

Proline-PRODH metabolism promotes placental angiogenesis via the mtROS-STAT3-bFGF/VEGF-A axis

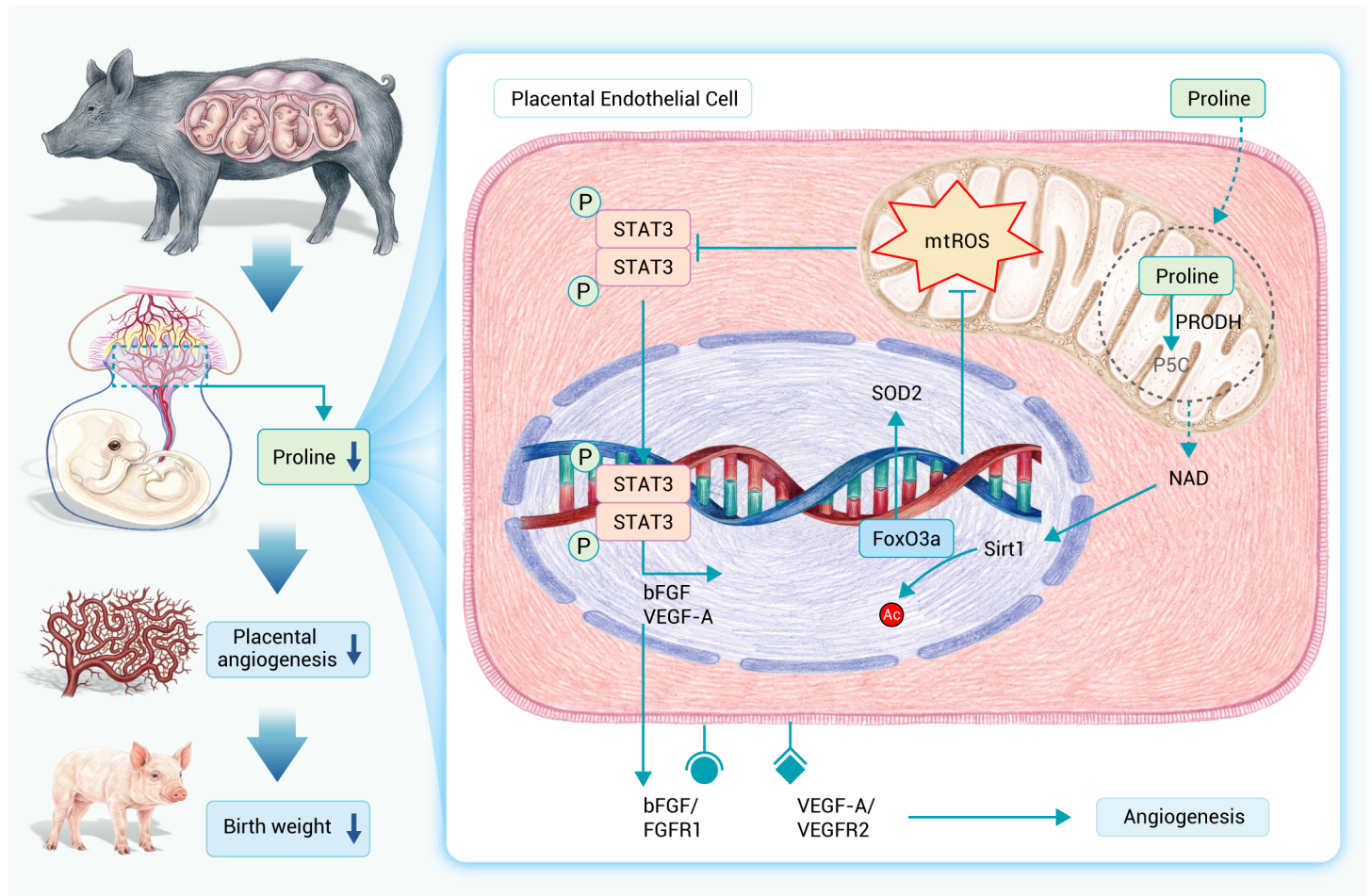
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Dietary proline (Pro) deficiency damages placental angiogenesis and raises low birth weight risk.
- Proline regulates placental angiogenesis via mitochondrial reactive oxygen species (mtROS).
- mtROS regulates the activation of signal transducer and activator of transcription 3 and angiogenic factors.
- Pro dehydrogenase metabolism maintains mtROS homeostasis via sirtuin 1/superoxide dismutase 2 pathways.

Proline-PRODH metabolism promotes placental angiogenesis via the mtROS-STAT3-bFGF/VEGF-A axis

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Functional amino acids have been shown to regulate fetal and placental development. However, little is known about the role and mechanism of proline (Pro) in placental angiogenesis. Here, we analyzed amino acid profiles and vascular development in pig placentae at different gestational time points, and found that decreased placental Pro availability was associated with impaired angiogenesis. In a nutrient-restricted mouse model, dietary Pro deficiency reduced placental proline availability, further increased the incidence of low birth weight (LBW), and exacerbated placental vascular defects. Mechanistically, Pro regulates mitochondrial reactive oxygen species (mtROS) homeostasis and subsequently promotes placental angiogenesis through the mediation of phospho-signal transducer and activator of transcription 3 (p-STAT3^{Tyr705})-basic fibroblast growth factor (bFGF)/vascular endothelial growth factor A (VEGF-A) pathway. Moreover, Pro-proline dehydrogenase (PRODH) metabolism maintains mtROS homeostasis through glutathione-independent and Sirtuin 1/superoxide dismutase 2-dependent pathways. Collectively, these results demonstrate that dietary Pro plays a crucial role in placental angiogenesis and identify the Pro-PRODH-mtROS-STAT3-bFGF/VEGF-A axis as a potential target for the prevention of LBW.

INTRODUCTION

In pig production, approximately 15% to 25% of piglets suffer from low birth weight (LBW, defined as a birth weight being lower than the 10th percentile of gestational age).¹ LBW piglets exhibit higher morbidity and mortality before weaning and slower post-weaning growth. Placental vascular development is essential for maintaining adequate blood flow, ensuring the supply of nutrients and oxygen necessary for fetal growth.² Defects in placental vascularization often reduced fetal weight accumulation and even intrauterine growth restriction (IUGR).³ Therefore, understanding the mechanisms regulating placental vascular development may provide insights into preventing or treating LBW.

Fetal and placental development requires functional amino acids. Supplementation with arginine (Arg) or citrulline, for instance, is particularly suitable in resource-constrained settings and has been shown to enhance placental vascular development and improve birth outcomes.² Interestingly, reductions in placental and fetal growth, whether due to natural variation or malnutrition-induced growth retardation, are associated with decreased placental proline (Pro) availability and Pro dehydrogenase (PRODH) activity.⁴ This highlights Pro as a key functional amino acid whose role in placental vascular development warrants particular attention.

Recent studies have delineated four key metabolic fates of Pro in the placenta: (i) mitochondrial PRODH-mediated oxidation to Pro-pyrroline-5-carboxylate (P5C) feeding into the tricarboxylic acid (TCA) cycle for energy production; (ii) conversion to polyamines via Ornithine for placental angiogenesis; (iii) incorporation into L-Prolyl-tRNA^{Pro} for collagen and extracellular matrix formation ensuring structural stability; (iv) and hydroxylation to 4-hydroxyproline for post-translational modifications and cellular redox homeostasis.⁵⁻⁹ However, current understanding of Pro function in the placenta remains fragmented. In particular, the integrated mechanisms by which Pro

coordinates mitochondrial metabolism, redox signaling, transcriptional regulation, and angiogenic pathways during placental development remain largely unclear. Given the high incidence of LBW and its close association with impaired placental vascularization, as well as the emerging link between Pro metabolism and placental function,^{10,11} Therefore, this study aimed to characterize alterations in amino acid profiles associated with LBW, examine the relationship between Pro availability and placental vascular development, and elucidate the underlying mechanisms by which Pro metabolism regulates angiogenic signaling in the placenta.

MATERIALS AND METHODS

Determination of sample sizes in the sow study and mouse study across treatment groups and all outcome variables (Figure S1).

Placental samples of sows

For non-full-term sows (primiparity), placentae and amniotic fluid were obtained from 8 sows with different gestational days after anesthesia and dissection [day 33 of gestation (G33d), 3 sows; G50d, 2 sows; G90d, 2 sows].

For full-term sows (primiparity), 132 full-term placentae were obtained from 132 sows after delivery and one placenta was collected from each sow. When the sows farrowed, umbilical cords were tied with a short silk line and labeled with a numbered tag to match individual piglets with their placentae.¹² About 10 g of placental tissue was separated from each placenta at 3-4 cm from the insertion point of the umbilical cord, and the separated tissues were immediately frozen in liquid nitrogen or preserved in formalin. In this study, the mean birth weight of the 1,200 piglets was 1.4 ± 0.2 kg (mean ± standard error). Piglets weighing less than 2 standard deviations from the mean birth weight were defined as LBW (1.6-0.4) piglets. Sows were categorized into 2 classes based on the neonatal weight per litter: LBW (1.00 < average newborn weight < 1.2 kg) and NBW sows (average newborn weight > 1.4 kg). For a detailed classification of NBW and LBW piglet weights, see Figure 1 and Table S1.

Mouse models

Seven-week-old C57BL/6J male and female mice were acclimatized for a week, followed by mating them overnight. All mice were housed in a temperature/humidity-controlled environment (23 ± 3°C/70 ± 10%) on a 12-hour light/dark cycle with free available water and a standard AIN-93G diet unless otherwise specified. The C57BL/6J mice used in this study were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All animal experiments were performed according to procedures reviewed and approved by the Animal Care and Use Committee of South China Agricultural University. All dietary formulations used in this paper met the criteria of AIN93G unless specified. Mice were euthanized by carbon dioxide asphyxiation (CO₂) inhalation and cervical dislocation was performed as a secondary euthanasia procedure.

LBW fetus model was established as previously described, and the experimental procedure is shown in Figure S2A. Briefly, 8-week-old C57BL/6J male and female mice were mated overnight, and pregnancy was assigned the designation embryonic day 0.5 (E0.5) based on observation of a vaginal plug

