Identification of novel candidate factor SNX5 specific for pregnancy failure in Holstein heifers

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Abstract

Pregnancy loss predominantly occurs during periods between blastocyst hatching and conceptus (embryo plus extraembryonic membranes) implantation to the endometrium in cattle. Insufficient biochemical communication between conceptus and endometrium has been suspected as the primary cause for early embryonic losses. If molecules regulating this communication were identified, molecular mechanisms associated with early pregnancy success or loss could be better understood. To identify novel factors as detection markers of non-pregnant or females undergoing embryonic loss, blood sera from embryo-transferred heifers on day 7 (day 0 = day of estrus) were collected on day 17, 20, or 22, which were subjected to metabolome and global proteome iTRAQ analyses. On each sample, the metabolome analysis partly divided serum components into pregnant or not. In the iTRAQ analysis, heatmap analysis with 25 unique proteins separated into pregnant or not on day 20 or 22. Furthermore, receiver operating characteristic curve (ROC) analysis identified five candidate proteins detecting non-pregnant heifers, of which SNX5 in day 22 sera had the highest area under the curve (AUC), 0.983. We also detected SNX5 in day 22 sera from non-pregnant heifers using western blotting. These results suggest that high SNX5 in day 22 sera could predict early pregnancy loss in heifers.

Introduction

In cattle, pregnancy loss predominantly occurs during the period between blastocyst hatching and conceptus (embryo plus extraembryonic membranes) implantation to the endometrium[1]. Insufficient biochemical communication between conceptus and endometrium has been suspected as the primary cause for early embryonic losses. Numerous global analyses have been conducted to identify key molecules that govern feto-maternal communication, which could aid in establishing an optimal uterine environment for conceptus implantation to the endometrium[2–9]. If such molecules were identified, the molecular mechanisms associated with pregnancy success or loss during this period could be elucidated.

In our previous cattle study of the peri-implantation period, we identified a series of events required for the progression of conceptus implantation to placentation [10]: lymphocyte homing, the epithelial to mesenchymal transition (EMT), and dynamic changes in the production of interferon tau (IFNT), a cytokine required for the process of maternal recognition of pregnancy in ruminants. In our co-culture system, the treatment of bovine trophoblast cells and endometrial epithelial cells with uterine flushing fluids (UFs) obtained from pregnant day 17, 20 or 22 (day 0 = day of estrus, day of embryo implantation is initiated on days 19-19.5) induced lymphocyte homing and EMT of trophoblast cells and *in utero* gene expression pattern including IFNT on day 17, 20 or 22, respectively [11,12]. This indicates that the proper uterine environment necessary for the establishment of conceptus implantation could be defined biochemically. Although several global analyses during the peri-implantation period have also demonstrated changes in intrauterine transcripts and protein levels [12–15], factors that initiate and/or drive conceptus implantation to the endometrium have not yet been determined.

To discriminate pregnancy losses from cows successfully conceived and carrying pregnancy is pivotal for profitability in the cattle industry. We propose that earlier detection of pregnancy failure can allow for sooner preparation of the uterus/dam for the next pregnancy opportunity, resulting in the reduction of calving intervals. Several studies have explored to identify a factor specific for pregnancy establishment, but except pregnancy associated glycoproteins (PAGs), a definitive candidate has not yet been determined. Previously, IFNT has been considered as a strong candidate for the determination of pregnancy success. Except for inside the sheep uterine vein, IFNT has not been identified in the peripheral blood. Instead of IFNT, IFN-stimulated gene products have been examined to see whether one or two of those could be used for pregnancy diagnosis; however, factors specific to or a method of convenient pregnancy testing have not yet been determined or developed.

Pregnancy establishment requires a series of dynamic physiological events and the conceptus goes through not only its elongation, but also attachment, adhesion to the uterine endometrium, followed by placentation. Because of dynamic changes in ruminants' conceptuses *in utero*, some changes in transcripts and/or proteins could also be seen in the peripheral blood. We, therefore, hypothesized that the presence or absence of conceptuses in the uterus could be identified through changes in the peripheral blood compositions, which could be minor changes in proteins and/or metabolites. To test this hypothesis, blood samples taken from Holstein heifers, which went through embryo transfer (ET) procedure on day 7, and later the status of pregnancy or non-pregnancy (NP) were evaluated. Serum samples from the control heifers, which did not go through ET procedure, were also obtained. All serum samples obtained during estrous cycle and peri-implantation periods were subjected to protein global analysis and metabolome analysis, from which their expression profiles were evaluated.

