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Research Article

Maternal L-proline supplementation enhances fetal survival, placental development, and nutrient transport in mice†

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Abstract

L-Proline (proline) in amniotic fluid was markedly increased during pregnancy in both pigs and sheep. However, in vivo data to support a beneficial effect of proline on fetal survival are not available. In this study, pregnant C57BL/6J mice were fed a purified diet supplemented with or without 0.50% proline from embryonic day 0.5 (E0.5) to E12.5 or term. Results indicated that dietary supplementation with proline to gestating mice enhanced fetal survival, reproductive performance, the concentrations of proline, arginine, aspartic acid, and tryptophan in plasma and amniotic fluid, while decreasing the concentrations of ammonia and urea in plasma and amniotic fluid. Placental mRNA levels for amino acid transporters, including *Slc36a4*, *Slc38a2*, *Slc38a4*, *Slc6a14*, and Na^+/K^+ ATPase subunit-1α (*Atp1a1*), fatty acid transporter *Slc27a4,* and glucose transporters *Slc2a1* and S*lc2a3*, were augmented in proline-supplemented mice, compared with the control group. Histological analysis showed that proline supplementation enhanced labyrinth zone in the placenta of mice at E12.5, mRNA levels for *Vegf*, *Vegfr*, *Nos2*, and *Nos3*, compared with the controls. Western blot analysis showed that proline supplementation increased protein abundances of phosphorylated (p)-mTORC1, p-ribosomal protein S6 kinase (p70S6K), and p-eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), as well as the protein level of GCN2 (a negative regulator of mTORC1 signaling). Collectively, our results indicate a novel functional role of proline in improving placental development and fetal survival by enhancing placental nutrient transport, angiogenesis, and protein synthesis.

Summary Sentence

Proline supplementation enhances placental nutrient transport, angiogenesis, and protein synthesis, which are critical for fetal survival in mice.

Key words: fetal survival, general control nonderepressible 2, L-proline, mammalian target of rapamycin complex 1, mice, nutrient transporters, placenta.

Abbreviations

4E-BP1:	eukaryotic translation initiation factor 4E-binding protein 1
ATF4:	activating transcription factor 4
Atp1a1 :	Na^+/K^+ ATPase subunit-α1
eIF2α:	eukaryotic initiation factor 2α
GCN2:	general control nonderepressible 2
HPLC:	high-performance liquid chromatography
IUGR:	intrauterine growth restriction
mTORC1:	mammalian target of rapamycin complex 1
Nos2 :	nitric oxide synthase 2
Nos3 :	nitric oxide synthase 3
p70S6K:	ribosomal protein S6 kinase
Slc2a1 :	solute carrier family 2 member 1
Slc2a3 :	solute carrier family 2 member 3
Slc6a14 :	solute carrier family 6 member 14
Slc27a4 :	solute carrier family 27 member 4
Slc36a1 :	solute carrier family 36 member 1
Slc36a4 :	solute carrier family 36 member 4
Slc38a1 :	solute carrier family 38 member 1
Slc38a2 :	solute carrier family 38 member 2
Slc38a3 :	solute carrier family 38 member 3
Slc38a4 :	solute carrier family 38 member 4
Vegf :	vascular endothelial growth factor
Vegfr :	vascular endothelial growth factor receptor

Introduction

Embryonic loss and fetal death during gestation period are major reproductive problems in humans and animals [1, 2]. A variety of factors impact the outcome of embryonic and fetal survival, including gestational age [3], limited uterine capacity [1], placental insufficiency [4], maternal nutrition [5], and reproductive hormones [6]. As the building blocks of tissue proteins, amino acids play a major role in conceptus survival, growth, and development [5, 7, 8]. Thus, insufficient protein intake during pregnancy is associated with placental insufficiency, embryonic loss, intrauterine growth restriction (IUGR), as well as increased risk for developing metabolic disorders in the offspring later in life [8].

The placenta is responsible for the delivery of nutrients from mother to fetus and, therefore, is a major determinant of fetal growth [9]. Placental insufficiency can reduce the activity and expression of amino acid transporters, therefore limiting the availability of nutrients for the fetus and contributing to IUGR, embryonic loss, and fetal death in mammals [1, 10–12]. Accumulating evidence shows that a deficiency of specific amino acids, such as L-arginine and L-glutamine, impairs not only neonatal survival and development but also embryonic development [13–15]. Consistently, L-arginine or L-glutamine supplementation during gestation can improve reproductive performance and enhance embryonic growth and survival [16], indicating a functional role of amino acids in gestating mammals [1, 17].

L-Proline (proline) is an imino acid with versatile roles, such as enhancing protein synthesis by activating mTOR signaling [18], regulating gene expression [19], scavenging free radicals, and maintaining intracellular redox state [20]. In addition, proline serves as a major nitrogenous substrate for the synthesis of polyamines, key regulators of DNA and protein synthesis, cell proliferation and differentiation, in both the small intestine and placentae of gestating pigs [21–23] and sheep [24]. It has been reported that the concentration of proline in amniotic fluid was markedly increased during pregnancy in both pigs and sheep [24, 25]. Moreover, reduced placental and fetal growth is associated with reductions in placental proline transport in gestating dams with either naturally occurring or malnutrition-induced growth retardation [19]. All these lines of indirect evidence indicate a crucial role of proline in fetal nutrition and metabolism. However, in vivo data that support this concept are not available.

