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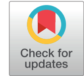
Proteomic profile alterations inporcine conceptuses during early stages of development



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A B S T R A C T

The dynamic embryo development during the early stages of gestation requires precise molecular changes, including proteomic ones. We aimed to find unique proteins for porcine conceptuses specifically during the peri-implantation period, *i.e.* on days 15–16 of pregnancy. The proteomic profile of these conceptuses was compared with conceptuses at an earlier stage of the development, *i.e.* collected during maternal recognition of pregnancy on days 12–13 of pregnancy. The 2DE, gel image analysis, and MALDI TOF mass spectrometry were used 500 protein spots were annotated as common to conceptuses harvested during both studied periods. Proteomic profile of the conceptuses collected during the peri-implantation period contains 24 unique proteins. Identified unique for the peri-implantation period proteins are involved in adhesion processes, cadherin, and actin-binding, and actin filament organization, extracellular matrix organization, and cytoskeleton organization. Systemic analysis of identified proteins confirmed their involvement in cell adhesion and cytoskeletal organization as being two major affected functions. The unique proteins might be recognized as factors conditioning the proper peri-implantation embryo development and gaining competences for implantation. In further studies, BRCA1 might be considered as a candidate for a potential marker of embryonic competences for implantation in pigs.
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1. Introduction

The reproductive success in mammals requires the proper intrauterine embryo-maternal crosstalk. This communication is certainly dependent on the embryonic and uterine secretory proteins and membrane ligands action [1–3]. The inadequate or insufficient molecular embryo-maternal crosstalk occurred mainly during the peri-implantation period, which may cause pregnancy failure [4]. The dynamic development of conceptuses during early pregnancy is undoubtedly outstanding. Soon after entering the uterine horns, embryos adopt spheroid form (diameter 1–2 mm) which transforms to the ovoid and the tubular one (diameter 9–20 mm) [4]. The rapid elongation of porcine embryos occurs between days 10 and 12 of pregnancy enabling embryos to transform to the filamentous form (length >100 mm) [4,5]. It was established that during days 11–12 of pregnancy, when the morphology of conceptuses is between tubular and filamentous form, the proteomic profile of these conceptuses contains 35 unique proteins [5]. These proteins are associated with cell proliferation, differentiation, apoptosis, and embryo-maternal signaling [5]. Until days 12–13 of gestation, the porcine embryos remain free-floating in the uterine lumen then on day 13 of pregnancy appose to the uterine luminal uterine epithelium of the endometrial tissue [4,6]. The trophoblast adhesion, which follows its apposition, requires the presence of components of the extracellular matrix (ECM), cadherins and integrins [7]. We believe that reaching this stage of embryonic development (conceptuses are at filamentous forms on days 15–16 of pregnancy) requires abundant proteomic changes in conceptuses which are crucial for normal implantation and thus, pregnancy maintenance.

We hypothesize that the proteomic profile of conceptuses on days 12–13 of gestation when they remain free-floating in the uterine lumen and continue their slow elongation significantly differs from the proteomic profile of conceptuses on days 15–16 of

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gestation when the embryos start to attach to the endometrial surface. Thus, the study aimed to compare proteomic profiles of porcine conceptuses harvested on days 12–13 of pregnancy with the proteomic profile of conceptuses on the days 15–16 using 2DE, gel image analysis, and MALDI TOF mass spectrometry. The results of the present study have the potential to complete knowledge concerning the proteomic profile of porcine conceptuses from days 12 to 16 of pregnancy and may be useful to determine the stage-specific markers of early pregnancy development in this species.

2. Experimental procedures

2.1. Animals and embryos collection

All experiments were approved by the Animal Ethics Committee, University of Warmia and Mazury in Olsztyn, Poland. Post-pubertal crossbred pigs (Large White × Polish Landrace) in the early stages of pregnancy, weighing 90–110 kg were used. Gilts were naturally fertilized on the second day of estrus which was designated as the first day of pregnancy. The animals were slaughtered on days 12–13 ($n = 4$), and 15–16 ($n = 4$) of pregnancy in a commercial abattoir. After slaughter, corpus lutei from both ovaries of each pig were counted and totaled between 11 and 15 in both groups: pigs on days 12–13, and days 15–16 of pregnancy. The entire conceptuses (embryos and extraembryonic membranes) were flushed from each uterine horn with 20 ml of sterile phosphate-buffered saline (PBS). Flushed cconceptuses were examined morphologically to confirm the stage of the pregnancy [1]. The number of conceptuses was not calculated. The collected conceptuses were not separated on embryonic disc and extraembryonic membranes. Conceptuses were pooled from each pig and centrifuged for 10 min at 300 × g, washed with PBS, and again centrifuged in the same conditions to separate uterine secretory proteins. Then, conceptuses were snap-frozen in liquid nitrogen and stored at -80 °C until required for further steps of the analysis.

