Comparative analysis of the transcriptome and proteome during mouse placental development

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ABSTRACT

The condition of the placenta is a determinant of the short- and long-term health of the mother and the fetus. However, critical processes occurring in early placental development, such as trophoblast invasion and establishment of placental metabolism, remain poorly understood. To gain a better understanding of the genes involved in regulating these processes, we utilized a multi-omics approach, incorporating transcriptome, proteome, and phosphoproteome data generated from mouse placental tissue collected at two critical developmental timepoints. We

found that incorporating information from both the transcriptome and proteome identifies genes associated with timepoint-specific biological processes, unlike using the proteome alone. We further inferred genes upregulated based on the proteome data but not the transcriptome data at each timepoint, leading us to identify 27 genes that we predict to have a role in trophoblast migration or placental metabolism. Finally, using the phosphoproteome dataset, we discovered novel phosphosites that may play crucial roles in the regulation of placental transcription factors. By generating the largest proteome and phosphoproteome datasets in the developing placenta, and integrating transcriptome analysis, we uncovered novel aspects of placental gene regulation.

KEYWORDS: placenta, trophoblast invasion, proteomics, transcriptomics, phosphoproteomics, multi-omics, mouse, development.

INTRODUCTION

Normal placental development is essential for a successful pregnancy and fetal growth, as the placenta performs the role of several organ systems[1]. It provides nutrients and oxygen to the growing fetus, eliminates waste from the fetal blood supply, and secretes hormones that maintain the pregnancy[1]. Soon after implantation, specialized cells of the placenta (trophoblasts) invade the uterine tissue, ensuring attachment of the fetus to the mother[1]. Trophoblasts also invade uterine spiral arteries, remodeling them to increase blood supply to the placenta[1]. In order to maximize the surface area available for nutrient and gas exchange between maternal and fetal blood, fetal blood vessels within the placenta form a branching structure, known as the villous tree, which is in direct contact with maternal blood[1].

Defects in these early placental processes are linked to multiple disorders. For example, abnormal trophoblast invasion has been associated with preeclampsia (PE)[2], a hypertensive pregnancy disorder that can cause significant liver and kidney damage and lead to maternal and

fetal mortality[2]. In addition, defects in trophoblast invasion or placental metabolism are associated with intrauterine growth restriction (IUGR), where the fetus is too small for gestational age due to a deficient supply of nutrients and oxygen[3].

However, it is difficult to access normal human placentas during early development, while trophoblasts are migrating and nutrient transport is being established. Therefore, the mouse is frequently used as a model for understanding placental biology. Although some differences exist, such as in the anatomical structure, mouse placentation shares many similarities with human placentation[4]. The placentas of both species are classified as hemochorial (fetal cells come in direct contact with maternal blood), and possess invasive subtypes of trophoblasts[4]. Moreover, there are many conserved signaling pathways between them, such as Hypoxia Inducible Factor signaling[5] and Wnt signaling[6]. Thus, we utilized the mouse model to investigate the molecular pathways involved in early placental development.

At embryonic day (E)7.5 in mouse, blood flow has not yet been established and invasion-associated genes, such as Matrix metallopeptidase-9 (Mmp9)[7], are highly expressed. On the other hand, metabolism of nutrients from maternal blood occurs after blood flow establishment, around E9.5[8]. Many genes are important for regulating migration and metabolism in the placenta. For example, MMPs have a role in trophoblast migration[9], and peroxisome proliferator-activated receptor-γ (PPARγ) has a role in metabolism[10]. However, we still do not fully understand how trophoblast migration and placental metabolism are regulated on a genomic level.

Most genome-wide investigations in the placenta employ transcriptome profiling, because it is sensitive and cost-effective. However, studies have shown that mRNA may not serve as a reliable proxy for protein expression[11–15]. Nevertheless, few studies have been carried out in the placenta that integrate proteome and transcriptome data[16–18]. Furthermore, despite the importance of

posttranslational modifications, such as phosphorylation, in modulating protein function, only two studies have previously profiled the placental phosphoproteome at the level of the entire cell[19,20], whereas other publications have focused on phosphorylation of plasma membrane proteins[21] or of mitochondrial proteins[22]. None of these studies utilized the phosphoproteome to complement proteome and transcriptome profiling in the developing placenta.

We used transcriptomics, proteomics, and phosphoproteomics to identify expression differences between the E7.5 and E9.5 placenta. We identified upregulated proteins that were unchanged at the transcript level, and upregulated transcripts that were unchanged at the protein level. Based on these data, we identified novel genes that we predict to be involved in trophoblast migration and placental metabolism. In addition, we discovered novel phosphorylation events on transcription factors (TFs) known to regulate placental processes. This analysis furthers our understanding of gene expression dynamics in the mouse placenta, reveals novel phosphorylation events, and implicates new candidates that may have a role in trophoblast migration or placental metabolism.

MATERIALS AND METHODS

A list of used materials and reagents can be found in the Supplementary Materials and Methods.

Ethical statement

All animals were treated under protocol 11-14-7898-M approved by the Iowa State University Institutional Animal Care and Use Committee.

Sample collection

Timed-pregnant CD-1 mice were obtained from Charles Rivers Labs and dissected at E7.5 to obtain ectoplacental cones (EPCs) and at E9.5 to obtain placentas as described by Martin and Cockroft[23]. Staging of the mouse embryos was done according to Theiler criteria[24]. For each biological replicate at E7.5 (3 replicates total), ~60 EPCs were collected and combined from ~6

pregnant mice. Biological replicates did not overlap in any litters. For each biological replicate at E9.5 (3 replicates total), four placentas were collected from one pregnant mouse. The placentas and EPCs were snap frozen in liquid nitrogen and stored at -80ºC until extraction.