Results

Metabolic analysis of bovine peripheral blood during the peri-implantation period

To understand metabolic profiles between pregnant and NP heifers during peri-implantation periods, sera from peripheral blood in cyclic days 0, 7, 14 and 17 (n = 3) or pregnant days 17, 20, and 22 (n = 17) heifers were subjected to metabolome analysis. Through principal component analysis (PCA), serum components were divided into different days (Fig. 1a). PCA also divided either sera from heifers with and without ET, or heifers pregnant and NP (Fig. 1a). Heatmap with days 17, 20, and 22 sera also partly divided into pregnant and NP heifers (Fig. 1b). We further investigated each metabolic data on day 17, 20, or 22 (Fig. 1c). On all data on day 17, 20, or 22, serum components were partly divided into pregnant and NP groups. However, changes in serum components in each sample differed among days 17, 20, and 22. In receiver operating characteristic curve (ROC) analysis with day 17 metabolic data, changes in L-serine [area under the curve (AUC) 0.85], L-glutamic acid (AUC 0.83), L-phenylalanine (AUC 0.82), and L-asparagine (AUC 0.78) were significant between pregnant and NP heifers ($P < 0.05$) (Fig. 1d). In day 20 ROC analysis, 1-methylhistidine (AUC 0.87), L-lysine (AUC 0.82), and n-methylethanolamine (AUC 0.73)

were detected ($P < 0.05$) (Fig. 1e). Furthermore, ROC analysis with day 22 data identified ($P < 0.05$) L-alanine (AUC 0.92), 1-hexadecanol (AUC 0.9), and 2-aminoisobutanoic acid (AUC 0.83) as the amino acid contents that differed between pregnant and NP heifers (Fig. 1f).

Comparison of serum composition between pregnant, NP heifers and those with estrous cycle

We next investigated serum metabolic data from NP heifers, which were compared with those from the estrous cycle. First, we compared serum metabolites among cyclic day 0, 7, 14, or 17. The heatmap and PCA showed that estrous cycle data were divided into different cyclic days (Fig. 2a). In the serum metabolite comparison of day 17 pregnant or NP heifers with those of cyclic ones, components on cyclic days were separated from those of day 17 pregnant or NP heifers (Fig. 2b). However, components on day 17 were not divided from those of NP heifers. On days 20 comparison, components of cyclic days were completely separated from those of day 20 pregnant or NP heifers (Fig. 2c). Components on day 20 could be further divided into pregnant and NP groups. Similar to D20, components on cyclic days were separated from those of day 22 pregnant or NP heifers (Fig. 2d). Components on day 22 could also be divided into pregnant and NP heifers.

Global proteome analysis of sera from day 20 pregnant and NP heifers

In addition to the metabolic profiles, we investigated the proteome profile of sera from day 20 pregnant and NP heifers. PCA did not divide protein components in day 20 pregnant heifers from those of NP ones (Fig. 3a). Heatmap with 25 unique proteins separated protein components in day 20 NP heifers except for one sample (Fig. 3b), of which ROC analysis identified 5 candidate proteins signifying pregnancy failure ($P < 0.05$): angiotensinogen (AGT; AUC 0.93), cadherin-5 (CDH5; AUC 0.87), fibronectin alpha chain (FGA; AUC 0.92), ADAM metallopeptidase with thrombospondin type 1motif 13 (ADAMTS13; AUC 0.85), and histidine-rich glycoprotein (AUC 0.9) (Fig. 3c). Furthermore, the network analysis with all protein profile identified impacting proteins: FGA, FGB, FGG, thrombospondin-1 (THBS1), bromodomain containing 9 (BRD9), and C-X-C motif chemokine ligand 4 (PF4) (Fig. 3d).

Identification of specific proteins in day 22 blood sera detecting NP heifers

We next investigated the proteome profile of sera from day 22 pregnant or NP heifers. Like day 20 sera, PCA did not divide protein components on day 22 pregnant from those of NP heifers (Fig. 4a). Heatmap analysis with 25 unique proteins separated serum protein components in NP heifers from those of day 22 samples except for some (Fig. 4b), of which ROC analysis identified five candidate proteins detecting NP heifers ($P < 0.05$): sorting nexin 5 (SNX5; AUC 0.98), acetyl-CoA carboxylase alpha (ACAC; AUC 0.95), cleavage and polyadenylation specific factor 6 (CPSF6; AUC 0.95), damage specific DNA binding protein 1 (DDB1; AUC 0.93), and serum response factor binding protein 1 (SRFBP1) (Fig. 4c). To examine whether these five proteins could be used as markers to detect NP heifers, day 22 peripheral serum samples were subjected to western blotting analysis. SNX5 in sera from NP heifers was higher than that in the pregnant heifers (Fig. 4d). However, ACAC, CPSF6, DDB1, and SRFBP1 in sera did not differ between day 22 pregnant and NP heifers. Western blots were again executed using another antibody specific for SNX5,

resulting that SNX5 in the NP heifers was higher than that of pregnant heifers on day 22 (Fig. 4e). Moreover, the network analysis with day 22 protein profiles identified impacting proteins: component C3 (C3), thyroglobulin (TG), c-type lectin domain family 12 member B (CLEC12B), ankyrin repeat and SOCS box protein 17 (ASB17), and beta-2-glycoprotein1 (APOH) (Fig. 4f).