2.2. Proteins extraction and proteome profiling

For the proteomics study, samples of conceptuses prepared as in Section 2.1, were extracted directly into the rehydration buffer (8 M urea, 2% CHAPS, 0.002 % Bromphenol blue, 0.28 % dithiothreitol, 0.5 % ampholytes 3–10 pH (IPG) buffer). Extracts were then centrifuged at 13,000 rpm for 15 min and supernatants were used for two-dimensional gel electrophoresis (2DE). For each gravid pig, one 2DE gel was generated.

The 2DE, gel image analysis, and MALDI TOF mass spectrometry were performed as was described earlier [8]. In the first dimension, isoelectrofocusing was performed using 18 cm linear Immobiline DryStrips with a pH range of 3–10 in an IPGPhor instrument (GE Healthcare) by passive rehydration for 10 h at 20 °C and following electrophoresis (50 V for 3 h, 1000 V for 1 h, and 5000 V for 10 h or until reaching 32,000 Vhrs). Before the second dimension SDS-PAGE, the IPG strips were equilibrated in two steps with equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30 %, glycerol, 2% SDS, 0.002 % bromophenol blue). The first step includes incubation with the equilibration buffer containing DTT, and the second step depends on incubation with the equilibration buffer containing iodoacetamide. The second dimension SDS-PAGE was performed in Ettan Dalt Six electrophoresis system (GE Healthcare). At least four good quality 12 % SDS-PAGE gels were generated for each sample and were stained with silver nitrate. Protein spots were analyzed using Image Master Platinum v5.0 software (GE Healthcare). A Student's t-test was performed to ensure the statistical significance of the spot selection ($P < 0.05$). Generated 2DE gels were divided into two groups. Group A included the gels representing the proteome of tubular and filamentous, free-floating in the uterine lumen conceptuses harvested on days 12–13 of pregnancy ($n = 4$). Group B included the gels representing the proteome of filamentous conceptuses at the beginning of attachment to the endometrium, harvested on days 15–16 of pregnancy ($n = 4$). Proteins which had the unique expression pattern between embryos harvested at different stages of pregnancy were selected for identification by mass spectrometry. The protein spots then were visible only in 2DE gels made for conceptuses harvested on days 12–13 or 14–15 of pregnancy will be further called unique, which can be understood as the expression level of the specific protein is detectable in one of the compared groups and in other group is below the level of detection.

2.3. Protein identification

Protein spots were excised from the gels, destained and subjected to in-gel digestion with trypsin (modified sequence-grade, porcine, Promega). Tryptic peptides were concentrated and desalted on a μC18 ZipTip (Millipore Billerica). Peptides were directly eluted with 50 % acetonitrile containing α-cyano-4-hydroxycinnamic acid (CHCA) matrix onto the metal target and analyzed by MALDI TOF MS on Micromass' M@LDI-Reflectron instrument (Waters, Milford, USA). Embedded Micromass software (MassLynx ™ Software v4.0) was used to process the mass spectra. Peptides spectra were internally calibrated using autolytic peptides from the trypsin (842.510, 1,045.564 and 2,211.105 Da). To identify proteins we performed searches in the NCBI database using the ProFound search engine. One missed cleavage, alkylation with iodoacetamide and partial oxidation of methionine were allowed. Search parameters were set on mass tolerance of 0.3 Da, pI and M_r defined by the migration position of a spot in the 2D gel, and '*Mammalia*' was selected for species search. "Mammalia" was selected in searches because the "*Sus scrofa*" entries which could be used for mass spectrometry searches are fewer in comparison to "*Mammalia*". Therefore, the use of "*Mammalia*" for searches allowed better matching of the mass spectra with proteins. The significance of the identification was evaluated according to the probability value, Z-value, number of the matched peptides and sequence coverage.

2.4. Systemic analysis

Protein names were translated into Gene Ontology terms (bioDBnet biological DataBase network (http://biodbnet.abcc.ncifcrf.gov). The Panther Classification System was used to identify functional domains affected by the identified proteins. Relationships between these identified proteins were explored by Cytoscape tool 3.7.0 (cytoscape.org). MiMI plug-in was used to extract relevant proteins and genes from public databases. We used databases with deposition of *Sus scrofa* genes and proteins interactome data, *e.g.* UniProt. The network was viewed in Cytoscape. A Fisher's exact test was used to calculate the P-value determining the network connectivity and any connections with $P < 0.05$ were considered.