Based on the forgoing, we hypothesized that dietary supplementation with proline may enhance fetal survival by promoting placental nutrient transport and protein synthesis. This hypothesis was tested with the gestating mice, a widely used animal model for studying human reproduction [2, 26].

Materials and methods

Chemicals

Proline and L-alanine were purchased from Sangon Biotech (Shanghai, China). Antibodies against mammalian target of rapamycin complex 1 (mTORC1, catalog no. #2972), phosphorylated (p)-mTORC1 catalog no. #2971, eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1, catalog no. #9452), p-4E-BP1 (catalog no. #13396), eukaryotic translation initiation 2α (eIF2α, catalog no. #2103), p-eIF2α (catalog no. #3597), ribosomal protein S6 kinase (p70S6K, catalog no. #9202), p-p70S6K (catalog no. #9234), general control nonderepressible 2 (GCN2, catalog no. #3302), and activating transcription factor 4 (ATF4, catalog no. #11815) were products of Cell Signaling Technology (Beverly, MA) unless otherwise stated. β-Actin (catalog no. SC-47778) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were procured from Huaxingbio Biotechnology Co. (Beijing, China). Amino acid standards for high-performance liquid chromatography (HPLC) analysis were obtained from Sigma-Aldrich (St. Louis, MO). Unless indicated, all other chemicals were obtained from Sigma-Aldrich.

Mice and diets

This study was conducted in accordance with the guidelines for animal protocols approved by Institutional Animal Care and Use Committee of China Agricultural University. Virgin female C57BL/6J mice aged 8 weeks were obtained from Beijing Huafukang Bioscience Co. Inc (Beijing, China). During the whole experimental period, mice were individually raised in an environment with the temperature set at 23 °C and a 12-h light/dark cycle. The mice had free access to feed and water. Mice with normal estrus cycles as determined by daily vaginal smears were used for the study. The presence of a vaginal plug was considered as embryonic day 0.5 (E0.5). A total of 48 pregnant mice were assigned randomly to either control or proline group (n = 24 dams/group). The mice were fed an AIN-93G purified diet [27, 28] supplemented with or without 0.50% (wt: wt) proline. This dose of proline was based on results of our pilot study showing that supplementation with 0.5% proline to the basal diet

Table 1. Amino acid composition of the basal diet (as-fed basis).

Items	Content, %
Alanine	0.52
Arginine	0.53
Aspartic acid + Asparagine (1:0.66, g/g)	1.29
Cystine	0.36
Glycine	0.30
Glutamic acid + Glutamine (1:1.19, g/g)	3.74
Histidine	0.45
Isoleucine	0.88
Leucine	1.70
Lysine	1.33
Methionine	0.49
Phenylalanine	0.80
Proline	1.80
Serine	0.92
Threonine	0.75
Tryptophan	0.22
Tyrosine	0.79
Valine	1.14

improved reproductive performance without exerting adverse effect. The isonitrogenous control diet was the basal diet supplemented with 0.39% L-alanine and 0.11% cornstarch. The amino acid content in the basal diet was analyzed by HPLC as previously described [1], and is summarized in Table 1. Half of the mice in each group were euthanized at embryonic day 12.5 (E12.5) for fetal survival and placental analysis, whereas the others remained to be fed the control or proline-supplemental diet until delivery for reproductive performance analysis. Feed intakes and body weight changes of dams during the experimental period were recorded.

Blood sample, placenta, and amniotic fluid collection

At E12.5, 12 mice from each group were randomly selected and intraperitoneally anesthetized with sodium pentobarbital (40 mg/kg body weight). Blood samples from retro-orbital were collected, and plasma was obtained after centrifugation at 3000 g for 15 min. Plasma samples were stored at –80°C until analysis for amino acids, ammonia, and urea. After blood samples were collected, uterine horns were immediately exposed, and the number and weight of fetuses were recorded. Placentae and amniotic fluid were obtained and stored at –80 °C until later analysis.

Analysis of amino acids, ammonia, and urea in plasma and amniotic fluid

Amino acids in plasma and amniotic fluid were analyzed by HPLC methods involving pre-column derivatization with o-phthaldialdehyde as previously described [1]. Ammonia and urea were determined by using assay kits from Nanjing Jiancheng Biochemistry (Nanjing, China).

Quantitative real-time PCR analysis

Total RNA was extracted with the Trizol reagent (CWBio Biotech Co., Beijing) and reverse-transcribed by using a cDNA archive kit (TIANGEN, China). Quantitative real-time qPCR was performed to determine the mRNA levels of genes in placentae. Primer sequences used in this study are shown in Supplemental Table S1. *β-Actin* was used as an internal control for normalization.