Sample processing and two-dimensional liquid chromatography coupled with tandem mass spectrometry (2D-LC-MS/MS)

Protein extraction, digestion, tandem mass tag (TMT) labeling, and phosphopeptide enrichment were carried out according to previously published methods[25–28]. A detailed description of sample processing and 2D-LC-MS/MS is provided in the Supplementary Materials and Methods. The LC parameters for transfer of peptides from the C18 trap column to the Strong Cation Exchange (SCX) column as well as the LC parameters for 2D SCX-Reversed Phase (SCX-RP) separation are provided in Supplementary Tables S1 and S2, respectively.

Database search and false discovery rate (FDR) filtering

The raw data were analyzed using MaxQuant version 1.6.0.16[29]. Spectra were searched against the GRCm38.p5 Ensembl genome assembly, which was complemented with reverse decoy sequences and common contaminants by MaxQuant. Carbamidomethyl cysteine was set as a fixed modification while methionine oxidation and protein N-terminal acetylation were set as variable modifications. Digestion parameters were set to "specific" and "Trypsin/P;LysC". Up to two missed cleavages were allowed. A false discovery rate less than 0.01 at both the peptide spectral match and protein identification level was required. The "match between runs" feature of MaxQuant was not utilized. Raw data files and MaxQuant search results have been deposited in the Mass Spectrometry Interactive Virtual Environment (MassIVE) repository: https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp with dataset identifier: MSV000082849

Data normalization and filtering

The unmodified proteome ("proteinGroups.txt" MaxQuant output) intensities were normalized using Normalyzer global median intensity (MedI) normalization[30]. Using Perseus[31] version 1.6.1.1, we imported the data and then filtered out potential contaminants and reverse peptides. In addition, only proteins that were quantified in all samples were kept in the unmodified proteome (leaving 6,919 proteins). For the phosphoproteome ("Phospho (STY)Sites.txt" MaxQuant output), we used Perseus to filter out contaminants, reverse peptides, and Class II and III phosphosites (localization probability < 0.75) to leave only high confidence phosphorylation events. We then expanded the table before normalizing so as not to introduce bias. Normalization was carried out as it was for the unmodified proteome. We imported the normalized dataset to Perseus and filtered out phosphosites not quantified in all biological replicates of at least one timepoint (leaving 6,161 Class I phosphosites). We then imputed the missing values from a normal distribution.

Statistical analysis

Perseus was used for statistical analysis and for generating heatmaps. Hierarchical clustering heatmaps were generated using Perseus's default settings (Euclidean distance, average linkage, k-means processing, 300 clusters, and maximum of 10 iterations) after Z-score normalization without grouping the replicates. Pairwise scatterplots with Pearson correlations between biological replicates were generated using the Perseus Multi scatter plot function. Differential expression for both the whole proteome and the phosphoproteome was determined using a two-sample Student's t-test with Perseus's default settings (p-values were corrected with a permutation-based FDR with an FDR cutoff of < 0.05 and 250 permutations). Proteins and phosphosites were considered differentially expressed if the *q*-value (i.e. FDR) was < 0.05 and the fold change was $\geq \log_2(1.2)$ for upregulation at E7.5 and $\leq -\log_2(1.2)$ for upregulation at E9.5.

Transcriptome analysis

Analyzed mRNA-seq data generated with three biological replicates from each timepoint was obtained from Tuteja et al.[32] (two files: one taking into account each isoform and one averaging across isoforms). Fragments per kilobase of transcript per million mapped read (FPKM) values < 1 were considered not expressed, and the data was filtered to remove genes with FPKM < 1 at both timepoints. Differential expression was determined based on statistical significance (q-value < 0.05) and on a fold change $\geq \log_2(1.5)$ (upregulated at E9.5) or $\leq -\log_2(1.5)$ (upregulated at E7.5).

Proteome-transcriptome integration

The Ensembl IDs provided by MaxQuant were converted to Mouse Genome Informatics (MGI) symbols (provided in the mRNA-seq analysis) using the biomaRt R package[33]. Ensembl IDs that were not mapped (~23) were manually converted using the gene name. Venn Diagrams were generated with the VennDiagram R package[34].

Functional Enrichment Analysis

WebGestalt[35] was used for functional enrichment analysis. The reference gene set was "genome_protein-coding", the Functional database was "geneontology" and the functional database name used was "Biological_Process". Advanced parameters were left as defaults, except that significance level was set to an FDR cutoff of 0.05.

Phosphoproteome analysis

Known phosophosites were identified based on the July 2, 2018 update of the phosphorylation site dataset from PhosphoSitePlus[36]. Conservation of phosphosites was found by running BLAST on the phosphopeptide sequence (obtained from the "sequence window" column in the "phospho (STY)Sites.txt" file) in Uniprot[37].

Kinase motifs were identified on differentially accumulating sites by running our dataset pre-aligned to Serine or Threonine residues on Motif-X[38] using the following parameters: width = 13,

occurrences = 20, significance = 0.000001, and the background database was IPI mouse proteome. Following the identification of the motifs, we used literature searches, combined with the Phosida tool[39] and Human Protein Reference Database[40], to identify putative kinases associated with each motif.