2.5. Validation experiment

The validation of proteomic analyses was performed using immunofluorescence staining. Conceptuses collected during days 12–13 and 15–16 of pregnancy were first put on SuperFrost microscope slides and fixed with 4% paraformaldehyde for 15 min at room temperature (RT). Then, slides were washed in TBS-T (3 × 5 min, RT) and covered with a blocking buffer containing 30 % donkey serum in PBS with 1% TRITON X100 for 1 h at 4 °C. Next, slides were washed in triplicates in TBS-T (5 min, RT) and covered with primary antibodies anti-BRCA1 (Abcam, ab213929) according to the manufacturer's protocol. Incubation in primary antibodies

was conducted overnight at 4 °C. The next day slides were washed in TBS-T (3 × 5 min, RT) and covered with secondary antibodies AlexaFluor 555 donkey anti-rabbit (Thermofisher Scientific, 1:1500 in PBS, 1 h 4 °C). Subsequently, slides were washed in PBS (3 × 10 min, RT) and mounted with Fluoroshiled with DAPI (Sigma Aldrich, Germany). The next day slides were observed using a BX51 Olympus microscope and photographs were taken at magnification 400 × . Data were collected from 20 fields of each slide. The intensity of the immunofluorescence signal was calculated with Cell^F software and normalized with the intensity of the immunofluorescence signal of negative controls for each group (Olympus, Japan).

3. Results

3.1. Proteome profiling of embryos

Two-dimensional gel electrophoresis (2DE) was used to identify proteins that were unique for conceptuses collected on days 12–13 of pregnancy and for those collected at the peri-implantation period (days 15–16 of pregnancy). We performed image analysis to detect protein spots common for each experimental group. After gel image analysis and statistical analysis, the range of 500 protein spots was annotated as common to both groups. For comparison of the proteome maps in 2D gels, only protein spots that were unique for each group were considered for further identification. Fig. 1 shows representative gels for each experimental group, with indications of the migration positions of the identified proteins. When compared to the proteome of conceptuses collected at different stages of development, we identified five spots that were unique to conceptuses harvested on days 12–13 of pregnancy and 107 spots that were unique to conceptuses harvested on days 15–16 of pregnancy. Out of 112 unique spots, we identified 24 proteins unique only to conceptuses harvested at the peri-implantation (days 15–16 of pregnancy). Identification data for other proteins did not pass the required significance, probably due to under-representation of *Sus scrofa* proteins is the NCBI database. The list of these proteins is shown in Table 1.

3.2. Systemic analysis

To investigate the relationship between the 24 identified proteins, a network of interactions involving these proteins was generated. The generated network incorporated 17 of the 24 proteins. Afadin (MLLT4), dual specificity mitogen-activated protein kinase 7 (MAP2K7) and breast cancer type 1 susceptibility protein homolog (BRCA1) created separate structures in the whole network, which indicates that these proteins do not have a direct connection to other proteins in the network. Moreover, proteins leucine-rich repeat-containing 16A (LRRC16A) and coiled-coil domain-containing protein 88B (CCDC88B) are located at a distance to the whole network in comparison to the remaining proteins, which indicates a lower level of connection to the other proteins (Fig. 2).

To identify clusters of potential key regulators, the generated network (Fig. 2) was explored for the presence of subnetworks (Fig. 3). Subnetworks represent nodes with higher connectivity between them, as compared to other nodes in the whole network. The detection of subnetworks allows the detection of specific regulatory processes integrated with the whole network. Eleven subnetworks were extracted (Fig. 3). The subnetworks were mostly involved in the following functions: binding processes, transcription, DNA repair and replication, cell cycle regulation, signaling processes, metabolism, transport, and apoptosis. Many proteins involved in subnetwork 3 take part in estrogen receptor binding and activity, response to estradiol and estrogen, androgen and androgen receptor binding, and in utero embryonic development. It is worth mentioning that one protein – afadin – is involved in two subnetworks (subnetworks 6 and 8). To classify the identified proteins into biological categories, the Panther Classification System was used. The classification of the identified proteins is shown in Tables 2 and 3.

3.3. Validation experiment

The abundance of BRCA1 protein was observed in all collected conceptuses and was significantly higher ($P \leq 0.05$) in conceptuses collected on days 15–16 of pregnancy when compared to conceptuses collected on days 12–13 of pregnancy (Fig. 4A, B). Thus, the validation experiment confirmed the results obtained *via* proteome profiling.