Table 2. Fetal survival of C57BL/6J mice fed a purified diet supplemented with or without L-proline between E0.5 and E12.5.[1]

Items	Control[2]	Proline[2]
Total fetus number, n/litter	7.7 ± 0.22	9.1 ± 0.19*
Live fetus number, n/litter	7.4 ± 0.23	8.8 ± 0.25*
Placental weight, mg	56.3 ± 0.93	57.9 ± 1.04
Fetal weight, mg	59.0 ± 0.24	61.7 ± 0.90
Maternal feed intake, g/d	2.99 ± 0.04	3.06 ± 0.03

[1]Data are means ± SEMs, n = 12. *Different from the control group, $P < 0.05$.

[2]Control, L-alanine-supplemented diet; Proline, L-proline-supplemented diet.

Placental morphological analysis

The placentae harvested at E12.5 were fixed in 4% paraformaldehyde. The tissues were dehydrated, embedded in paraffin wax, and cut into 4 μm thickness cross-sections. Serial sections were stained with hematoxylin and eosin (H&E) and photographed using a fluorescence microscope (Olympus IX50) linked to the NIS-ELEMENTS F3.2 software. The areas of decidua, labyrinth, and junctional zone were determined as previously described [29]. A minimum of six fields for each placental section, 12 placentas per group, were randomly selected for the morphometric qualification of placentae. The areas of labyrinth zone, junctional zone, and maternal decidua were expressed as the percentage of the total placental area.

Western blot analysis

Frozen placentae were homogenized in a ceramic mortar with liquid N_2 and then lysed in the ice-cold radioimmunoprecipitation assay lysis buffer containing 50 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 0.1% SDS, 1.0 mmol/L phenylmethanesulfonyl fluoride (PMSF), 1.0 mmol/L Na_3VO_4, and 1.0 mmol/L NaF. The concentration of protein was measured with a bicinchoninic acid protein assay kit (Applygen Technologies). Equal amounts of proteins (50 μg) were separated on 10% SDS-PAGE gels, transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA), and blocked in 5% nonfat milk (for nonphosphorylated proteins) or 5% bovine serum albumin (for phosphorylated proteins) for 1 h at 25°C. The membranes were incubated with a primary antibody overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The protein bands were incubated with an enhanced chemiluminescence kit (Huaxingbio, Beijing, China). Images were developed by using the ImageQuant LAS 4000 mini system (GE Healthcare). Quantification of band density was determined by using the Image-Pro Plus 6.0 software (Media Cybernetics). β-Actin was used as a loading control.

Statistical analysis

Values are expressed as means ± SEMs. The difference between the two groups was determined by the unpaired Student *t*-test with the use of GraphPad PRISM version 7.0 (GraphPad Software, Inc., San Diego, CA). $P \leq 0.05$ was considered to indicate statistical significance.

Results

Fetal survival and reproductive performance of mice

As shown in Table 2, mice fed with a proline-supplemented diet had a greater number of total fetuses ($P < 0.05$) and live fetuses ($P < 0.05$)

Table 3. Reproductive performance of C57BL/6J mice fed a purified diet supplemented with or without L-proline during pregnancy.[1]

Items	Control[2]	Proline[2]
Litter size, n	7.5 ± 0.25	8.9 ± 0.25*
Live-born mice, n/litter	7.2 ± 0.27	8.5 ± 0.31*
Individual birth weight of born mice, g	1.27 ± 0.01	1.28 ± 0.01
Individual birth weight of mice born alive, g	1.27 ± 0.01	1.28 ± 0.01
Maternal feed intake, g/d	3.17 ± 0.04	3.24 ± 0.03

[1]Data are means ± SEMs, n = 12. *Different from the control group, $P < 0.05$.

[2]Control, L-alanine-supplemented diet; Proline, L-proline-supplemented diet.

at E12.5, as compared with the controls. Proline supplementation had no effect on maternal feed intake, fetal weight, or placental weight at E12.5 (Table 2). In line with the data at E12.5, dietary supplementation with proline enhanced ($P < 0.05$) litter size and the number of live-born mice at term, without affecting the birth weight of pups and maternal feed intake during the experimental period (Table 3).

Concentrations of amino acids, ammonia, and urea in plasma and amniotic fluid

Compared with controls, the concentrations of proline, arginine, aspartic acid, and tryptophan were increased ($P < 0.05$) in the plasma and amniotic fluid of proline-supplemented mice at E12.5 (Table 4). The concentration of ornithine in plasma and that of glutamine in the amniotic fluid of proline-supplemented mice were also increased ($P < 0.05$) as compared with controls. Concentrations of other amino acids in maternal plasma and amniotic fluid did not differ between the two groups of mice (Table 4). In addition, concentrations of ammonia and urea were decreased ($P < 0.05$) in the maternal plasma and amniotic fluid of proline-supplemented mice (Table 5), as compared with controls.

Placental nutrient transporters in mice

Dietary proline supplementation enhanced ($P < 0.05$) mRNA levels of solute carrier family 36 member 4 [*Slc36a4*, a proline transporter], Na^+/K^+ ATPase subunit-α1 (*Atp1a1*), as well as neutral amino acid transporters of the solute carrier family 38 member 2 (*Slc38a2*) *and Slc38a4* in placental tissues (Figure 1A), as compared with the control group. In contrast, proline supplementation had no effect on *Slc36a1*, *Slc38a1*, and *Slc38a3* (Supplemental Figure S2). In comparison with the controls, the mRNA levels for solute carrier family 2 (glucose transporter) member 1 (*Slc2a1*), *Slc2a3*, and fatty acid transporter (solute carrier family 27 member 4, *Slc27a4*) were enhanced ($P < 0.05$) in proline-supplemented mice (Figure 1B).