Discussion

Using PCA and the heatmap with the 25 unique factors from the metabolome analysis, serum components were divided into days 17, 20, and 20 pregnant from those of NP heifers. On each pregnant day sample, serum components were partly divided into pregnant and NP groups. Notably, serum components from cyclic day 0, 7, 14, or 17 were distinctive, which also differed from those of days 17, 20, or 22 NP heifers. These indicate that serum components in NP heifers differ from those of cyclic ones and may dynamically change during the peri-implantation periods. In the proteome iTRAQ analysis, the heatmap with 25 unique proteins mostly separated into pregnant and NP heifers on day 20 or 22. Furthermore, ROC analysis identified five candidate proteins detecting heifers not pregnant, of which SNX5 in day 22 sera had the highest AUC, 0.983. Using western blotting, we detected SNX5 in day 22 sera from NP heifers. These results indicate that sera on pregnant day 22, 2 ~ 3 days after conceptus attachment to the maternal endometrium is initiated, well reflect the differences between pregnant and NP heifers during the early pregnancy period, and suggest that high levels of SNX5 in peripheral sera on day 22 could predict pregnancy failure or females undergoing embryonic loss in ET heifers.

SNX5 encodes a member of the sorting nexin family. Members of this family contain a phox (PX) domain, a phosphoinositide binding domain, and are involved in intracellular trafficking. SNX5 protein functions in endosomal sorting, the phosphoinositide signaling pathway, macropinocytosis, and micropinocytosis [16,17]. The macropinocytosis and micropinocytosis are considered essential for providing nutrients from a mother to the fetus. In humans, the trophoblast cells possess various transporters for glucose, amino acids, and fatty acid and are believed to preferentially utilize these low molecular-weight nutrients. However, maternal exposure to nutrient insufficiency will cause restricted nutrient supply to the placenta. The macropinocytosis is adapted as an alternative means of nutrient source to allow sustained fetal growth, which is greatly enhanced during amino acid shortage [18,19]. In addition, it has been reported that SNX5 stimulated autophagy during viral infection [20]. In cattle, amino acids are essential for the survival and development of embryo [14,21]. Furthermore, amino acid concentrations in uterine fluids during early pregnancy differ in fertile and subfertile dairy cows [22]. In this study, the metabolome analysis showed that the serum levels of several amino acids differed between pregnant and NP heifers. These observations suggest that the increase in amino acid usages for placental and fetal growth recruit more plasma amino acids to the uterus in pregnant animals, but in the NP heifers, which experienced early embryonic loss, these recruitments do not occur, resulting in transient increase in some amino acids and SNX5 in the plasma.

Pregnancy-associated glycoproteins (PAGs), produced by mono-, bi- or multi-nucleated trophectodermal cells, are released into maternal circulations. PAGs with a high concentration in peripheral blood during early pregnancy are the target factors for pregnancy test, which is the commercially available ELISA detection for domestic ruminants [23]. This detection method using the PAGs antibodies enables cows to discriminate pregnancy or not on day 28 after insemination. PAGs are pepsin-like aspartic proteinases expressed by mononuclear trophoblast and by binuclear trophoblast cells in cattle and sheep [24–26], although PAG function as active proteases is unclear. PAGs are expressed after conceptus implantation and expression increases throughout pregnancy with peak blood concentrations at near parturition [24]. Recently, in addition to PAGs, several studies have been attempting to identify specific markers for discriminating success and failure of pregnancy in ruminants [22,27–32]. However, these outcomes have not yet reached practical application. This study identified SNX5 as a novel target for discrimination between pregnant and NP heifers. Furthermore, SNX5 is detectable using blood serum on pregnant day 22, indicating that SNX5 could be usable as a pregnancy test prior to the ELISA test with PAG antibodies. Whereas, it has not been determined where SNX5 has been expressed and how SNX5 functions during pregnancy. Further investigation is required to understand the molecular mechanisms by which SNX5 affects the success or failure of pregnancy.

In conclusion, this study shows that the results from global metabolome analysis with blood sera on days 17, 20, and 22 are partly divided into pregnant and NP heifers. The proteome iTRAQ analysis also separated serum protein components between pregnant and NP heifers on day 20 or 22. Furthermore, ROC analysis identified five candidate proteins detecting pregnancy failure, of which SNX5 was detected as the novel target for discrimination between pregnant and NP heifers. These results indicate that peripheral blood on pregnant day 22 can be used for a pregnancy test, and suggest that high levels of serum SNX5 on day 22 are predictive of females undergoing embryonic loss in heifers and possibly cows.