RESULTS AND DISCUSSION

Analysis of the mouse placental proteome at E7.5 and E9.5

To identify protein pathways mediating trophoblast migration and placental metabolism, we assayed the mouse placental proteome at two developmental timepoints, E7.5 and E9.5. We extracted proteins from three independent biological replicates per timepoint using urea-based extraction, and digested them into proteins using filter aided sample preparation (FASP)-based on-filter digestion[25]. The peptides were then labeled with six-plex TMT[41,42] and a 45 μg aliquot of the labeled peptides was used directly to quantify protein abundance (i.e. non-modified proteome) by 2D-LC-MS/MS (Supplementary Scheme S1). With stringent FDR cutoffs (0.01 at both the peptide spectral match and protein identification level), and after removing potential contaminants, reverse peptides, and proteins that were not quantified in all of the samples, we identified 6,919 proteins in the placenta at E7.5 and E9.5 (Figure 1a; Supplementary Table S3). To the best of our knowledge, this is the largest proteome obtained from a placental sample. The previous largest proteome was ~4,700 proteins[17] (Supplementary Table S4). To check the quality of the data, we generated pairwise Pearson correlations between the samples. The average correlation between biological replicates was ~0.998, which demonstrated the high reproducibility of our datasets (Supplementary Figure S1). This was further supported using hierarchical clustering, which resulted in grouping of the biological replicates for each timepoint (Figure 1b).

To determine if there were differences between the proteome at E7.5 and E9.5, we performed differential expression analysis. We designated proteins as differentially expressed if they had a *q*-value < 0.05 and their fold change was $\geq \log_2(1.2)$ (upregulated at E7.5 and downregulated at E9.5) or $\leq -\log_2(1.2)$ (upregulated at E9.5 and downregulated at E7.5). We identified a total of 1,834 differentially expressed proteins (Supplementary Table S5), of which 799 were upregulated at E7.5 and 1,035 were upregulated at E9.5 (Figure 1c). To gain a better understanding of the timepoint-specific processes, we determined which Gene Ontology Biological Process (GOBP) annotations were enriched in each set of differentially expressed proteins. At E7.5, the upregulated proteins were significantly associated with the process of coagulation (Figure 1d), which can be linked to trophoblast function. For example, trophoblast cells secrete many cytokines, which are known to affect coagulation[43], to protect the fetus from local attack[44]. In addition, some hemostatic proteins, such as urokinase-type plasminogen activator (uPA), act to stimulate trophoblast invasion[45]. Finally, the coagulation terms may also reflect the involvement of trophoblasts in maintaining hemostasis at the maternal-fetal interface[46]. Therefore, while the coagulation term is relevant, it is not specific to one function of trophoblasts at E7.5.

The 1,035 proteins upregulated at E9.5 were mainly associated with cellular adhesion and translation (Figure 1d). The enrichment of adhesion terms may be related to branching morphogenesis of placental villi that occurs at E9.5[47], or the continuous process of trophoblast migration. Again, while adhesion terms are relevant, they are not specific to one function of trophoblasts at E9.5, and some of the adhesion terms are also significant at E7.5 (Figure 1d).

