# Unbiased placental secretome characterization identifies candidates for pregnancy complications

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Running title: Placenta secretome identifies biomarkers of pregnancy health

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## Abstract

Pregnancy requires adaptation of maternal physiology to enable normal fetal development. These adaptations are driven, in part, by the production of placental hormones. Failures in maternal adaptation and placental function lead to pregnancy complications including abnormal birthweight and gestational diabetes. However, we lack information on the full identity of hormones secreted by the placenta that mediate changes in maternal physiology. This study used an unbiased approach to characterise the secretory output of mouse placental endocrine cells and examined whether these data could identify placental hormones that are important for determining pregnancy outcome in humans. Secretome and cell peptidome analyses were performed using mass spectrometry on cultured primary trophoblast and fluorescence-activated sorted endocrine cells from pregnant mouse

dams, and a placenta secretome map containing 319 proteins was generated. Gene ontology analyses showed that a high proportion of placental secretome proteins are involved in metabolic and immune modulation, signalling and growth. The majority of proteins identified are also expressed by human placenta and several have been reported to be dysregulated in pregnancy complications. Moreover, in proof of concept studies, we found that the relative abundance of secreted placental proteins (sFLT1/MIF and ANGPT2/MIF ratios) was increased in women at 12 weeks of pregnancy, prior to diagnosis of gestational diabetes. Finally, we identified several transcription factors that may be important for controlling hormone production by the placenta and associate with pregnancy outcome. Thus, our observations suggest that analysis of the placental secretome could lead to the identification of new placental biomarkers of pregnancy complications.

## Introduction

The placenta forms the functional interface between the mother and fetus that is essential for fetal development and growth during pregnancy. It is responsible for secreting a plethora of endocrine meditators that induce local and systemic changes in the mother to enable fetal nutrient and oxygen transfer and prevent immunological rejection of the fetus[1]. Aberrant placental function can lead to insufficient or inappropriate adaptations in maternal physiology, with consequences for pregnancy outcome and with immediate and lifelong impacts on the health of both the mother and child[2]. Indeed, placental malfunction is a leading cause for the development of pregnancy complications, such as preeclampsia (PE), gestational diabetes mellitus (GDM) and intrauterine growth restriction (IUGR). Combined, these complications affect up to 6-8% of pregnancies in the UK (https://www.gov.uk/government/statistics/birth-characteristics-england-and-wales-2014).

Typically, these complications are diagnosed in the second or even the third trimester of gestation, after the complication has already manifested. Moreover, current diagnosis methods, namely blood pressure and proteinuria evaluation for PE, oral glucose tolerance test for GDM and uterine fundal height and ultrasound measures for IUGR are performed at a specific time/s in gestation and the development of the complication may not be detected in some cases. Therefore, the identification of novel placental biomarkers for earlier detection and improved diagnosis of pregnancy complications is highly desirable. Moreover, the illumination of placental biomarkers may aid in the design of novel therapeutic targets for pregnancy complications.

The notion that placental biomarkers may provide diagnostic or prognostic value for pregnancy complications is a long-standing and supported concept. For instance, detection of the placental hormone, chorionic gonadotropin is used to confirm pregnancy, reduced levels of pregnancy-associated plasma protein-A (PAPP-A) in the maternal circulation are predictive of IUGR and PE[3,4] and an imbalance in placental derived angiogenic regulators, like soluble fms-like tyrosine kinase 1 (sFLT1) and placental growth factor (PIGF) can be predictive of PE[5]. However, studies in experimental animals have demonstrated that the production of many other protein hormones by the placenta could also be important in determining pregnancy outcome. In rodents, placental lactogens/prolactins (PL/PRL), growth hormone (GH) and insulin-like growth factor 2 (IGF2) modulate maternal insulin and glucose levels during pregnancy[1,6,7] and perturbed expression of these proteins by the placenta have been associated with GDM and abnormal fetal growth in humans[8-10]. The placenta also produces a wide variety of cytokines throughout pregnancy[10,11], which contribute to the low grade systemic inflammation and induction of maternal insulin resistance that normally occurs in the second half of pregnancy and some data suggest that placental cytokine production is aberrant in women with poor pregnancy outcomes like PE, GDM and IUGR[12]. Additionally, the placenta secretes inhibins, activins and relaxins, which aid in the adaptation of the endocrine, renal, circulatory and immune systems of the mother during gestation[13,14]. Finally, the placenta secretes proteases, inhibitors of peptidases, binding proteins and soluble forms of receptors for steroids, growth factors, cytokines and circulating factors, like lipoproteins, which contribute to the pleiotropic endocrine regulation of maternal physiology during gestation and show some predictive value for conditions like IUGR[15-18]. Thus, there is likely a constellation of protein mediators secreted by the placenta that facilitate maternal adaptations and ensure adequate fetal growth, required for a healthy pregnancy outcome.