Materials And Methods

Ethics statement

All animal procedures in this study were performed in accordance with the guidelines of the Committee for Experimental Animals at Zen-noh Embryo Transfer Center (Hokkaido, Japan), with the approval of the Institutional Animal Care and Use Committee of Zen-noh Embryo Transfer Center (Approval number: ZET20190628). All animals used were raised and kept at this center throughout the course of this experimentation. This study was carried out in compliance with the ARRIVE guidelines.

Collection of bovine blood samples

Estrous synchronization, superovulation, and embryo transfer (ET) processes were performed as previously described [33]. Recipient heifers (14~16 months old, n = 20) were given a single injection of 0.75 mg cloprostenol to synchronize their estrous cycles and the heifers exhibited behavioral estrus 40-48 h

after the injection (n = 17, day 0 = day of estrus). For ET processes, day 7 embryos were collected from super-ovulated and artificially inseminated (AI) Japanese black cows (3~5 years old, n = 5). Two blastocysts each derived from the superovulation/AI procedure were then transferred non-surgically into the uterine horn of Holstein heifers, ipsilateral to the corpus luteum, on day 7 of the estrous cycle. Blood samples were collected on pregnant days 17, 20, and 22. In addition, blood samples from a group of heifers (14~16 months old, n = 3), which went through the same estrous synchronization without ET procedure, were collected on days 0, 7, 14, and 17 of the estrous cycle. The diagnosis of pregnancy or non-pregnancy (NP), indicative of embryonic loss, was done by real-time B-mode ultrasonography (Convex scanner HS-1500, Honda electronics Co. Ltd., Toyohashi, Japan) on days 30 of gestation, from which blood samples were divided into pregnant or NP group. After sorting of blood samples, albumin was removed from serum samples by ProMax Albumin Removal Kit (Polysciences, Warrington, PA, USA).

iTRAQ analysis

A global analysis of proteins using iTRAQ analysis was performed as described previously [12]. Briefly, serum samples from ET-heifers on day 17, 20 or 22 and those without ET were resuspended in 30 µl iTRAQ lysis buffer (50 mM TAEB, 0.1 % SDS). Total protein (100 µg) was subjected to trypsin digestion and then reacted with appropriate iTRAQ reagent according to the manufacturer's instructions. Sample fractionation was performed with an Agilent 3100 OFFGEL Fractionator (Agilent Technologies, Santa Clara, CA, USA). Furthermore, mass spectrometry analysis was performed with a Thermo Scientific LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Mascot software was used to simultaneously identify and quantify proteins.

Western blot analysis

Serum samples were separated through SDS-PAGE and were then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After blocking with Block Ace reagent (DS Pharma Biomedical, Osaka, Japan), membranes were incubated with goat polyclonal anti-SNX5 (1:2000, ab5983, abcam, Tokyo, Japan), rabbit polyclonal anti-SRFBP1 (1:2000, ab109598, abcam), rabbit polyclonal anti-FAS (1:2000, ab22759, abcam), rabbit polyclonal anti-DDB1 (1:2000, ab97522, abcam), rabbit monoclonal anti-CPSF6 (1:2000, ab175237, abcam), rabbit polyclonal anti-FUT8 (1:500, ab115925, abcam), mouse monoclonal anti-ACAC (1:2000, ab205883, abcam), rabbit polyclonal anti-SNX5 (1:2000, SAB2102260, Sigma-Aldrich, Tokyo, Japan), rabbit polyclonal anti-FUT8 (1:2000, LS-C145608, Life Span BioSciences, Seattle, WA, USA), or rabbit polyclonal anti-ISG15 (1:2000, Origene Technologies, Rockville MD, USA) antibody. Immunoreactive bands were detected using enhanced chemiluminescence (EMD Millipore, Temecula, CA, USA) after incubation with horseradish peroxidase labeled anti-mouse, rabbit, or goat IgG (1:5000, Vector Laboratories, Burlingame, CA, USA). Signals were detected using C-DiGit Blot Scanner (LI-COR) and then band density was assessed with Image Studio DiGit software (version 5.2) [34].

Metabolome analysis

A serum metabolomics analysis was performed using GC/MS as described previously [35] with some modifications. In brief, a sample of 50 µl of serum was mixed with 5 µl of 1 mg/ml 2-isopropylmalic acid (Sigma-Aldrich) in distilled water as an internal standard, and 250 µl of methanol–chloroform–water (2.5:1:1) mixture. Then samples were lyophilized, and added with 40 µl of 20 mg/ml methoxyamine hydrochloride (Sigma-Aldrich), dissolved in pyridine for oximation. After mixing, the samples were shaken for 90 min at 30°C. Next 20 µl of N-methyl N-trimethylsilyl-trifluoroacetamide (GL Science, Tokyo, Japan) was added for trimethylsilylation, and the mixture was incubated at 37°C for 45 min. The sample was subjected to GC/MS (GCMS QP2010-Ultra; Shimadzu, Kyoto, Japan). The Shimadzu Smart Metabolites Database (Shimadzu) was used to identify metabolites. Samples were normalized by a pooled sample from control group. A metabolic pathway analysis was performed using MetaboAnalyst [36]. Metabolites that significantly diffed between two groups were subjected to an enrichment analysis (http://www.metaboanalyst.ca/faces/upload/EnrichUploadView.xhtml).

