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Study on the pathogenesis of autoimmune-type recurrent spontaneous abortion by establishing a new mouse model

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ABSTRACT

Objective: To establish a new mouse model for autoimmune-type recurrent spontaneous abortion (AI-RSA) and demonstrate the potential role of intrauterine immunization with β2GP-1-like antigen in AI-RSA, we performed an intrauterine injection of human β2GP-1 in BALB/c mice and unrelated protein, adjuvants, and normal saline (NS) as controls. The mean number of embryos implanted (MNEI), embryo loss rate (ELR), mean embryo bulk (MEB), and mean placental weight (MPW) were analyzed. Compared with the control mice, BALB/c mice injected with human β2GP-1 showed increased anti-β2GP-1 and MPW. Moreover, BALB/c mice immunized with human β2GP-1 exhibited hypercoagulability and vascular thrombus formation in the placenta. Electron microscopy confirmed the existence of platelet aggregation, mitochondrial swelling, and endothelial cell necrosis in the placentas of BALB/c mice immunized with human β2GP-1. These finding indicated that intrauterine immunization with β2GP-1 successfully induced AI-RSA in mice. Increased anti-β2GP-1 antibody could independently induce hypercoagulability, vascular endothelial injury, and vascular thrombus formation in the placenta, which led to AI-RSA.

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Introduction

Anti-phospholipid antibody syndrome (APS) has been established as one of the most frequent causes of autoimmune-type recurrent spontaneous abortion (AI-RSA). APS is characterized by vascular thrombosis and/or pregnancy morbidity, in association with anti-cardiolipin antibodies (ACAs) and/or lupus anti-coagulant (LA) and/or anti-β2GP-1 antibodies [1].

Anti-β2GP-1 antibody is a biomarker for the diagnosis of APS. More than 10 studies have confirmed that an increased IgG anti-β2GP-1 antibody level is an independent risk factor for venous thrombosis and fetal loss [2–4]. "Intra-placental thrombosis" has traditionally been presumed to play a role in the fetal loss in APS patients [5], but no direct evidence has been reported. To clarify the pathogenic role of anti-β2GP-1 antibodies in both thrombosis and fetal loss, several animal models have been established for the study of APS since the 1990s [1]. In a previous study, mouse footpad immunization with human β2GP-1 resulted in elevated antibodies against negatively charged phospholipids, fetal loss, prolonged activated partial thromboplastin time (APTT), and thrombocytopenia, without evidence for thrombus formation [2]. Recently, Arad et al. found that anti-β2GP-1 antibodies from patients with APS induced arterial thrombus formation in a mouse model [3]. However, no available animal model reproduces the full range of clinical manifestations observed in the human syndrome. Most studies have shown either fetal loss or thrombosis, not both.

Herein, we immunized BALB/c mice by intrauterine injection with human β2GP-1 and included unrelated protein, adjuvants, and normal saline (NS) as controls. BALB/c mice injected with β2GP-1 developed high concentrations of anti-β2GP-1 antibodies. Moreover, they also showed both fetal loss and thrombus formation in the placenta. This study demonstrates that anti-β2GP-1 antibodies independently induce fetal resorption and thrombus formation in the placenta, which may be the primary mechanism of recurrent abortion.

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Mice

Six- to 8-week-old female BALB/c mice were purchased from the Laboratory Animal Center of Shanghai, The Chinese Academy of Science. Genetic monitoring confirmed that the mice were up to the international standard of quality. The study was approved by the Institutional Review Board of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University.

Intrauterine injection method

A self-designed injection syringe was used for intra-uterine injection. The TIP (Axygen, USA) was heated for 3–5 s using a Spirit Lamp, and the melted tips were then elongated to 3 cm and a diameter of 1 mm (Fig. 1A). We termed it an intrauterine injection syringe when it was attached to a 1 ml injection syringe. Using this intrauterine injection syringe, the antigen and the control formulations were injected into the uterine cavity through the vagina and cervix (Fig. 1B). Dye injection was first used to test this injection method. We observed that the uterus and not the peritoneal tissue filled with dye when the needle was manipulated gently (Fig. 1C).