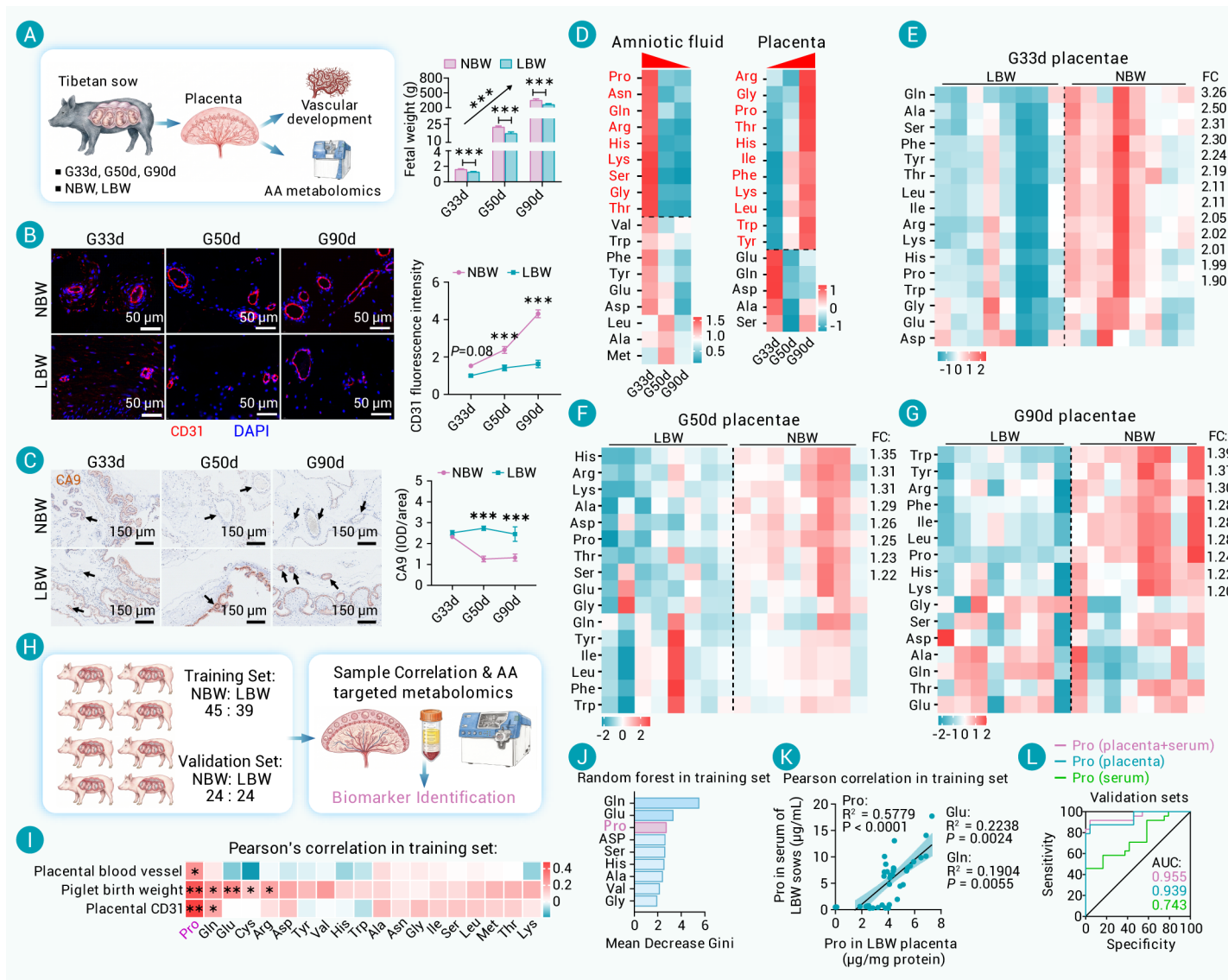


Figure 1. Placental poor vascularization in sows is associated with insufficient placental Pro abundance (A) Schematic diagram of the collection and analysis of placental samples from sows at different gestation stages (n = 8 fetuses from 2-3 litters per group). (B) Representative immunofluorescence staining images of placental CD31 (an endothelial cell marker in blood vessels) in NBW and LBW fetuses of sows on G33d, G50d, and G90d (n = 6 placenta from 2-3 litters per group). Scale bars, 50 μ m. (C) Representative immunohistochemistry staining images of placental CA9 in NBW and LBW fetuses of sows on G33d, G50d, and G90d (n = 6 placenta from 2-3 litters per group). IOD, integrated optical density. The blood vessels are indicated using black arrows. Scale bars, 150 μ m. (D) Heat map analysis of amino acid content changes in amniotic fluid (left) and placenta (right) of NBW fetuses (n = 4). (E-G) Heat map analysis of placental amino acids between NBW and LBW placenta of sows on G33d, G50d, and G90d (n = 8 placenta from 2-3 litters per group). Values represent FC. (H) Targeted metabolomics analysis of amino acid abundance in serum and placenta from full-term pregnant sows in training (NBW: LBW = 45: 39) and validation sets (NBW: LBW = 24: 24). (I) Pearson correlation coefficient analysis between the levels of amino acid in placenta and neonatal weight, placental vascular density, and placental CD31 in training set. These parameters are based on the data from Table S1, with red color for a positive correlation and blue color for a negative correlation. The color intensity is proportional to the strength of Pearson correlation. (J) Identification of key amino acids by the random forest method of NBW and LBW placenta in training set. (K) The correlation of amino acids concentrations from the placenta and maternal serum of LBW sows in training set (n = 39). (L) ROC curve analysis of the discriminatory power of placental Pro abundance in full-term pregnant sows to predict LBW fetuses in validation set. In this figure, LBW stands for low body weight or low birth weight. Data were analyzed with two-way ANOVA followed by post hoc Bonferroni tests (A-C) and unpaired t test (D-G) and presented as mean $\pm$ SEM unless indicated. * P < 0.05, ** P < 0.01, *** P < 0.001.

and housed individually. Pregnant mice were randomly allocated to 2 groups (n = 10 mice per group): one group received a normal 18% crude protein (CP) diet (NBW group); another group received a 7% low-protein/isocaloric diet to induce LBW fetus (LBW group) as previously reported. The formulas of the two diets are presented in Table S2.

For the experiment using a low-protein diet-induced LBW fetal model combined with dietary Pro deprivation, the experimental procedure is shown in Figure 2A. Briefly, pregnant C57BL/6J mice were randomly allocated to 3 groups (n = 10 mice per group) and fed amino acid-defined purified diets: group 1 received a diet containing 18% CP with Pro (18% CP + Pro diet, NBW + Pro group); group 2 received a diet containing 7% CP with Pro (7% CP + Pro diet, LBW + Pro group); group 3 received a diet containing 7% CP without Pro (7% CP - Pro diet, LBW -Pro group). The 7% CP + Pro and 7% CP - Pro diets

were isonitrogenous and isocaloric, with equivalent levels of all amino acids except Pro and alanine (Ala). The detailed compositions of the three diets are provided in Table S3.

All E18.5 mice were anesthetized by isoflurane followed by cervical dislocation or decapitation for blood collection, and the placenta and fetuses were collected and separated. The weight and length of each fetus and its corresponding placenta were recorded. The placental tissue samples were snap-frozen in liquid nitrogen immediately and stored at -80°C, or fixed in 4% paraformaldehyde for further analysis. The LBW mice were defined as fetuses 2 SDs lower than the average birth weight. Placental efficiency is defined as the ratio of fetal weight to placental weight, which reflects the placenta's capacity to supply nutrients.

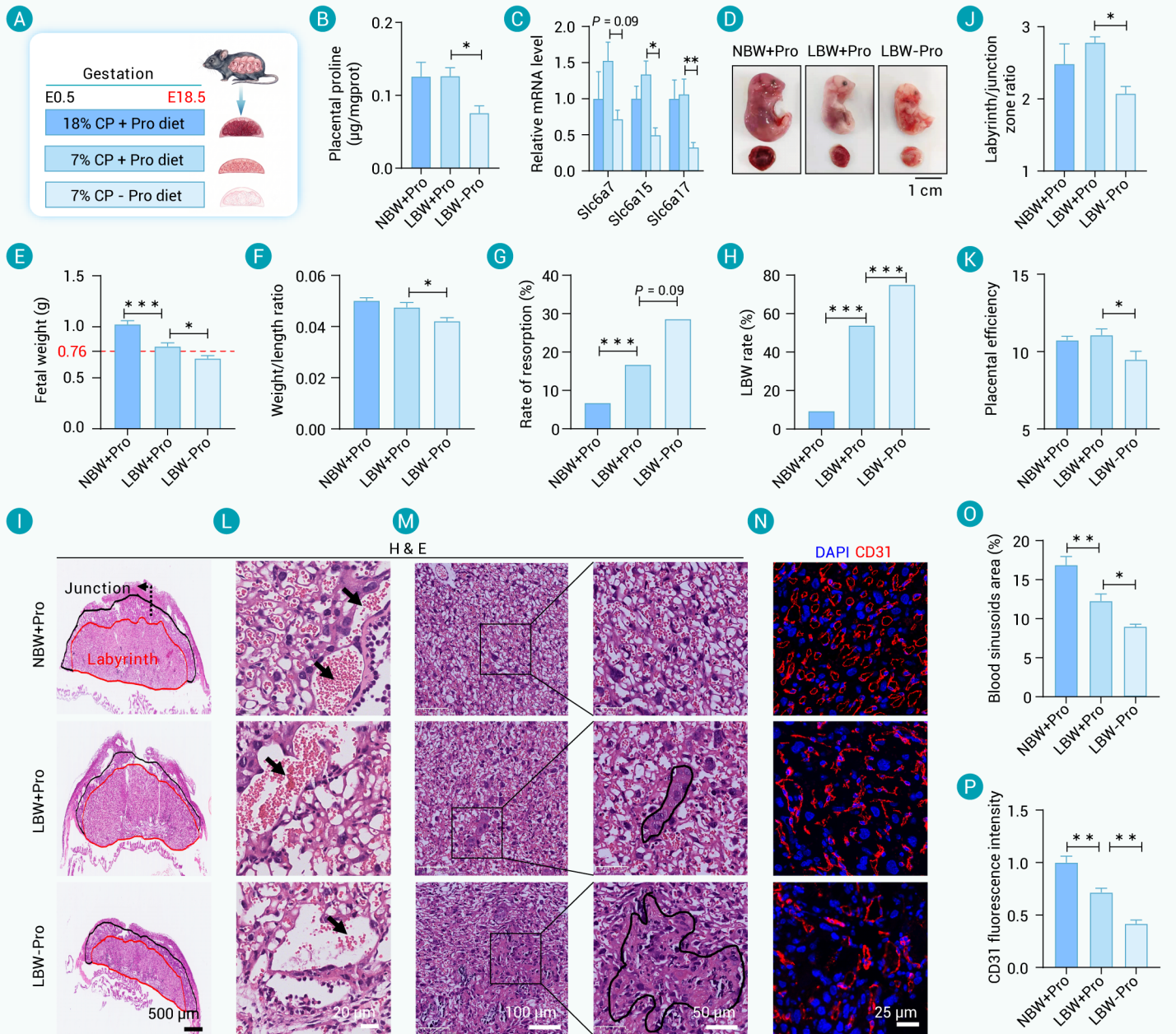


Figure 2. Dietary Pro deprivation during pregnancy exacerbates nutritionally restricted-induced pregnancy impairment and impaired placental angiogenesis (A) Schematic of fetal low birth weight induced by nutritional restriction with and without Pro in the diet during pregnancy ($n = 10$ mice). In this and following figures: NBW + Pro, maternal diet contains 18% CP with Pro; LBW + Pro, maternal diet contains 7% CP with Pro; LBW - Pro, maternal diet contains 7% CP without Pro (Pro deprivation). (B) Placental free Pro levels ($n = 8-10$ placentae from 8-10 litters per group). (C) Relative mRNA expression of Pro transporters in placentae (*Slc6a7*, *Slc6a15*, and *Slc6a17*) ($n = 6$ placentae from 6 litters per group). (D-E) Fetal weight and fetal/placental morphology at E18.5 ($n = 10$ litters per group). The LBW mice (0.76 g) were defined as fetuses 2 SDs lower than the average birth weight. (F) Weight (g)/length (cm) ratio of fetus at E18.5 ($n = 10$ litters per group). (G-H) Rates of resorption and LBW at E18.5 ($n = 10$ litters per group). The LBW mice (birth weight < 0.76 g) were defined as fetuses 2 SDs lower than the average birth weight. (I-J) Representative H&E staining of midsagittal placental tissue sections, with labyrinth zone and junctional zone marked with a black arrow and a red arrow, respectively ($n = 6$ placentae from 6 litters per group). Scale bars, 500 μm. (K) Placental efficiency at E18.5 ($n = 10$ litters per group). Placental efficiency = fetal weight (g)/placental weight (g); reflecting the nutritional supply capacity of the placenta. (L & O) Representative H&E staining and quantification of placental blood sinusoid area (black arrows) ($n = 6$ placentae from 6 litters per group). Scale bars, 20 μm. (M) Representative H&E staining of the labyrinth zone of mice placentae and typical collagen deposition lesions (marked by black lines) ($n = 6$ placentae from 6 litters per group). Scale bars, 100 and 50 μm. (N & P) Representative immunofluorescence staining images of CD31 of placental labyrinth zone and quantification ($n = 6$ placentae from 6 litters per group). Scale bars, 25 μm. Data were analyzed with one-way ANOVA followed by post hoc Tukey's tests (B-F, and I-P), and chi-square test (G-H) and presented as mean $\pm$ SEM unless indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Stattic or tetrahydro-2-furoic acid (T2FA) intrauterine injection

Briefly, at E15.5, mice were anesthetized with 0.015 mL/g body weight of Avertin [2.5% tribromo ethyl alcohol and 2.5% tert-amyl alcohol in phosphate buffered saline (PBS)], followed by making a 2-cm midline incision in the lower abdomen. Then, 20 μL of Stattic (20 μM), T2FA (1 mM) or DMSO was injected into each placenta on one side of the uterus and 20 μL of PBS/placenta on the other side of the uterus was used as a control. Be careful not to inject into the fetal sac. The abdomen was closed in 2 layers, with 4-0

polyglactin sutures at the peritoneum and wound clips at the skin. After killing the mice at E18.5, their placentae were collected and fetal parameters were recorded.

Cell culture

Porcine vascular endothelial cells (PVECs) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in 1640 medium supplemented with 10% fetal bovine serum, 250 μM hypoxia

mimic CoCl_2 , 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin in a 5% CO_2 incubator at 37°C . Notably, 250 μM cobalt chloride (CoCl_2) was used in this study to mimic physiological hypoxia during normal placental development, instead of inducing pathological hypoxia.¹³ Results from the concentration gradient assay showed no significant difference in angiogenic potential between the hypoxia-treated group and the control group. The treatments were detailed in the legend of each figure.

