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Placensin is a glucogenic hormone secreted by human placenta

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Abstract

FBN1 encodes asprosin, a glucogenic hormone, following furin cleavage of the C-terminus of profibrillin 1. Based on evolutionary conservation between FBN1 and FBN2, together with conserved furin cleavage sites, we identified a peptide hormone placensin encoded by FBN2 based on its high expression in trophoblasts of human placenta. In primary and immortalized murine hepatocytes, placensin stimulates cAMP production, protein kinase A (PKA) activity, and glucose secretion, accompanied by increased expression of gluconeogenesis enzymes. *In situ* perfusion of liver and *in vivo* injection with placensin also stimulate glucose secretion. Placensin is secreted by immortalized human trophoblastic HTR-8/SVneo cells, whereas placensin treatment stimulates cAMP-PKA signaling in these cells, accompanied by increases in MMP9 transcripts and activities, thereby promoting cell invasion. In pregnant women, levels of serum placensin increase in a stage-dependent manner. During third trimester, serum placensin levels of patients with gestational diabetes mellitus are increased to a bigger extent compared to healthy pregnant women. Thus, placensin represents a placenta-derived hormone, capable of stimulating glucose secretion and trophoblast invasion.

Keywords gluconeogenesis; hormone; invasiveness; placensin
Subject Category Metabolism
DOI 10.15252/embr.201949530 | Received 24 October 2019 | Revised 13 March 2020 | Accepted 27 March 2020 | Published online 24 April 2020
EMBO Reports (2020) 21: e49530

Introduction

In addition to mediating nutrient and waste exchange between maternal and fetal compartments, the placenta is also a hormone-producing organ, capable of regulating maternal and fetal metabolism and hormonal balance [1–3]. With the mother maintaining metabolic homeostasis for herself and the fetus, pregnancy is characterized by stage-dependent increases in insulin resistance with minimal changes in serum glucose levels [4,5]. During normal pregnancy, there is a progressive increase in the insulin/glucose ratio based on the oral glucose tolerance test and hyperinsulinemic–euglycemic clamp [6]. In addition, 6–9% of pregnant women developed gestational diabetes mellitus (GDM) [7], showing increased risk for high blood pressure and preeclampsia [8] as well as for future development of type II diabetes in mothers and children [9,10].

A glucogenic peptide hormone asprosin was recently identified in the *FBN1* gene following cleavage of the C-terminal region of profibrillin 1 by the furin protease [11]. Asprosin is secreted by white adipose tissues and stimulates the liver to release glucose into the circulation. Based on evolutionary conservation of furin cleavage sites and asprosin-like sequences in the C-terminal region of *FBN2* in diverse vertebrates, we discovered a paralogous peptide hormone encoded by *FBN2* and investigated its expression and biological actions. Due to the high expression of *FBN2* in human placenta, this asprosin-like peptide hormone was named placensin. We generated recombinant placensin proteins and demonstrated the ability of placensin to stimulate glucose release by hepatocytes and invasiveness of trophoblasts. We further found that serum placensin, unlike glucogenic glucagon, showed stage-dependent increases during human pregnancy with further elevation in GDM patients.

Results

Comparison between *FBN1* and *FBN2* genes

We compared human *FBN2* and *FBN1* gene structures (Fig 1A). *FBN1* has 66 exons, and asprosin is encoded by the last two exons following a cleavage site for the protease furin [12,13]. In contrast, *FBN2* has 65 exons and an asprosin-like sequence (placensin) was

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found in exons 64 and 65, following a furin cleavage site predicted using the PiTou algorithm [14]. As shown in Fig 1B, human FBN2 encodes placensin (133 residues) that is highly homologous to asprosin (140 residues) with 47% identity and 67% similarity. In diverse vertebrates ranging from fish to human, furin cleavage sites and predicted placensin sequences are highly conserved, especially in the C-terminal region of placensin (Figs 1C and EV1A).

FBN2 tissue expression pattern

We investigated *FBN2* expression in diverse human adult tissues. Similar to findings in the Bio-GPS and Human Protein Atlas databases (Fig EV1B and C) and consistent with an earlier report [15], *FBN2* transcripts were found almost exclusively in the placenta, with much lower levels in diverse other tissues (Fig 2A). In contrast, *FBN2* transcripts in mouse tissues showed a wide expression pattern (umbilical cord > ovary > others) but were low in placenta (Fig 2B). RT–PCR analyses using common primers indicated that *FBN2* transcripts in human placenta were nearly 50-fold higher than its murine counterpart (Fig 2C), consistent with findings showing placenta as the mammalian organ with the highest evolutionary diversity in structure and function [16,17]. Also, serum asprosin levels were low and showed small increases during the third trimester (Fig EV1D). We generated polyclonal antibodies against recombinant placensin. In human placenta, immunostaining using placensin antibodies showed higher positive signals in cytotrophoblasts as compared with syncytiotrophoblasts at 8–9 weeks of pregnancy (Fig 2D, same as published RNA-seq analyses Fig EV1E) and in both cyto- and syncytiotrophoblasts at 38 weeks of pregnancy (Fig 2D). In term pregnancy placenta, immunostaining showed co-localization of placensin and furin signals (Fig EV1F). Immunoblotting of placental villi from term pregnancy using placensin antibodies further indicated immuno-reactive bands with M.W. of ~ 20 kDa corresponding to placensin in three patients (Fig 2E). Treatment with N-glycosidase decreased the sizes of placensin bands during normal pregnancies, consistent with the prediction of one N-linked glycosylation site (residue 29) in the placensin coding region.

Expression of recombinant placensin

Using a prokaryotic expression vector, we generated *Escherichia coli*-derived placensin with tandem GST (glutathione S-transferase) and His tags appended at its N-terminus. Recombinant placensin was retained in the GST glutathione Sepharose 4B column and then eluted by removing the GST tag using PreScission protease (Fig 2F4). Eluted placensin was further purified using a Nickel column, followed by electrophoresis and immunoblotting (Fig 2F, right panel; Fig 2G(a), ~ 16 kDa band). For eukaryotic cell expression, we constructed an adenoviral vector using the placensin cDNA. After infection of CHO cells with adenoviruses encoding placensin, secreted placensin was found as a ~ 20 kDa band following immunoblotting (Fig 2G(b)). Further treatment of CHO cell-derived placensin with N-glycosidase yielded a lower size band (Fig 2G(c), ~ 16 kDa), confirming that the recombinant placensin is a glycoprotein.

Glucogenic actions of placensin

Based on the glucogenic action of asprosin [11], we isolated primary hepatocytes from mice and cultured AML12 cells, a differentiated, non-transformed hepatocyte cell line derived from mice transgenic for transforming growth factor-a [18]. As shown in Fig 3A, treatment of hepatocytes with placensin, like glucagon, increased cAMP production (at 10 min) in a dose-dependent manner for both primary hepatocytes and AML12 cells. Placensin also stimulated PKA activity (at 100 ng/ml for 30 min) in both cell types. At 5 h after treatment, placensin stimulated glucose secretion by both cell types, accompanied by increases in transcript levels for PEPCK (phosphoenolpyruvate carboxykinase) and G6Pase (glucose 6-phosphatase) (Fig 3B), genes important in gluconeogenesis. Furthermore, treatment of primary hepatocytes with paralogous asprosin stimulated cAMP and glucose production (Fig EV2A), whereas treatment with glucagon also stimulated glucose production with no additive effects when both placensin and glucagon were used (Fig EV2B). In contrast, insulin suppressed placensin stimulation of glucose secretion (Fig EV2B). Unlike hepatocytes, treatment of rat intestinal epithelial IEC6 [19] and dog kidney MDCK cells [20] with placensin did not increase cAMP production (Fig EV2C). Furthermore, *in situ* perfusion of liver from adult mice showed placensin stimulation of glucose output (Fig 3C), whereas *in vivo* injection of adult mice with placensin increased serum glucose levels, showing major increases at 15 and 30 min after injection (Fig 3D).

