) Statistical analysis of protein levels in placental homogenates studied by Western blot (Newman-Keuls test). Each dot represents a placenta. Below, representative blots: MMP-9 complex (190 kDa) and pro-MMP-2 (72 kDa) were detected (β-Actin: loading control). (B) Statistical analysis of gelatinolytic activities of MMP-9 and MMP-2 in placental culture supernatants analyzed by zymography. Each dot represents a placenta. Representative stained gels are shown

maternal age and the cross-breeding. Interestingly, its expression was increased in spongiotrophoblast and in the invasive trophoblast cells in placentae from multiparous females of all combinations (Figure 3A). WB studies showed a band compatible to plasminogen (92 kDa) and demonstrated that its expression in multiparous placentae from any combination was twofold higher than that detected in samples from primiparous females (PY and PO vs MO of any combination, $P<.001$, Figure 3B).

3.4 | Placentae from multiparous females from the abortion-prone model express a high proportion of the low molecular weight urokinase-type PA (LMW-uPA)

We next studied the local expression of plasminogen activators. Cells expressing tPA were observed solely in the spongy zone by IHC in all groups, while the statistical analysis of WB patterns neither showed differences (Figure 4A), indicating that tPA is not regulated by parity status, male genotype, or pregnancy success.

On the other hand, IHC studies showed that uPA/PLAU is mildly expressed in spongiotrophoblast in the primiparous groups regardless of maternal age. Instead, placentae from multiparous females of any cross-breeding increased uPA expression both in spongiotrophoblast and also in the invasive trophoblast tissue (Figure 5A). The high uPA expression observed in placentae from all multiparous groups was statistically confirmed by semiquantitative DB analyses (Figure 5B). However, when placental homogenates were analyzed by WB, samples from multiparous females of the syngeneic mating and of the allogeneic low fetal loss model showed a band at the expected molecular weight of the two-chain uPA form (54 kDa), also called high molecular weight (HMW) uPA. Placentae from multiparous females of the high fetal loss model showed the same band at 54 kDa, but also a band at 33 kDa compatible with the β chain of uPA, known as low molecular weight (LMW) uPA which was not detected in the other multiparous groups (Figure 5C). Densitometric analysis of these blots showed that LMW-uPA contributes to the half or more of the total placental uPA (data not shown).

4 | DISCUSSION

In the current study, we first compared the influence of a fourth pregnancy on the in vitro invasiveness of trophoblast suspensions from three different mouse combinations. The results demonstrated that placentae obtained from the fourth pregnancy of females belonging to the low fetal loss model (CBA/J×BALB/c), the high fetal loss one (CBA/J×DBA/2), or the CBA/J syngeneic combination showed an