Detection of angiogenic capacity in PVECs

Angiogenesis assay was performed according to standard methods.¹⁴ Tube formation assay, trans-well migration assay, and wound healing scratch assay were used to measure the angiogenic capacity of PVECs. Briefly, 2×10^6 PVECs inoculated in 6-well plates were subjected to the indicated treatments. For tube formation assay, the 96-well plates were precoated with 50 μL Matrigel (BD Company), then seeded with 2×10^4 PVECs for each well, and incubated with normal or conditioned medium with serum-free medium at 37°C for 6 h. Finally, endothelial tube formation was observed and photographed using an Olympus inverted microscope. For Trans-Well migration assay, the chemotactic migration of PVECs was assayed using a trans-well chamber equipped with a polycarbonate filter with a diameter of 6.5 mm and a pore size of 8 μm . Then, PVECs were suspended in corresponding medium to a final concentration of 1×10^5 cells/mL. Next, 100 μL of PVECs suspension was placed in the upper wells of the chamber, while the lower chamber was filled with 600 μL medium containing 10% FBS. After incubation at 37°C for 48 h, the cell culture inserts were collected. The PVECs on the upper side of the filters were removed with cotton-tipped swabs, while the PVECs on the underside of the filters were fixed with 4% formaldehyde for 30 min. Finally, the polycarbonate filters were stained with crystal violet for 20 min and counted in five randomly chosen fields. For wound healing scratch assay, 4×10^6 PVECs were seeded onto 6-well plates and incubated until a fused monolayer was formed. Then, cells were scratched with a 200- μL pipette tip and treated as indicated in the legend of the corresponding figure. After 24 h of treatment, images of the wounded area were captured using an Olympus inverted microscope. Angiogenic capacity were quantified by ImageJ software.

Histology and microscopy

Histology and microscopy were performed on paraffin sections and cell sample using previously reported methods.¹⁵ For Hematoxylin-Eosin staining, placental samples were fixed in 4% paraformaldehyde overnight, followed by dehydration, paraffin-embedding, sectioning at 5 μm thickness, and staining with hematoxylin and eosin using standardized procedures. The total area of vessels was traced with a projecting microscope (Olympus CX41) and then corrected using the measured total placental stromal area. For mice placenta, the borders of junction zone and labyrinth zone were manually drawn by 2 independent investigators and quantified with the Case Viewer software. Then, to estimate blood sinusoids area in placental labyrinth, 4 fields were selected in each section. Moreover, the degree of placental pathology was assessed according to previously reported criteria.³ For immunohistochemical staining, sections were deparaffinized, boiled in antigen-retrieval solution, treated with primary antibodies in blocking buffer at 4°C overnight, treated with secondary antibody for 2 h at room temperature in blocking buffer, and stained with Vectastain ABC KIT and DAB KIT. Immunostaining images were taken using a projecting microscope (Olympus CX41). The positive areas of each sample were measured using ImageJ software. For immunofluorescence staining, the fixed, permeabilized, and sliced samples were blocked with Quickblock blocking buffer for Immunol-staining, followed by incubation successively with primary and secondary antibodies. Stained samples were viewed using a confocal fluorescence microscope (Zeiss). For slides from PVECs and sow placenta, 3-4 fields per slide were randomly selected for quantification. For slides from mice placenta, 3 fields within labyrinth zone per slide were randomly selected for quantification. The fluorescence intensity [platelet endothelial cell adhesion molecule (CD31), phospho-signal transducer and activator of transcription 3 (p-STAT3^{Tyr705}), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor A (VEGF-A)] was quantified using the Image J software (National Institutes of Health, Bethesda, Maryland). Details of the primary antibodies are provided in Table S4.

Targeted metabolomics assays

Measurements of amino acids, glutathione (GSH), and glutathione disulfide (GSSG) in the cell, conditioned media, or placenta were performed by using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Metabolites were extracted according to standard methods. Briefly, 200 μL of serum, 100 mg of placenta, or 1×10^7 cells were mixed with 800 μL of acetonitrile. Then, the samples were homogenized, sonicated, centrifuged, dried and re-solubilized, followed by using 800 μL of supernatant for HPLC-MS/MS analysis. Data analysis was performed with Xcalibur software.

Untargeted metabolomics assays

Untargeted metabolomics assays of PVECs were performed at Shanghai Biotree Biotech Co. Ltd. Briefly, PVECs (1.5×10^7 cells per well) were treated as indicated in the legend of the corresponding figure, cryo-quenched, and collected. Each sample was added with 1 mL of acetonitrile and then homogenized, sonicated, dried and re-solubilized. Subsequently, the supernatant was transferred to a glass vial for GC-MS analysis. Data analysis was performed with Lims2.

Stable isotope tracing

Isotope tracing was performed as previously reported.¹⁶ Briefly, PVECs were grown in 100-mm dishes until 90% density, then changed to Pro-deficient medium with 5 mM $[\text{U-}^{15}\text{N}]\text{-Pro}$. After 24h of treatment, PVECs were washed twice with phosphate-buffered saline and harvested in 1.5 mL 80% (v/v) methanol solution. Macromolecules and debris were removed by centrifugation, followed by adding an internal standard (50 nmol of 2-oxobutyrates), completely evaporating the supernatant with aqueous metabolites and derivatization for 30 min at 42°C in 100 μL of a trimethylsilyl donor (Tri-Sil, Thermo). Finally, samples were analyzed by GC-MS using an HP-5MS column (Agilent Technologies) in an Agilent Intuvo gas chromatograph coupled to an Agilent 5997B mass spectrometer under following conditions. Helium was used as the carrier gas at a flow rate of 1.2 mL/minute. One microliter of sample was injected in split mode (split 1:1) at 270°C . After injection, the GC oven was held at 100°C for one min, up to 300°C at 3.5°C/min , and then increased to 320°C at 20°C/min and held for 5 min at 320°C . The MS system operated under electron impact ionization at 70 eV and the MS source and quadrupole were held at 230°C and 150°C , respectively. The detector was used in scanning mode, and the scanned ion range was 10-650 m/z. Mass isotopomer distributions were determined by integrating the appropriate ion fragments for each metabolite using MATLAB correction for natural abundance.¹⁷ The mass isotopomer distribution analysis was used to measure the fraction of each metabolite pool that contained every possible number of ^{15}N atoms. Isotope tracing was conducted by Profleader Co., Ltd. (Shanghai, China).

Gene silencing or over expression for PVECs

Gene silencing for PVECs was used for siRNA transfection. All siRNA were designed and synthesized by GenePharma Co., Ltd (Shanghai, China). PVECs at 30% to 35% confluency were transfected with 25-nM siRNA targeting the target gene or a nontargeting negative control (si-NC) using Lipofectamine 3000 (ThermoFisher) as instructed by the manufacturer. After 6 h of transfection, the medium was changed to fresh RPMI 1640 containing 10% FBS, followed by incubating the cells for another 48 h before further experiments. Gene overexpression was performed by transfecting the pcDNA3.1(+)-Mam-VEGF-A or pcDNA3.1(+)-Mam-bFGF into PVECs using Lipofectamine 3000 according to the manufacturer's protocol. The siRNA sequences and overexpression plasmids were listed in Table S4.

RNA isolation and quantitative real-time polymerase chain reaction (q-PCR) analysis

Total RNA from PVECs was extracted with the reagent box of Total RNA Kit according to the manufacturer's instructions and then quantified with Nanodrop 2000, followed by reverse-transcribing the high-quality RNA to cDNA using Primer Script™ RT reagent Kit based on the protocols (Abclonal, China). RT-qPCR was performed via SYBR Green on the Quant Studio 6 Real-Time PCR System (Thermo Fisher Scientific, America). Fold change was assessed through the $2^{-\Delta\Delta\text{Ct}}$ method using $\beta\text{-actin}$ or tubulin beta class I

(*TUBB*) for normalization. The primers were listed in Table S4.

Enzyme linked immunosorbent assay (ELISA) assay

The culture supernatants and cell were collected, and the levels of bFGF, VEGF-A, PRODH, carbonic anhydrase 9 (CA9), γ -glutamyl cysteine synthetase (λ GCS), superoxide dismutase 2 (SOD2), succinate dehydrogenase (SDH, CII), and ATPase (CV) were determined using ELISA assay kits (CUSABIO or Jiangsu Meimian industrial Co. Ltd) according to the manufacturer's protocols.

Chromatin immunoprecipitation (ChIP)-q-PCR

ChIP assay was performed using the ChIP kit (Cell Signaling Technology). Briefly, after the indicated treatments, cells were cross-linked with 1% formaldehyde for 10 min and quenched with 125 mM glycine. Next, the samples were subjected to enzymatic digestion and sonication to generate 200–500 bp fragments. Immunoprecipitation was performed using p-STAT3^{Tyr705} antibody (Table S4). Purified DNA was subjected to qPCR analysis using specific primer bFGF and VEGF-A (Table S4). Data were normalized to the corresponding DNA input control.

Western blot (WB) analysis

The tissues and cells were washed with iced-cold PBS and lysed in radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors (Beyotime). Protein concentration was quantified using a bicinchoninic acid protein assay kit (Beyotime) as instructed by the manufacturer, and an equal amount of protein was subjected to SDS-polyacrylamide gel electrophoresis (ACE Biotechnology). Next, proteins were transferred onto polyvinylidene difluoride membranes, followed by incubation with primary and secondary antibodies, and visualized using chemiluminescent reagent. The optical densities of the signals on the membranes were quantified by ImageJ software. The antibodies used in this study are listed in Table S4.

Mitochondrial observation

Mitochondrial observation was performed according to the standard protocols. After treatments, cells were washed in ice-cold PBS and fixed in 2.5% glutaraldehyde. Then, the cells were gently scraped and placed in 1.5 ml EP tubes, followed by fixing the cells in 1% osmium tetroxide, dehydration in a gradient ethanol series, and encapsulation in epoxy resin. Next, samples were made into ultra-slices (60 nm) and stained with uranyl acetate and lead citrate. Finally, the ultra-slices of samples were viewed using a transmission electron microscope.

Reactive oxygen species (ROS) and mitochondrial ROS (mtROS)

After the appropriate treatment, PVECs (1.5×10^7 cells per well, 10-cm petri dishes) were washed with cold PBS, then incubated with 20 μ M of 2,7-dichlorofluorescein diacetate or 5 mM of MitoSox (Invitrogen) at 37°C for 30 min. The fluorescence intensity corresponding to ROS and mtROS was obtained by zymography or flow cytometry. For the determination of placental mtROS in mice, mitochondria from fresh placentas were isolated for mtROS determination using differential centrifugation methods.¹⁸ Briefly, tissue was homogenized using a Dounce homogenizer and centrifuged at 4000 rpm for 3 min at 4°C, followed by collecting the supernatant and centrifugation at 10000 rpm for 10 min at 4°C. Next, the precipitate was sequentially resuspended in Mito I and Mito I buffer to obtain mitochondria, followed by incubating the mitochondria (0.4 mg/mL) in 96 well plates with respiration buffer, SOD, HRP, and succinate, and starting the reaction by adding Amplex Red to the sample wells. Appropriate controls (blank wells without mitochondrial protein or Amplex red) were used. Hydrogen peroxide production was recorded in real-time using a plate reader at 555/581 nm excitation/emission for 30 min at 25°C. Results are plotted as an average of production over the 30 min read frame.

Intracellular adenosine triphosphate (ATP) measurement

PVECs (2×10^6 cells per well, 6-well plates) were lysed in the ATP assay buffer and were centrifuged for 5 min (4°C, 12,000 g). Then, the supernatant was collected to test the ATP level according to the manufacturer's protocol (Beyotime, China).

Mitochondrial membrane potential determination

The mitochondrial membrane potential was determined using the mitochondrial membrane potential assay kit (Beyotime) according to the manufacturer's instructions.

RNA stability assay

PVECs (1×10^6 cells per well, 12-well plates) were treated with 5 μ g/mL actinomycin D (AD) to inhibit global mRNA transcription, followed by collecting the cells at the indicated time points. Total RNA was extracted for reverse transcription, and the expression levels of genes of VEGF-A and bFGF were analyzed by PCR and qPCR.

Protein stability assay

A cycloheximide (CHX)-based assay was used to analyze the protein turnover of VEGF-A and bFGF in PVECs. Briefly, PVECs (2×10^6 cells per well, 6-well plates) were treated with 7 μ g/mL CHX and harvested at the indicated time points. Next, the cells were lysed by RIPA buffer (Beyotime) containing phosphatase inhibitors, followed by WB analysis of the cell lysates with anti-VEGF-A, anti-bFGF, and anti- β -actin as indicated. Finally, the VEGF-A, bFGF, p-STAT3^{Tyr705} protein levels relative to β -Actin or STAT3 were quantified using the Image J software.