Placensin secretion by placental cells and promotion of cell invasiveness

We tested several human trophoblastic cell lines for cellular content of placensin using immunoblotting (Fig 4A) and found placensin immunoreactivity in HTR-8/SVneo cells derived from human trophoblasts overexpressing simian virus 40 large T antigen [21], with negligible levels in BeWo, JAR, and JEG3 cells. In addition, human embryonic kidney-derived 293T cells also showed no expression. Using polyclonal antibodies against recombinant placensin as the capturing reagent and a monoclonal antibody against a synthetic placensin fragment (1–96 residues) as the detection reagent, we prepared an ELISA to monitor placensin levels. We found time-dependent increases in placensin secretion by HTR-8/SVneo cells upon culture but negligible secretion by JAR cells (Fig 4A). We also subjected conditioned media of HTR-8/SVneo cells to immunoprecipitation using specific antibodies, followed by mass spectrometry analyses. As shown in Fig EV3A, several placensin fragments were detected. We further suppressed FBN2 expression by HTR-8/SVneo cells. As shown in Fig 4B, knockdown of FBN2 gene expression in HTR-8/SVneo cells using siRNA (SiFBN2), but not control siRNA (SiC), suppressed FBN2 transcript levels, together with decreased placensin secretion based on ELISA and immunoblotting. Furthermore, treatment with recombinant placensin stimulated cAMP production by HTR-8/SVneo cells in a dose-dependent manner, together with increases in PKA activity (Fig 4C). In addition, the paralogous asprosin also stimulated invasiveness of HTR-8/SVneo cells (Fig EV3C), whereas placensin stimulated cAMP production by placental BeWo and JEG3, but not JAR, cells (Fig EV3D). Because HTR-8/SVneo cells have been used to

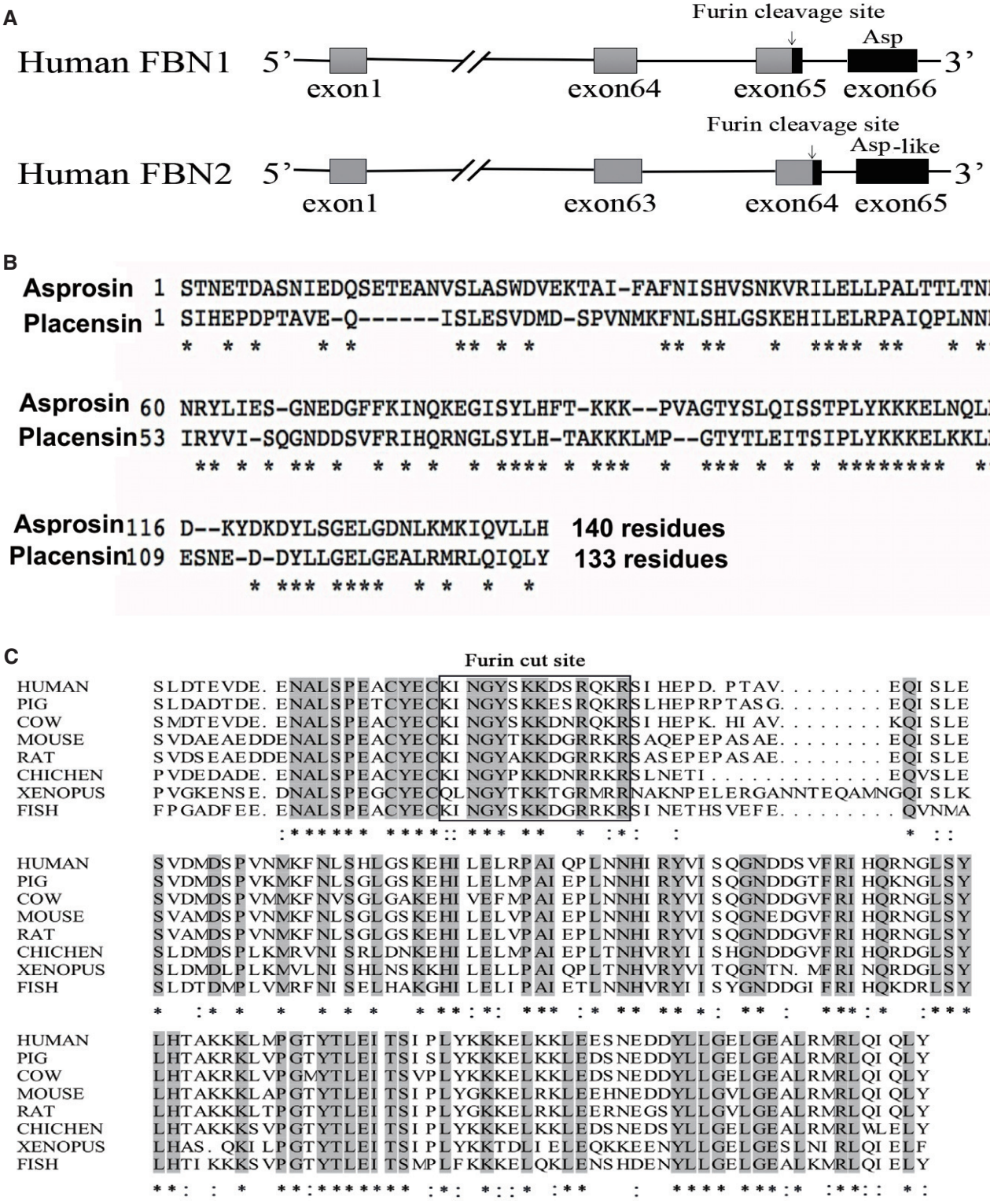

Figure 1. Prediction of a peptide hormone in the C-terminal region of *FBN2*.

A Exon–intron structures for human *FBN1* and *FBN2* genes. Conserved furin cut sites are indicated. Asprosin (Asp) is encoded by exons 65–66 of the *FBN1* gene, whereas a conserved Asp-like sequence is found in exons 64–65 of *FBN2*.

B Sequence comparison between C-terminal regions of human *FBN1* and *FBN2*, encoding asprosin, and placensin, respectively. Asterisks indicate identical residues.

C Conservation of FBN2 furin cleavage sites and C-terminal regions among diverse vertebrate species. Boxed area represents conserved furin cleavage site. Asterisks indicate identical residues conserved in all species examined, colons indicate highly conserved residues.