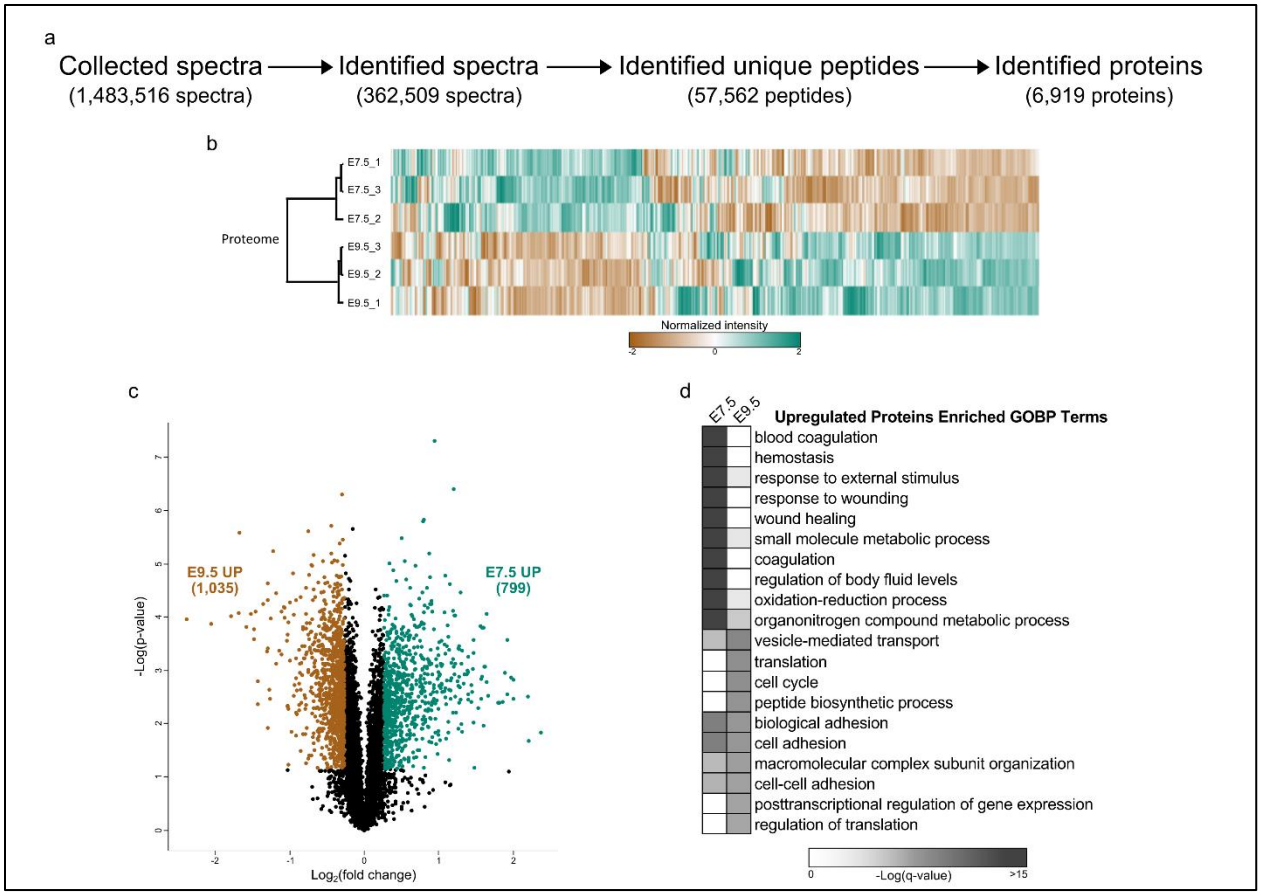

Figure 1. a) Whole proteome summary of acquired spectra, peptides, and proteins. b) Hierarchical clustering, based on Euclidian distance, of the proteome data from E7.5 and E9.5 placenta. Three biological replicates were carried out for each timepoint. The gradient reflects normalized intensity values of E7.5 (positive values; green) relative to E9.5 (negative values; brown). c) Scatterplot showing proteins upregulated at E7.5 (green) and at E9.5 (brown), based on a fold change of $\geq \log_2(1.2)$ (upregulated at E7.5) or $\leq -\log_2(1.2)$ (upregulated at E9.5) and a permutation-based *q*-value cutoff of < 0.05. d) Heatmap showing the top ten most significant Gene Ontology Biological Process (GOBP) terms associated with upregulated proteins at each timepoint. Color gradient represents the significance based on Benjamini-Hochberg *q*-value. E, embryonic day; UP, upregulated protein.

Comparison of timepoint-specific proteomes and transcriptomes

To complement the proteomic analysis, we utilized mRNA-seq data generated in placenta at the same two timepoints, which we previously published[32]. We focused this analysis on the 6,170 genes that were detected in both the proteome dataset and the transcriptome dataset. 1,178 of these genes were upregulated at E7.5 at the protein or transcript level (Figure 2a), of which only 21.3% were upregulated at both levels (Figure 2a). At E9.5, 1,295 genes were upregulated at the protein or transcript level (Figure 2b), of which only 24.7% were upregulated at both levels (Figure 2b). These data agree with previous findings that mRNA may not be a reliable proxy for protein levels[11–15] and that genes can exhibit differential expression at the transcript and/or protein level[48]. There could be many reasons for these discrepancies, such as translational efficiency and protein degradation[49].

To investigate whether genes that are upregulated at both the transcript and protein levels are related to timepoint-specific functions, we examined their functional annotations. We observed a stronger enrichment of migration terms at E7.5 compared to E9.5 and a stronger enrichment of metabolism terms at E9.5 compared to E7.5 (Figure 2c). Migration terms at E7.5 are more specific than the coagulation terms that were enriched when we only used proteomic data (Figure 1d). As discussed in the previous section, enrichment of coagulation terms can reflect many aspects of placental development at E7.5, unlike migration terms, which are likely specific to only trophoblast migration. Additionally, lipid metabolism terms at E9.5 are more specific to this timepoint than the adhesion terms that were enriched when we only used proteome data (Figure 1d). These results indicate that combining information from the proteome and transcriptome can identify more specific functions related to the timepoints than using data from the proteome alone.