4. Discussion

Detection of proteins unique for conceptuses harvested on days 12–13 as compared to those harvested at days 15–16 gives some insight into the development of porcine embryos. We report here detection of five protein spots unique for conceptuses harvested on days 12–13 of pregnancy and 107 protein spots that were unique

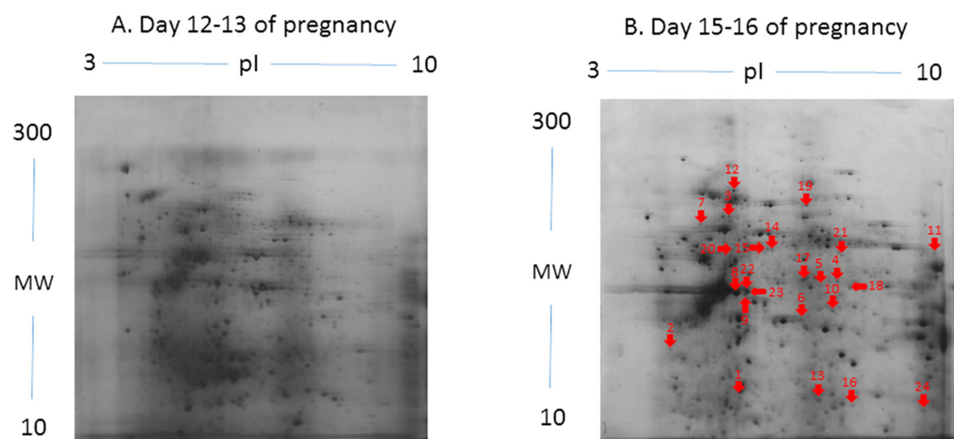

Fig. 1. The images of representative 2DE gels used for proteome profiling of porcine conceptuses harvested on days 12-13 (A), and 15-16 (B) of pregnancy. The images show the separation of proteins extracted from porcine conceptuses with the indication of migration positions of identified proteins. Directions of isoelectric focusing and SDS-PAGE are indicated on the top and the side of each gel.

Table 1
The list of unique proteins identified in porcine embryos harvested on days 15-16 of pregnancy when compared to embryos harvested on days 12-13 of pregnancy. Annotations: (1) Names of the proteins are as in the NCBI database, with annotation of the corresponding GO terms for the identified proteins. (2) - (8) Probability, Z-value, number of matched peptides, sequence coverage, pI and mass values were obtained through database searches.

Spot no.	Protein identity (1)	NCBI accession no. (2)	Proba-bility (3)	Z score (Est'dZ) (4)	Matched peptide (5)	Sequence coverage (%) (6)	pI (7)	MW (kDa) (8)
1	titin, isoform CRA_b	EAX11015.1	1.0e+000	1.67	36	7	7.8	76.92
2	leucine-rich repeat-containing protein 16A isoform 1	XP_003263275.1	1.0e+000	1.23	17	15	7.2	122.08
3	WD repeat domain 52, isoform CRA_c	EAW79642.1	9.9e-001	1.56	53	20	5.3	215.12
4	nuclear pore complex protein Nup205	XP_003261424.1	1.0e+000	1.49	22	14	5.8	230.20
5	Coiled-coil domain-containing protein 41	EGW12191.1	1.0e+000	2.43	26	25	6.1	82.32
6	afadin	XP_002747234.2	1.0e+000	1.33	28	21	6.1	202.68
7	collagen, type VI, alpha 3, isoform CRA_a	EAW71103.1	1.0e+000	0.97	18	12	5.8	238.89
8	probable ATP-dependent RNA helicase DDX60-like	XP_001373688.2	1.0e+000	1.17	24	17	8.1	191.99
9	centromere protein J	AEI84875.1	1.0e+000	1.38	16	20	8.1	61.25
10	MORC family CW-type zinc finger protein 4 isoform 2	XP_003414863.1	1.0e+000	1.05	12	16	6.6	106.64
11	hypothetical protein PANDA_008266	EFB20728.1	1.0e+000	1.11	19	14	7.7	194.61
12	ADAMTS-like 3	XP_002721508.1	1.0e+000	1.67	22	15	9.5	191.83
13	FERM and PDZ domain-containing protein 1	XP_003940107.1	1.0e+000	1.08	22	15	5.2	174.18
14	human BRCA1 homolog	AAB17113.1	1.0e+000	1.31	21	15	5.5	201.73
15	RUN and FYVE domain-containing protein 2-like	XP_003501055.1	9.8e-001	1.22	19	22	5.5	78.78
16	kinesin-like protein KIF14-like, partial	XP_002809692.2	1.0e+000	1.17	25	26	9.1	117.84
17	E3 ubiquitin-protein ligase TTC3	NP_001103237.1	1.0e+000	0.92	22	12	6.3	228.74
18	ubiquitin-protein ligase E3B	XP_003994904.1	1.0e+000	1.41	18	16	9.0	124.52
19	myosin XVIIIB, isoform CRA_c	EAW59704.1	1.0e+000	1.43	28	13	6.3	235.65
20	coiled-coil domain-containing protein 88B-like	XP_003515068.1	9.9e-001	1.03	19	14	5.2	165.25
21	mitogen-activated protein kinase 4	XP_548811.3	1.0e+000	1.20	13	31	5.3	66.65
22	FRAS1-related extracellular matrix protein 1	XP_002819829.1	1.0e+000	1.18	20	11	5.5	245.96
23	uncharacterized protein LOC100932970	XP_003770262.1	9.7e-001	1.01	15	20	11.5	62.60
24	hypothetical protein LOC100717135	XP_003477705.1	9.9e-001	0.94	16	14	11.2	151.30