Statistical analysis

All data are presented as mean $\pm$ standard error of the mean (SEM) or standard deviation (SD). Statistical analyses were performed using the GraphPad Prism 9.3 statistics software unless otherwise stated. The individual biological samples for each test are given in the corresponding legend. Gene Set Enrichment Analysis (GSEA) and Pearson or Spearman correlation coefficient analysis was performed using the OmicStudio tools at <https://www.omicstudio.cn/tool>. The rates of resorption and LBW were analyzed using the Chi-square test. Statistical analysis methods were chosen based on the design of each experiment and indicated in the corresponding figure legends. Briefly, for continuous variables, the Shapiro-Wilk test was used to assess the normality of the data and the Brown-Forsythe test was used to assess the variance of the data. If the data had uniform variance and normality, two-sided unpaired Student t-tests were used for two-group comparisons of means, and one-analysis of variance (ANOVA) followed by post hoc Tukey's tests or two-way ANOVA followed by post hoc Bonferroni tests were used for multi-group comparisons of means. Otherwise, two-group comparisons were analyzed using the Welch-corrected unpaired t-test for normally distributed data without equal variance or the Mann-Whitney U test for non-normally distributed data; multiple group comparisons were analyzed using Kruskal-Wallis for non-normally distributed data. Data in figures are presented as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS

Pro is lower in LBW placentae and linked to poor placental angiogenesis

Porcine placental vascular development is characterized by vasculogenesis prior to G35d (vasculogenesis), followed by nonbranching-angiogenesis (angiogenesis) until term.² However, it is still unclear whether either or both are responsible for the abnormal vascular development in LBW placentae. Therefore, we collected placentae of normal and low birth weight fetuses (NBW and LBW) from sows at G33d, G50d, and G90d for further analysis (Figures 1A & S1). Fetal weight gain was accompanied by maturation of the placental vascular system, with vascular development (assessed by platelet endothelial cell adhesion molecule, CD31) consistently higher in NBW placentae than in LBW placentae. Angiogenesis during mid-to-late gestation was identified as the key contributor to this difference (Figure 1B). Moreover, compared to NBW placentae, LBW placentae exhibit a higher hypoxic microenvironment during the angiogenesis phase (Figure 1C), where hypoxia is detrimental to endothelial function as a pathologic phenomenon during mid to late gestation.¹³ Therefore, impaired angiogenesis is the main event leading to reduced placental vascular development in LBW placentae. Next, we analyzed differences in amino acids availability in placentae. First, in the temporal sequence, 9 amino acids were decreased in the amniotic fluid at G50d and G90d, whereas 11 amino acids were increased in the placentae at G50d and G90d compared with G33d (Figure 1D). In contrast, when stratified

by body weight phenotype, amino acid levels were globally reduced in LBW placentae: 13 amino acids were decreased at G33d, 8 at G50d, and 9 at G90d relative to NBW placentae (Figures 1E–G). In another larger cohorts, full-term placental samples from term sows were used for targeted metabolome analysis of amino acid abundance, and they were divided into training and validation sets (Figure 1H). In the training set, compared with NBW placentae, LBW placentae had decreased placental vascular density and mRNA expression of *CD31*, but increased hypoxia (Table S1). Notably, only Pro, Arg, and glutamine (Gln) levels were significantly decreased in LBW placentae (Table S1). Further Pearson correlation analysis showed that Pro had the most significant correlation with placental vascularization parameters and neonatal weight (Figure 1I). Then, based on the amino acid data of the training set, a random forest algorithm was used to establish an LBW diagnosis and prediction model based on amino acid abundance, and Gln, glutamate (Glu), and Pro were found to be the top 3 key amino acids (Figure 1J). Notably, Pro levels in serum and placenta from the same LBW sows were strongly correlated ($R^2 = 0.5779$, $P < 0.0001$) (Figure 1K), suggesting Pro as a potentially valuable diagnostic biomarker for LBW fetuses. Subsequently, we assessed this potential by plotting the receiver operating characteristic (ROC) curve of Pro concentrations in the validation set. ROC analyses showed that Pro in placenta or placenta plus serum was effective in discriminating newly diagnosed LBW sows from NBW sows, with the area under the curve (AUC) values being 0.939 and 0.955, respectively (Figure 1L). Collectively, these findings suggested that Pro is linked to poor placental angiogenesis and LBW progression.

Dietary Pro deprivation reduces placental Pro availability and angiogenesis in LBW fetus model

Next, we induced NBW and LBW models in pregnant C57BL/6 mice by feeding either a normal 18% CP diet or a low 7% CP isocaloric diet (Figure S1 & S2A, Table S2). The rationale for the use of this animal model can be summarized as follows: (I) Maternal undernutrition during gestation reduces placental and fetal growth of both domestic animals and humans. Available evidence suggests that fetal growth is most vulnerable to maternal dietary deficiencies of nutrients, especially protein, during the peri-implantation period and the rapid placental development period, and in animal agriculture, fetal undernutrition frequently occurs worldwide.¹⁹ (II) In pigs, the restrictive feeding strategy can lead to disproportionate nutrient availability at the uterine horn, which could result in 15 to 20% of LBW piglets with severely reduced postnatal survival and growth performance.² (III) Similarly, maternal undernutrition due to limited food availability or lack of dietary protein is a risk factor for fetal growth restriction, especially in developing countries.² (IV) A low-protein diet has been widely used to induce LBW animal models, which can also replicate features of LBW or IUGR placentae,¹⁵ such as abnormal placental vascularization, hypoxia, and reduced amino acid availability (Figures S2B–L). These findings suggested that deficiency of multiple amino acids (including Pro) may be an important cause of the LBW and placental insufficiency phenotypes.

To clarify the causal link between Pro availability and impaired angiogenesis in LBW placentae, dietary Pro deprivation was achieved using an amino acid-defined diet that was completely devoid of Pro (LBW - Pro diet). This design ensured the LBW + Pro and the LBW - Pro diets were identical in total nitrogen level, caloric density, and concentrations of all other amino, except that Ala was used to maintain isonitrogenous balance (Figures 2A & S1, Table S3). Based on the LBW model, compared with dietary Pro supplementation (LBW + Pro) group, dietary Pro deprivation (LBW - Pro group) was found to reduce placental Pro availability, as indicated by lower levels of placental Pro and mRNA expression of Pro transporters [solute carrier family 6 members 7, 15, and 17 (*Slc6a7*, *Slc6a15*, and *Slc6a17*)] (Figures 2B–C). Importantly, a more severe impairment of pregnancy was observed in mice fed the Pro-deficient diet versus mice fed the Pro-supplementary diet. Specifically, fetal weight was further reduced in mice fed Pro-deficient diet (average fetal weight reached the range of LBW fetuses) and the incidence of embryo resorption and LBW fetuses was further elevated (Figures 2D–H). Moreover, the development of the labyrinth zone was impaired in mice fed the Pro-deficient diet, which was associated with further impairment of placental efficiency (Figures 2I–K). The maldevelopment of the placental labyrinth zone

was also reflected by vascular infarction associated with exacerbated collagen deposition and decreased CD31 immunofluorescence intensity (Figures 2L–P). Therefore, we consider that the reduced placental Pro availability in LBW mice model induced by nutritional restriction leads to further impaired angiogenesis, thereby exacerbating the development of LBW fetuses, and these results demonstrate a causal relationship between Pro and placental angiogenesis.

Pro supports angiogenesis via VEGF-A and bFGF

Due to the physiological hypoxic state existing in the placenta, PVECs were cultured under physiological hypoxic conditions (250 μ M hypoxia mimic CoCl_2) to explore the regulatory effect of Pro.¹³ Then, primary screening tests were performed by measuring tube formation, transwell migration assay of the migratory number, wound healing assay of migratory distance, and the expression of angiogenesis-related genes of PVECs. The results showed that Pro deficiency reduced angiogenesis (Figures 3A–B). However, re-supplementation of exogenous Pro into the culture medium significantly alleviated the inhibition of angiogenesis induced by Pro deficiency, with the angiogenic capacity being basically restored to the normal level (Figures S3A–C). Furthermore, subsequent q-PCR, ELISA, immunofluorescence, and WB analyses confirmed that the changes in angiogenic capacity were associated with the expression of angiogenesis-related genes, including increased protein expression and secretion of VEGF-A and bFGF, as well as activation of the receptors phospho-VEGF Receptor 2 (p-VEGFR2^{Tyr1175}) and phospho-FGF Receptor 1 (p-FGFR1^{Tyr653}) (Figures 3C–F). We hypothesized that whether Pro promotes angiogenesis in PVECs through the mediation of VEGF-A or/and bFGF could be tested by using small interfering RNA (si-VEGF-A and si-bFGF) and overexpressed plasmids (OE-bFGF and OE-VEGF-A) to regulate the expression of VEGF-A and bFGF (Figures S3D–E). We found that simultaneous silencing of VEGF-A and bFGF reduced the migration and tube formation ability of the Pro-supplemented group to the level of the Pro-deficient group, which could be restored by supplementing bFGF and VEGF-A recombinant proteins in the medium (Figure 3G). In addition, simultaneous overexpression of bFGF and VEGF-A could also fully restore the migration and tube formation ability under Pro deprivation (Figure S3F). These findings indicate that Pro supports angiogenesis via VEGF-A and bFGF.

bFGF/VEGF-A mediated Pro supports angiogenesis through p-STAT3^{Tyr705} activation

Next, we explored by which signaling molecules or transcription factors Pro regulates the expression of bFGF and VEGF-A. The effect of Pro on the kinetics of bFGF and VEGF-A mRNA and protein degradation was first excluded. After inhibiting mRNA and protein synthesis using AD and CHX, no significant difference was observed in the degradation of these mRNA and protein in PVECs with or without Pro (Figures S4A–B). This indicated that transcriptional activation is the main reason for the upregulation of VEGF-A and bFGF expression in Pro-stimulated PVECs.

Placental angiogenesis and its related gene expression are related to the activation of mammalian target of rapamycin (mTOR),²⁰ STAT3,²¹ protein kinase B (Akt),¹⁵ hypoxia-inducible factor-1 α (HIF-1 α),²² and nuclear factor κ B (NF- κ B) signaling,²³ so we analyzed the activation of these pathways in PVECs with and without Pro. We found that Pro supplementation promoted the activation of mTOR, STAT3, and Akt based on the high ratio of p-mTOR/t-mTOR, p-STAT3^{Tyr705}/t-STAT3, and p-Akt/Akt, but inhibited the HIF-1 α and NF- κ B pathways (Figure 4A). In addition, STAT3 acts as a nuclear transcription factor, and the nuclear p-STAT3^{Tyr705} level and nuclear translocation also increased in the presence of Pro (Figures S4C–D). Then, Rapamycin (15 μ M), MK2206 (1 μ M), and Stattic (5 μ M) were used to inhibit mTOR, STAT3, and Akt, respectively. Notably, inhibition of p-STAT3^{Tyr705} but not p-mTOR and p-Akt completely eliminated the promotion of cell migration and angiogenesis by Pro treatment (Figure 4B). Moreover, ChIP-qPCR analysis showed the increased p-STAT3^{Tyr705} enrichment in the gene body regions of VEGF-A and bFGF after Pro treatment (Figure 4C), indicating that increased VEGF-A and bFGF expression is more likely the result of STAT3 transcriptional activation. To further validate the importance of STAT3 in Pro-mediated high VEGF-A and bFGF production, the activation of this axis in PVECs was manipulated, and inhibition of p-STAT3^{Tyr705} was confirmed to completely reverse the