Statistical analysis

All experimental data represent the results obtained from three or more independent experiments each with triplicate assays. Data were expressed as the mean ± SEM. A *P*-value < 0.05 was considered statistically significant.

Declarations

Declaration of competing interest

The authors declare no conflicts of interest.

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Author contributions

K.K., R.B., and K.I. conceived and designed the experiments; K.K., R.B., and K.N. performed the experiments and analyses. K.K. and K.I. wrote the manuscript. K.K., R.B., Y.M., T.S., K.N., M.H., and K.I. coordinated the project and contributed to the data analysis and results interpretation. All authors read and approved the final manuscript.

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Figures

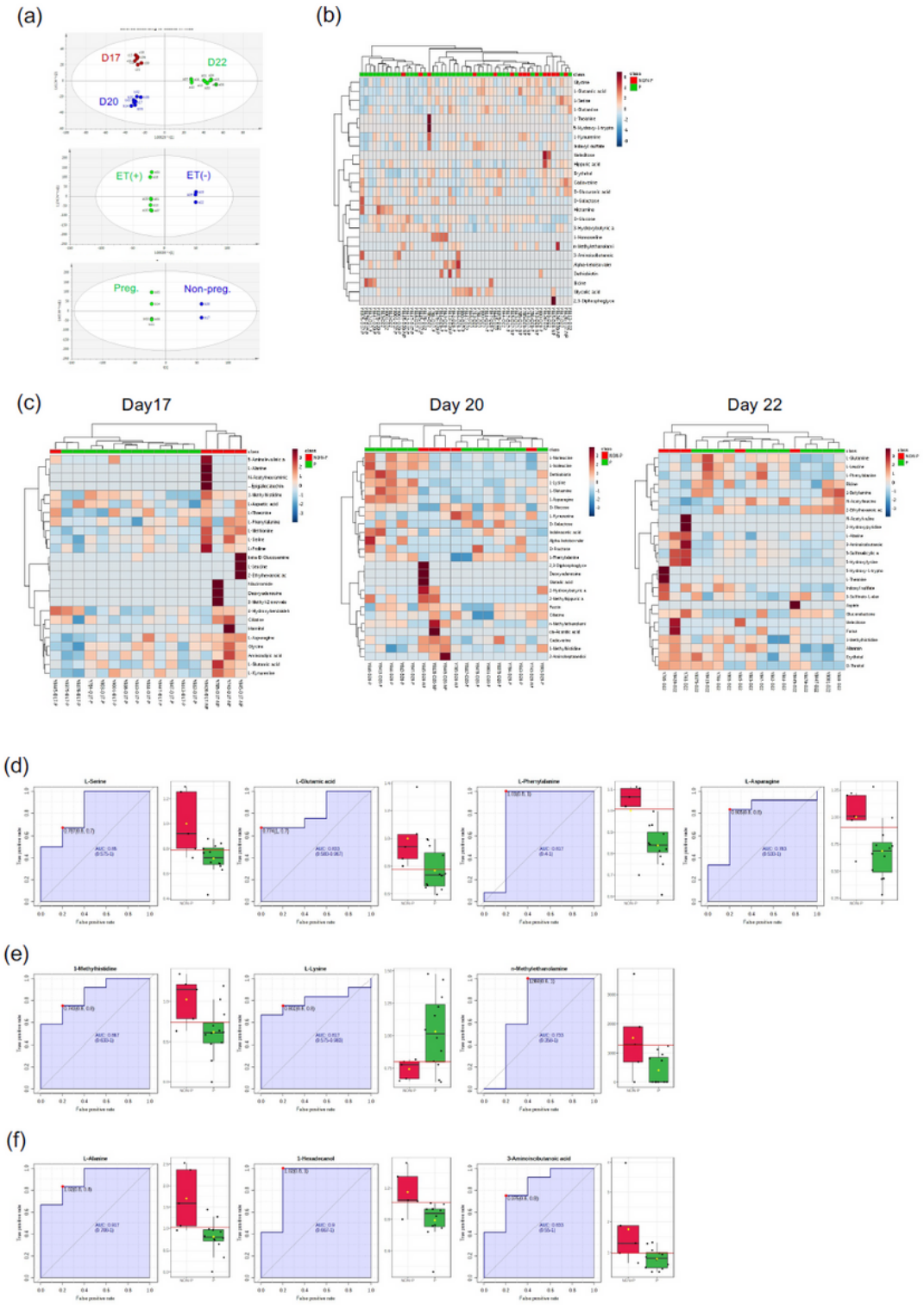

Figure 1

Metabolic analysis of bovine peripheral blood sera during the peri-implantation period. (a) Biplot produced by the principal component analysis (PCA) of metabolome factors in bovine blood sera. Differences are found between days of pregnancy (top), ET (+) and ET (-) (middle), and pregnancy and non-pregnancy (NP) (bottom). (b) Heatmap analysis with days 17, 20, and 22 sera. High-concentration factors are shown in red and low-concentration factors are shown in blue. P: sera from pregnant heifers,