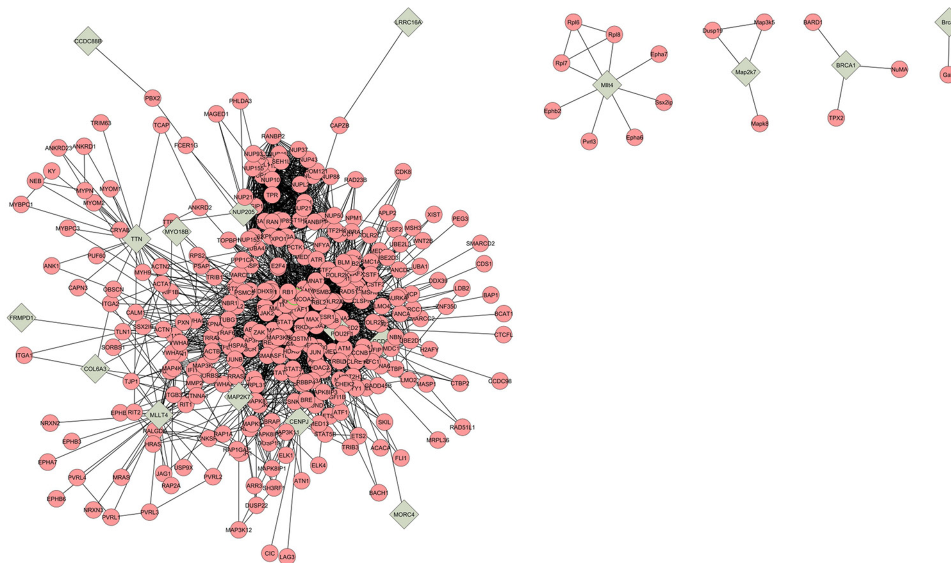

Fig. 2. The network of unique proteins identified in porcine conceptuses harvested on days 15-16 of pregnancy when compared to embryos harvested on days 12-13 of pregnancy. The network is shown to indicate the overall structure of the network. Diamonds represent proteins identified by proteome profiling and cycles show network components that interact with the identified proteins. Annotation of the network components are in GO terms, and the network was created in Cytoscape.