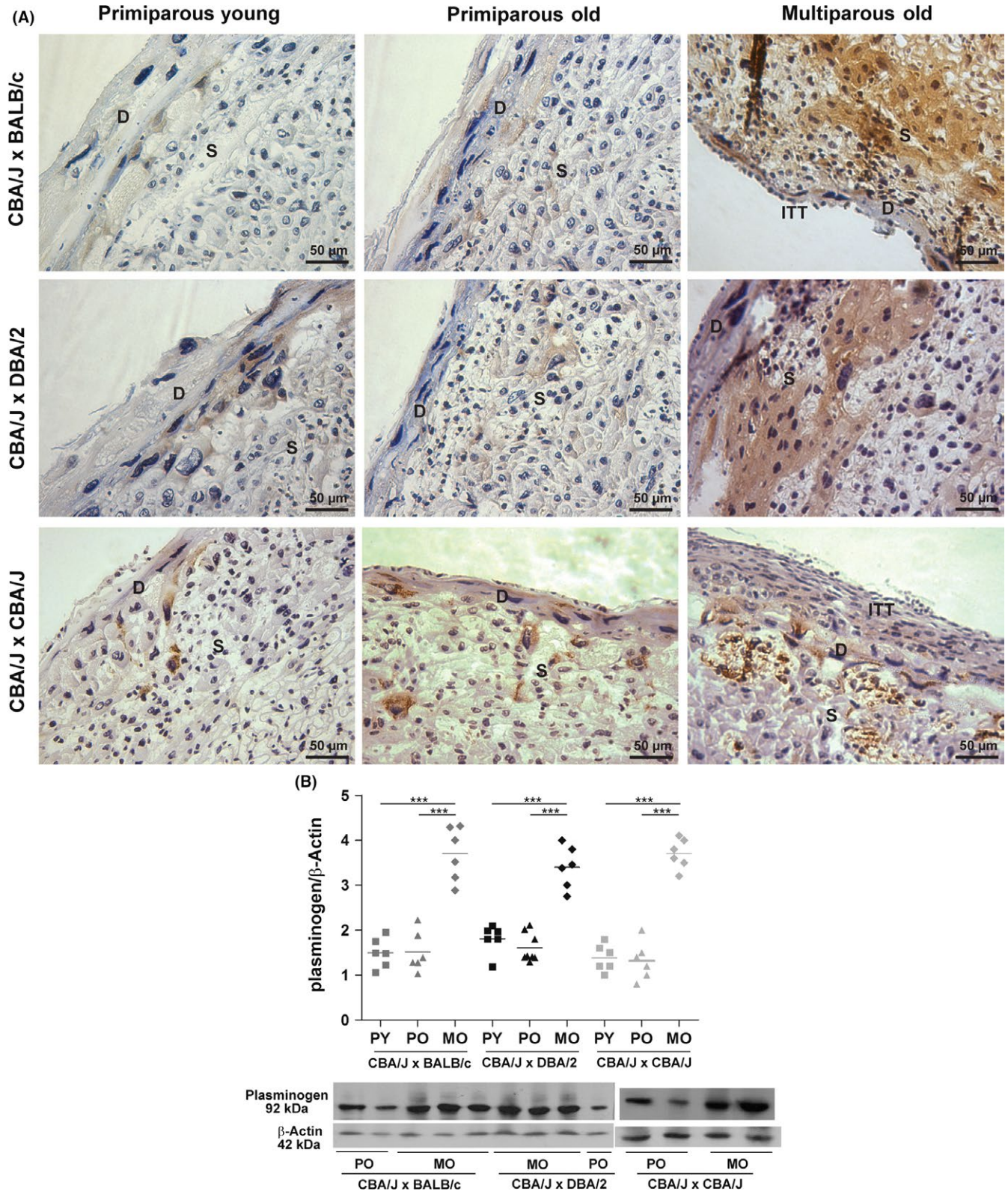


FIGURE 3 Plasminogen expression in placental tissue. Placentae were obtained from primiparous young (PY), primiparous old (PO), and multiparous old (MO) CBA/J females mated with BALB/c (CBA/J×BALB/c, low fetal resorption model), DBA/2 males (CBA/J×DBA/2, abortion-prone model), or CBA/J males (CBA/J×CBA/J); each group n=6. (A) Immunohistochemical detection. Staining of plasminogen was detected in the invasive trophoblast tissue (ITT) and spongiotrophoblast (S). Decidua (D) is also indicated. (B) Expression of plasminogen in placental homogenates by Western blot. Graph: Statistical analysis is shown (Newman-Keuls test, ***P<.001). Plasminogen expression was related to β-Actin expression. Each dot represents a placenta. Below, representative blots are shown