NON-P: sera from NP heifers. (c) Heatmap analysis with each of day 17, 20, or 22 sera. High-concentration factors are shown in red and low-concentration factors are shown in blue. P: sera from pregnant heifers, NON-P: sera from NP heifers. (d-f) Receiver operating characteristic curve (ROC) analysis was performed to assess the predictive power of variables and to measure the optimum cutoff point for NP heifers in peripheral blood sera from day 17 (d), 20 (e), or 22 (f). Box plot shows individual sample values from pregnant and NP heifers.

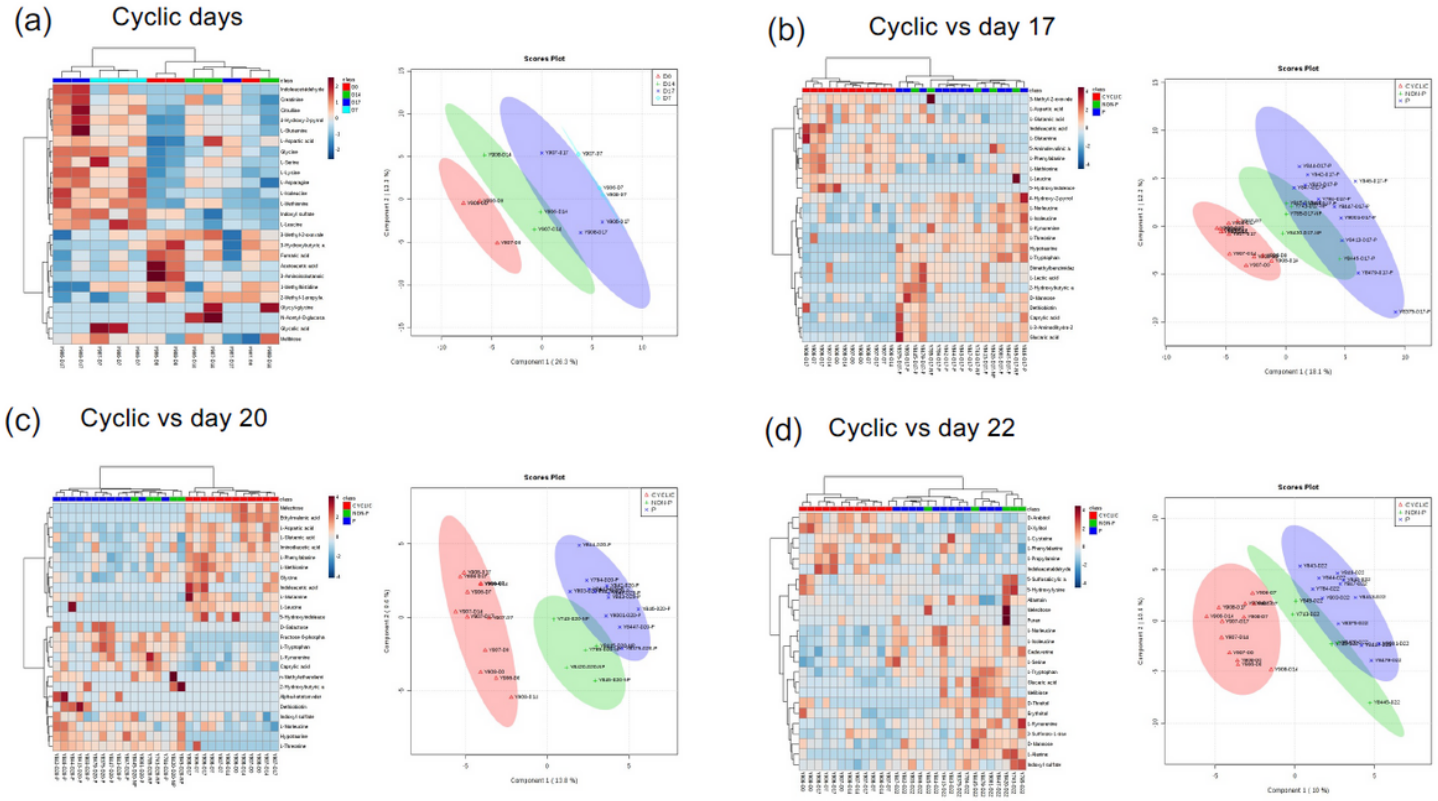

Figure 2

Comparison between pregnant and NP heifers and those with estrous cycle. (a) Heatmap analysis with blood sera from heifers with estrous cycle (days 0, 7, 14, and 17). Biplot produced by PCA of metabolome factors in sera from heifers with estrous cycle; days 0, 7, 14, and 17. (b-d) Heatmap and PCA with sera from estrous cycles compared with those on day 17 (b), 20 (c), or 22 (d). CYCLIC: serum samples from heifers with estrous cycle, P: sera from pregnant heifers, NON-P: sera from NP heifers.