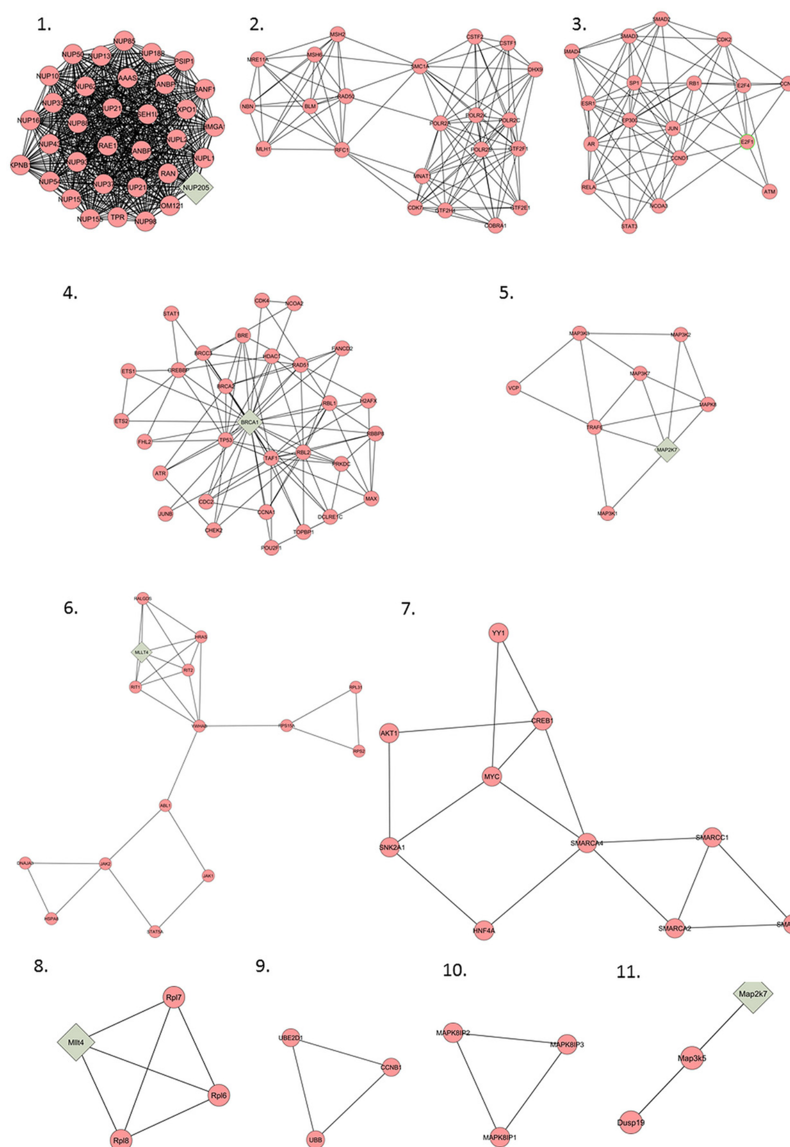

Fig. 3. Subnetworks formed by the proteins involved in the main network of unique proteins identified in porcine conceptuses harvested on days 15-16 of pregnancy when compared to conceptuses harvested on days 12-13 of pregnancy. Diamonds represent proteins identified by proteome profiling and cycles show network components that interact with the identified proteins and with each other. The proteins included in the subnetworks are mainly involved in transcription, DNA repair and replication, signaling processes and cell cycle.

Table 2
Classification of unique proteins identified in porcine conceptuses harvested on days 15-16 of pregnancy when compared to embryos harvested on days 12-13 of pregnancy, in terms of the biological process using gene ontology (GO) terms.

Biological process	Proteins
cellular component organization or biogenesis	CCDC88b, TTN, BRCA1, NUP205
cellular process	NIUP205, MAP2K7, FREM1, BRCA1, TTN, CCDC88b, ADAMTSL3
developmental process	MAP2K7, MYO18B, TTN
immune system process	LRRC16A
localization	CDC88b
metabolic process	LRRC16A, BRCA1, TTN, ADAMTSL3
multicellular organismal process	MYO18B, TTN
reproduction	CENPJ
response to stimulus	MAP2K7, LRRC16A, BRCA1
biological regulation	MAP2K7, BRCA1

for conceptuses harvested on days 15–16 of pregnancy. Among these spots were indicated 24 defined, unique proteins for conceptuses harvested on days 15–16 of pregnancy when compared to conceptuses harvested on days 12–13 of pregnancy. The uniquely expressed proteins play an important role in adhesion processes, cadherin, and actin-binding, actin filament organization, extracellular matrix and cytoskeleton organization, *e.g.* titin (TTN), afadin (MLLT4), leucine-reach repeat-containing

Table 3
Classification of unique proteins identified in porcine conceptuses harvested on days 15-16 of pregnancy when compared to embryos harvested on days 12-13 of pregnancy, in terms of the molecular function using gene ontology (GO) terms.

Molecular function	Proteins
binding	MAP2K7, LRRC16A, TTN, CCDC88B, ADAMTL3
catalytic activity	MAP2K7, BRCA1, CENPJ, ADAMTSL3
receptor activity	CENPJ
signal transducer activity	MAP2K7
structural molecule activity	NUP205, TTN

protein 16A isoform 1 (LRRC16A), collagen, type VI, alpha 3, isoform CRA_a (COL6A3), myosin XVIIIB, isoform CRA_c (MYO18B) and FRAS-1 related extracellular matrix protein 1 (FREM1) [9–12]. Among proteins that are unique for conceptuses on days 15–16 of pregnancy breast cancer type 1 susceptibility protein homolog (BCRA1) was also determined. Previously, it was found that knockout *BRCA1* (-/-) mouse conceptuses are developmentally retarded and the presence of BCRA1 has been suggested as a marker of implantation competency in this species [13,14]. The validation procedure showed that for further research we can propose testing BRCA1 for distinguishing the developmental stage of embryos, as the content of this protein in embryos collected on days 15–16 of pregnancy is significantly higher than in embryos collected on days 12–13 of pregnancy. This result can indicate that on days 12–13 of pregnancy conceptuses can start gaining competence for implantation, but the significant increase of the content of implantation markers, like BRCA1, is visible on days 15–16.