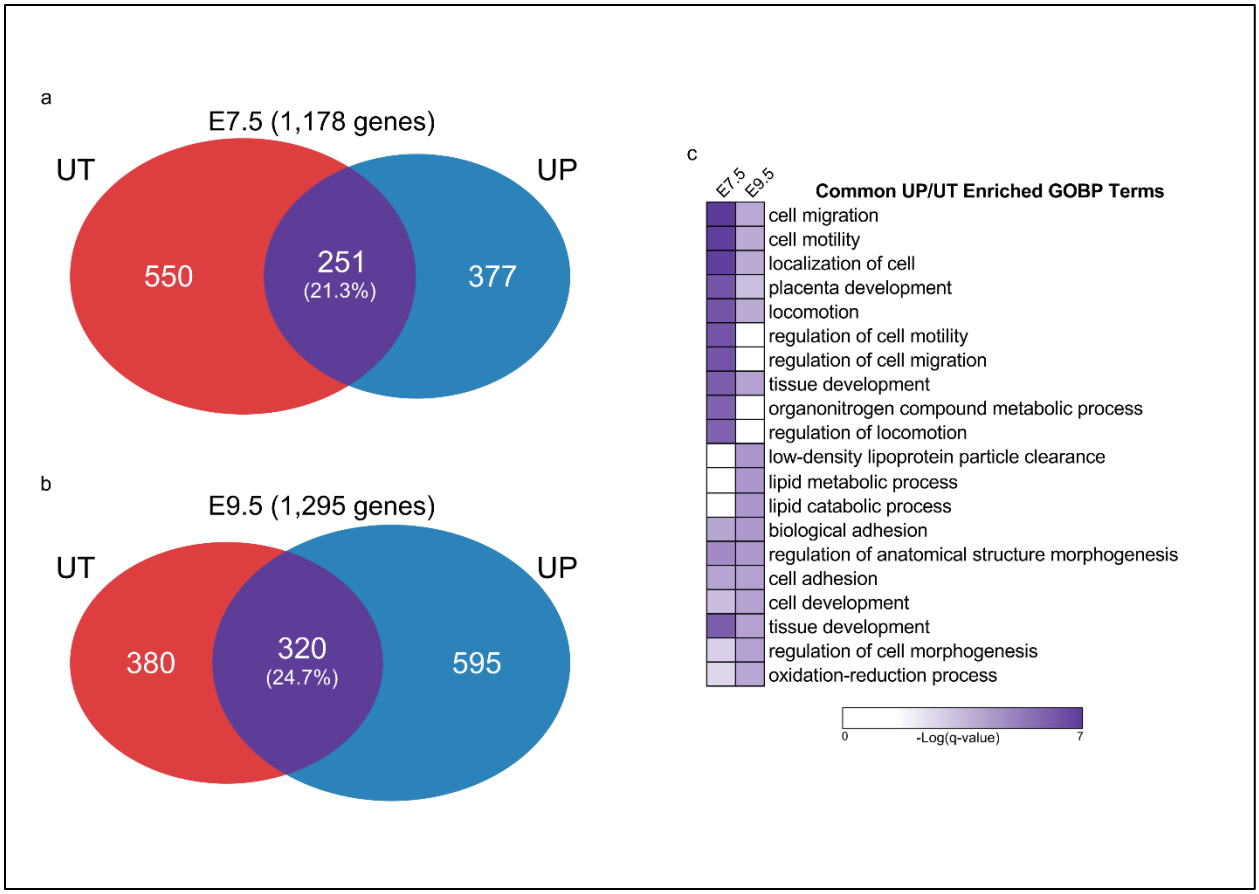

Figure 2. Overlap of upregulated proteins (UPs) and upregulated transcripts (UTs) for genes detected at both the protein and transcript levels at E7.5 (a) and at E9.5 (b). c) Heatmap showing the top ten most significant Gene Ontology Biological Process (GOBP) terms associated with genes upregulated at both the protein and transcript levels at each timepoint (purple overlap in a and b). Color gradient represents the significance based on Benjamini-Hochberg *q*-value. E, embryonic day.

Investigation of differentially expressed proteins unique to the proteome

We next wanted to investigate if genes upregulated only at the protein level included genes previously unknown to be important for specific placental functions. These genes may have been overlooked by other analyses because of their lack of differential expression at the transcript level,

which is the most commonly used way to assess global gene expression. To identify potentially novel genes important for placental function at E7.5, we focused on the term "locomotion", because of its relatedness to cell migration. While this term was not one of the top ten (Figure 1d), it was significant (q-value < 0.05). At E7.5, 39 of the 377 genes upregulated exclusively at the protein level were associated with the "locomotion" term. To increase stringency of which genes were considered exclusively upregulated in the E7.5 proteome, we excluded genes that had any transcript isoforms upregulated at E7.5 (instead of using the average isoform expression per gene). We also excluded genes that were significant at the transcript level, even if they had a low fold-change (instead of only considering genes with fold ≥ 1.5 as significant). This left 30 genes that we were confident were upregulated at E7.5 only at the protein level (Table 1). For 29 of the 30 genes, we were able to identify literature describing their involvement in migration or invasion in non-placental contexts, such as leukocyte migration or cancer cell invasion. Of those, five were already known or implicated in trophoblast invasion, according to previous studies (Table 1). Our literature search identified no such known roles in the placenta for the remaining 24 genes, even though they were associated with invasion/migration in other contexts (Table 1). These genes are potentially novel regulators of trophoblast migration that could either inhibit or promote the process. While inhibitors of invasion are often secreted by the decidua (the uterine tissue surrounding the placenta and embryo[50]), some, such as Gata3[51] and Timps[52], are also expressed in trophoblasts. Among the genes we identified, Atp8a1[53,54] and Coro1a[55,56] promote cancer and white blood cell migration, respectively, and therefore may also promote trophoblast migration. On the other hand, Usp33 inhibits colorectal[57] and breast[58] cancer cell migration, and therefore may also be an inhibitor of trophoblast migration. Additional evidence from the literature also supports our prediction that the genes have a role in trophoblast migration. For example, Lsp1[59],

Snca[60], and Ppbp[61] are implicated in PE. In addition, Workalemahu et al.[62] found SNPs in Prkca that were significantly associated with risk of placental abruption, which has been linked to abnormal trophoblast invasion[63].

Table 1. Genes that are upregulated only at the protein level at E7.5 (using stringent criteria) and are associated with the "locomotion" Gene Ontology term are shown and are predicted to have a role in placental cell migration. For each gene, we determined if it has a known role related to cell migration or trophoblast migration.

Gene	Known related role	Known role in trophoblast migration?
Fermt3	Cancer cell invasion[64,65] and natural killer cell migration[66]	No
Atp8a1	Cancer cell invasion[54] and ovarian cell migration[53]	No
Tspo	Cancer cell invasion[67,68]	No
Coro1a	Neutrophil[55] and lymphocyte[56] migration	No
Gpx1	Cancer cell invasion[69,70]	No
Hyal2	Cancer cell invasion[71,72]	No
Lsp1	Cancer cell invasion[73] and macrophage migration[74]	No
Usp33	Cancer cell invasion[57,58]	No
Prkca	Cancer cell invasion[75,76]	No
Rras	Breast epithelial cell migration[77] and endothelial cell adhesion[78]	No
Snca	Cancer cell invasion[79,80]	No

Stx4a	Cancer cell invasion[81] and macrophage migration[82]	No
Slirp	Cancer cell invasion[83]	No
Pf4	Neutrophil[84], monocyte[85], and cancer cell[86,87] migration	No
Plgrkt	Monocyte migration[88,89]	No
Ppbp	Cancer cell invasion[90,91]	No
Golph3	Cancer cell invasion[92]	No
Rps6kb1	Cancer cell invasion[93]	No
Lamb2	Astrocyte migration[94]	No
Sord	Cancer cell invasion[95]	No
Syk	Cancer cell invasion[96–98]	No
Apoa1	Macrophage migration[99,100]	No
Anxa1	Cancer cell invasion[101] and myoblast migration[102]	No
Mpp1	Neutrophil migration[103]	No
Cd34	Cancer cell invasion[104]	No direct role known (involved in lumen formation by invasive trophoblasts[105])
Col3a1	Cancer cell invasion[106,107]	No direct role known (highly expressed in extracellular matrix[108–110])
Gab1	Cancer[111] and epithelial[112] cell invasion	No direct role known (though it has been implicated in trophoblast invasion[113])
Dcn	Cancer cell invasion[114]	No direct role known (but expressed in invasive trophoblast cells in different

		stages/conditions of placentation[115,116])
Tgfb1	Cancer cell invasion[117]	Yes[118–121]. Inhibits[118,119] or promotes[120,121] invasion.
Chmp3	No known role in migration	-