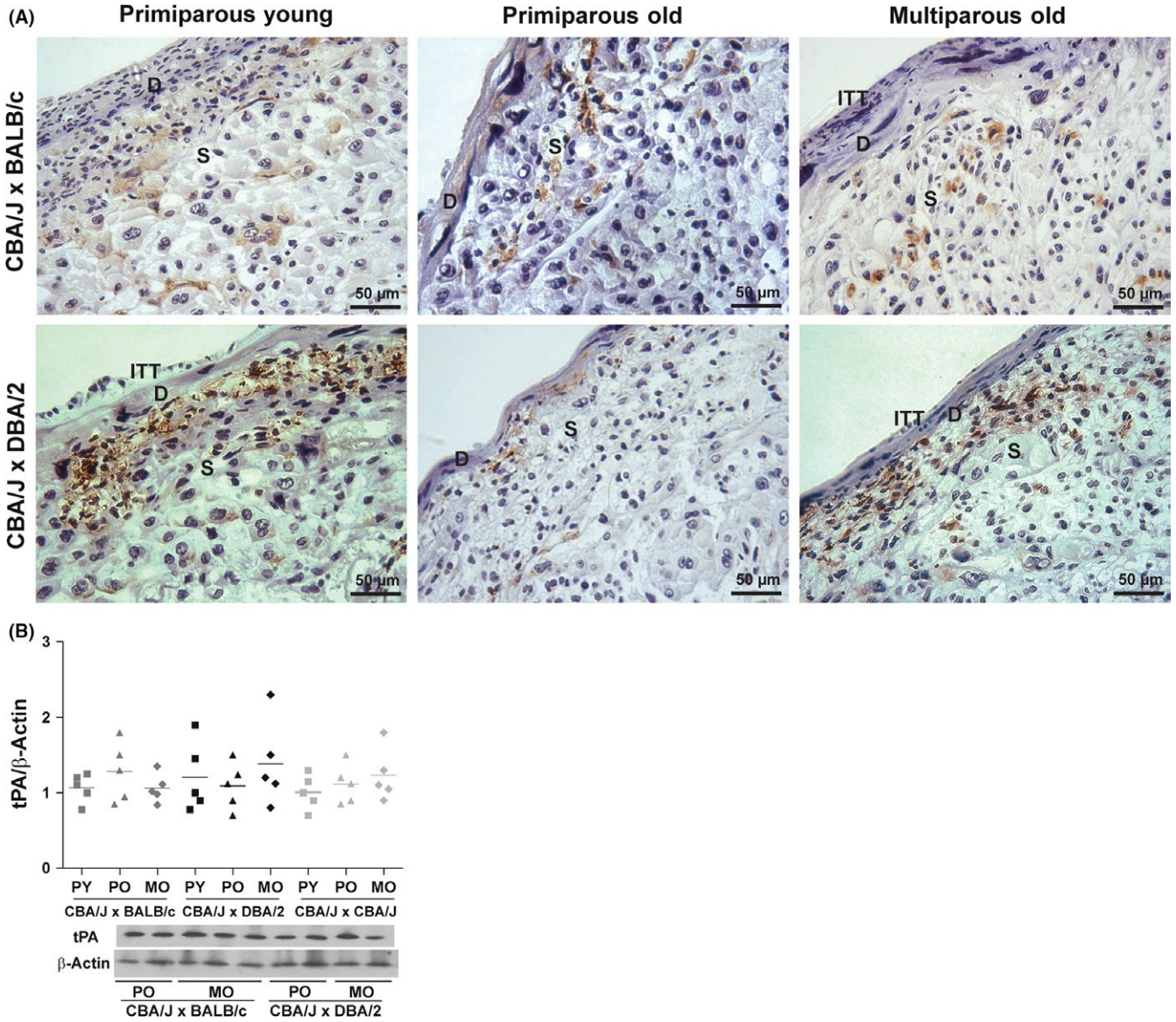
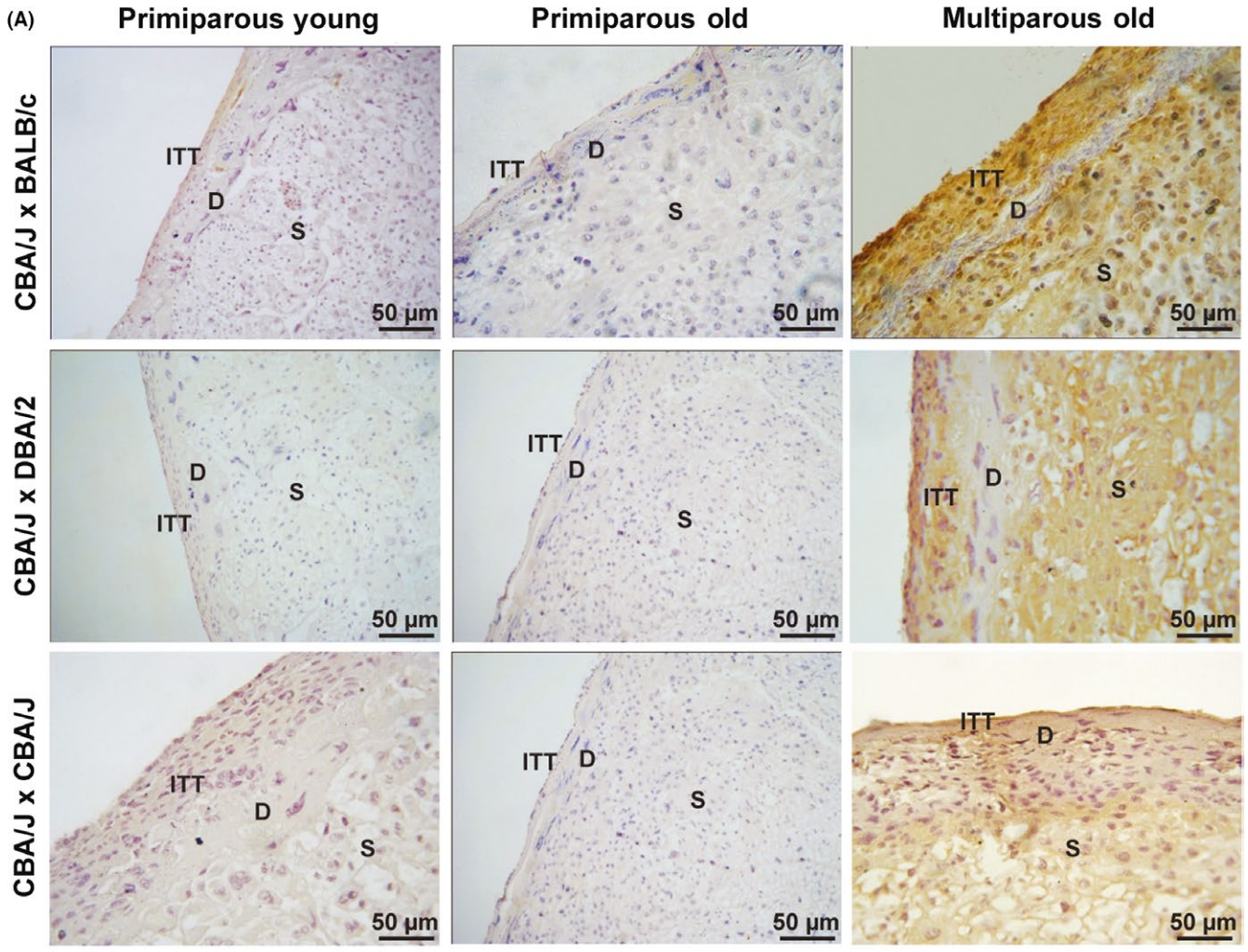