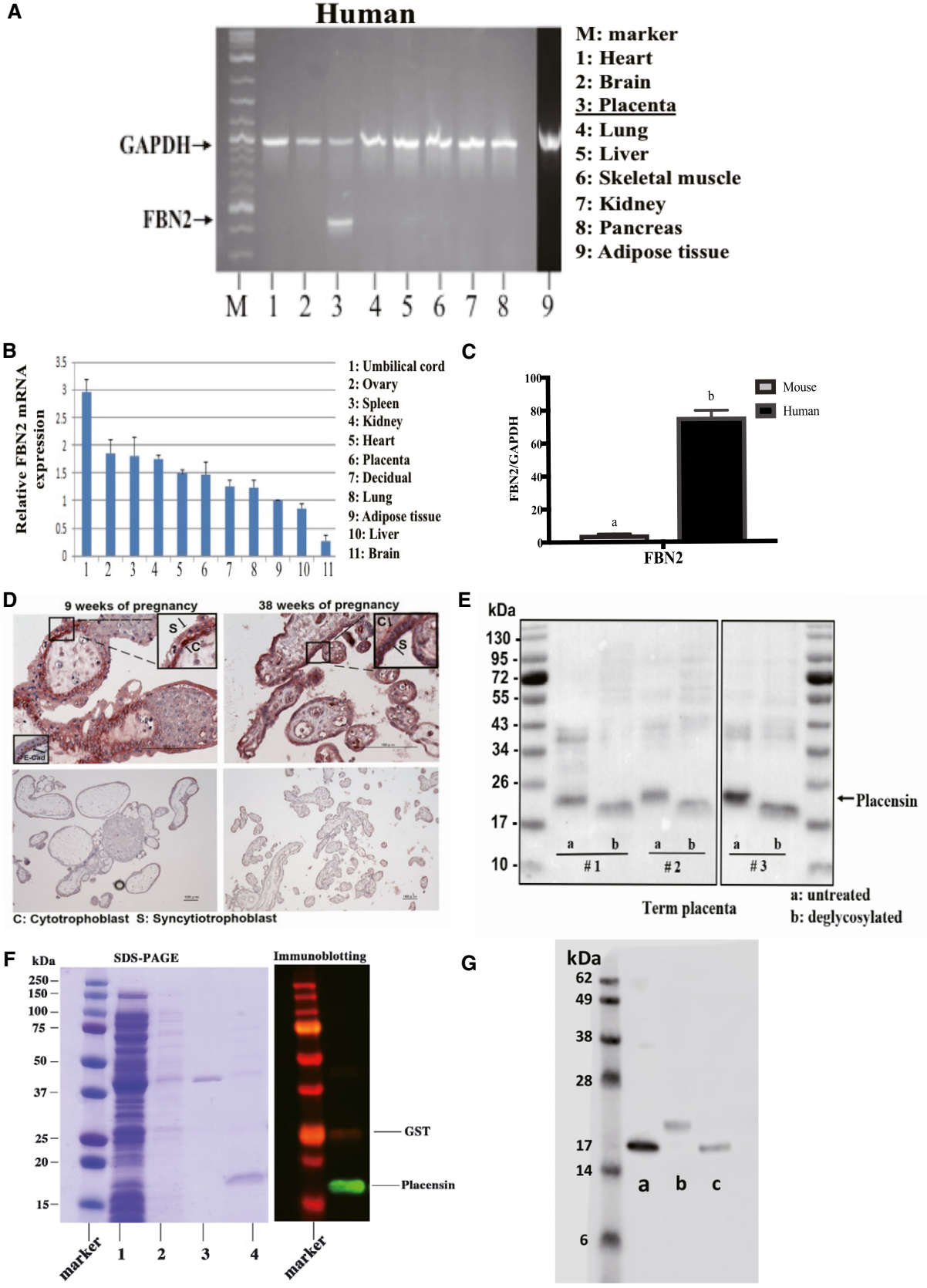

Figure 2.

Figure 2. High expression of *FBN2* and placensin in human, but not mouse, placenta.

A *FBN2* mRNA levels in multiple human tissues. Human tissue cDNAs were used as templates for PCR of *FBN2*, followed by agarose gel analyses. *GAPDH* levels served as loading controls (biological replicates, $n = 2$).

B *FBN2* mRNA levels in mouse tissues based on quantitative RT–PCR. Data were normalized based on levels in adipose tissues.

C Comparison of *FBN2* transcript levels between human and mouse placentas. Common primers for *FBN2* and *GAPDH* in both species were used for RT–PCR analyses (biological replicates, $n = 3$).

D Immuno-histochemical detection of placensin in human placental villi obtained from women at 9 and 38 weeks of pregnancy. During early pregnancy, placensin immunostaining was found in cytotrophoblasts (c), whereas syncytiotrophoblasts (s) showed weak staining. At 38 weeks of pregnancy, strong placensin signals were detected in both syncytiotrophoblasts and cytotrophoblasts (arrows). Staining using E-cadherin antibodies confirmed the identity of cytotrophoblasts (inset showing higher magnification E-cadherin (E-Cad) staining). Lower panels depict sections stained with nonimmune IgG as negative controls. Scale bars, 100 μm.

E Immunoblotting of placensin in human placental villi (term pregnancy). Samples from three individual patients were processed with or without N-glycosidase pre-treatment to reveal untreated and deglycosylated placensin.

F For prokaryotic cell expression, a cDNA fragment corresponding to residues 2,779–2,912 of the human FBN2 coding region was appended with GST and 6-histidine tags and subcloned into the PGEX-6P-1 vector for expression in *Escherichia coli*. Left panel: Coomassie Blue staining of proteins in the SDS–PAGE gel before and after digestion with the PreScission protease. 1: Flow-through; 2: washes; 3: before PreScission treatment; 4: after PreScission treatment. Right panel: Immunoblotting using placensin antibodies.

G Immunoblotting of recombinant placensin generated in *E. coli* and CHO cells. For CHO cell expression, placensin cDNA sequence was subcloned into an adenoviral vector pAV[Exp]-CMV downstream of the IgK signal peptide under the control of the CMV promoter for infection of cells before immunoblotting of media 2 days later. Recombinant placensin proteins secreted from CHO cells (b) showed higher molecular weight than those from bacteria (a). Following treatment with N-glycosidase to remove N-linked carbohydrate side chains (c), prokaryotic- and eukaryotic cell-derived placensin proteins showed similar sizes.

Data information: Bars are shown as mean ± SEM. For C: a,b, groups with different letters were significantly different ($P < 0.05$) by parametric unpaired Welch's *t*-test.

investigate trophoblast invasiveness [22], we treated HTR-8/SVneo cells with placensin for 24 h to test the expression of key matrix metallopeptidases (MMPs) and cell invasion. At 24 h after placensin treatment, HTR-8/SVneo cells showed increased transcript levels (Fig 4D) and enzyme activities for MMP9, but not MMP2, without affecting cell proliferation (Fig 4E). We then cultured these cells for another 24 h and demonstrated that placensin pre-treatment enhanced HTR-8/SVneo cell invasion based on a Transwell assay (Fig 4F).