Placental morphology and vascularization

The mouse placenta consists of the maternal decidua, the junctional zone, and the labyrinth zone. To explore whether the increased fetal survival and reproductive performance were associated with changes in placental morphology, placentae at E12.5 were collected and stained. We observed a decreased ratio of decidua area/total area ($P < 0.05$), and an increased ratio of labyrinth area/total area ($P < 0.05$) in proline-supplemented mice (Figure 2A and B). Placental angiogenesis is critical for fetal survival and growth, in which several factors including vascular endothelial growth factor (VEGF),

Table 4. Concentrations of amino acids in plasma and amniotic fluid of C57BL/6J mice fed a purified diet supplemented with or without L-proline between E0.5 and E12.5.[1]

Items	Plasma (μmol/L)		Amniotic fluid (μmol/L)	
	Control[2]	Proline[2]	Control[2]	Proline[2]
Arginine	123 ± 2.2	154 ± 9.5*	450 ± 16.3	546 ± 18.3*
Aspartic acid	20 ± 2.0	27 ± 2.1*	53 ± 10.2	86 ± 6.5*
Glutamine	479 ± 41.5	489 ± 29.9	1308 ± 65.0	1487 ± 21.9*
Ornithine	46 ± 3.1	59 ± 4.2*	114 ± 4.7	117 ± 4.7
Proline	143 ± 9.6	180 ± 6.4*	879 ± 65.7	1253 ± 136.1*
Tryptophan	69 ± 6.6	107 ± 9.5*	78 ± 3.2	94 ± 3.1*
Alanine	523 ± 51.1	448 ± 89.1	1304 ± 97.8	1460 ± 89.4
Asparagine	23 ± 1.5	24 ± 2.9	213 ± 14.0	247 ± 5.8
Citrulline	59 ± 6.1	61 ± 4.8	17 ± 0.6	19 ± 0.6
Glutamate	71 ± 4.5	85 ± 6.3	137 ± 32.3	198 ± 18.0
Glycine	142 ± 9.3	167 ± 6.3	585 ± 61.1	606 ± 27.4
Histidine	20 ± 0.8	24 ± 4.3	277 ± 27.7	310 ± 7.2
Hydroxyproline	12 ± 0.7	16 ± 1.2	48 ± 9.7	54 ± 3.8
Isoleucine	89 ± 7.1	100 ± 9.6	230 ± 14.4	244 ± 13.2
Leucine	84 ± 8.3	99 ± 17.0	491 ± 39.7	539 ± 24.2
Lysine	411 ± 56.0	335 ± 46.5	1636 ± 33.7	1547 ± 59.6
Methionine	49 ± 3.7	50 ± 4.7	165 ± 13.4	179 ± 9.3
Phenylalanine	29 ± 2.1	28 ± 4.1	215 ± 16.0	237 ± 11.0
Serine	51 ± 5.8	57 ± 5.6	806 ± 56.1	868 ± 26.6
Taurine	810 ± 34.3	791 ± 65.9	1727 ± 174.8	1937 ± 123.7
Threonine	416 ± 45.5	467 ± 34.8	615 ± 31.2	608 ± 37.8
Tyrosine	30 ± 2.5	38 ± 4.6	232 ± 23.5	256 ± 13.5
Valine	154 ± 15.4	166 ± 15.9	677 ± 38.7	708 ± 40.6

[1]Data are means ± SEMs, n = 12. *Different from the control group, $P < 0.05$.

[2]Control, L-alanine-supplemented diet; Proline, L-proline-supplemented diet.

Table 5. Concentrations of ammonia and urea in plasma and amniotic fluid of C57BL/6J mice fed a purified diet supplemented with or without L-proline between E0.5 and E12.5.[1]

Items	Plasma		Amniotic fluid	
	Control[2]	Proline[2]	Control[2]	Proline[2]
Ammonia, μmol/L	229 ± 5.82	198 ± 6.49*	218 ± 13.35	179 ± 9.16*
Urea nitrogen, mmol/L	8.6 ± 0.26	7.6 ± 0.37*	9.2 ± 0.12	7.8 ± 0.29*

[1]Data are means ± SEMs, n = 12. *Different from the control group, $P < 0.05$.
[2]Control, L-alanine-supplemented diet; Proline, L-proline-supplemented diet.