Immunization

Fifteen BALB/c female mice were immunized in the uterine cavity with 10 μg of human β2GP-1 (Cell Sciences, USA) mixed with CFA (Sigma, USA). One week later, the mice were immunized again with 10 μg of human β2GP-1 in IFA (Sigma, USA). The unrelated protein and adjuvants were injected into the uterine cavity as controls (6–15 mice/group). Mice immunized with β2GP-1 were observed in four replicate groups. Ten days after active immunization, BALB/c female mice were paired overnight with males. Mating was evidenced by the appearance of a vaginal plug the following morning.

Evaluation of pregnancy outcome

All plugged females were sacrificed by cervical dislocation on days 12–14, the uteri were removed, and the total number of implantations and resorption sites were recorded, as described by Bertoja et al. [4]. The MNEI was calculated as the number of total fetuses divided by the total number of mice. The ELR was calculated as the number of resorbed fetuses divided by the number of resorbed and full-term fetuses. The MPW (g) was calculated as the total placenta weight divided by the number of full-term fetuses. The MEB was calculated using the following formula: MEB (mm^3) = 0.5 × length-diameter × short $diameter^2$.

ELISA

Blood samples were collected on day 14, and the serum levels of ACA and anti-β2GP-1 antibodies were measured by ELISA. The ACA ELISA (EUROIMMUN, Germany) was performed according to the manufacturer's protocol, The intra- and inter-assay coefficients of variation are respectively 7.5% and 10.5%. The minimum detectable amount of the assayed substance is 2 U/ml. Anti-β2GP-1 activity was detected by a solid-phase ELISA similar to a previous report [4]. Plates were coated with purified β2GP-1 (5 μg/ml) in PBS overnight. The coated plates were then blocked with 2% BSA and incubated with serial dilutions of test serum. After washing, the plates were incubated with a diluted goat anti-mouse IgG HRP conjugate (1/5000) and developed with 1 mg/ml p-nitrophenyl-phosphate in diethanolamine buffer for 15–20 min. The intra- and inter-assay coefficients of variation are respectively 8.0% and 11.3%, the minimum detectable amount of the assayed substance is 2 U/ml.

Blood cell counts and coagulation studies

Platelets from mouse blood samples were counted using the Sysmex XT-1800i Automated Hematology Analyzer. Anticoagulant activity was evaluated by the APTT, prothrombin time (PT), and fibrinogen (FIB) level obtained using the Sysmex CA-7000 Automated Blood Coagulation Analyzer.

Placental pathology

Pathological changes in the placenta were observed by H&E staining and electron microscopy (PHILIP CM-120). The presence of thrombus was evaluated independently by two pathologists (Dr. Yao and Dr. Yan).

Statistical analysis

Data are expressed as the mean ± SD. ANOVA followed by post hoc test was carried out for the statistical analysis. The p values < 0.05 were considered statistically significant.

Characterization of serological features of BALB/c mice following intrauterine injection

Six- to eight-week-old female BALB/c mice were immunized with β2GP-1 via intrauterine injection. Fourteen days after the injection, we examined the concentrations of anti-β2GP-1 antibody and ACAs in the serum from mice in the CFA/β2GP-1 group and the control group. The concentration of anti-β2GP-1 antibodies in the CFA/β2GP-1 group was significantly higher compared to the control group ($p < 0.05$), whereas there were no significant differences in the ACA concentrations among all groups ($p > 0.05$) (Fig. 1D). These results indicated that intrauterine injection successfully induced a high anti-β2GP-1 antibody concentration in BALB/c mice.