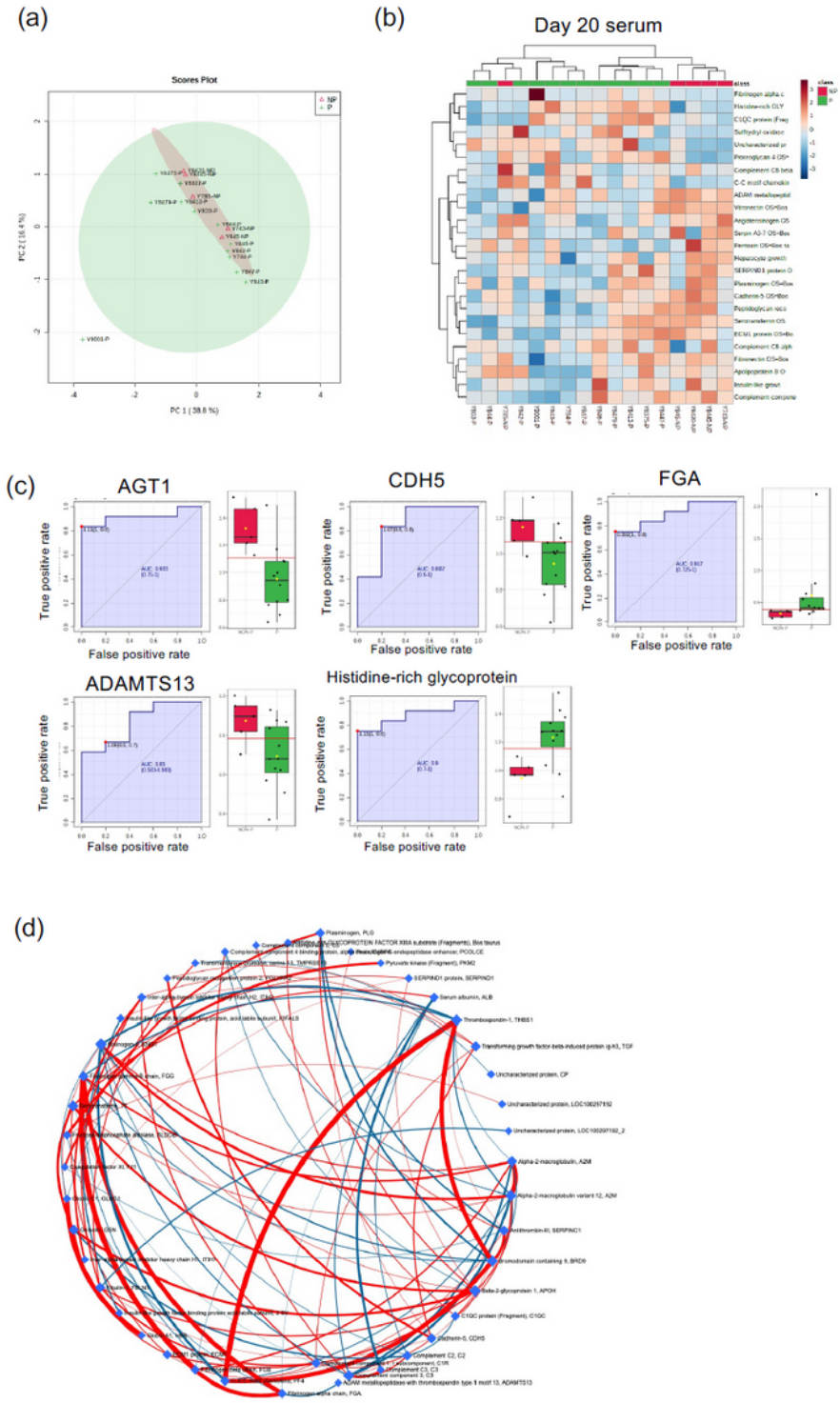

Figure 3

Global proteome analysis with bovine day 20 peripheral blood sera. (a) Biplot produced by PCA of proteins identified by iTRAQ proteome analysis in day 20 blood sera. (b) Heatmap analysis with proteins in day 20 sera. High-concentration factors are shown in red and low-concentration factors are shown in blue. P: sera from pregnant heifers, NP: sera from NP heifers. (c) ROC analysis was performed to assess the predictive power of variables and to measure the optimum cutoff point in day 20 sera for NP heifers.

Box plot shows individual sample values from pregnant and NP heifers. (d) Network analysis with proteins identified in day 20 sera by iTRAQ. Red or blue lines indicate positive or negative regulation, respectively.