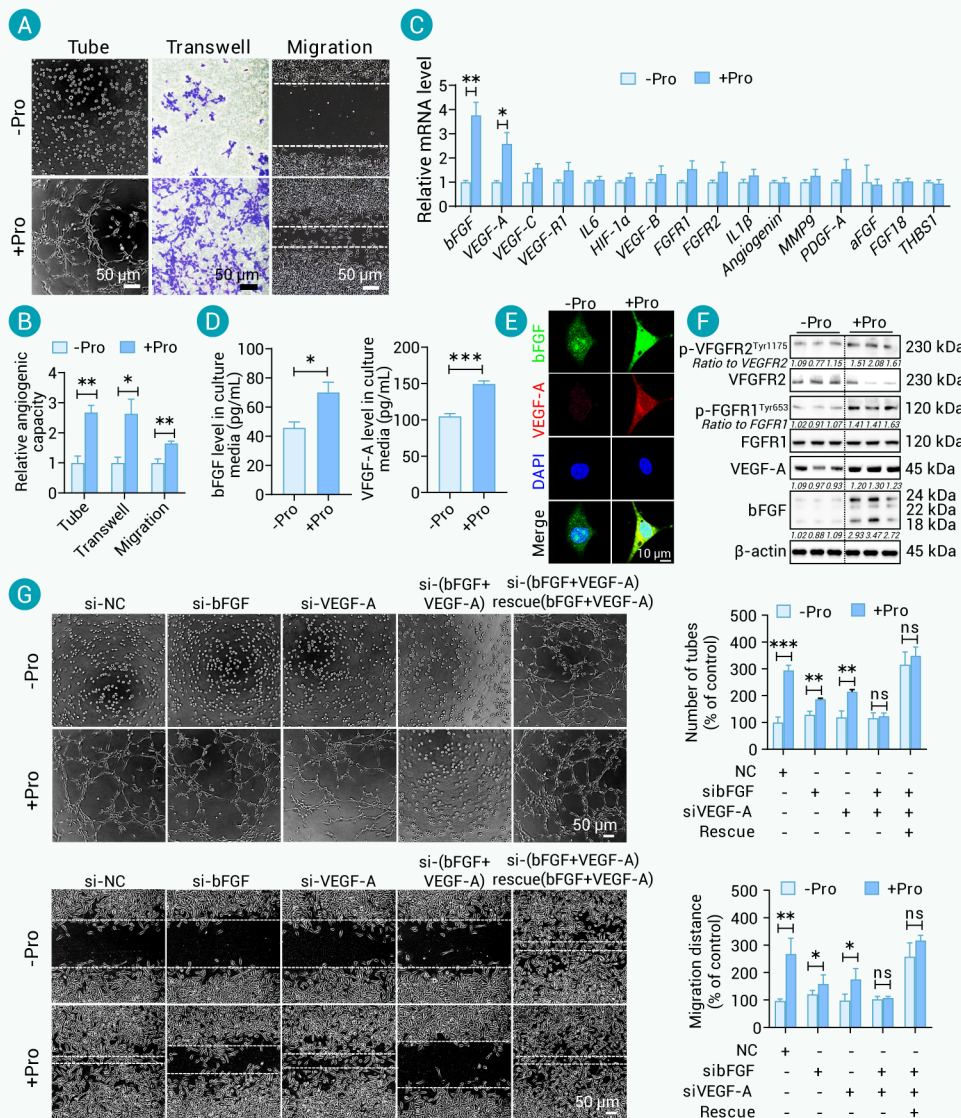


Figure 3. Pro depends on VEGF-A and bFGF expression to support angiogenesis (A-B) Representative images and quantification of tube formation (left), transwell migration assay of the migratory number (middle), and wound healing assay of migratory distance (right) in PVECs treated for 24h under hypoxia in medium without (-Pro) or with (+Pro) 5 mM Pro (n = 3-4). Scale bars, 50 μm. Results represent two independent experiments. (C) Relative mRNA expression of angiogenesis-related genes (n = 4). (D) The concentration of VEGF-A and bFGF in culture media (n = 4). (E) Confocal microscopy of bFGF (green), VEGF-A (red), and DAPI (blue) in PVECs (n = 3-4). Scale bars, 10 μm. (F) The protein abundance of p-VEGFR2^{Tyr1175}, VEGFR2, p-FGFR1^{Tyr653}, FGFR1, bFGF, and VEGF-A in PVECs (n = 3). (G) Representative images and quantification of tube formation (top), and wound healing assay of migratory distance (bottom) in PVECs treated as indicated (n = 3-4). PVECs were treated with VEGF-A silencing and/or bFGF silencing, and medium supplemented with VEGF-A (100 ng/mL) and bFGF (100 ng/mL) recombinant proteins in the absence and presence of exogenous Pro (5 mM) under hypoxia. Scale bars, 50 μm. Data were analyzed with unpaired t test (A-F) and two-way ANOVA followed by post hoc Bonferroni tests (G) and presented as mean ± SEM unless indicated. *P < 0.05, **P < 0.01, ***P < 0.001.

found to perform an important role in STAT3 protein stability.²¹ In the present study, the degradation of p-STAT3^{Tyr705} protein was observed to be slower in PVECs treated with Pro versus those without Pro (Figure 5A), indicating that Pro increases the p-STAT3^{Tyr705} stability to maintain p-STAT3^{Tyr705} activation. Consistently, compared with Pro deprivation, Pro supplementation improves mtROS homeostasis, as indicated by a reduction in the levels of ROS and mtROS, and an increase in the levels of adenosine triphosphate (ATP), protein expression of SDHA and ATPB, mitochondrial number and area, and mitochondrial membrane potential in PVECs (Figures 5B-F). Data from porcine term placentae also confirmed the presence of marked oxidative

promotion of VEGF-A and bFGF expression by Pro (Figures 4D-E & S4E). These findings indicate the importance of the p-STAT3^{Tyr705}-bFGF/VEGF-A axis in regulating Pro-promoted angiogenesis in PVECs.

In a mouse model of LBW fetus, p-STAT3^{Tyr705} was activated in the placenta of the Pro-supplementary group, consistent with the changes in bFGF and VEGF-A expression (Figure S4F-G), so we wanted to know the role of p-STAT3^{Tyr705} in Pro-mediated placental angiogenesis. Mice models of LBW fetuses were subjected to Pro-deficient diet (LBW-Pro) or Pro-containing diet (LBW+Pro) throughout gestation, followed by intrauterine injection of p-STAT3^{Tyr705} inhibitor at E15.5 (LBW+Pro+Static) (Figures 4F & S1). Inhibition of placental p-STAT3^{Tyr705} reduced fetal weight in LBW+Pro+Static group to that of LBW-Pro group (Figure 4G), coupled with an increase in the rates of resorption and LBW to those of the LBW-Pro group (Figure 4H). The placental angiogenic phenotype showed lower placental blood sinusoid area and CD31 levels in LBW+Pro+Static group than in LBW+Pro group (Figures 4I-L), suggesting an association between p-STAT3^{Tyr705} and Pro-supported placental angiogenesis. Inhibition of placental p-STAT3^{Tyr705} also reduced the protein expression of placental VEGF-A and bFGF in placenta (Figure 4M). Collectively, both *in vitro* and *in vivo* model demonstrated that bFGF/VEGF-A mediated Pro supports angiogenesis through p-STAT3^{Tyr705} activation.

Pro improves mtROS homeostasis to maintain angiogenesis and p-STAT3^{Tyr705}-bFGF/VEGF-A pathway

The next question is how Pro regulates p-STAT3^{Tyr705} activation. In our previous studies, oxidative stress, especially the mtROS imbalance, was

stress in LBW placentae, particularly an imbalance in ROS homeostasis (Table S1). Moreover, in a mouse model of LBW fetuses, Pro deprivation increased placental ROS and mtROS levels compared with Pro supplementation (Figures 5G-H).

Next, the involvement of ROS or mtROS in Pro-mediated p-STAT3^{Tyr705} activation was confirmed by analyzing the levels of angiogenesis and p-STAT3^{Tyr705} activation in Pro-treated PVECs in the presence of whole-cell component ROS scavengers (N-acetylcysteine, NAC, 1 mM), mitochondria-targeted ROS scavengers (MitoQ, 0.5 μM), and mitochondrial ROS generator MitoParaquat (MitoPQ, 20 μM). Importantly, Pro supplementation failed to promote tube formation and cell migration in the presence of mitochondrial ROS generator MitoParaquat (MitoPQ), but the presence of mitochondria-targeted ROS scavengers (MitoQ) restored the impaired tube formation and cell migration induced by Pro deprivation under hypoxia (Figure 5I). As expected, the p-STAT3^{Tyr705}-bFGF/VEGF-A axis showed the same changes, as indicated by WB analysis: (i) in the presence of MitoPQ, Pro supplementation did not promote p-STAT3^{Tyr705}-bFGF/VEGF-A axis expression; (ii) the impairment of the p-STAT3^{Tyr705}-bFGF/VEGF-A axis caused by Pro deprivation was recovered in the presence of MitoQ (Figure 5J). Together, Pro maintains angiogenesis and p-STAT3^{Tyr705}-bFGF/VEGF-A pathway primarily by improving mtROS homeostasis.

Pro regulates mtROS homeostasis via PRODH catabolism pathway

Despite our clarification of the mechanisms by which Pro activates cell signaling (Figures 4-5), how Pro regulates mtROS homeostasis in PVECs