Intrauterine injection with human β2GP-1 leads to a high rate of abortion

To identify the effect of increased anti-β2GP-1 antibody on embryo resorption, all pregnant mice were sacrificed 12–14 days after plug formation, and the embryos and the placenta were further analyzed. We found that the rate of resorption (23.36%) in the CFA/β2GP-1 group was significantly higher compared to the control groups (4.23–10.19%). Moreover, BALB/c mice immunized with 10 μg of β2GP-1 developed a significantly lighter and smaller placenta and embryos compared to the CFA/BSA, CFA, and NS groups ($p < 0.05$) (Fig. 1E). Representative resorbed fetuses are shown in Fig. 1E. No significant difference in the MNEI was observed among all groups (Table 1). These results suggested that the intrauterine injection of β2GP-1 induced fetal resorption and had no obvious effect on fertility or embryo implantation. Interestingly, a Pearson correlation analysis demonstrated no significant correlation between the anti-β2GP-1 antibody concentration and the rate of embryo loss in the CFA/β2GP-1 group ($r = 0.369$, $p = 0.176$).

Coagulation state

Previous reports on the effect of anti-β2GP-1 antibodies on PT, FIB, and platelet count were controversial [2,3]. In our intrauterine injection mouse model, we found that pregnant mice injected with β2GP-1 had a higher level of FIB ($p < 0.05$) (Table 2). However, the PT, APTT and platelet count did not significantly differ between β2GP-1-immunized mice and the other groups (Table 2).