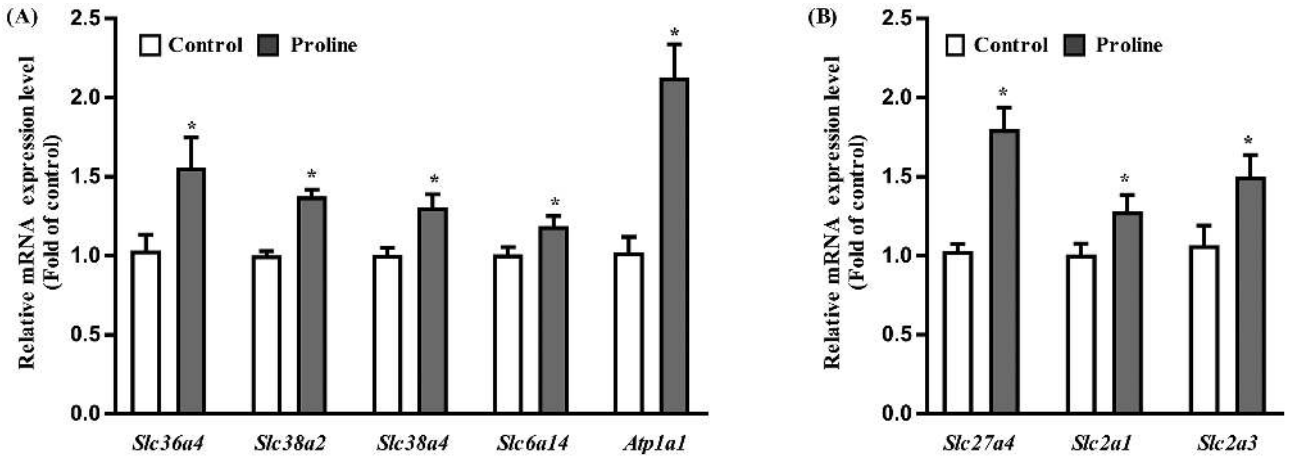

Figure 1. Abundances of mRNAs for transporters in the E12.5 placentae of C57BL/6J mice fed a purified diet supplemented with or without L-proline between E0.5 and E12.5. Values are means ± SEMs, n = 12. *Different from the control group, $P < 0.05$. Control, L-alanine-supplemented diet; Proline, L-proline-supplemented diet; E, gestational day; *Atp1a1*, Na^+/K^+ ATPase subunit-α1; *Slc2a1*, solute carrier family 2 member 1; *Slc2a3*, solute carrier family 2 member 3; *Slc6a14*, solute carrier family 6 member 14; *Slc27a4*, solute carrier family 27 member 4; *Slc36a4*, solute carrier family 36 member 4; *Slc38a2*, solute carrier family 38 member 2; *Slc38a4*, solute carrier family 38 member 4.

fibroblast growth factor (FGF), and nitric oxide (NO) are involved [30]. Quantitative real-time PCR showed that the mRNA levels of *Vegf*, *Vegfr*, *Nos2*, and *Nos3* were upregulated ($P < 0.05$) in the placentae of proline-supplemented mice at E12.5 (Figure 2C), as compared with the controls.

Placental proteins of mTORC1 and GCN2 pathways

Western blot was performed to determine the activation of mTORC1 signaling in placental tissues of mice supplemented with or without proline. Proline supplementation enhanced protein levels for p-mTORC1 (Figure 3A), as well as p-p70S6K (Figure 3B) and p-4E-BP1 (Figure 3C), two downstream targets of mTORC1. Further study showed that the protein abundance of GCN2, a negative regulator of mTORC1, was reduced ($P < 0.05$) in the placental tissue of proline-supplemented mice (Figure 3D). Consistently, the protein abundances of p-eIF2α (Figure 3E) and ATF4 (Figure 3F), two downstream targets of GCN2, were reduced ($P < 0.05$) in the placental tissues of proline-supplemented mice.

Discussion

Proline is a major amino acid for the synthesis of protein, arginine, and polyamines in the conceptus [19]. In the present study, we found that dietary supplementation with proline enhanced fetal survival at both E12.5 and reproductive performance at term. Further study showed that this effect of proline was associated with increases in placental transport, vascular development, and activation of mTORC1 (a critical signaling pathway for protein synthesis), as well as the suppression of the GCN2-eIF2α-ATF4 signaling pathway in the placental tissue. This is the first in vivo study showing a functional role of proline on fetal survival and placental development in animals.

Abundant nutrients in amniotic fluid, such as amino acids and polyamines, are critical for survival and development of fetus and placental growth in mammals [25]. An elevated concentration of proline in the amniotic fluid of pregnant dams [24, 25] as well as a positive correlation between reduced fetal growth and decreased placental proline transport in gestating dams [19] suggests a dietary requirement of proline for fetal survival and development. However, in vivo data to support this concept are not available. To test our hypothesis, virgin C57BL/6J mice were fed a purified diet [27, 28] supplemented with or without 0.5% proline. Of note, proline supplementation increased the number of fetuses at E12.5 (Table 2) and the number of live-born mice at term (Table 3), without affecting the birth weight of pups.