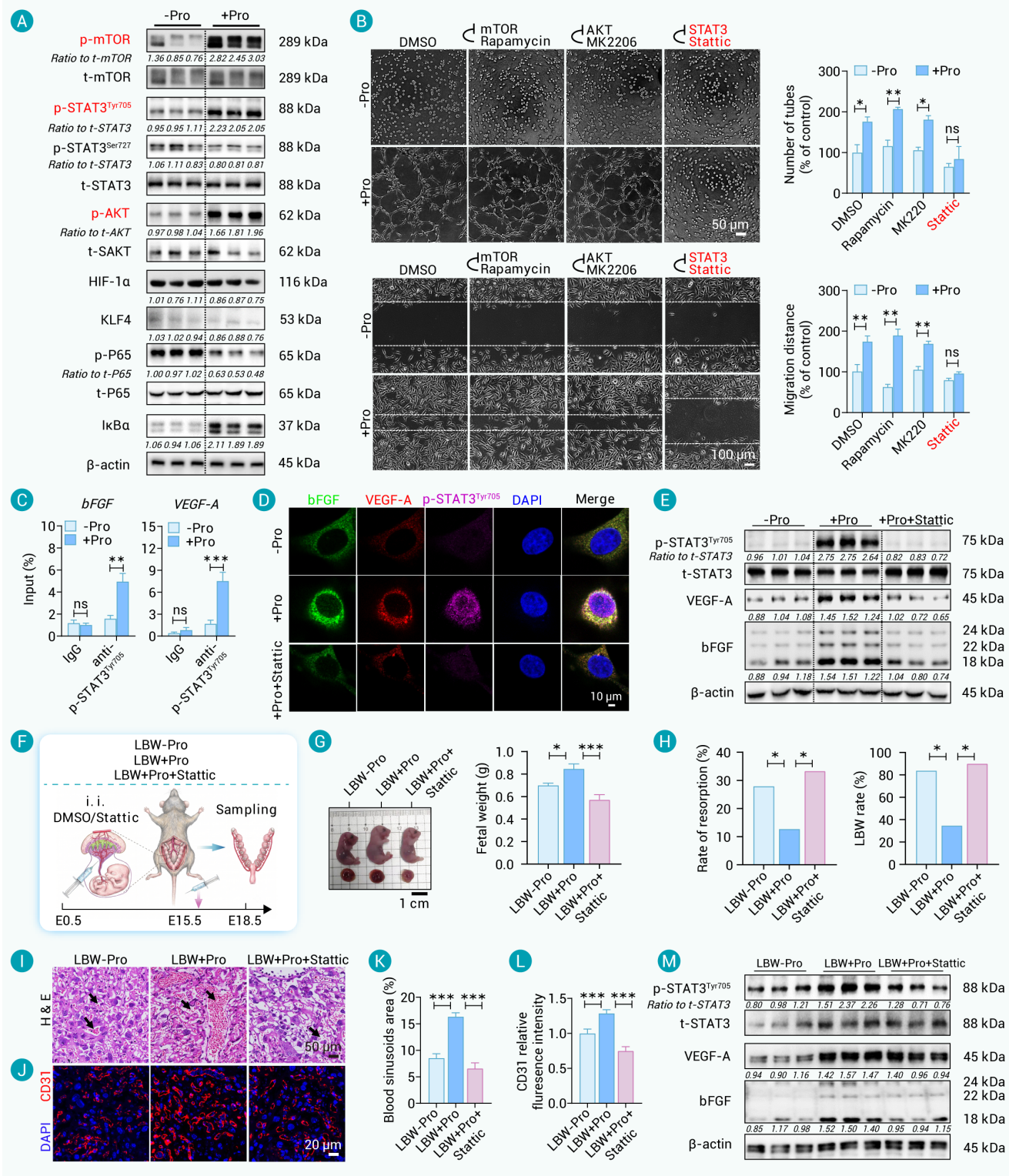


Figure 4. Phosphorylated p-STAT3^{Tyr705}-bFGF/VEGF-A pathway mediates Pro promotion of placental angiogenesis (A) Western blot analysis and quantification of the protein expression of p-mTOR, p-STAT3^{Tyr705}, p-STAT3^{Ser727}, p-AKT, HIF1α, KLF4, p-P65, and IκBα in PVECs treated for 24h under hypoxia in medium without (-Pro) or with (+Pro) 5 mM Pro. (B) Representative images and quantification of tube formation (top), and wound healing assay of migratory distance (bottom) in PVECs treated as indicated. PVECs were treated with Rapamycin (15 μM), MK2206 (1 μM), or Stattic (5 μM) in the absence and presence of exogenous Pro (5 mM) under hypoxia (n = 3-4). Scale bars, 50 and 100 μm. (C) ChIP-qPCR analysis of p-STAT3^{Tyr705} enrichment at the promoters of the *bFGF* and *VEGF-A* gene in PVECs under the indicated treatments (n = 3). (D) Confocal microscopy of p-STAT3^{Tyr705} (purple), bFGF (green), VEGF-A (red), and DAPI (blue) in PVECs under the indicated treatments (n = 3-4). Scale bars, 10 μm. (E) Western blot analysis and quantification of the protein expression of p-STAT3^{Tyr705}, VEGF-A, and bFGF in PVECs under the indicated treatments (n = 3). (F) Schematic of the inhibition of placental p-STAT3^{Tyr705}, i.e. mice were injected intrauterine with Stattic at E15.5 (n = 6 mice). In this and following figures unless indicated: LBW-Pro, maternal diet during pregnancy contains 7% CP without Pro; LBW+Pro, maternal diet during pregnancy contains 7% CP with Pro; LBW+Pro+Stattic, maternal diet containing 7% CP with Pro during pregnancy and intrauterine injection of p-STAT3^{Tyr705} inhibitor. (G) Fetal weight and fetal/placental morphology at E18.5 (n = 10 litters per group). (H) Rates of resorption and LBW at E18.5 (n = 10 litters per group). (I & K) Representative H&E staining and quantification of placental blood sinusoid area (black arrows) (n = 6 placenta from 6 litters per group). Scale bars, 50 μm. (J & L) Representative immunofluorescence staining images of CD31 of placental labyrinth zone and quantification (n = 6 placenta from 6 litters per group). Scale bars, 20 μm. (M) Western blot analysis and quantification of the protein expression of p-STAT3^{Tyr705}, VEGF-A, and bFGF in placenta (n = 6 placenta from 6 litters per group). Data were analyzed by unpaired t test (A), two-way ANOVA followed by post hoc Bonferroni tests (B-C), one-way ANOVA followed by post hoc Tukey's tests (D-G, and I-M), and chi-square test (H) and presented as means ± SEM unless indicated. *P < 0.05, **P < 0.01, ***P < 0.001.

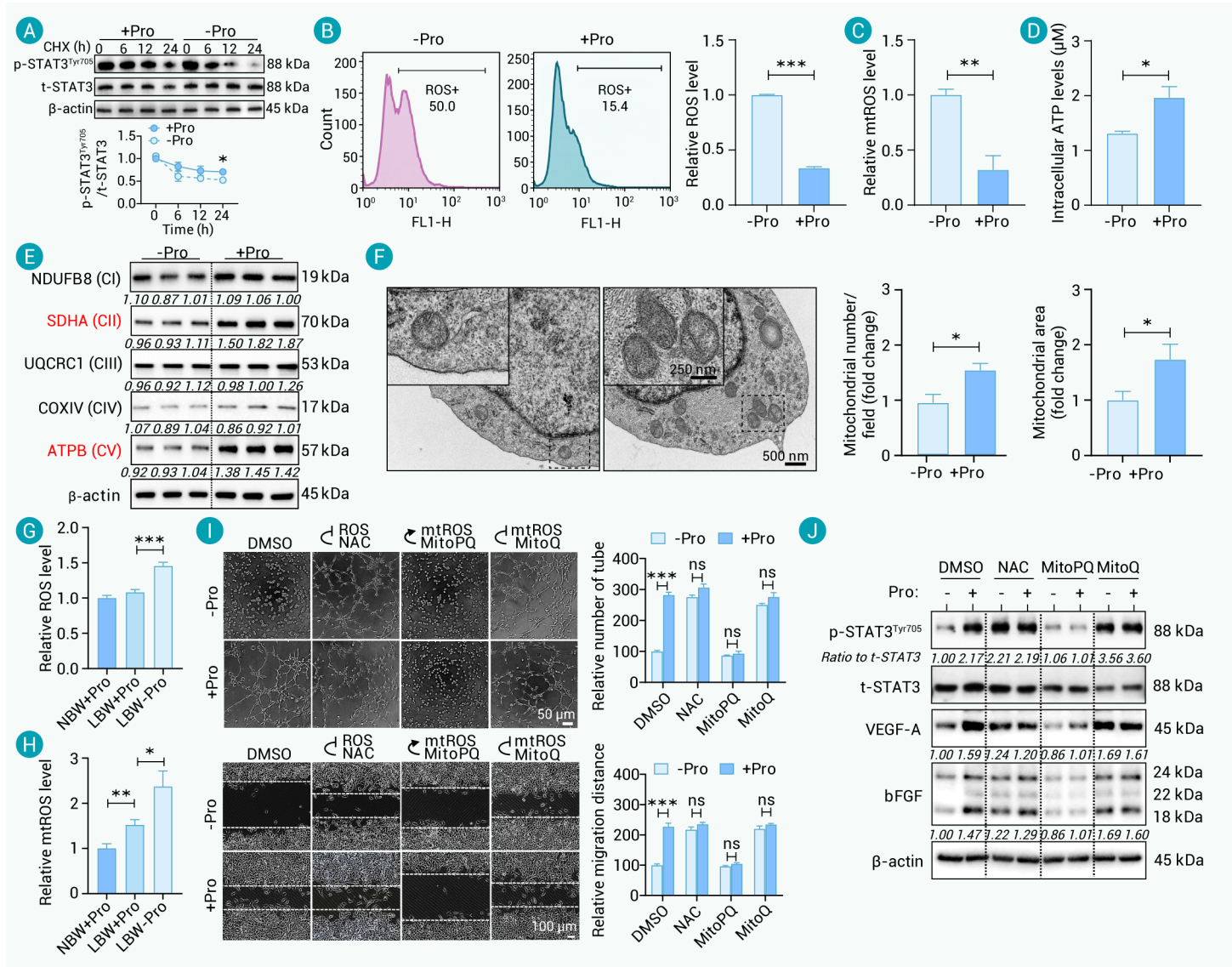


Figure 5. Pro improves mtROS homeostasis to maintain p-STAT3^{Tyr705}-bFGF/VEGF-A pathway (A) Protein turnover assay. PVECs were treated with or without Pro under hypoxia were incubated with 7 μ M CHX for the indicated intervals. WB was employed to observe the p-STAT3 protein levels ($n=3$). In this and following figures unless indicated: -Pro, medium without Pro; +Pro, medium with 5 mM Pro; Hypoxia, 250 μ M hypoxia mimic CoCl₂. (B) Relative intracellular ROS levels in PVECs treated for 24h under hypoxia in medium without (-Pro) or with (+Pro) 5 mM Pro ($n=3$). (C) Relative intracellular ROS levels in PVECs under the indicated treatments ($n=5$). (D) Intracellular ATP levels in PVECs under the indicated treatments ($n=6$). (E) Western blot analysis and quantification of the protein expression of NDUFB8 (CI), SDHA (CII), UQCRC1 (CIII), COXIV (CIV), and ATPB (CV) under the indicated treatments ($n=3$). (F) Transmission electron microscopy observation for mitochondrial number and area in PVECs under the indicated treatments ($n=5$). Scale bars, 250 and 500 nm. (G-H) Relative placental ROS and mtROS levels in mice ($n=6$ placenta from 6 litters per group). Schematic diagram for the description of treatments in mice is presented in Figure 2A. (I) Representative images and quantification of tube formation (top), and wound healing assay of migratory distance (bottom) in PVECs treated as indicated. PVECs were treated with NAC (1 mM), MitoPQ (20 μ M), or MitoQ (0.5 μ M) in the absence and presence of exogenous Pro (5 mM) under hypoxia ($n=3-4$). Scale bars, 50 and 100 μ m. (J) Western blot analysis and quantification of the protein expression of p-STAT3^{Tyr705}, VEGF-A, and bFGF in PVECs treated as in (I) ($n=3$). Data were analyzed by two-way ANOVA followed by post hoc Bonferroni tests (A & I-J), unpaired t test (B-F), one-way ANOVA followed by post hoc Tukey's tests (G-H), and presented as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

remains unclear. Pro metabolism assisting redox homeostasis has been shown to be required for the normal function of hypoxic cancer cells.²⁴ The intracellular Pro levels of PVECs were substantially upregulated after 24 h of hypoxic culture in Pro-containing medium (Figure S5A). Additionally, the mRNA expression levels of several known enzyme genes including *PRODH*, *P4HA2*, and *PARS2* were upregulated in the presence of Pro (Figures 6A & S5B). Therefore, we hypothesized that Pro catabolism regulates mtROS homeostasis. To test this hypothesis, Pro catabolism was blocked by using inhibitors, such as T2FA, Lesinurad, halofuginone (HF) and 1,4-dihydropyridine-4-one-3-Carboxylic acid (DPCA) (Figure 6A), which can block *PRODH*, ornithine-delta-aminotransferase (OAT), prolyl-tRNA synthetase (PRS) and prolyl-4-hydroxylase (P4H), inhibiting Pro catabolism into Glu, ornithine (Orn), L-prolyl-tRNAPro in protein synthesis and 4-hydroxyproline (4-OH Pro) in collagen, respectively.⁵⁻⁹ We found that T2FA, HF, and Les could block Pro depletion in the medium (Figure S5C). Importantly, inhibition of

PRODH abolished the effect of Pro supplementation on maintaining mtROS homeostasis, as evidenced by elevated mtROS (Figure 6B) and ROS (Figure S5D) levels, reduced mitochondrial membrane potential (Figure 6C) and decreased activities of mitochondrial complexes II and V (Figures 6D-E). Furthermore, only in the presence of T2FA did Pro supplementation fail to sustain tube formation and cell migration (Figures 6F-G). Moreover, the p-STAT3^{Tyr705} activation and the protein expression of VEGF-A and bFGF promoted by Pro supplementation was reduced due to *PRODH* inhibition (Figures 6H-I). These results showed that Pro regulates mtROS homeostasis and subsequent angiogenesis via *PRODH* catabolism pathway in PVECs.

In a mouse model of LBW fetuses, dietary Pro deprivation was also shown to reduce *PRODH* mRNA expression in placenta (Figure 6J). Then, whether the placental Pro-*PRODH* catabolism pathway regulates mtROS homeostasis and subsequent angiogenesis in *in vivo* LBW model was tested by blocking *PRODH* activity in placenta through intrauterine injection of T2FA