Previously, it was determined that in porcine embryos collected at their earlier developmental stages, *i.e.* on days 11–12 of pregnancy, many of the identified proteins are involved in cell proliferation and differentiation, cytoskeleton organization, metabolism, and stress response [5]. Therefore, in the current study, we documented and confirmed that the proteomic profile of porcine conceptuses alters dynamically during the early stages of embryo development. It is known that during early pregnancy also uterus undergoes morphological and molecular adaptations, creating a suitable intrauterine milieu for embryos development [7,15–21]. The recent studies showed that endometrium collected from pigs during the peri-implantation period (days 15–16 of pregnancy) expressed differently 589 genes comparing to the endometrium collected from pigs during the luteolysis (days 15–16 of the estrous cycle) [17]. Encoded, by these genes with the altered expression, proteins were found to be involved in *i.a.* cell adhesion, cell communication, and developmental processes and enriched mostly pathways of cell adhesion molecules, and steroid hormone biosynthesis [17]. Moreover, the proteomic studies determined that during the peri-implantation period the expression of the mucin glycoprotein (Muc-1) on the surface of endometrial epithelial cells reduces and the expression of cadherins and integrins that are involved in trophoblast adhesion to the surface of the endometrium [7,15]. Further studies showed that among the unique proteins for endometrium collected during days 15–16 of pregnancy compared to days 12–13 od pregnancy there are Ras GTPase-activating protein 4 isoform 1 (RASA4) and thrombospondin 1, isoform CRA_b (THBS1) [20]. RASA4 was found to switch the Ras-MAPK pathway and therefore, to elevate intracellular calcium content [22]. THBS1, as a component of THBS1 proteins family, support calcium-dependent cell attachment, induce cell proliferation and migration as well as cytoskeletal organization [23–26]. Therefore, the preparation of the endometrium to the process of implantation appears to be connected with increased adhesion capacity, cell communication, and steroid hormones synthesis and metabolism Thus, the previous and the present study document that on days 15–16 of gestation both endometrium and embryos acquire similar competencies, including the increased adhesion capacity, for successful implantation.

Among proteins identified as unique for conceptuses harvested on days 15–16 of pregnancy, comparing to conceptuses harvested on days 12–13 of pregnancy we identified titin, collagen type 6 and afadin. Previously, defective myofibril assembly was observed in titin knockout mice, which suggests the role of this protein in the cytoskeleton organization [27]. Cadherin binding, actin filament binding, regulation of the cytoskeleton and cell adhesion are major functions in which protein building subnetwork 6 and 8 are involved. Among these proteins in both subnetworks, there is afadin – a protein identified in the current study. Abnormal cell adhesion may cause pregnancy failure. Thus, the proteins identified in our study may be important for embryo attachment and pregnancy maintenance during the peri-implantation period.

It is noteworthy that there are some similarities while comparing proteins identified in the current study and results obtained from the analysis of transcriptomic profiles of embryos collected during days 15–16 of pregnancy [28]. We reported previously that the expression of *COL6A3* and *TTN* was increased in embryos during the peri-implantation period in response to a restricted diet applied to females during the peri-conceptional period indicating the impact of environmental factors on these genes expression [28]. The Gene Ontology analysis showed that *COL6A3*, encoding collagen, type VI, alpha 3 is involved in cellular components (CC) terms - in the extracellular exosome (GO:0031012) and extracellular space (GO:0005615) categories, as well as in molecular functions (MF) terms - in serine-type endopeptidase inhibitor activity (GO:0004867) category [28]. Unfortunately, in the literature, there is a lack of data concerning

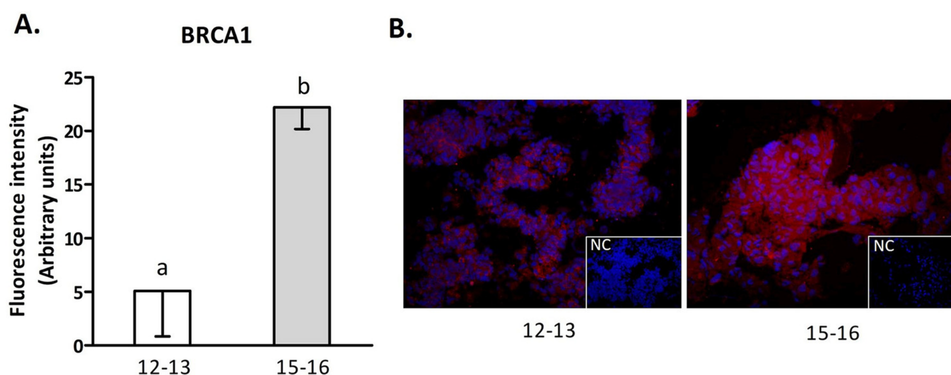

Fig. 4. The relative abundance (A) and immunolocalization (B) of BRCA1 in conceptuses collected on days 12-13 and 15-16 of pregnancy. Data concerning relative abundance (A) are shown as mean ± SEM. Different lower-case letters (a, b) indicate statistically significant differences in protein abundance in conceptuses collected on days 12-13 *vs.* 15-16 of pregnancy. For immunolocalization (B) photographs were taken under magnification 400×.