In consistence with improved reproductive performance, plasma concentrations of aspartic acid and tryptophan, as well as ornithine and arginine (two metabolites of proline) [19, 20], were markedly elevated in proline-supplemented mice. Importantly, the concentrations of aspartic acid, glutamine, arginine, proline, and tryptophan in amniotic fluid, an important source of nutrients for embryonic development, were also greater in proline-supplemented mice, indicating an increased availability of amino acids for fetal development. It has been reported that dietary arginine supplementation in gestating rats improves fetal survival [31]. Arginine and glutamine

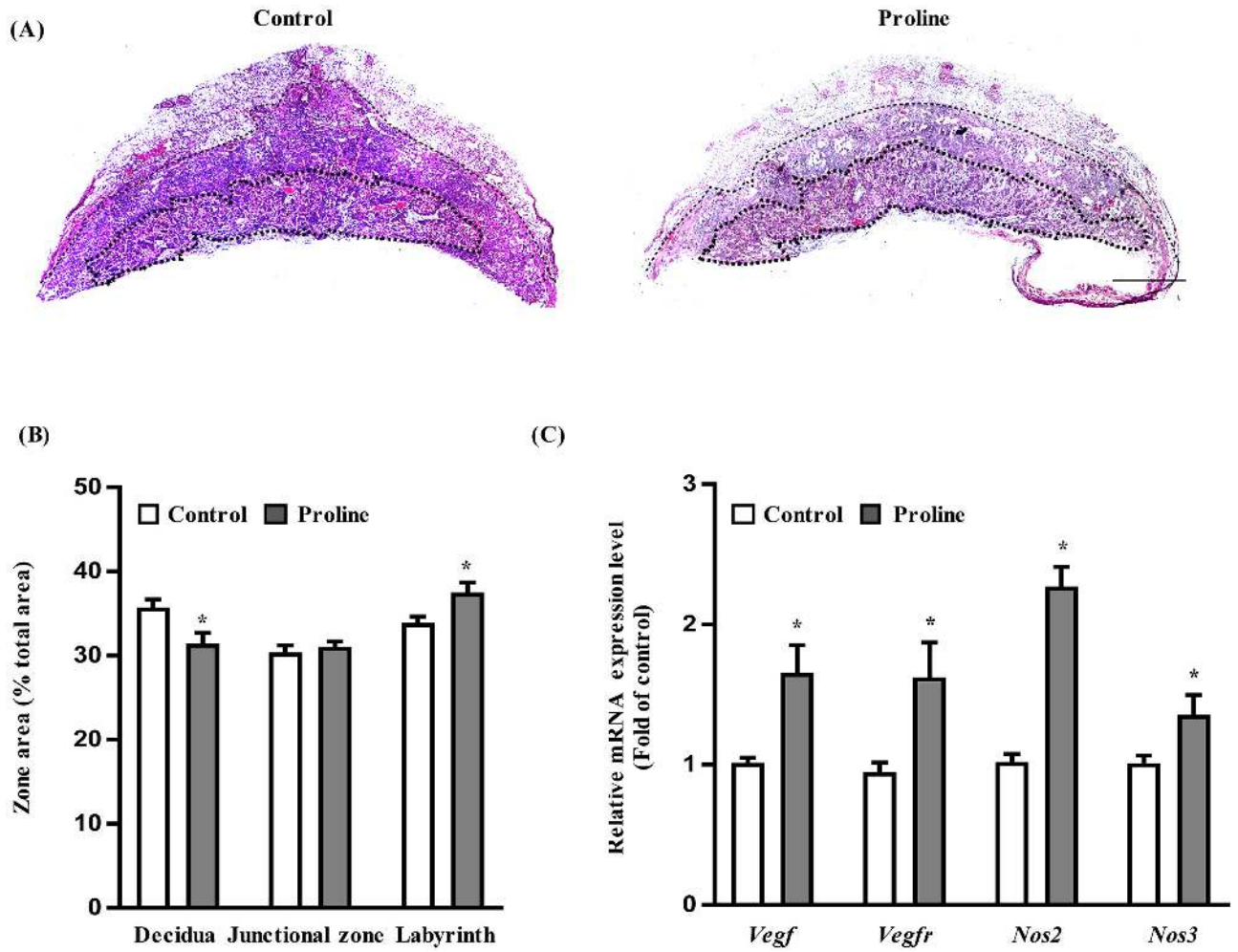

Figure 2. Placental morphology (A, B) and vascularization (C) in the E12.5 placentae of C57BL/6J mice fed a purified diet supplemented with or without L-proline between E0.5 and E12.5. Values are means ± SEMs, n = 12. *Different from the control group, $P < 0.05$. Scale bars = 0.5 mm. Control, L-alanine-supplemented diet; Proline, L-proline-supplemented diet; E, gestational day; *Vegf*, vascular endothelial growth factor; *Vegfr*, vascular endothelial growth factor receptor; *Nos2*, nitric oxide synthase 2; *Nos3*, nitric oxide synthase 3.