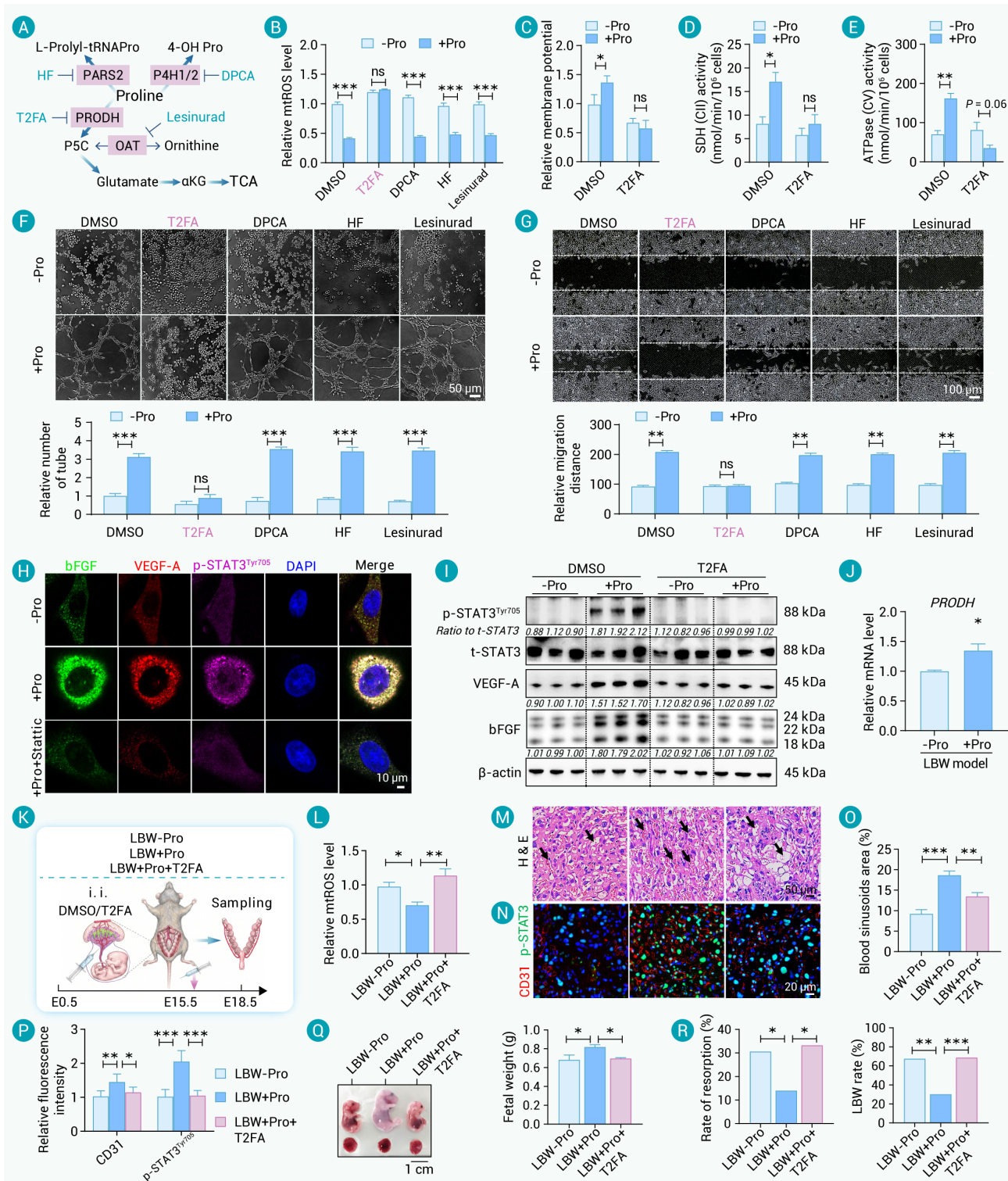


Figure 6. Pro regulates mtROS homeostasis and angiogenesis via PRODH catabolic pathway and gestational nutritional restriction (A) Schematic diagram of the 4 catabolic pathways of Pro and corresponding specific inhibitors (DMSO: vehicle control; T2FA: PRODH inhibitor, 5 mM; DPCA: P4HA inhibitor, 10 μ M; HF: OAT inhibitor, 1 μ M; Lesinurad: PARS2 inhibitor, 10 μ M). PVECs were treated with T2FA, DPCA, HF, or Lesinurad in the absence and presence of 5 mM Pro under hypoxia. (B) Relative mtROS levels in PVECs under the indicated treatments (n = 5). (C) Relative mitochondrial membrane potential in PVECs under the indicated treatments (n = 6). (D-E) The activities of Succinate dehydrogenase (SDH, CII, D) and ATPase (CV, E) in PVECs under the indicated treatments (n = 6). (F-G) Representative images and quantification of tube formation (F) and migratory distance (G) in PVECs under the indicated treatments. Scale bars, 50 and 100 μ m. (H) Confocal microscopy of p-STAT3^{Tyr705} (purple), bFGF (green), VEGF-A (red), and DAPI (blue) in PVECs under the indicated treatments (n = 3-4). Scale bars, 10 μ m. (I) Western blot analysis and quantification of the protein expression of p-STAT3^{Tyr705}, VEGF-A, and bFGF in PVECs under the indicated treatments (n = 3). (J) Relative mRNA expression of *PRODH* in placenta (n = 6 placenta from 6 litters per group). Placenta were collected from the dietary proline deprivation experiment shown in Figure 2. (K) Schematic of the inhibition of placental PRODH, i.e. mice were injected intrauterine with T2FA at E15.5 (n = 6 mice). In this and following figures: LBW-Pro, maternal diet during pregnancy contains 7% CP without Pro; LBW+Pro, maternal diet during pregnancy contains 7% CP with Pro; LBW+Pro+T2FA, maternal diet containing 7% CP with Pro during pregnancy and intrauterine injection of T2FA. (L) Relative placental mtROS levels in mice (n = 6 placenta from 6 litters per group). Scale bars, 50 μ m. (M & O) Representative H&E staining and quantification of placental blood sinusoid area (black arrows) (n = 6 placenta from 6 litters per group). Scale bars, 50 μ m. (N & P) Representative immunofluorescence staining images of CD31 and p-STAT3^{Tyr705} of placental labyrinth zone and quantification (n = 6 placenta from 6 litters per group). Scale bars, 20 μ m. (Q) Fetal weight and fetal/placental morphology at E18.5 (n = 6 litters per group). Scale bars, 1 cm. (R) Rates of resorption and LBW at E18.5 (n = 6 litters per group). Data were analyzed by two-way ANOVA followed by post hoc Bonferroni tests (B-I), unpaired t test (J), one-way ANOVA followed by post hoc Tukey's tests (L & O-Q), and chi-square test (R) and presented as means $\pm$ SEM unless indicated. * P < 0.05, ** P < 0.01, *** P < 0.001.

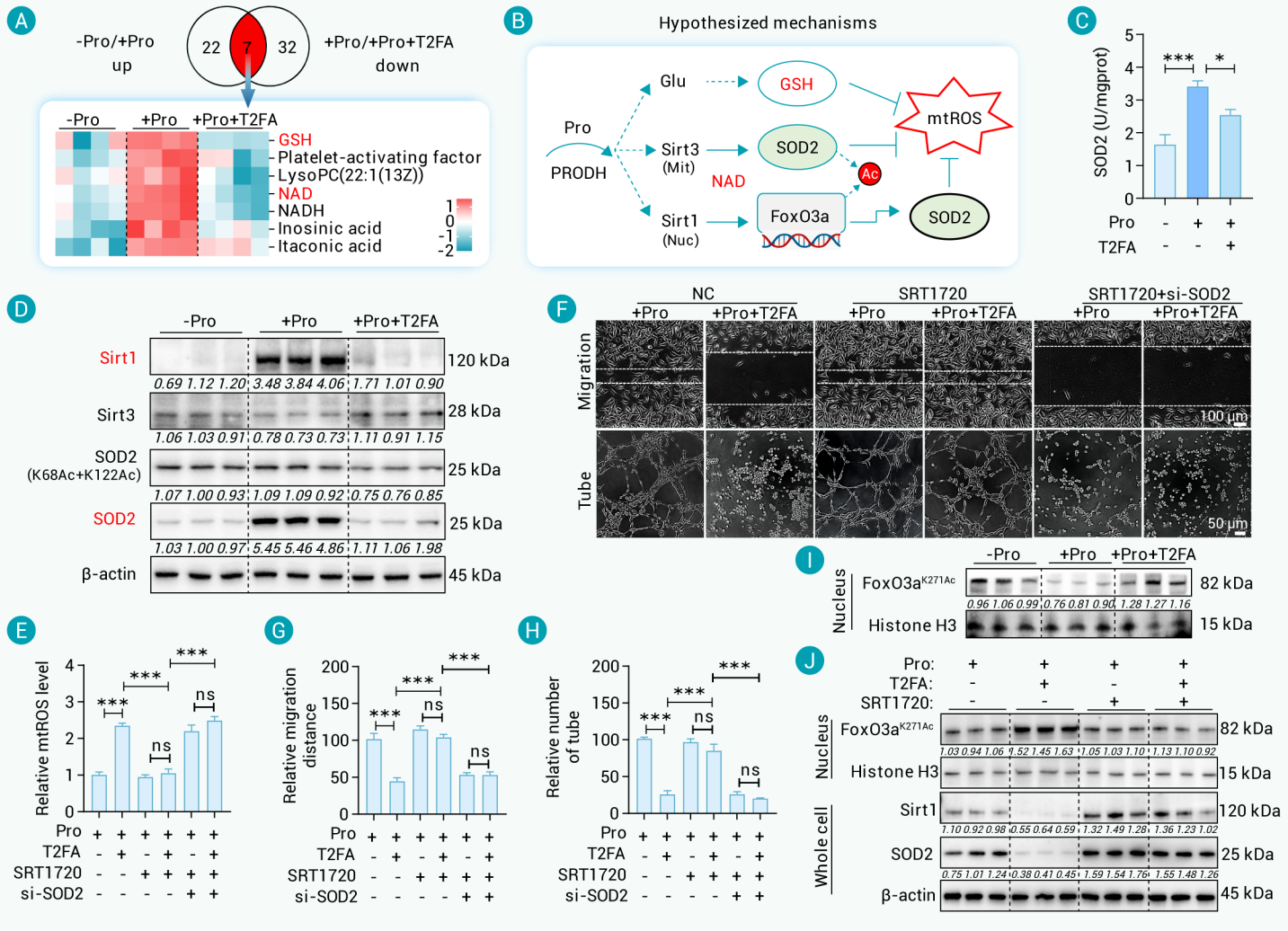


Figure 7. Pro-PRODH catabolism maintain mtROS homeostasis in Sirt1-SOD2-dependent pathway (A) Heat map analysis of 7 metabolites upregulated in Pro group and downregulated in Pro+T2FA group in PVECs (n = 4). Under hypoxia conditions, PVECs were treated with exogenous Pro deficiency (-Pro) or medium with 5 mM Pro with or without T2FA (5 mM) (+Pro or +Pro+T2FA) for 24 h. (B) Hypothesized mechanisms for the regulation of mtROS by Pro-PRODH catabolism. (C) The activities of SOD2 in PVECs treated as in (A) (n = 4). (D) Western blot analysis and quantification of the protein expression of Sirt1, Sirt3, SOD2^{K68Ac+K122Ac}, and SOD2 in PVECs treated as indicated (n = 3). (E) Relative mtROS in PVECs treated as indicated (n = 5). (F-H) Representative images and quantification of tube formation, and wound healing assay of migratory distance in PVECs treated as indicated (n = 3-4). Scale bars, 100 and 50 μm. (I) Western blot analysis of nuclear FoxO3a^{K271Ac} in PVECs treated as in (A) (n = 3). (J) Western blot analysis of FoxO3a^{K271Ac} expression in the nucleus and Sirt1 and SOD2 expression in whole cell (n = 3). Data were analyzed by one-way ANOVA followed by post hoc Tukey's tests and presented as means ± SEM unless indicated. **P* < 0.05, ****P* < 0.001.

(Figures 6K & S1). As expected, T2FA caused an increase of placental mtROS levels in the LBW+Pro group to the levels in the LBW-Pro group (Figure 6L) and blocked Pro-mediated placental angiogenesis (blood sinusoids area and CD31 fluorescence intensity) and p-STAT3^{Tyr705} activation (Figures 6M-P). Moreover, in the presence of T2FA, fetal weight and rates of resorption and LBW were also similar between the LBW+Pro group and LBW-Pro group (Figures 6Q-R). These findings indicate that Pro regulates mtROS homeostasis and subsequent angiogenesis via PRODH catabolism pathway under LBW model.

Pro-PRODH catabolism maintain mtROS homeostasis in GSH-independent and Sirtuin 1 (Sirt1)-superoxide dismutase 2 (SOD2)-dependent pathways

The underlying mechanism of PRODH catabolism pathway-mediated effect of Pro on PVECs was explored by using untargeted metabolomics to determine intracellular metabolites under the conditions of Pro-deficiency (-Pro), Pro-supplementation (+Pro), and Pro-supplementation in the presence of T2FA (+Pro+T2FA). Both Pro treatment and Pro-PRDOH catabolism inhibition reshaped cellular metabolic profile in PVECs (Figures S6A-I). The differential metabolites in the -Pro vs +Pro and +Pro vs +Pro+T2FA comparison groups were significantly enriched in Arg and Pro metabolism, Pentose and glucuronate interconversion, Taurine and hypotaurine metabolism, Ascorbate

and aldarate metabolism, and GSH metabolism pathway (Figures S6J-K). The candidate critical differential metabolites were selected by the standard: increased in +Pro versus -Pro but decreased in +Pro+T2FA versus +Pro. Only 7 differential metabolites were eligible (fold change > 2, VIP > 1, *P* < 0.01), including GSH, Platelet-activating factor, LysoPC(22:1(13Z)), nicotinamide adenine dinucleotide (NAD), NADH, Inosinic acid, and Itaconic acid (Figure 7A). Considering that GSH and NAD are important components in maintaining cellular redox^{25,26} we hypothesized and determined that they might be involved in Pro-PRODH catabolism to maintain mtROS homeostasis through the GSH or NAD-Sirts-SOD2 mechanisms (Figure 7B).

We first tried to reveal the reason for the increase in GSH. PRODH blockade was found to result in intracellular Pro accumulation accompanied by decreases in Glu, Gly, and GSH levels and GSH:GSSG ratio as well as λGCS and GSS enzyme activities (Figure S7A). These results suggest that Pro-PRODH catabolism enhances GSH levels through the pathways of *de novo* and salvage synthesis of GSH. Moreover, we used [U-¹⁵N]-Pro isotope tracer analyses to determine Pro-PRODH catabolism was incorporated into Glu and further used for *de novo* GSH synthesis. Unexpectedly, in the presence or absence of T2FA, both Glu and GSH were labeled at a low or even absent proportion (1.36 % and 0%, respectively) (Figure S7B). Meanwhile, T2FA reduced the fraction of Pro derived Glu but did not affect GSH (Figure S7B). Importantly, reduction of GSH levels by silencing λGCS and GSR did not

completely abolish the effects Pro in regulating mtROS homeostasis, and GSH treatment did not completely reverse the high mtROS level induced by the inhibition of Pro-PRODH catabolism (Figures S6L-M & S7C). Together, Pro-PRODH catabolism maintains mtROS homeostasis through a GSH-independent mechanism.