Increases in serum placensin during pregnancy and in GDM patients

We obtained serum samples from patients at second trimester and term pregnancy for immunoblotting analyses. As shown in Fig 5A, higher levels of immuno-placensin bands of ~ 20 kDa were found in sera of term pregnancy samples as compared with those from second trimester. Also, serum from GDM patients showed immuno-placensin band of ~ 20 kDa (Fig EV4). We further performed immunoprecipitation of term pregnancy sera before confirmation of placensin fragments using mass spectrometry (Fig EV3B). Using the placensin ELISA, we demonstrated that the ELISA showed < 0.1% cross-reactivity with related asprosin and unrelated hCG (Fig EV5A). We recruited 148 pregnant women at different gestational stages together with 26 gestational diabetes mellitus (GDM) patients at second and third trimesters. As shown in Fig 5B, there were stage-dependent increases in serum placensin levels throughout normal pregnancy with first-trimester sera showing the lowest levels. Comparing serum placensin levels between normal and GDM patients at the same weeks of pregnancy, GDM patients showed higher serum placensin levels as compared with normal pregnant women (Fig 5B). In contrast, serum glucagon showed minimal increases during pregnancy progression and in GDM patients (Fig 5B), consistent with earlier findings [23]. Although there were no differences in patient ages among different groups, major increases in body mass indexes (BMI) were found for GDM patients as compared with patients with normal pregnancy (Fig 5C).

Discussion

In addition to its role as a structural protein, *FBN2*, like *FBN1*, encodes a peptide hormone following furin cleavage of the C-terminal region of profibrillin 2. Placenta-derived placensin is secreted by trophoblasts and acts as a paracrine hormone to increase cAMP-

Figure 3. Placensin stimulation of cAMP production, PKA activity, and glucose release in hepatocytes and promotion of glucose secretion *in vivo*.

A Prokaryotic cell-derived placensin stimulated cAMP production (at 10 min), and PKA activity (at 30 min) by primary mouse hepatocytes and hepatocyte-derived AML12 cells (biological replicates, $n = 5$).

B Placensin stimulation of glucose secretion (at 5 h) and key gluconeogenesis gene transcripts (at 1 h) in both cell types (biological replicates, $n = 4$). Transcript levels for *PEPCK* and *G6Pase* were determined using quantitative RT–PCR. Treatment with glucagon (10 nM) served as positive controls.

C Liver from adult female mice was perfused *in situ* with placensin (1.0 μg/pulse) or PBS before measurement of hepatic glucose output from inferior vena cava (biological replicates, $n = 3$).

D Adult female mice were injected intraperitoneally with placensin (30 μg/injection) with blood sampling at different time points for glucose levels (biological replicates, $n = 5$).

Data information: Bars are shown as mean ± SEM. For A and B, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ by ANOVA followed by Brown–Forsythe and Welch's test; for C, $^{*}P < 0.05$, $^{****}P < 0.0001$, significant differences between placensin injection groups and corresponding control groups by parametric unpaired Welch's *t*-test; for D, $^{**}P < 0.01$, significant differences between placensin injection groups and corresponding control groups by nonparametric Mann–Whitney *t*-test, and $^{*}P < 0.05$ and $^{****}P < 0.0001$, significant differences between control groups and placensin injection groups by parametric unpaired Welch's *t*-test.
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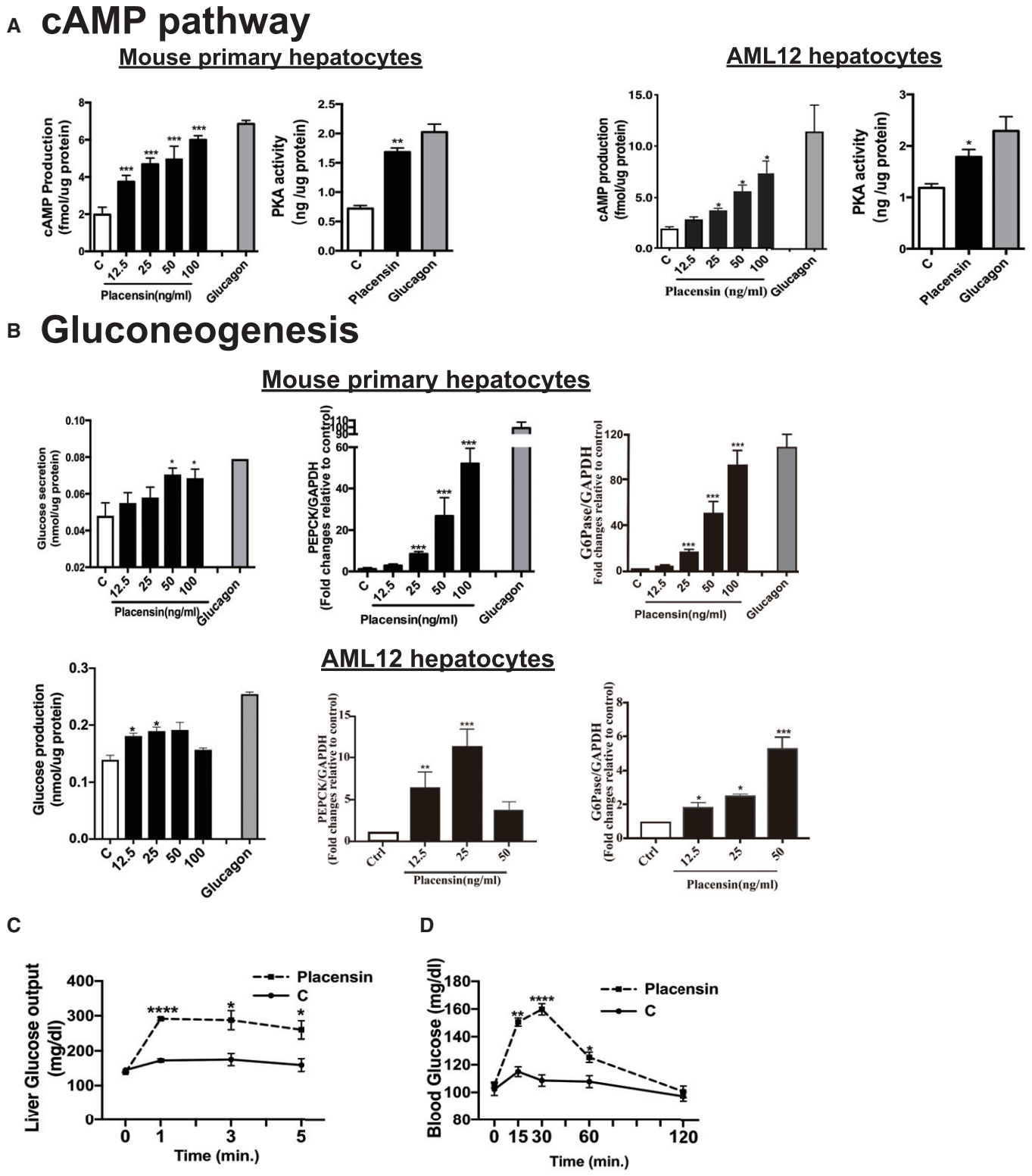


Figure 3.

PKA signaling in trophoblasts and to promote trophoblast invasiveness mediated by increases in MMP9 activity. Placensin secreted into maternal circulation could stimulate cAMP release, PKA activity, glucose secretion, and gluconeogenesis by hepatocytes. The growth of the embryo within the mother's womb has a major impact on the ability of mothers to balance their metabolism, and placensin likely plays important roles in maintaining metabolic homeostasis during human pregnancy.