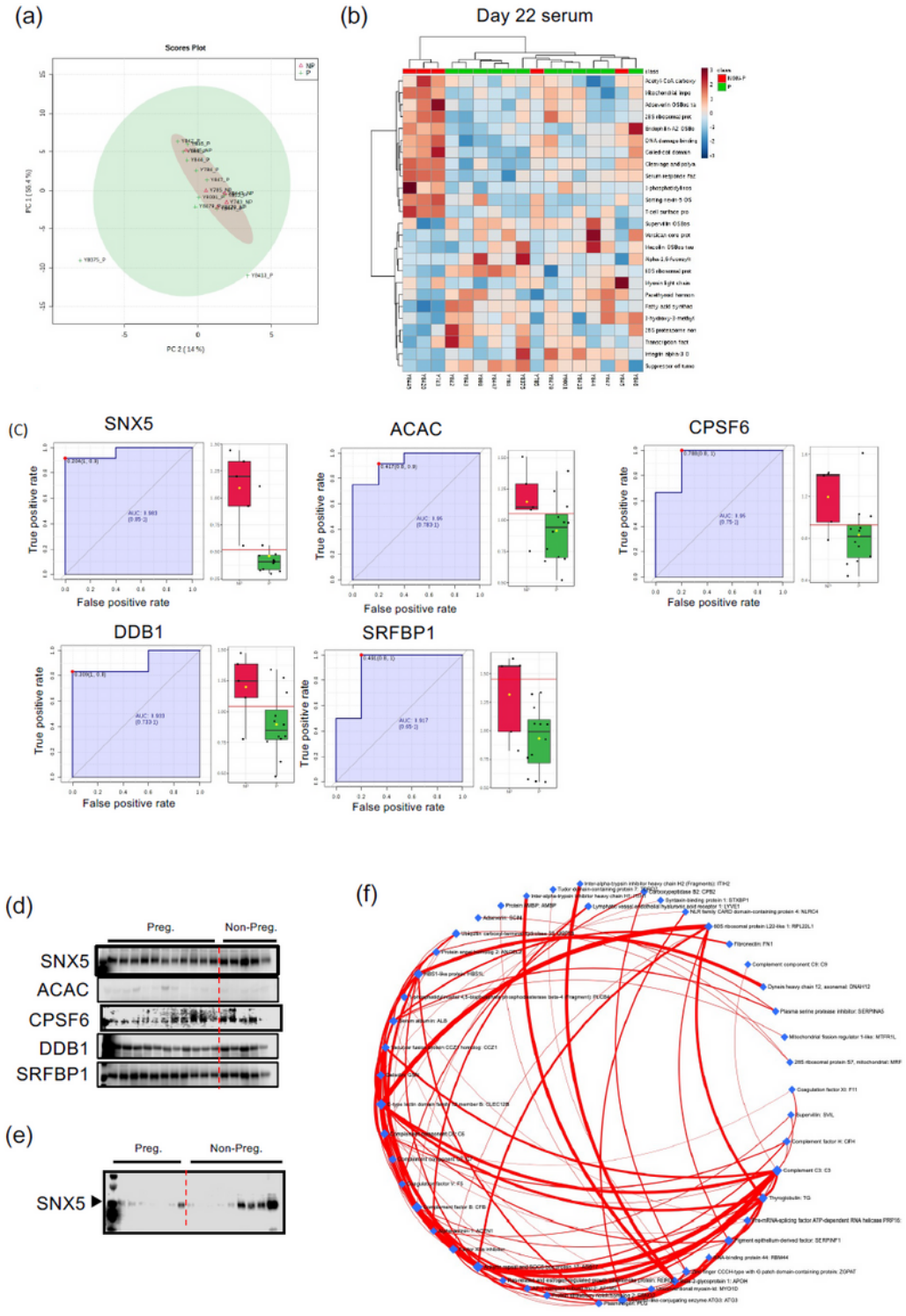

Figure 4

Identification of specific proteins in day 22 peripheral blood sera for NP heifers. (a) Biplot produced by PCA of proteins identified by iTRAQ proteome analysis in day 22 blood sera. (b) Heatmap analysis with

day 22 proteins in sera. High-concentration factors are shown in red and low-concentration factors are shown in blue. P: sera from pregnant heifers, NP: sera from NP heifers. (c) ROC analysis was performed to assess the predictive power of variables and to measure the optimum cutoff point in day 22 sera for NP heifers. Box plot shows individual sample values from pregnant and NP heifers. (d, e) The sera from pregnant or NP heifers on day 22 were subjected to western blotting, which revealed the presence of identified proteins; SNX5, ACAC, CPSF6, DDB1, or SRFBP1. (f) Network analysis with identified protein data on day 22 by iTRAQ. Red line indicates positive regulation.

Supplementary Files

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