For genes that were upregulated only at the protein level at E9.5, we focused on the significantly enriched GOBP term, "liver development," (q-value < 0.05). We focused on this term because the liver plays a central role in metabolism, a process that is more active in the placenta at E9.5, after blood flow establishment. Therefore, genes that are associated with this term may have a function in placental metabolism. Eleven of the 595 genes upregulated exclusively at the protein level at E9.5 were associated with the term. After applying the same stringent filtering criteria we did at E7.5, eight genes remained (Table 2). For four of these eight, we were able to identify literature related to their involvement in metabolism in a non-placental context. While one of these genes, Igf2r, has been associated with metabolic functions in the placenta[122,123], three genes, Rps6ka1[124], Upf2[125], and Gnpnat1[126], have a known role in metabolic processes, but have no known role in metabolism in the placenta (Table 2).

Table 2. Genes that are upregulated only at the protein level at E9.5 (using stringent criteria) and are associated with the "liver development" Gene Ontology term are shown and are predicted to have a role in placental metabolism. For each gene, we determined if it has a known role related to metabolism or specifically placental metabolism.

Gene	Known related role	Known involvement in placental metabolism?
Rps6ka1	Insulin signaling[124]	No
Upf2	Liver metabolism[125]	No

Gnpnat1	Insulin signaling[126]	No
Igf2r	Fetal growth[122], insulin resistance[123]	Implicated[116,127]. Receptor for Igf2, inhibiting its activity. Igf2 promotes nutrient transport to the fetus.
Rpl32	No known role in metabolism	-
Tgfbr3	No known role in metabolism	-
Tyms	No known role in metabolism	-
Pkd2	No known role in metabolism	-

Analysis of the mouse placental phosphoproteome at E7.5 and E9.5

Given the critical role of phosphorylation in modulating protein function, we performed phosphoproteome profiling at the two timepoints. For this, 300 μg of the pooled TMT-labeled peptides was subjected to phosphopeptide enrichment using Titansphere Phos-TiO_2 beads prior to 2D-LC-MS/MS analysis. To maximize the quality of our dataset, we filtered out potential contaminants, reverse peptides, and phosphosites that were not detected in all biological replicates of at least one timepoint. We quantified the level of 6,161 Class I (localization probability ≥ 0.75), 1,343 Class II (0.75 > localization probability ≥ 0.5), and 44 Class III (localization probability < 0.5) phosphorylation sites arising from 2,140 phosphoproteins (Figure 3a). For further analyses, we only considered the 6,161 Class I (localization probability ≥ 0.75) phosphosites (Supplementary Table S6). To the best of our knowledge, this is the largest scale phosphoproteome in the placenta[19,21,22]. The reproducibility of our dataset was demonstrated by an average pairwise Pearson correlation of ~0.98 between biological replicates (Supplementary Figure S2) and by the close clustering of each timepoint's biological replicates (Figure 3b). We identified a total of 1,775

differentially accumulating phosphorylation sites, of which 830 were upregulated at E7.5 and 945 were upregulated at E9.5 (Supplementary Table S7). The majority of the phosphosites were doubly phosphorylated, and only a fraction were triply (or more) phosphorylated (Figure 3c). As expected, most of the phosphorylation events occurred on Serines (S), and only a few were detected on Tyrosines (Y; Figure 3d).