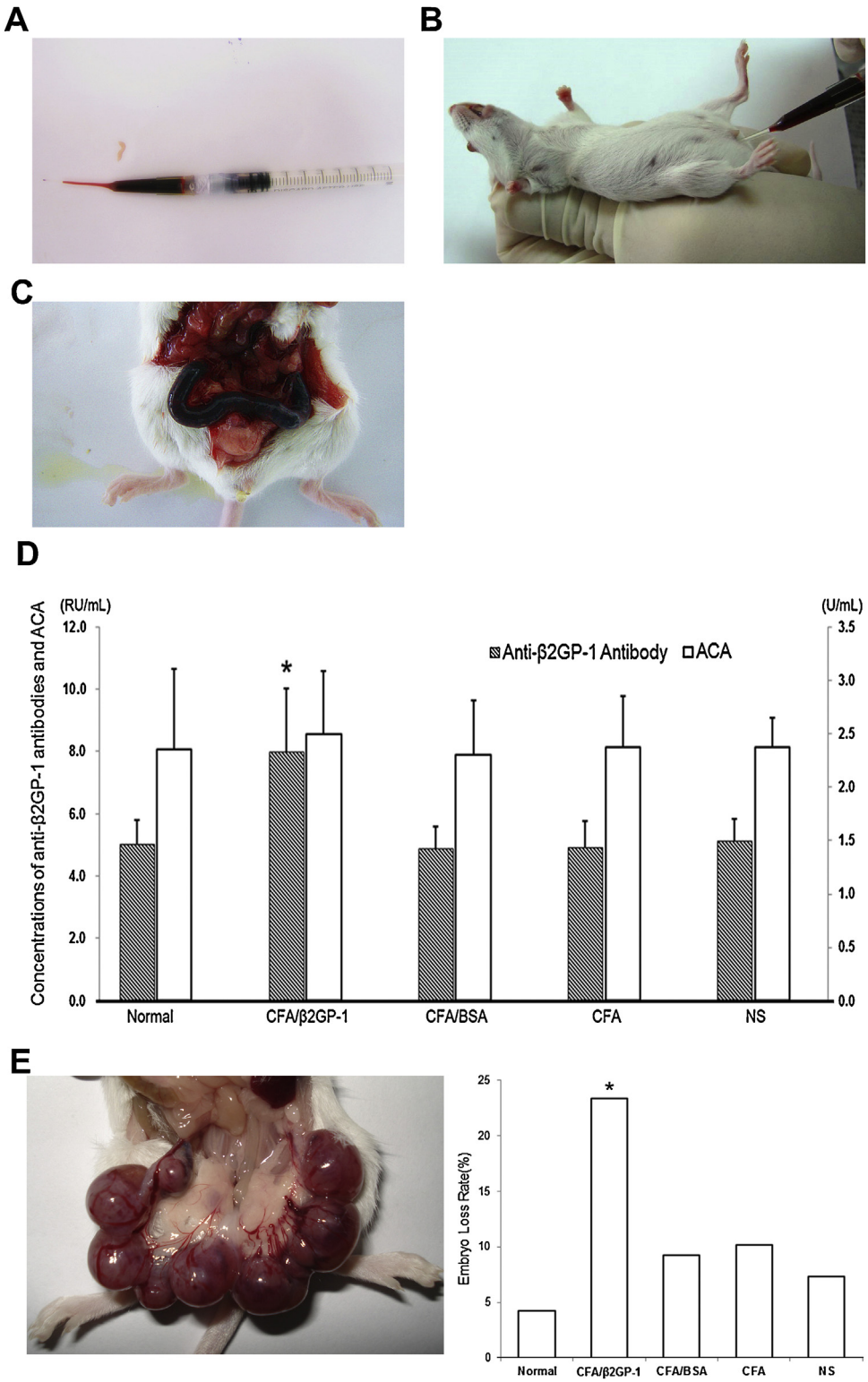

Fig. 1. Establishment of the recurrent abortion model. (A) Self-designed plastic injector; (B) intrauterine injection of a pregnant mouse; (C) dye staining of uterus following dye injection. The uterus was full of dye, but the peritoneal tissue was not stained; (D) concentration of anti-β2GP-1 antibodies and ACAs in sera obtained from BALB/c female mice. Compared with the control groups, the concentration of anti-β2GP-1 antibodies in the CFA/β2GP-1 group was significantly higher; and (E) typical uterus from a mouse immunized with β2GP-1(right). The arrow indicates resorption. The bar shows the percentage of fetal loss in the different groups (left). The *p* value was obtained from ANOVA followed by post hoc test. $^{*}p < 0.05$.

Thrombus formation in the placenta of BALB/c mice following the intrauterine injection of β2GP-1

Previous studies have shown that anti-β2GP-1 antibody can induce thrombus formation. To determine whether immunization with β2GP-1 can induce thrombus formation in the placenta and disrupt the blood supply to the fetus, we analyzed the histological characteristics of placentas from BALB/c mice following the intrauterine injection of β2GP-1 or control preparations. H&E staining showed vascular thrombosis in the placentas of almost all

Table 1
Effects of active immunization in the uterine cavity on the outcome of pregnancy.

	N	MNEI	MPW (g)	MEB (mm^3)
Normal	15	9.5 ± 1.4	0.096 ± 0.005	0.174 ± 0.026
CFA/β2GP-1	15	9.5 ± 2.8	0.089 ± 0.008*	0.160 ± 0.039*
CFA/BSA	6	9.0 ± 1.4	0.099 ± 0.007	0.173 ± 0.029
CFA	13	8.3 ± 1.4	0.095 ± 0.004	0.175 ± 0.047
NS	10	9.6 ± 1.6	0.097 ± 0.005	0.171 ± 0.041

* $p < 0.05$ compared with other groups. The *p*-values were obtained by ANOVA and SNK-q test.

Table 2
Platelet count, PT, and FIB in all groups.

	Platelet count. no. (ml)	PT (s)	APTT (s)	FIB (g/l)
Normal	392.13 ± 28.28	18.09 ± 4.83	52.51 ± 16.16	1.20 ± 0.16
CFA/β2GP-1	382.73 ± 16.82	14.79 ± 2.58	49.95 ± 16.14	1.45 ± 0.16*
CFA/BSA	397.40 ± 22.07	17.83 ± 3.86	56.56 ± 14.64	1.23 ± 0.19
CFA	392.67 ± 26.72	17.88 ± 3.23	53.50 ± 7.12	1.28 ± 0.21
NS	398.10 ± 32.15	17.79 ± 3.54	56.15 ± 9.91	1.23 ± 0.15

* $p < 0.05$ compared with the other groups. The *p*-values were obtained by ANOVA and SNK-q test.

mice injected with β2GP-1 (Fig. 2A), whereas few thrombi were observed in the placentas of mice injected with unrelated protein, adjuvants and NS (Fig. 2B). Fisher's exact test demonstrated a significant difference in thrombus formation among β2GP-1-immunized mice and the control groups ($p < 0.05$, Fig. 2C). A previous study showed that anti-lipid antibody could disrupt the trophoblast and induce thrombosis in the placenta.