are endogenous substrates for proline synthesis [19]. Maternal proline supplementation might spare the utilization of these two amino acids for proline synthesis, therefore increasing their availability in amniotic fluid. An active uptake of glutamine and arginine from amniotic fluid can enhance nucleic acid biosynthesis, which is required by rapidly dividing cells [32]. Additionally, arginase, a critical enzyme for polyamine synthesis, is present in amniotic fluid [33]. Elevated proline and arginine might enhance the production of polyamines, which are key regulators of placental angiogenesis, trophoblast growth, and embryogenesis [21–25] to support embryonic survival. It is intriguing that the concentration of tryptophan was higher in proline-supplemented mice, but the underlying mechanism is unknown. Based on the biochemical pathways of tryptophan metabolism, it is possible that proline supplementation may enhance the production of glutamate and alpha-ketoglutarate, which in turn increases the production of pyruvate and alanine, thereby reducing tryptophan catabolism in a tissue-specific manner. More studies are required to test this hypothesis. Furthermore, the concentrations of ammonia and urea in plasma and amniotic fluid, which are major metabolic endpoint products of amino acids, were reduced in proline-supplemented mice. It is well known that high levels of ammonia are toxic to embryonic survival in animals [34]. Oxidation of proline in maternal tissues generates ornithine, which is essential for hepatic and intestinal synthesis of urea from ammonia [19]. A reduced concentration of ammonia following proline administration might be another mechanism responsible for enhanced fetal survival.

Mammalian TOR is a key nutrient sensor to support placental growth, fetal survival, and growth [15, 35]. Restricting amino acid availability in the maternal diet during pregnancy has been reported to impair fetal survival and development due to downregulation of placental mTOR signaling [15, 35]. In the present study, we found that the phosphorylation of mTORC1 was enhanced, whereas that for GCN2 (a negative regulator of mTORC1) was repressed in the placenta of proline-supplemented mice. Generally, the activation of GCN2 in response to amino acid deficiency leads to (a) the phosphorylation of the α-subunit of eukaryotic initiation factor 2 alpha (eIF2α) to block protein translation and (b) upregulation of ATF4 expression to promote cellular adaptation to the nutritional insult [36]. An interaction between mTORC1 and GCN2 signaling acts together to regulate amino acid homeostasis [37]. The activation of mTORC1, as well as the elevated concentrations of glutamine, aspartic acid, arginine and tryptophan in amniotic fluid, indicated