NAD, as a substrate of Sirts, alters the enzyme activity by altering the acetylation modification of mitochondrial proteins.²⁷ SOD2 is the main ROS scavenging enzyme localized in mitochondria to maintain mtROS, and its activity can be regulated by the NAD-Sirts axis.²⁸ Specifically, Sirt3 and Sirt1 regulated SOD2 enzymatic activity by promoting SOD2 deacetylation and SOD2 transcriptional expression, respectively (Figure 7B). We found that T2FA reversed the Pro-mediated high enzymatic activity of SOD2 (Figure 7C). Further WB analysis confirmed that changes in the enzymatic activity of SOD2 were associated with the expression of Sirt1 and SOD2, rather than Sirt3-mediated deacetylation of SOD2 (Figure 7D). The importance of Sirt1-SOD2 axis in mediating Pro-PRODH catabolic regulation of mtROS homeostasis and angiogenesis was further investigated by activating Sirt1 and silencing SOD2 using Sirt1-specific agonist (SRT1720, 0.5 μ M) and si-RNA (si-SOD2), respectively. Sirt1 agonist rescued T2FA-induced mtROS homeostatic imbalance and impaired angiogenesis, and this rescue was blocked by SOD2 silencing (Figures 7F-H & S6N). Sirt1 has been reported to mediate the transcriptional activation of SOD2 via deacetylation of the nuclear transcription factor Forkhead box O3a (FoxO3a),^{29,30} so we assessed the acetylation level of FoxO3a in the nucleus after Pro and/or T2FA treatment. Pro treatment reduced the acetylation level of FoxO3a but this effect was eliminated by T2FA (Figure 7I). Notably, activation of Sirt1 reversed T2FA-induced FoxO3a hyperacetylation and low SOD2 expression (Figure 7J). These findings indicate that Pro-PRODH catabolism maintains mtROS homeostasis under hypoxia in the Sirt1-SOD2-dependent pathway, where SOD2 activity may be regulated by FoxO3a deacetylation-mediated expression.

Reduced Sirt1-SOD2-ROS axis is associated with impaired angiogenesis and reduced Pro-PRODH metabolic activity in pig and human placentae

Next, we analyzed the association of Sirt1-SOD2 axis activity, PRODH metabolic activity and angiogenesis in porcine cohorts (training sets). LBW placentae consistently exhibit lower Sirt1, SOD2, NAD and PRODH enzyme activity or levels (Figures S8A-D). Moreover, the Sirt1-SOD2 axis and PRODH enzyme activity were negatively correlated with placental ROS and positively correlated with placental vascular density and fetal weight, indicating their importance in pregnancy outcomes related to placental angiogenesis and redox homeostasis (Figure S8E).

DISCUSSIONS

Functional amino acids such as Arg and Gln have been well established as crucial regulators of fetal and placental development.² However, the role and mechanism of Pro in placental angiogenesis remains poorly understood. Here, we analyzed the amino acid metabolic profile of sows and mouse placentae and found that Pro availability was down-regulated in LBW placentae and it was associated with increased placental hypoxia and poor placental angiogenesis. Obtaining Pro from the microenvironment is crucial for organisms in stressful conditions (such as ischemic injury, wound healing, and hypoxic tumors formed due to insufficient blood supply and nutrient deficiency) and in situations of nutrient deprivation.³¹ Unlike Arg and Gln, which can be efficiently synthesized de novo in most tissues, Pro biosynthetic capacity is limited in the placenta.⁴ Under nutritional restriction or gestational stress, endogenous Pro production is insufficient to meet demand, making placental Pro availability highly reliant on maternal dietary intake.¹⁰ In the present study, Pro levels in serum and placenta were strongly correlated, supporting its practicality as a nutritionally intervenable target. We further found that dietary Pro deprivation reduced placental Pro availability in the LBW model, suggesting that dietary Pro complements the placental Pro pool. This is consistent with our recent study in pigs, in which placental Pro availability was markedly decreased in obese sows but could be restored by dietary Pro supplementation.¹⁰ Importantly, placental reduced Pro availability exacerbates impaired pregnancy and impaired placental angiogenesis in the LBW model. Mechanistically, we found that Pro regulates mtROS homeostasis and subsequently promotes placental angiogenesis through the mediation of

p-STAT3^{Tyr705}-bFGF/VEGF-A pathway. Additionally, Pro-PRODH metabolism maintains mtROS homeostasis through the Sirt1/SOD2-dependent pathway. These findings support the emerging paradigm that Pro functions as a conditionally essential amino acid.

Redox homeostasis in placental endothelial cells, particularly the maintenance of mtROS balance, is essential for the expression of angiogenesis-related factors. In our previous study, NOX2 was shown to impair placental angiogenesis by generating mtROS, which destabilized the transcription factor p-STAT3 and consequently reduced VEGF-A expression.²¹ Although maternal nutrition may influence placental redox status, dietary antioxidant supplementation has been shown to have limited efficacy in preventing oxidative stress-associated impairment of placental angiogenesis.³² In the present study, we demonstrate that Pro enhances the transcriptional activity of the p-STAT3^{Tyr705}-VEGF-A/bFGF axis by maintaining mtROS homeostasis, thereby preserving normal placental vascularization. These findings reinforce the central role of STAT3 in placental vascular development. However, the precise mechanistic link between mtROS and STAT3 activation remains unclear. Previous studies suggest that mtROS accumulation can inhibit Janus kinase, an upstream activator of STAT3, and can also upregulate SHP-1, a protein phosphatase that negatively regulates STAT3 activity.³³⁻³⁵ Accordingly, the antioxidative effect of Pro may facilitate STAT3 activation at multiple points within this signaling cascade.

The relationship between Pro-PRODH catabolism and mitochondrial function, particularly mtROS homeostasis, is complex and context-dependent.³⁶ During Pro-PRODH catalysis, electrons are transferred to flavin adenine dinucleotide (FAD) at the enzyme's active site. Depending on the cell type and metabolic state, these electrons can either directly reduce oxygen to form superoxide or enter the electron transport chain via cytochrome c to generate ATP.³⁶ Our results indicate that exogenous Pro preserves the functional integrity of the mitochondrial respiratory chain without exacerbating oxidative stress. Moreover, elevated Pro availability has been shown to alleviate oxidative stress.³⁷ Mitochondrial ROS scavenging is mediated by both non-enzymatic systems, such as GSH, and enzymatic systems, such as SOD2. SOD2, localized in the mitochondrial matrix, plays a critical role in eliminating mitochondrial O₂-.²⁸ However, prior to this, the exact source of the antioxidant capacity of Pro remained unclear. Our findings suggest that under hypoxic conditions, Pro-PRODH catabolism promotes mtROS clearance in placental vascular endothelial cells primarily via SOD2 rather than GSH, providing a mechanistic explanation for Pro's antioxidant function.

SOD2 activity is regulated by protein deacetylation. For example, Sirt3 and Sirt1 modulate SOD2 activity by promoting SOD2 deacetylation and SOD2 transcriptional expression, respectively.²⁷ In this study, Pro-PRODH metabolism was found to increase NAD levels and further promote Sirt1-mediated high SOD2 expression. Previous studies reported that adverse placentation and poor pregnancy outcome may be due to premature placental aging caused by Sirt1 deficiency.³⁸ In the present study, the NAD-Sirt1-SOD2 axis and PRODH enzyme activity were negatively correlated with placental ROS and positively correlated with placental vascular density and fetal weight, further highlighting the importance of Sirt1 in pregnancy outcomes related to placental development and redox homeostasis. In addition, how Sirt1 is associated with the high activity of SOD2 enzyme was initially explored in the present study. A previous study has shown that Sirts-mediated post-translational FoxO3a modification regulates its transcriptional activity, allowing the expression of pro-angiogenic factors to support the development of vascular network in growing tumors.³⁹ In this study, Sirt1 agonist was shown to reverse the high FoxO3a acetylation level and low SOD2 expression caused by PRODH metabolism inhibition. These findings indicate that Pro-PRODH catabolism maintains mtROS homeostasis under hypoxia through the Sirt1-SOD2-dependent pathway, where the SOD2 activity may be regulated by FoxO3a deacetylation-mediated expression. However, reports on the role of placental FoxO3a in placental angiogenesis are rather scarce. Therefore, it is interesting to further reveal the link between Pro metabolism and the FoxO3a-SOD2 axis.

Pro-PRODH metabolism exhibits multiple metabolic fates depending on tissue type and physiological context. In plants, Pro catabolism via PRODH can be bypassed under normal growth conditions without overt phenotypic consequences.⁴⁰ In contrast, the porcine placenta displays a high capacity for Pro

catabolism, including P5C-Orn pathway, which can contribute to polyamine biosynthesis essential for cell proliferation.⁴¹ Paradoxically, the retinal pigment epithelium tends to export Pro-derived TCA intermediates (Glu) for use by the retina despite the use of Pro at a high rate.⁵ In our study, although Pro catabolism increased Glu production, stable isotope tracing revealed that PVECs under hypoxic conditions were inefficient in utilizing Pro to fuel the TCA cycle. This may be explained by the inhibitory effects of hypoxia on mitochondrial respiration, which limit TCA cycle activity, thereby suppressing the P5C-Glu pathway.³¹ Notably, we also observed that Pro-PRODH metabolism elevates cellular NAD and NADH levels, suggesting a broader metabolic role beyond carbon metabolism. The Kynurenine pathway (KP) is the sole de novo NAD biosynthetic pathway, generating NAD from ingested tryptophan (Trp).⁴² Our unpublished data indicate that exogenous Pro increases the metabolic utilization of Trp by cells, which can be reversed by PRODH inhibition. These findings suggest that Pro-PRODH catabolism may act as a metabolic switch to modulate the Trp-KP-NAD axis in the placenta, linking amino acid metabolism to redox and energy homeostasis. Further studies are required to validate this hypothesis and clarify the mechanistic details.

There are several limitations to the present study. (i) Placenta-specific STAT3 or PRODH knockout mouse models have not been established. Moreover, trophoblast-specific knockout models are hampered by high embryonic lethality, and placenta endothelial cell-specific knockout mice are currently unavailable.⁴³ Accordingly, we employed relatively cost-effective yet less efficient siRNAs and pharmacological inhibitors to investigate the functions of these key genes in mouse models and cell lines, with rigorous validation performed to ensure the reliability of our *in vivo* observations. (ii) Due to the lack of commercial standards for P5C, the direct downstream metabolite of Pro, we did not quantify P5C levels using metabolomics or stable isotope tracing, nor did we perform P5C supplementation experiments to verify its regulatory role in mtROS production. (iii) We did not reveal the complete metabolic destination of Pro in the placenta or PVEC cell lines.

CONCLUSIONS

In this study, we analyzed amino acid profiles and vascular development in pig placentae at different gestational time points, and found that decreased placental proline availability was associated with impaired angiogenesis. In a nutrient-restricted mouse model, dietary proline deficiency reduced placental proline availability, further increased the incidence of LBW, and exacerbated placental vascular defects. Mechanistically, Pro regulates mtROS homeostasis and subsequently promotes placental angiogenesis through the mediation of p-STAT3^{Tyr705}-bFGF/VEGF-A pathway. Moreover, Pro-PRODH metabolism maintains mtROS homeostasis through GSH-independent and Sirt1/SOD2-dependent pathways. Collectively, these results demonstrate that dietary Pro plays a crucial role in placental angiogenesis and identify the Pro-PRODH-mtROS-STAT3-bFGF/VEGF-A axis as a potential target for the prevention of LBW.

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AUTHOR CONTRIBUTIONS

Conceptualization: CQT, JP, YLY; Methodology: SBH, DYW, CJH; Investigation: SBH, DYW, ZHC, KDM; Visualization: SBH; Supervision: CQT; Writing-original draft: SBH; Writing-review & editing: CQT, JP, YLY. All the authors read and agreed to publish the final version of the manuscript.

DECLARATION OF INTERESTS

Yulong Yin is an Editorial Board member of The Innovation Life and was blinded from reviewing or making final decisions on the manuscript. Peer review was handled independently of this member and their research group. The other authors declare no conflicts of interest. All data needed to evaluate the conclusions in the paper are presented in the paper and/or the Supplementary Information.

ETHICAL STATEMENT AND PATIENT CONSENT

The experimental protocol adopted in this study was reviewed and approved by the Animal Care and Use Committee of South China Agricultural University (2023G017).