We used electron microscopy to explore the detailed pathological changes in the placenta induced by increased anti-β2GP-1 antibodies. Electron microscopy confirmed the existence of platelet aggregation, mitochondrial swelling, and endothelial cell necrosis in the placentas of mice injected with β2GP-1 (Fig. 2D).

APS is an autoimmune disorder caused by antiphospholipid antibodies, and 15% of APS patients exhibit RSA [6]. The

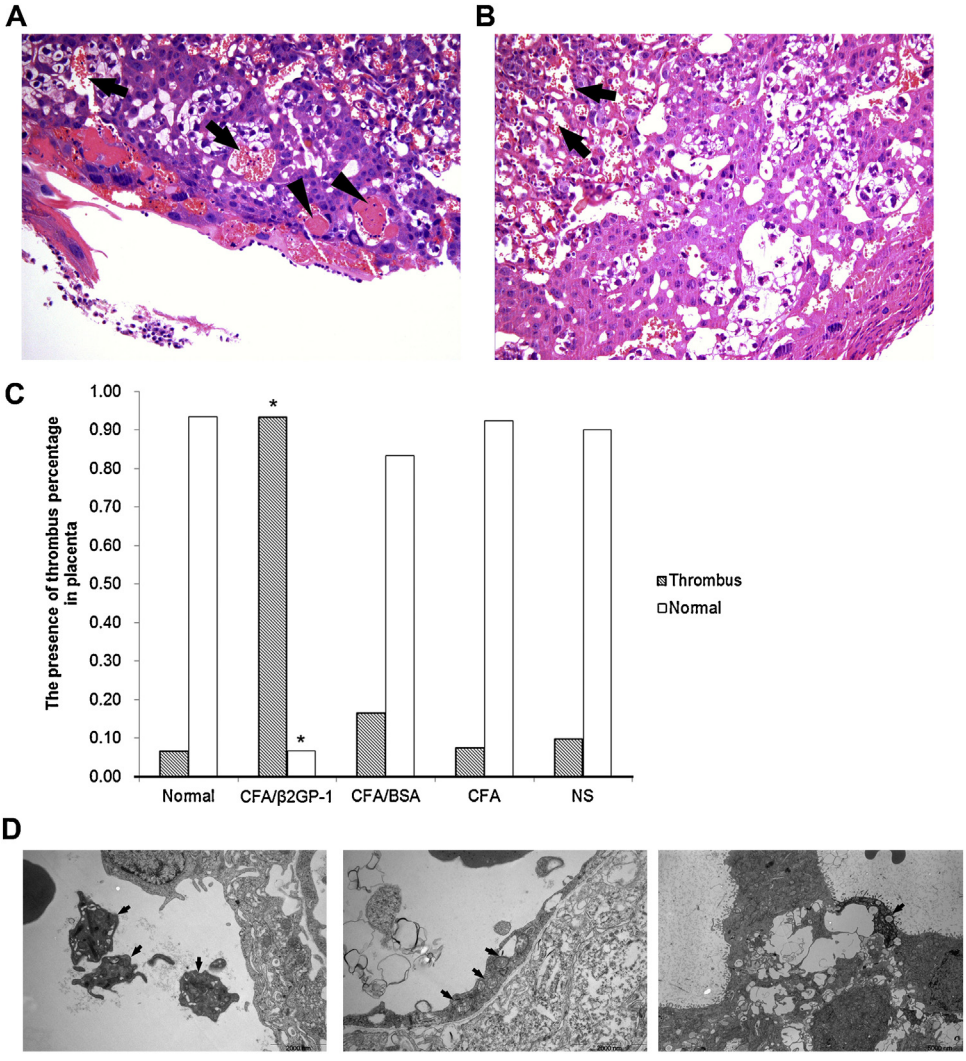

Fig. 2. Pathological analysis of placental tissue (H&E). (A) H&E staining of placental tissue from the AI-RSA mouse model, light microscopy (200×). Normal blood vessels filled with RBCs are indicated with arrows. No inflammatory cell invasion was observed. (A) Red thrombus is indicated by the arrowhead, with inflammatory cell infiltration; (B) H&E staining of placental tissue from control groups, light microscopy (200×). Normal blood vessels filled with RBCs are indicated with arrows. No inflammatory cell invasion was observed; (C) the percentage of placentas with thrombus formation. The percentage of mice with a placental vascular thrombus in the AI-RSA mouse model was 93.3% ($n = 15$), and the percentages of mice with a placental vascular thrombus in the normal, CFA/BSA, CFA, and NS groups were 6.7% ($n = 15$), 16.7% ($n = 6$), 7.7% ($n = 13$) and 10.0% ($n = 10$), respectively. The *p* value was obtained from ANOVA followed by an appropriated post hoc test. *$p < 0.05$; (D) the ultrastructure of placental tissue from the murine AI-RSA model was analyzed by electron microscopy. Right: platelet aggregation is indicated with arrows (13,500×). Middle: mitochondrial swelling is indicated with arrows (17,500×). Left: endothelial cell necrosis is indicated with an arrow (4200×).

mechanism of fetal loss in women with APS is still unknown, although it is hypothesized that placental thrombosis causes infarction and eventual fetal death. However, these proposed roles in APS are only based on observations of extensive placental infarction and thrombosis in failed pregnancies in women with APS [7–9]. Further investigations are needed to better understand the mechanisms leading the production of these antibodies and how antiphospholipid antibodies induce RSA.

Reproductive tract infection is a major cause for the risk of abortion [10–13]. Intrauterine infection provides the chance that the immune system contacts with the mimicry antigen. It is supposed reproductive tract infection with some bacteria or viruses with β2GP-1 mimicry molecules might induce APS [14,15]. In this study, we established an intrauterine injection method to induce high anti-β2GP-1 antibody