The placenta plays important roles in transporting nutrients, oxygen, wastes, and metabolites between mother and fetus, thus substituting functions of gut, lung, kidney, and liver for the fetus. It also secrets essential hormones to regulate functions of the mother's ovary and metabolic organs. Although our studies demonstrated the ability of placensin to regulate functions of murine

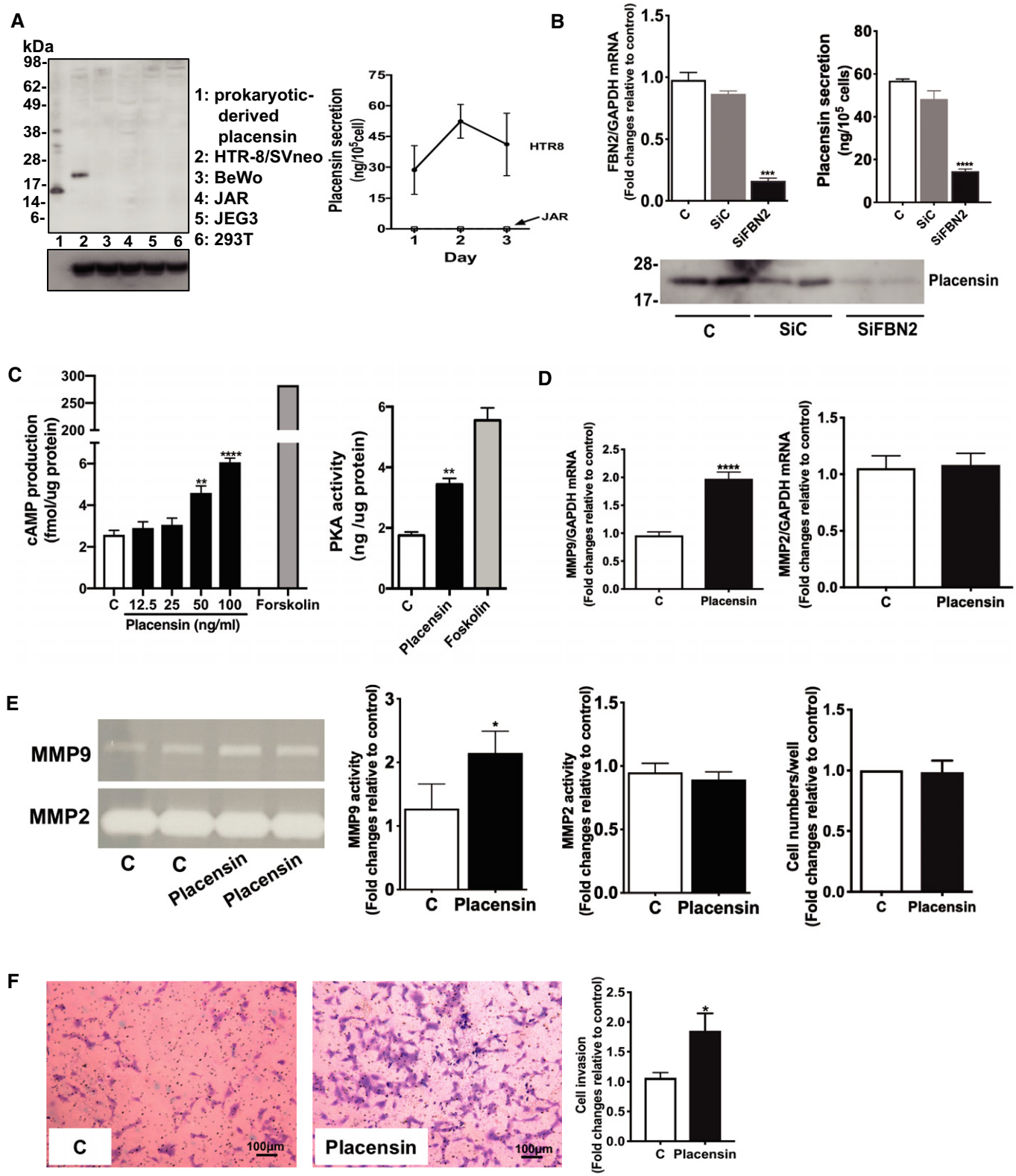


Figure 4.

Figure 4. Placensin secretion and promotion of trophoblastic cell cAMP production, PKA activity, MMP9 expression, and invasion.

A Left panel: Immunoblotting of placensin content in HTR-8/SVneo, but not BeWo, JAR, and JEG3, cells. Right panel: Time-dependent secretion of placensin by HTR-8/SVneo, but not JAR, cells monitored using an ELISA (biological replicates, n = 4–5).

B Knockdown of FBN2 in HTR-8/SVneo cells led to decreases in FBN2 transcripts and placensin secretion. Cells were treated with FBN2 siRNA (SiFBN2; 20 nM) or control siRNA (SiC; 20 nM) for 6 h. Following media change, cells and conditioned media were collected at 48 h after incubation. Placensin transcripts and proteins were determined by RT–PCR and ELISA (biological replicates, n = 8), respectively. Lower panel: immunoblotting of secreted placensin by HTR-8/SVneo.

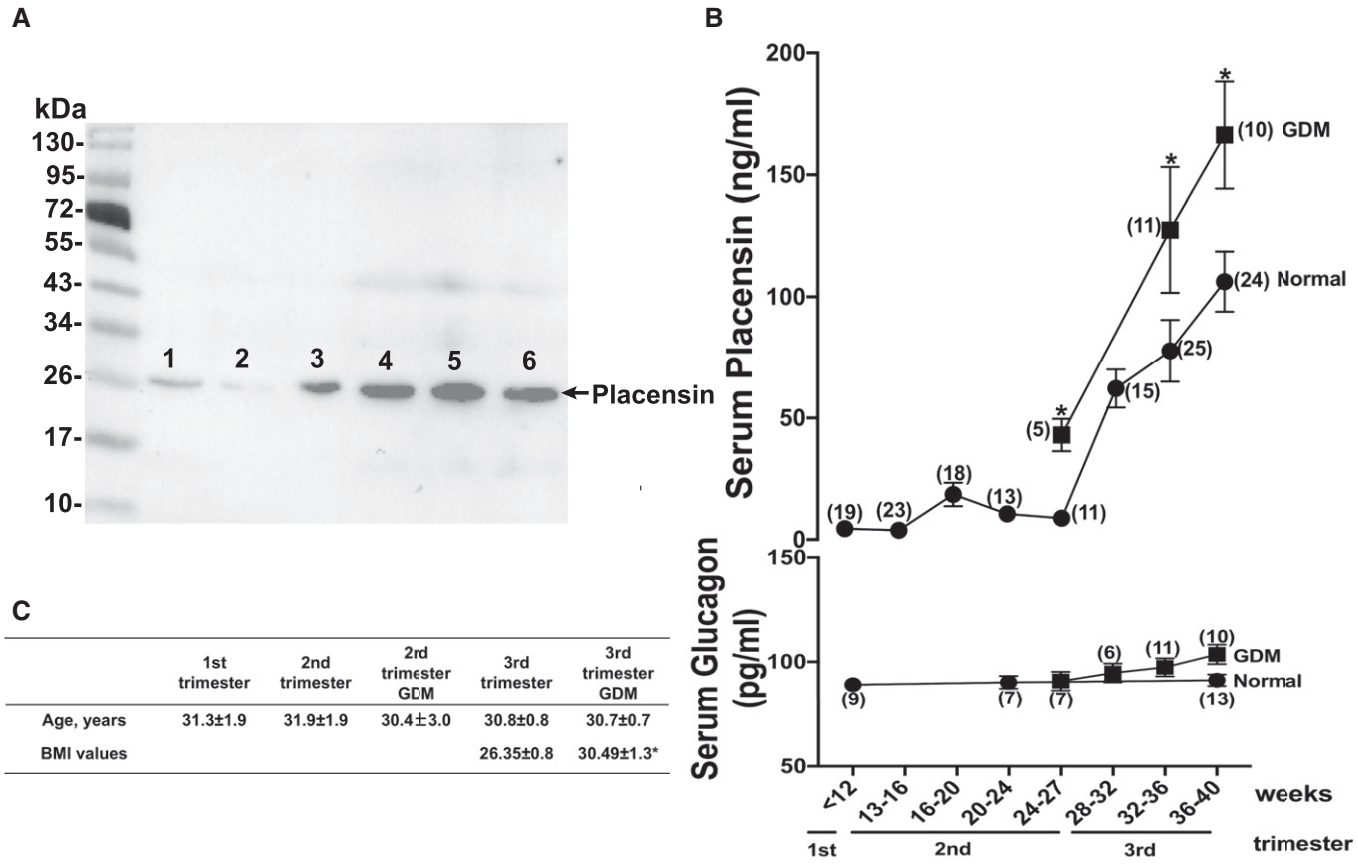
C Placensin stimulation of cAMP production and PKA activities by HTR-8/SVneo cells. Cells were treated with placensin for 10 min before cAMP measurement (biological replicates, n = 5). For PKA activities, cells were treated with placensin (50 ng/ml) for 30 min. Treatment with forskolin (10 μM) served as positive controls.