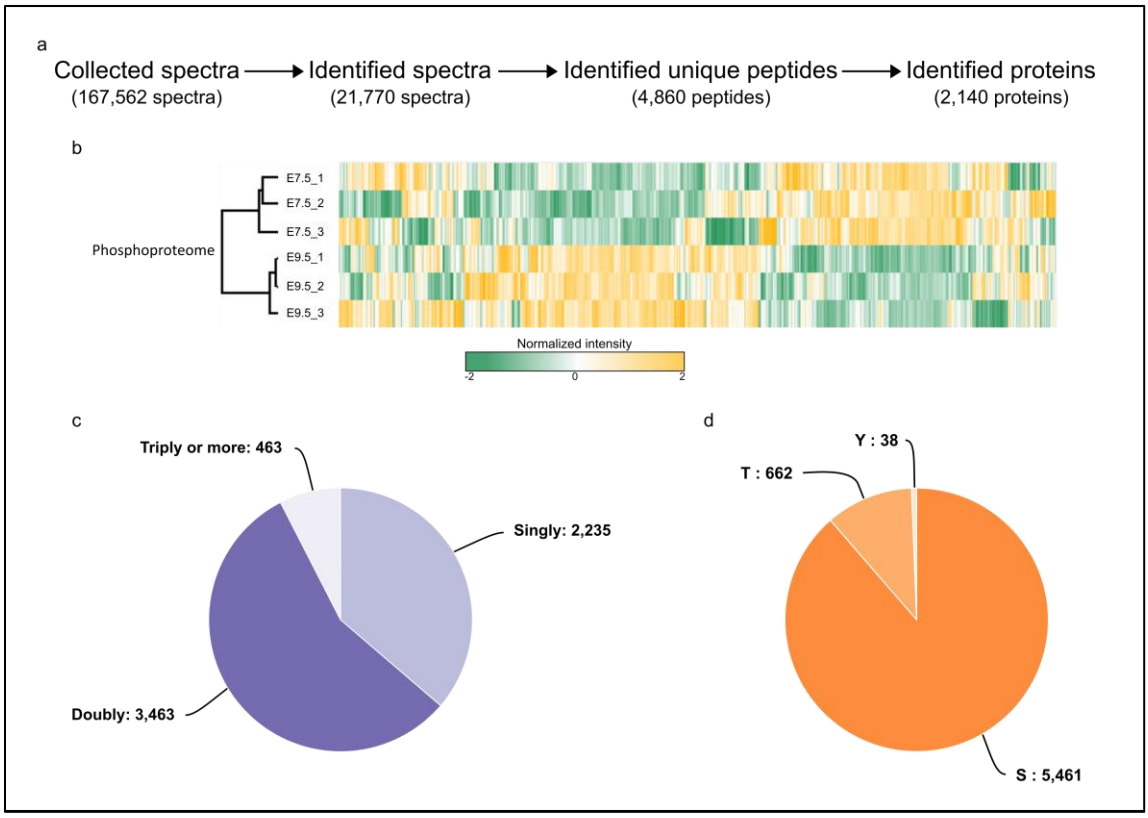

Figure 3. a) Phosphoproteome summary of acquired spectra, peptides, and proteins. b) Hierarchical clustering, based on Euclidian distance, of the phosphoproteome data from E7.5 and E9.5 placenta. Three biological replicates were carried out for each timepoint. The gradient reflects normalized intensity values of E7.5 (positive values; yellow) relative to E9.5 (negative values; green). c) Number of unique phosphopeptides with 1 (singly), 2 (doubly), or 3 (triply) or more phosphorylation events. d) Number of unique phosphorylation events on Serines (S), Threonines

(T), and Tyrosines (Y). The pie charts in c) and d) represent data from all Class I phosphosites in the filtered dataset (6,161 phosphosites).

Discovery of novel phosphosites

We next focused on information that can only be provided by the phosphoproteome. When comparing the phosphosites in our dataset to data on PhosphoSitePlus[128], we found that about 13% (830/6,161) of them were novel phosphosites, previously unknown to be phosphorylated in any mouse tissue (Figure 4a). For unknown sites that were differentially accumulating (see Methods; Supplementary Table S7), we further determined whether any of them were located on placental TFs, and if so, whether the site was conserved in human. The conservation criterion was added because functional phosphorylation events are more likely to be conserved than events without characterized functions[129]. At E7.5, we identified four phosphosites on placental TFs: Ets2 S300, Nfat5 S229, Satb2 S13, and Snai1 S92 (Figure 4b). Snai1 and Nfat5 are implicated in PE[130,131], while Ets2 and Satb2 are important for trophoblast stem cell renewal[132,133]. The sites on Nfat5, Satb2, and Snai1 were previously found to be phosphorylated in human on NFAT5 S229, SATB2 S13, SNAI1 S92, but had no prior evidence of phosphorylation in mouse (Figure 4b).

At E9.5, unknown sites that were differentially accumulating and located on placental TFs were Elf3 S230, Elf3 S232, Elf3 S235, and Zeb1 S1004 (Figure 4b). Elf3 is a member of the Ets TF family, which is important for normal placentation[134–137], and is differentially expressed under different placental conditions[32,110,138,139]. Zeb1 has been implicated in angiogenesis and placental maturation based on its expression patterns[140]. Three of the four phosphosites, Elf3 S232, Elf3 S235, and Zeb1 S1004, were previously identified in human (corresponding to ZEB1 S1022, ELF3 S212, and ELF3 S215 in human, respectively; Figure 4b). The novel sites identified in this

analysis could play crucial roles in modulating protein function and therefore placental development.