the significance of TTN in the regulation of embryo development. However, it could not be excluded that COL6A3 and TTN are, among others, the crucial proteins involved in the successful attachment of the conceptuses to the endometrium during the peri-implantation period.

To identify clusters of potential key regulators, the subnetworks were analyzed. The subnetwork analysis allowed gaining insight into mechanisms engaged by the identified proteins. The network retrieved 339 proteins with evidence of interaction with the identified proteins. Our results demonstrated that in subnetwork 3, five proteins (estrogen receptor 1, ESR1; androgen receptor, AR; retinoblastoma-associated protein, RB1; nuclear receptor coactivator 3, NCOA3 and G1/S-specific cyclin-D1, CCND1) are involved in estrogen and androgen binding, estrogen, and androgen receptor binding and its signaling pathway and in the response to estrogens. The protein involved in the cellular response to E_2 is also BRCA1 [29], identified in our study as unique to embryos harvested on days 15–16 of pregnancy.

It is worth mentioning that in the subnetwork 3 as well as in subnetworks 2 and 4, there are proteins which are involved *in utero* embryo development and somitogenesis. For example, mothers against decapentaplegic homolog 3 and 4 (SMAD 3 and SMAD 4) – in subnetwork 3, DNA mismatch repair protein (Msh2, MSH2) – in subnetwork 2 and DNA-dependent protein kinase catalytic subunit (PRKDC) – in subnetwork 4. Somitogenesis is a crucial process in vertebrate body segmentation during embryogenesis [30]. Thus, we suggest that these factors may be important for proper embryos development, particularly during the peri-implantation period.

In the current study, BRCA1 and MAP2K7 were identified as being unique in conceptuses during days 15–16 of pregnancy. According to databases and the current literature, both proteins play a role in the apoptotic process [31]. Programmed cell death is present in mammalian blastocysts, and its normal pattern is crucial for further development of the embryo [32]. Disruption of apoptosis in the blastocyst may lead to early embryonic death or the formation of anomalies in the fetus [33]. During later stages of normal embryo development, apoptosis plays a key role in the formation of the extraembryonic structures and the embryo itself. Apoptosis was also observed in fetal membranes [34]. There is also evidence, that mutation in the *BRCA1* gene in mice results in embryonic lethality and the developing embryos show signs of cellular proliferation defects associated with activation of the p53 pathway [35]. Gene knockout studies in mice reveal that several of the MAPK signaling events are essential for embryonic development [36]. The results of the above-mentioned studies and the known role of identified proteins in apoptosis suggest that BRCA1 and MAP2K7 are important for embryo development and the establishment of pregnancy.

5. Conclusions

In conclusion, many of the proteins identified during the peri-implantation conceptuses are involved in cytoskeletal organization and adhesion and thus, may play important roles in embryonic development and their preparation for implantation. We believe that the synthesis of these unique proteins in conceptuses is required for acquiring developmental competence for implantation. The next step of the study should be determining the effect of knock-out or silencing the expression of genes encoding these unique in conceptuses proteins to determine whether their deficiency deteriorates the developmental potential of embryos or disrupts the regular course of implantation. Moreover, we suggest that BRCA1 might be used in further studies as a potential marker of embryonic competence for implantation in pigs.

Author contributions

J. K. conception of the study, performance of main analyses and interpretation of data, preparation of a draft of the MS; E. M. D. performance of validation experiment, preparation of a draft of the MS and its edition; approval of the final version to be submitted; W. K taking part in the validation of the study, edition of figures and tables; A. Z. collection of biological samples, taking part in proteomic analyses, edition of MS for intellectual content; S.S. professional care of the proper performance of proteomic analyses, approval of the final version of the MS. A. F. the conception of the study, edition of MS for intellectual content; approval of the final version of the MS.

The first two authors contributed equally to the work described in this report.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experiments were approved by the Animal Ethics Committee University of Warmia and Mazury in Olsztyn, Poland – decision No. 54/2015/ DTN. This article does not contain any studies with human participants performed by any of the authors.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.repbio.2021.100481.

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