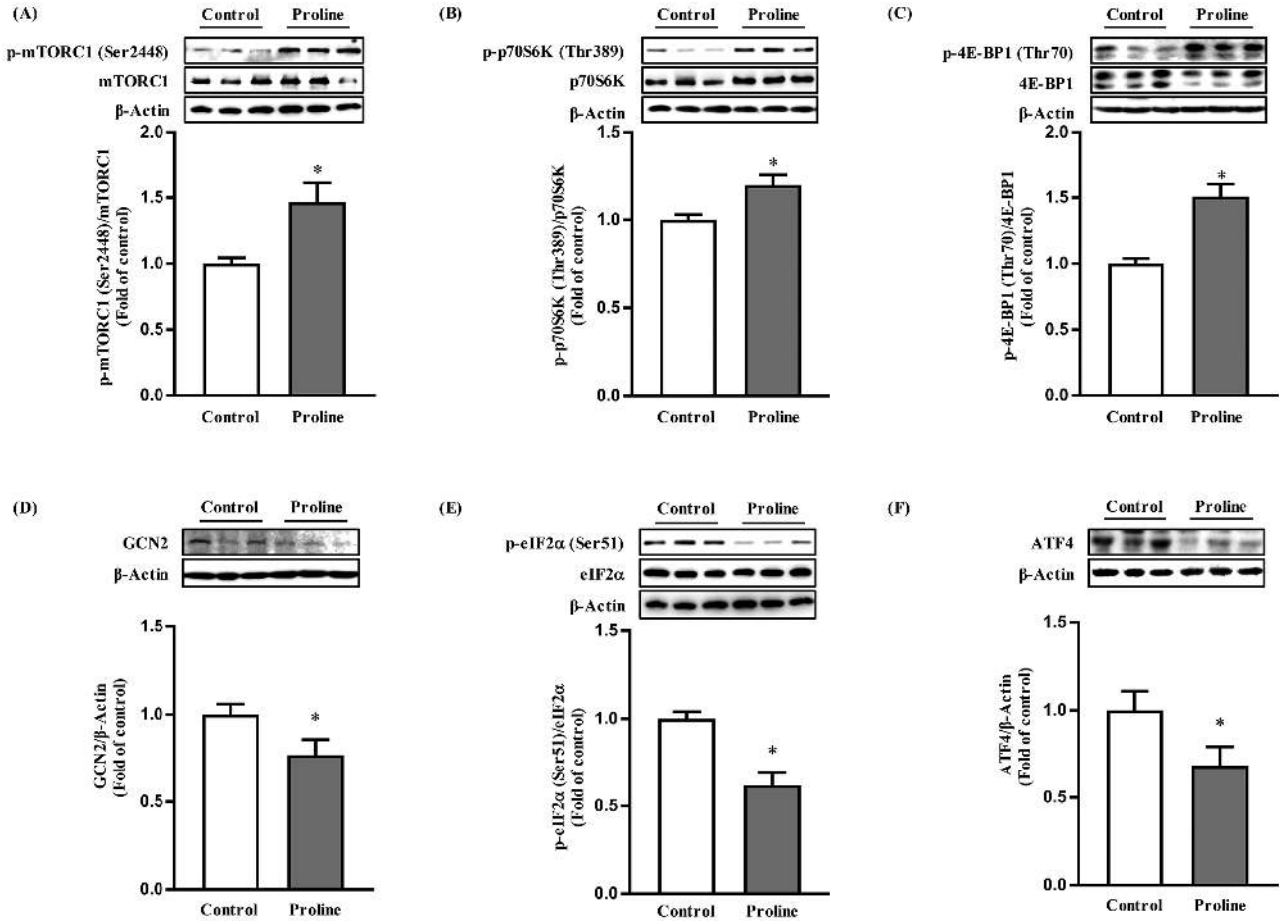

Figure 3. Protein abundance of mTORC1 (A), p70S6K (B), 4E-BP1 (C), GCN2 (D), eIF2α (E), and ATF4 (F) in the E12.5 placentae of C57BL/6J mice fed a purified diet supplemented with or without L-proline between E0.5 and E12.5. Representative western blots are protein abundance from three individual mice. Values are means ± SEMs, n = 12. *Different from the control group, $P < 0.05$. Control, L-alanine-supplemented diet; Proline, L-proline-supplemented diet; E, embryonic day; 4E-BP1, 4E-binding protein 1; ATF4, activating transcription factor 4; eIF2α, eukaryotic initiation factor 2α; GCN2, general control nonderepressible 2; mTORC1, mammalian target of rapamycin complex 1; p-4E-BP1, phosphorylated 4E-binding protein 1; p70S6K, ribosomal protein S6 kinase; p-eIF2α, phosphorylated eukaryotic initiation factor 2α; p-mTORC1, phosphorylated mammalian target of rapamycin complex 1; p-p70S6K, phosphorylated ribosomal protein S6 kinase.

enhanced protein synthesis for embryonic survival in mice. Considering that a large number of upstream signals, including nutrients, hormones, and growth factors [38–40], can activate mTORC1 or inhibit GCN2, further studies are needed to investigate how the multiple signaling pathways are integrated to improve embryonic survival and growth in mammals.

Placenta is the primary interface between the fetus and mother. This organ plays an important role in delivering nutrients and oxygen from mother to fetus to sustain fetal development and growth [30]. A number of protein transporters are expressed in the placenta to facilitate the transport of nutrients and other biological substances from maternal to fetal blood. To explore whether proline supplementation may regulate placental transport of nutrients, we determined the expression of amino acid, glucose, and fatty acid transporters in placental tissues. We found that the mRNA levels for *Slc36a4* (a proline transporter), neutral amino acids transporters (*Slc6a14*, *Slc38a2*, and *Slc38a4*), fatty acid transporter *Slc27a4*, as well as glucose transporter *Slc2a1* and *Slc2a3* were markedly enhanced in the placenta of proline-supplemented mice. Upregulation of nutrient transporters can improve the transfer of nutrients (including amino acids, fatty acids, and glucose) from mother to fetus, thereby contributing to placental development and fetal growth [5, 28, 41, 42].

Development and survival of the mammalian embryo can be influenced by placental size, morphology, blood flow, and vascularity [28, 30]. The mature mouse placenta is a layered structure that is composed of the maternal decidua, the junctional zone, and the labyrinth zone. The junctional zone separates the labyrinth from the maternal decidua, whereas the labyrinth zone provides the interface for exchange between maternal and fetal circulations [29, 30]. In the present study, we found that dietary proline supplementation enhanced the area of the labyrinth zone in the placenta, therefore constituting a greater surface for nutrient and oxygen exchange, as well as the removal of embryonic wastes. Importantly, we observed increases in the mRNA levels for *Vegf*, *Vegfr*, *Nos2*, and *Nos3* in the placenta of proline-supplemented mice, indicating an effect of proline supplementation on the vascular development of the placenta. Proline is a critical amino acid for embryonic and fetal survival in mammals. This finding has important implications for the use of feed-grade proline and its low-cost sources (e.g. meat and bone meal, feather meal, and milk byproducts [43–45]) to improve reproductive efficiency in livestock species.

In summary, results of the present study indicated that maternal proline supplementation enhanced fetal survival and reproductive performance in mice. The beneficial effect of proline was associated with increases in vascular development and protein synthesis in placental tissue, as well as placental transport of amino acids, glucose and fatty acids, and the availability of nutrients in the conceptus. These novel findings indicate a hitherto unrecognized functional role for proline in fetal survival in mice.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Table S1. Primer sequences used for quantitative real-time PCR.

Supplementary Figure S1. Abundances for mRNA levels of amino acid transporters in the E12.5 placentae of C57BL/6J mice fed a purified diet supplemented with or without L-proline between E0.5 and E12.5. Values are means ± SEMs, n = 12. Means without a common letter differ, $P < 0.05$. Control, L-alanine-supplemented diet; Proline, L-proline-supplemented diet; E, embryonic day; *Slc36a1*, solute carrier family 36 member 1; *Slc38a1*, solute carrier family 38 member 1; *Slc38a3*, solute carrier family 38 member 3.

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The authors' responsibilities were as follows. ZW, PT, and GW designed the research; NL, YZ, and JC conducted the research; NL, ZD, and YY analyzed the data; NL, ZW, and GW wrote the manuscript; ZW and GW had primary responsibility for final content. All authors have read and approved the final manuscript.

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