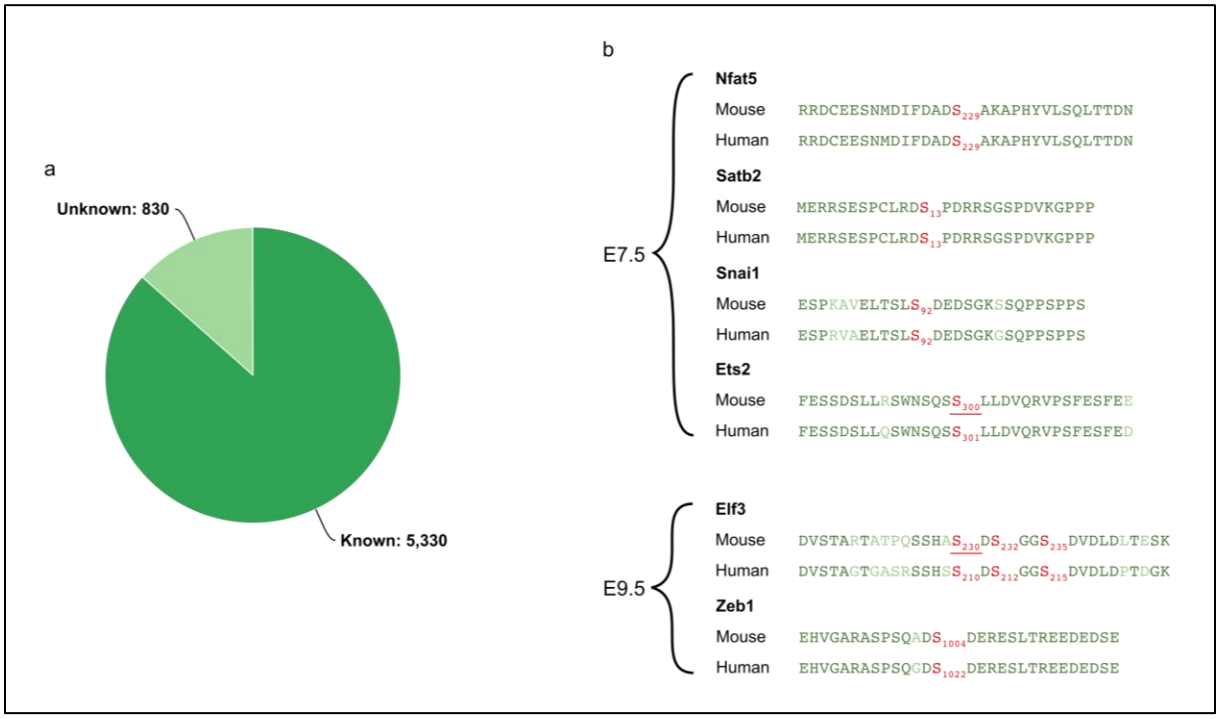

Figure 4. a) Proportion of previously characterized (dark green) and uncharacterized (light green) phosphosites in the phosphoproteome dataset, based on PhosphoSitePlus[128]. b) Transcription factors implicated in placental processes with previously unknown phosphorylation events, where the phosphorylated amino acid is conserved in human (red). Underlined phosphosites were previously unknown in human as well as mouse. Other conserved amino acids are shown in dark green letters, and non-conserved amino acids are shown in light green letters. E, embryonic day.

Identification of enriched phosphorylation motifs

Given the importance of kinases in cell signaling and gene expression regulation, we wanted to search for overrepresented phosphorylation motifs among differentially accumulating phosphosites. Using Motif-X[38], we found 20 significantly overrepresented motifs among the E7.5 upregulated phosphosites (UpP). One was a phosphothreonine motif, while the rest were centered

on phosphoserines (Supplementary Table S8). Among E9.5 UpPs, we identified 31 significantly overrepresented motifs, two of which were centered on phosphothreonines, while the remaining were phosphoserine-centered motifs. (Supplementary Table S8). We were not able to identify overrepresented phosphotyrosine motifs due to their low abundance in the data. We next determined the kinases associated with known motifs using a literature search (Supplementary Table S8). While there were shared motifs between E7.5 and E9.5, such as those for Glycogen synthase kinase 3 (Gsk3), Casein kinase II (Ck2), and Cyclin-dependent kinase (Cdk), there are substrate motifs unique to each timepoint. For example, the Mitogen-activated kinase substrate motif, Serine-Proline-x-x-Serine-Proline, where x is any amino acid, is only enriched at E7.5; and the substrate motif, Serine-x-x-x-Serine-Serine, for Inhibitor of nuclear factor kappa-B kinase is only enriched at E9.5. These results may indicate that timepoint-specific kinases regulate specific processes in the placenta.

Our analysis revealed nuanced gene expression regulation in the placenta: we found many upregulated transcripts that were unchanged at the protein level and vice versa. However, genes upregulated at both levels were strongly associated with known timepoint-specific functions, highlighting the importance of incorporating different measurements of gene expression. We utilized the differences between the proteome and the transcriptome to implicate novel genes involved in placental development. Finally, we complemented our analysis with phosphoproteome data, which allowed us to discover novel phosphosites that potentially play important roles in placental development.

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Author Contributions

M.A., G.S. and J.W. prepared samples for the LC/MS-MS run. J.W. and G.S. carried out the database search and FDR filtering of the data from LC/MS-MS. M.A., J.W. and G.T. contributed to data analysis design, and M.A. carried out the analysis. M.A., H.K., J.W. and G.T. contributed to the overall study design and interpretation of results. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

UP, upregulated protein; UT, upregulated transcript; UpP, upregulated phosphosite; E, embryonic day; FPKM, fragments per kilobase mapped; GOBP, Gene Ontology biological process; S, Serine; T, Threonine; Y, Tyrosine; TF, transcription factor.

SUPPLEMENTARY INFORMATION

The following files are available free of charge.

- Supplementary Materials and Methods and Supplementary Figures: Supplementary Scheme S1 and Supplementary Figures S1 and S2 (file type: DOCX):
    - Supplementary Scheme S1. General overview of sample processing steps until LC-MS/MS analysis.
    - Supplementary Figure S1. Pairwise correlation scatterplots of placental proteome samples between E7.5 replicates (a) and E9.5 replicates (b), with Pearson correlations.
    - Supplementary Figure S2. Pairwise correlation scatterplots of placental phosphoproteome samples between E7.5 replicates (a) and E9.5 replicates (b), with Pearson correlations.
- Supplementary Tables: Supplementary Tables S1-S8 (file type: XLSX):
    - Supplementary Table S1: Transfer of peptides from C18 loading (trap) column onto SCX.
    - Supplementary Table S2: Elution of peptides from SCX to 2nd C18 column (nanospray tip) and reverse-phase separation into the MS.
    - Supplementary Table S3: Whole proteome dataset after filtering and normalization.
    - Supplementary Table S4: Placenta whole proteome studies and number of proteins quantified in each study.
    - Supplementary Table S5: Differentially expressed proteins.
    - Supplementary Table S6: Class I phosphosites after filtering and normalization.
    - Supplementary Table S7: Differentially accumulating Class I phosphosites.
    - Supplementary Table S8: Enrichment of overrepresented kinase motifs among differentially accumulating phosphosites and the putative kinases associated with them.

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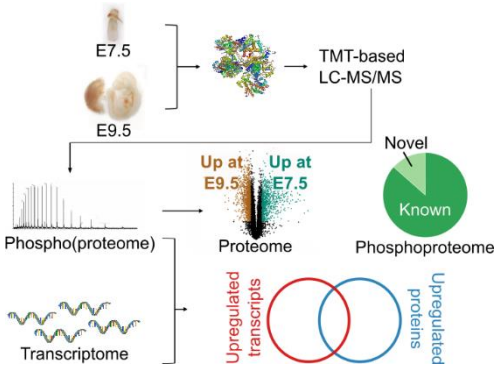
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