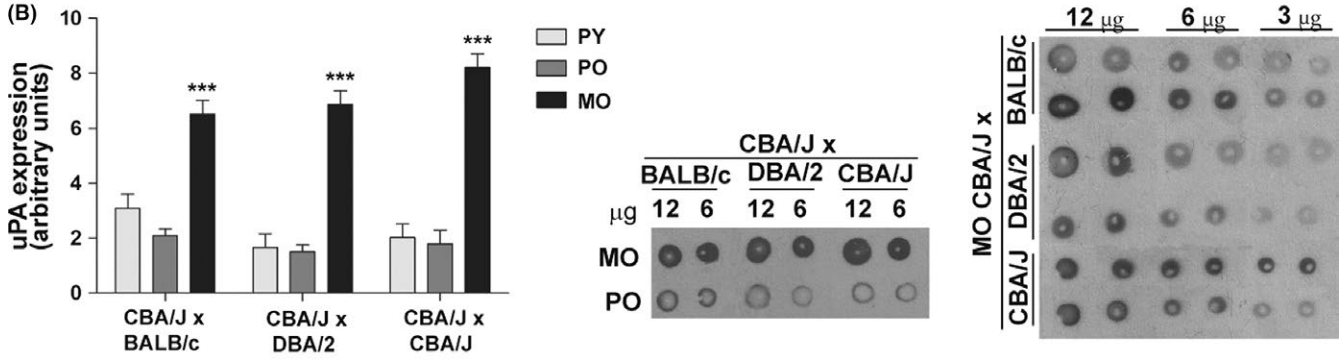