D Placensin stimulation of transcript levels for MMP9 but not MMP2 (biological replicates, n = 12). Cells were treated with placensin for 24 h before RT–PCR analyses of transcript levels.

E Placensin stimulation of MMP9, but not MMP2, activities without affecting cell proliferation. After 24 h of treatment with placensin, media were collected for zymographic and densitometric analyses as well as determination of total cell numbers (biological replicates, n = 4).

F Placensin stimulation of HTR-8/SVneo cell invasion. After placensin treatment for 24 h, cell invasiveness was determined using the Transwell assay (left panel: micrographs of cells; right panel: fold changes in number of migrated cells, biological replicates, n = 4). Cells were incubated with mitomycin C to rule out effects on cell proliferation. Scale bars: 100 μm. C: control.

Data information: Bars are shown as mean ± SEM. For B, ANOVA followed by Tukey's multiple comparison tests was used for comparison. For C, ANOVA followed by Brown–Forsythe and Welch's test was used for comparison. For D–F, parametric unpaired Welch's t-test was used. When comparing with control groups, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
Source data are available online for this figure.



C

	1st trimester	2nd trimester	2rd trimester GDM	3rd trimester	3rd trimester GDM
Age, years	31.3±1.9	31.9±1.9	30.4±3.0	30.8±0.8	30.7±0.7
BMI values				26.35±0.8	30.49±1.3*

Figure 5. Increases in serum placensin and minimal changes in glucagon levels during human pregnancy and in patients with gestational diabetes mellitus (GDM).

A Immunoblotting of serum placensin from patients at second trimester (1–3), term pregnancy (4–6) using specific placensin antibodies.

B Serum levels of placensin and glucagon at different weeks of pregnancy measured using specific ELISA in normal and GDM patients. Numbers in parentheses represent number of patients.

C Ages of patients at different gestational stages and BMI values for normal pregnant women and GDM patients.

Data information: Bars are shown as mean ± SEM. *P < 0.05, nonparametric Mann–Whitney's t-test was used for comparison between GDM groups with corresponding control groups.
Source data are available online for this figure.

hepatocytes and a human placental cell line, *FBN2* is highly expressed in human placenta, but lower in mouse placenta. We found high expression of placensin in cytotrophoblasts during early pregnancy consistent with recent single-cell RNA-seq profiling [24, Data ref: 25]. Of interest, another human pregnancy hormone, chorionic gonadotropin (hCG) produced by human placenta, was not found in rodent species [26]. Placensin and hCG are secreted by trophoblasts, consistent with major structural and functional divergences between human and rodent placentas during evolution [16,17]. Because placensin and associated furin cleavage sites are highly conserved in diverse mammals, future studies on tissue expression of FBN2 could reveal physiological roles of placensin in diverse animals.

Treatment with placensin increased glucose release and gluconeogenesis by hepatocytes, similar to the action of glucagon. During pregnancy, major changes in metabolic homeostasis took place due to increasing demand of the fetus and placenta. Maternal liver metabolism contributes to the increased energy demand of the placenta and the embryo by enhancing the production of glucose [27,28]. Since gluconeogenesis is minimal in the fetus, maternal glucose is the main source for the growing fetus [29]. Commensurate with increasing demand during late gestation, there is an increase in maternal glucose production and gluconeogenesis [27]. As pregnancy progresses, stage-dependent increases in insulin resistance were found [6,30,31]. Because diabetes-like changes in glucagon secretion were not found in normal pregnant women or those with gestational diabetes mellitus in present and earlier studies [32], observed increases in insulin resistance during pregnancy have been ascribed to increased production of placental hormones. However, longitudinal measurement of diverse placental hormones (hCG, estradiol, progesterone, human placental lactogen, and prolactin), together with leptin and cortisol, during different stages of pregnancy showed no correlation between levels for these hormones and pregnancy-associated increases in insulin resistance [33]. Our findings of a glucogenic placental hormone and stage-dependent increases in serum placensin levels during pregnancy progression provide an endocrine basis for pregnancy-associated increases in maternal glucose production.

After implantation, placental villous cytotrophoblasts, a population of progenitor cells, become highly invasive extravillous cytotrophoblasts or undergo cell fusion to form the multinucleated syncytiotrophoblasts [34]. *FBN2* is expressed in cytotrophoblasts during early pregnancy and in both cytotrophoblasts and syncytiotrophoblasts following cytotrophoblast differentiation and fusion. Glycosylated placensin was also found in extracts of term placentas and pregnant sera. Our findings of placensin secretion by HTR-8/SVneo cells and placensin stimulation of cAMP-PKA signaling, MMP9 expression, and invasion of HTR-8/SVneo cells suggest that placensin could be a paracrine/autocrine factor during placental differentiation. Of interest, earlier studies have demonstrated that HTR-8/SVneo cells are a mixture of trophoblasts and stromal/mesenchymal cells [35], likely closer to *in vivo* conditions. Because placensin is derived following cleavage of profibrillin 2 by the proprotein convertase furin co-localized with placensin in placental cells, our findings underscore results showing the essential role of furin during human trophoblast syncytialization [36]. In addition to its metabolic functions, placensin, like hCG [37], could play important roles in placental development.