levels in mice to explore the role of anti-β2GP-1 antibodies in RSA. Our ELISA results showed that anti-β2GP-1 antibody was highly induced in mice following the intrauterine injection of β2GP-1. However, no significant difference was found in the anti-ACA antibody concentration between mice that received an intrauterine injection of β2GP-1 and the control groups. These results indicated that the intrauterine injection of β2GP-1 could successfully and specifically induce a high anti-β2GP-1 antibody level in mice.

Interestingly, significantly higher ELR and lower MEB and MPW were found in mice injected with β2GP-1. Because anti-ACA antibody was not increased in this mouse model, these results also strongly indicate that anti-β2GP-1 antibody is an independent risk factor for RSA [16]. No significant difference in the MNEI was observed among the groups in our study, suggesting that the administration of human β2GP-1 to the mouse uterus does not affect fertility or embryo implantation in mice.

Autoantibodies are thought to affect coagulation, resulting in decidua vascular lesions, placental thrombosis, and infarction, which are harmful to placental function and cause embryo necrosis. As a result, they may be the primary pathophysiological mechanism behind AI-RSA [17,18]. Blood hypercoagulabity is commonly observed in patients with APS and manifests as plasma viscosity, blood viscosity, platelet aggregation, and increased platelet granule protein expression. The administration of aspirin and heparin as an anticoagulant therapy is often efficacious in APS patients [19,20]. Consistent with these characteristics of APS in humans, we found that the FIB level was significantly increased in mice immunized with β2GP-1 compared to the control groups. Moreover, vascular thrombosis was found by H&E staining in the placentas of mice immunized with β2GP-1. Electron microscopy confirmed the existence of platelet aggregation, mitochondrial swelling, and endothelial cell necrosis. It should be noticed that inflammation and complement activation might also have a crucial role based on in vivo studies using different mouse models of APS [21–24]. Further studied on this mouse model should be performed in the future.

In summary, our mouse model of the intrauterine injection of β2GP-1 induced anti-β2GP-1 antibody production and RSA. This induction demonstrated the pathogenic potential of anti-β2GP-1 antibodies and provided direct evidence that anti-β2GP-1 antibodies were able to induce blood hypercoagulability, placental vascular endothelial injury, vascular thrombosis, and cause RSA.

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