FIGURE 4 Expression of tissue-type plasminogen activator (tPA). Placentae were obtained from primiparous young (PY), primiparous old (PO), and multiparous old (MO) CBA/J females mated with BALB/c (CBA/J×BALB/c, low fetal resorption model), DBA/2 males (CBA/J×DBA/2, abortion-prone model), or CBA/J males (CBA/J×CBA/J); each group n=5. (A) Immunohistochemistry: Placentae express tPA only in the spongiotrophoblast (S). It is also indicated the invasive trophoblast tissue (ITT) and the decidua (D). (B) Protein levels of tPA were analyzed by Western blot. Graph: Statistical analysis, each dot represents a placenta. β-Actin was used as loading control. Below, representative blot is shown

increased in vitro invasiveness with respect to placentae from the first pregnancy of each combination regardless of the maternal age. These observations support histological evidences previously reported by our group.[7,8] Moreover, the present work demonstrated that the placental invasive capacity of multiparous placentae from the syngeneic combination and from the low fetal loss model was higher than that exhibited by the abortion-prone combination. These results suggest that the enhancement of placental invasiveness in multiparity could be related to pregnancy success and might explain differences in maternal thymocytes subpopulations previously reported.[18]

Second, as invasive trophoblasts express VEGF and its receptor VEGF-R1 (Flt-1), we compared the placental expression of VEGF-activated proteases such as matrix metalloproteases (MMP-2 and MMP-9), plasminogen (PA), and its activators (tissue type: tPA and urokinase type: uPA). The present data indicated that both the expression and activity of MMP-2 and MMP-9 are not influenced by any of the analyzed factors. However, the level of placental plasminogen expression was similar among the multiparous females regardless of the male genotype and pregnancy success, and higher with respect to their respective primiparous ones. For this reason, we next analyzed the expression of plasminogen activators such as tPA and uPA/PLAU. The aim was to investigate whether differences on plasminogen activation/expression could explain the enhanced invasiveness of multiparous placentae previously reported by us[7,8] and the lower invasiveness exhibited


(A)
Primiparous young
Primiparous old
Multiparous old
CBA/J x BALB/c
CBA/J x DBA/2
CBA/J x CBA/J
ITT
D
S
50 µm


(B)
uPA expression (arbitrary units)
PY
PO
MO
***
CBA/J x BALB/c
CBA/J x DBA/2
CBA/J x CBA/J
µg 12 6 12 6 12 6
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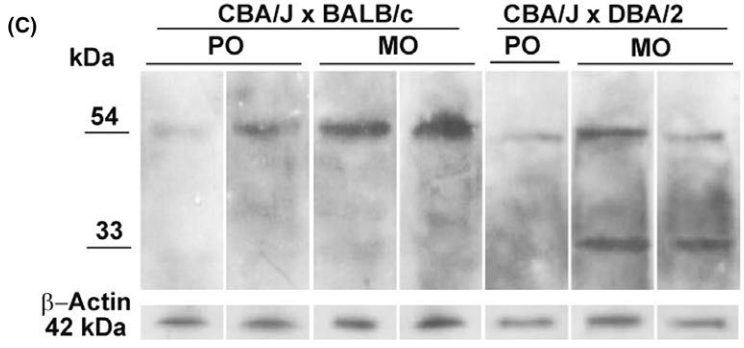
(C)
CBA/J x BALB/c
CBA/J x DBA/2
PO
MO
kDa
54
33
β–Actin
42 kDa

FIGURE 5 Study of urokinase-type plasminogen activator (uPA) expression. Placentae were obtained from primiparous young (PY), primiparous old (PO), and multiparous old (MO) CBA/J females mated with BALB/c (CBA/J×BALB/c, low fetal resorption model), DBA/2 males (CBA/J×DBA/2, abortion-prone model), or CBA/J males (CBA/J×CBA/J); n=5. (A) Immunohistochemistry: uPA was detected in spongiotrophoblast (S) and in the invasive trophoblast tissue (ITT) of multiparous placentae. Decidua: (D). (B) Protein levels of uPA were analyzed by dot blot (6 μg of placental homogenate). Statistical analysis is shown (Newman-Keuls test, ***P<.001). On the right, semiquantitative blots are shown. (C) Expression of uPA by Western blot

by multiparous placentae from the high fetal loss model with respect to the other combinations found in this work. Our findings indicated that tPA expression did not vary among the groups, and hence, it would not be involved in such effects. However, we detected an increase of uPA expression in placentae of multiparous females in all combinations. Nevertheless, in the high fetal loss model approximately 50% or more of the total protein corresponds to the LMW-uPA fragment, which lacks uPAR-binding domain but contains the protease domain.