The prevalence of gestational diabetes mellitus (GDM) in the United States is increasing to 9% [38]. Left untreated, GDM pregnancy could lead to fetal macrosomia, which in the absence of obstetric intervention leads to dystocia, posing a severe risk to both mother and child [39]. In addition to pregnancy complications, GDM is associated with long-term adverse effects in both mothers and their offspring. Cumulative incidence of postpartum type II diabetes in GDM mothers ranged from 2.6% at 6 weeks postpartum to over 70% up to 28 years postpartum [40]. Furthermore, hyperglycemia during pregnancy is associated with an increased risk of childhood obesity [41], abnormal glucose tolerance, hypertension, preeclampsia, and related metabolic syndromes [8,10]. Although the present screening for GDM relies upon established maternal risk factors and oral glucose tolerance tests [42], these tests were not conducted until late in the second trimester, which could expose a developing fetus to hyperglycemic conditions for a long period of time. Future prospective studies to monitor serum placensin levels during pregnancy progression could provide an early predictor for GDM. It is also of interest to monitor changes in serum placensin levels in patients with other pregnancy complications, including preeclampsia, pregnancy-induced hypertension, fetal growth restriction, ectopic pregnancy, and fetal chromosomal anomalies. Indeed, a peptide fragment of FBN2 was found as a serum marker for preeclampsia based on a mass spectrometric approach [43].

Due to their paralogues relationship, genetic analyses in mice suggest that fibrillin 1 and fibrillin 2 perform both overlapping and unique functions [44]. In humans, mutations in the *FBN1* gene result in skeletal, cardiovascular, and ocular features of the Marfan syndrome [45], whereas mutations in the *FBN2* gene result in skeletal features of congenital contractural arachnodactyly or Beals syndrome [46,47]. Gene targeting in mice has demonstrated that *FBN1* is required for postnatal cardiovascular functions [48] and metabolic processes [11], whereas *FBN2* is required for proper limb patterning [49]. The limb phenotype for mutant *FBN2* is likely due to malfunction of fibrillin 2 as a structural protein during fetal development. Placensin could promote adipogenesis similar to its paralog asprosin [11] and a genome-wide association study in man identified *FBN2* as a locus for extreme obesity [50]. Future screening of variations in *FBN2* gene promoter and placensin-coding region in non-pregnant obese individuals as well as in pregnant women with metabolic disorders and inadequate weight gains could allow a better understanding of placensin pathophysiology.

Accumulating validation of the developmental origin hypothesis [51] indicated that the foundation of many aspects of adult health is laid down *in utero* and gestational metabolic disorders such as diabetes mellitus can have severe adverse effects on fetal and neonatal outcomes [52]. Optimal secretion of placensin capable of stimulating hepatic glucose secretion to maintain metabolic homeostasis and promoting trophoblast invasiveness to establish adequate placentation is important for carrying the offspring to propagate the species. Further elucidation of the physiological and pathological roles of placensin in metabolic homeostasis during pregnancy could improve the health of both mothers and infants.

Materials and Methods

Animals and human subjects

C57BL/6 and BALB/C mice were purchased from Charles River Lab. (Wilmington, MA). Animals were housed under 12-h light/dark with free access to water and food, and then randomly divided into control and treatment groups. Biosafety and Animal Research Committees at Stanford University approved all experimental protocols. Measurements were done by the person who was blind to the treatments.

Collection of human serum and tissue samples from Han Chinese were approved by Human Subject Committees of First Affiliated Hospital of Zhengzhou University and St. Marianna University School of Medicine. Human term placentas were from patients following normal delivery. Serum samples were collected from 12 weeks (first trimester), 13–27 weeks (second trimester), and 28–40 weeks (third trimester) of pregnancy. To diagnose GDM, patients showed a fasting plasma glucose level of > 5.8 mmol/l in two or more tests, or a random plasma glucose of > 11.1 mmol/l. This was followed by confirmation using oral glucose tolerance tests. After oral loading of 75 g glucose, patients showed elevated serum glucose (0 h > 5.1 mmol/l; 1 h > 10 mmol/l; 2 h > 9.2 mmol/l; 3 h > 8.1 mmol/l) [53,54]. All patients have signed informed consent. Measurement of serum placensin and glucagon and characterization of placental placensin in patients have been registered in Chinese clinical trial registry (ChiCTR1800014372).

Recombinant placensin production in prokaryotic cells

A cDNA fragment corresponding to residues 2,780–2,912 of the human *FBN2* coding region after the furin cleavage site was subcloned into the PGEX-6P-1 vector (VWR). Placensin sequence was flanked, in the N-terminus, by the GST (glutathione S-transferase) sequence, followed by 6xHis tags, for expression in *E. coli*. After transformation of *E. coli* using the plasmid, cells were homogenized using high pressure homogenizer before collecting the supernatant at 20,000 × *g* for 90 min. The supernatant was allowed to bind to a glutathione Sepharose 4B column (GE Healthcare) for 2 h. After extensive washing of the column to remove contaminant proteins, PreScission protease (GE Healthcare) was added to cleave 6xHis-placensin from GST. After elution from the column, 6xHis-placensin was further purified using a Ni-NTA His-bind column (QIAGEN). 6xHis-placensin was then eluted from the column using a buffer containing 300 mM imidazole, followed by dialysis in PBS. Subsequently, recombinant proteins were added to the endotoxin removal resin (Thermo Fisher Scientific) for 2–3 times to minimize endotoxin contamination. Recombinant asprosin was purchased from Aviscera Bioscience (Santa Clara, CA).

Generation of recombinant placensin in eukaryotic cells and construction of adenoviral vectors

For eukaryotic cell expression, cDNA corresponding to the entire coding region of placensin was subcloned into the eukaryotic expression vector pEBMulti-Neo (Wako Lab. Chemicals) following the IgK signal peptide and 6xHis sequences. CHO cells were transfected with the expression vector, and conditioned media were subjected to immunoblotting. After confirming the secretion of placensin in eukaryotic cells, the placensin cDNA was subcloned into an adenoviral vector pAV[Exp]-CMV (Cyagen Biosciences Inc., Guangzhou, China) downstream of the IgK signal peptide under the control of the CMV promoter.

RT–PCR, immunostaining, immunoblotting, and histological staining

Total RNAs were extracted using an RNeasy Micro Kit, and cDNAs were synthesized using a iScript™ cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using iTaq SYBR Green SuperMix (Bio-Rad) in the StepOnePlus™ Real-Time PCR System (ThermoFisher) as follows: 15 min at 95°C, 45 cycles of 15 s at 95°C, and 60 s at 60°C. Relative abundance of specific transcripts was normalized based on GAPDH levels. Human multi-tissue RNA samples were obtained from Clontech (Mountain View, CA). Primers for quantitative RT–PCR analyses are listed in Table EV1.

For immunohistology staining, polyclonal antibodies against prokaryotic cell-derived recombinant placensin were generated in rabbits (Sino Biological Inc. Beijing, China) and tissue sections were incubated with antibodies. Mouse E-cadherin monoclonal antibody staining was performed using antibody from Santa Cruz Biotech (sc-8426, 1:100 dilution). For negative controls, nonimmune IgG (Dako) was used. For double immunofluorescence staining, mouse FBN2 monoclonal antibody was purchased from Abnova Corporation (M01, clone 1C2, the same antibody as used in mass spectrometry) and rabbit furin polyclonal antibody was purchased from Abcam (ab3467). For immunoblotting analyses, proteins were extracted using M-PER Mammalian Protein Extraction Reagent (Thermo) containing a protease inhibitor mixture before immunoblotting using placensin polyclonal antibodies (Sino Biological Inc. Beijing, China). Placensin from infected CHO cells or placental villi were also pretreated with N-glycosidase (50,000 units/ml, New England Biolabs) for 3 h to remove N-linked carbohydrate side chains.

Stimulation of hepatocyte functions

Primary mouse hepatocytes were isolated from 8-week-old BALB/C female mice fasted for 16 h, by a two-step EGTA/collagenase retrograde perfusion protocol and cultured as described [55]. Mouse differentiated, non-transformed hepatocyte AML12 cell line was obtained from ATCC (Manassas, VA) and maintained in DMEM/F-12 medium (Gibco) supplemented with insulin, transferrin, selenium (ITS; Sigma), and dexamethasone (1 μM) in a humidified atmosphere containing 5% CO_2 at 37°C. Before testing, cells were washed with PBS and suspended (2×10^6 per ml) in pre-warmed glucose-free DMEM medium (Gibco). Aliquots (10 μl) were dispensed on 48-well plate in 200 μl glucose production buffer consisting of glucose-free DMEM (pH 7.4 without phenol red, supplemented with 20 mM sodium lactate, and 2 mM sodium pyruvate) [56] with or without placensin or glucagon. After 5 h of incubation, glucose secreted into the media was determined with a glucose colorimetric assay (BioVision). Values were normalized to total protein content. Some cells were incubated for 10 min before cAMP determination using a cAMP assay kit (Cell Biolab). Other cells were incubated for 30 min before measuring PKA activity using the PKA kinase activity kit (Enzo Life Sciences, Inc.). Cells were also

used for real-time PCR determination of transcript levels for gluconeogenesis genes after 1 h of incubation. Cellular protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA). To evaluate placensin actions on intestinal and kidney cells, rat small intestine epithelial cell line IEC6 and dog kidney epithelial cell line MDCK were purchased from ATCC.

For *in situ* stimulation of hepatic glucose output, mice were anesthetized with isoflurane and liver was perfused *in situ* using PBS equilibrated with 95% O_2 and 5% CO_2 as described [57]. The perfusion was kept at a flow rate of 3.0 ml/min, and a pulse of 1 μg placensin in 150 μl PBS was injected. Outputs from the inferior caval vein were collected, and glucose levels were measured [58]. Each animal represents an independent experiment and was repeated in three animals. For *in vivo* studies, adult female C57BL/6 mice (8–10 weeks of age) were injected intraperitoneally with prokaryotic recombinant placensin (30 μg/injection in 150 μl PBS) with blood sampling at different time points by snipping the tail, followed by glucose measurement using blood glucose meter (Contour). Mice injected with PBS served as controls. For *in vivo* experiments, each animal represents an independent experiment and was repeated in five animals.

Regulation of HTR-8/SVneo cell functions

Human trophoblast-derived HTR-8/SVneo, BeWo, JAR, and JEG3 cells were obtained from ATCC. Cellular content of placensin was determined using immunoblotting, whereas secretion of placensin was monitored using an ELISA. HTR-8/SVneo cells were treated with recombinant placensin before measurement of cAMP production at 10 min, PKA activity at 30 min, or gene transcript levels at 24 h. To knockdown endogenous expression of FBN2, HTR-8/SVneo cells were transfected for 6 h with 20 nM FBN2 siRNA (Santa Cruz Biotechnology) using the Lipofectamine RNAiMAX transfection reagent (Thermo Fisher). Negative control siRNA was also used. After media change, knockdown efficiency was determined using RT–PCR at 24 h after transfection, whereas media placensin was monitored by a placensin ELISA and immunoblotting at 48 h after transfection.

Invasiveness of cells was determined using a Transwell assay [59]. Transwell cell culture inserts (8-μm pore size, 24 wells, BD Biosciences) were coated with 1 mg/ml of growth factor-reduced Matrigel and HTR-8/SVneo cells. Cells were preincubation for 24 h with placensin before measurement of MMP transcripts using RT–PCR and enzyme activities using zymographic analyses, together with cell number counting under optical microscope. For zymographic analyses [60], media from cells treated with placensin for 24 h were concentrated and loaded (15 μg/lane) onto 10% zymogram gels (Thermo Fisher) before overnight incubation and scanning for lysis bands. For the invasion assay, cells were washed and further treated for 30 min with media supplemented with 0.1% FBS and 10 μg/ml of mitomycin C, an inhibitor of cell proliferation [61]. Cells (1×10^5 cells/insert) were then incubated for 24 h against a gradient of 10% FBS. Non-invading cells were removed with a cotton swab from the upper side of the chamber. Migrated cells were fixed with cold methanol, and cell nuclei were stained with crystal violet (0.5%) for 30 min before washing. The invasion assay results were normalized with untreated controls and expressed as fold change in triplicates.

Establishment of ELISA for placensin and measurement of placensin in serum

We established placensin ELISA in which rabbit polyclonal antibodies against recombinant placensin were used as capturing antibodies, whereas a monoclonal antibody against C-terminal peptides of FBN2 (amino acids 2,776–2,876; Abnova, H00002201-M01) was conjugated to horseradish peroxidase for detection. The polyclonal antibodies were purified using prokaryotic recombinant placensin before use. At 100-fold excess, recombinant asprosin and hCG (human chorionic gonadotropin) showed < 0.1% cross-reactivity in the placensin ELISA as shown in Fig EV5A. In addition, inclusion of increasing serum samples from patients showed parallel dose–response curves as the standards (Fig EV5B). For serum glucagon levels, glucagon ELISA kit (R & D Systems) was used. For serum asprosin levels, a human asprosin ELISA kit (Abbexa, abx257694) was used.

Some serum samples and conditioned media of HTR-8/SVneo cells were subjected to immunoprecipitation before proteolytic digestion. Cleaved peptides were separated using a reversed-phase analytical column, followed by analyses using a mass spectrometer (Thermo Scientific). The collected data were analyzed using Byonic v3.3.11 (Protein Metrics).

Statistical tests

All *in vitro* experiments were repeated in at least three independent experiments. *In vivo* experiments were repeated in at least three independent experiments. All data analysis was conducted on GraphPad Prism 8.0 software. Bars are shown as mean ± standard error of the mean (SEM). Before data analysis, normal distribution tests and group variation estimations were conducted. For two groups' comparisons, if passed normality test, parameter unpaired *t*-test (if with significant variation: Welch's test) would be used; if not, nonparametric Mann–Whitney's *t*-test would be used. For multiple group comparisons, if passed normality test, parameter one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests (if with significant variation: Brown–Forsythe and Welch's ANOVA test) would be used; if not, nonparametric ANOVA (Kruskal–Wallis test) followed by Dunn's multiple comparison tests would be used.

Expanded View for this article is available online.

Acknowledgements

The synopsis image is created with BioRender. We thank Drs. Richard Pfuetzner, Qiangjun Zhou, Steven Wilson, and Austin Wang at Stanford University for help in recombinant protein purification. Funding: This work was supported by Key research and development project of Henan Education department (No. 182102310136 to Linli Hu); Science and Technology Research Project of Henan Province (No. 172102310396 to Guidong Yao).

Author contributions

YY, J-HH, L-LJ, L-LF, S-JW, G-DY, QY, YG, LL, TS, and YS performed experiments and data analyses; YY, J-HH, L-LJ, LLF, and AJWH designed experiments and wrote the manuscript; KK, L-LH, and Y-PS supervised clinical sample collection; all authors reviewed the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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