A number of reviews deal with the diverse biological functions of uPA, which not always require its proteolytic activity.[24-26] uPA is secreted as an inactive proenzyme (pro-uPA), which binds its own receptor (uPAR/PLAUR) and is subsequently cleaved by membrane-bound plasmin (or other proteases) to produce the active two-chain uPA form (HMW-uPA), which in turn increases the conversion of plasminogen to plasmin focusing the proteolytic activity to the cell membrane. Both pro-uPA and HMW-uPA share an apparent molecular weight of 54 kDa. In addition, uPA binding to its receptor promotes cell adhesion and chemotactic activity. A further cleavage of uPA releases the carboxy-terminal region that contains the catalytic domain of the molecule, known as LMW or β chain (MW: 33 kDa). Soluble LMW-uPA is able to activate plasminogen,[24,25] even though with lower efficiency than the HMW-uPA form.[27-29]

The CBA/J×DBA/2 mating was originally described as a high fetal resorption model because of an altered antipaternal immune response.[30] Later, Dixon et al.[31] also demonstrated failures of decidual arteriolar remodeling. Nowadays, it has also been described as a preeclampsia-like model.[32] In addition, we have reported a reduced number of implantation sites[33] and litter size regardless of the parity status.[18] Therefore, the high expression of the LMW-uPA form in placentae from the high fetal loss model would explain in part their lower invasiveness and poor gestational performance. In line with our previous works, the present study design consisted of working with the first and fourth pregnancies of each combination at 18.5 days of gestation, to set up an endpoint to investigate the multiparity hypothesis. Having obtained the present results, multiple new questions arise: (i) How do plasminogen and uPA regulate their expression during mouse pregnancy? (ii) Does the trophoblast invasion increase gradually with each gestation? (iii) Why do placentae from the high fetal loss model expresses the LMW-uPA form? (iv) Is it possible to improve trophoblast invasion in the high fetal loss model reducing the proportion of the less active LMW-uPA by passively transfecting HMW-uPA? (v) In that case, does this cross-breeding increase the number of live fetuses and decrease the abortion rate? To answer these questions, a new study design should be employed. Among the causes that could explain the higher expression of the LMW-uPA form, we hypothesize that CBA/J×DBA/2 placentae present dysregulations in PAI-2 (the major inhibitor of uPA) or in uPAR/PLAUR levels, which we are currently studying.

The immunoregulation of uPA expression and its relationship with trophoblast migration are poorly known. Some works reported such a role for certain pro-inflammatory cytokines. IL-1β has shown to induce secretion of uPA, PAI-1, and PAI-2 and in turn elevate the migration of first-trimester extravillous trophoblasts.[34] Moreover, elevated expression of uPA and PAI proteins in the presence of IL-1 has also been described in many different human cell types including endometrial stromal cells[35] and was shown to be associated with increased invasion of various cancer cell types.[36,37] On the other hand, IL-6 promotes trophoblast invasion,[1] and its expression was also showed to be related to uPA.[38,39]

The importance of an adequate trophoblast invasion for a healthy human gestation is widely accepted. In line with our present observations, human studies have shown that first-trimester endovascular trophoblast invasion is more extensive in parous women than in nulliparous ones.[40] Prior et al.[41] reported in an uncomplicated, low-risk cohort, that parous women had significantly improved fetal hemodynamic parameters compared with nulliparous women. Of note, primigravidity is considered a risk factor for preeclampsia and intrauterine growth restriction.[42]

5 | CONCLUSIONS

To sum up, repetitive mouse gestations in successful mating combinations would promote long-term changes in trophoblast invasion by means of VEGF, plasminogen, and HMW-uPA that would bring a beneficial effect to future gestations. This mechanism would be altered in the high fetal loss model.

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