Transcriptomic analyses has informed on the repertoire of hormones expressed by the human placenta in healthy and complicated pregnancies[19-22]. However, these studies are conducted mainly on samples obtained at delivery and involve analysis of pieces of placenta tissue, which is heterogeneous in nature and includes trophoblast, vascular, stromal and specialised immune cell types. Moreover, as powerful a tool it may be, transcriptomic analyses on their own may not be sufficient to identify the protein hormones secreted by the placenta. This is because genes can be subjected to post-transcriptional and post-translational regulatory mechanisms, such as alternative splicing, folding, transport, localization, degradation and secretion[23]. Thus, analysis of the secretome, the complete list of proteins secreted by the placenta, would be invaluable for identifying placental biomarkers of maternal and fetal wellbeing that could be altered prior to the manifestation of a pregnancy complication.

The mouse is a valuable species for defining the placental secretome. This is because hormone secretion by the placenta is principally performed by trophoblast endocrine cells that are conveniently arranged into a structure, termed the junctional zone. The junctional zone is also discrete from, and forms under distinct genetic instruction to, the labyrinthine zone, which performs substrate transport function in the mouse placenta. This is in contrast to humans, where the endocrine and transport functions of the placenta are carried out by the same region/cell type, the syncytiotrophoblast (STB), preventing the specific, sole examination of placental hormone production. The mouse also offers the key advantage that tools to selectively modify and isolate endocrine cells in the placenta are now available[24]. Moreover, despite some variations between mice and humans, many mouse-specific hormone genes are structurally similar to those in the human and perform similar functions (e.g. PRL/PL and GH genes)[1]. Furthermore, many gene and protein networks regulating placental development and function overlap in the two species[25,26].

Herein, we first established a method for obtaining primary cultures of mouse placental endocrine cells from which the cells and secretory output could be collected. As a complementary approach, we also employed fluorescence-activated cell sorting (FACS) to isolate endocrine cells from the placenta of mice. We used mass spectrometry to unbiasedly identify the proteins in our different mouse placental endocrine cell preparations and applied a bioinformatic pipeline to refine a placental secretome map. We then overlaid our placental secretome map to a compilation of RNA/protein expression databases publicly available for the human placenta in women with pregnancy complications, including PE, GDM and IUGR to identify secreted placental proteins that could be clinically important. As a proof of concept, we quantified the abundance of four secreted placental protein candidates (sFLT1, MIF, ANGPT2 and IGF2) and their ratios to one another in blood samples taken from women who had uneventful/healthy pregnancy outcomes and those who developed GDM (both populations were normotensive). We found that sFLT1 was altered in abundance in women with GDM and moreover, the ratios of sFLT1 or ANGPT2 to MIF were altered in the first trimester of pregnancy in women who went on to develop and be diagnosed with GDM in the second trimester of pregnancy. Finally, we identified several transcription factors that are predicted to be important for controlling endocrine function of the placenta and determining pregnancy outcome. Our methodology and novel placental secretome map may be useful in identifying additional placental biomarkers for pregnancy complications.

**Results:**

We first wanted to establish a method for obtaining primary cultures of mouse placental endocrine cells from which secretory output could be collected and unbiasedly characterised. We harvested placentas at day 16 of gestation from wild-type females mated with males expressing Cre-EGFP under the *Tpbpa* promoter, which is specifically active in the trophoblast endocrine cells of the junctional zone[24] (Figure 1A). Day 16 of gestation was chosen as this corresponds to when all the endocrine cells in the mouse placenta have differentiated and are non-proliferating. Moreover, this is when the junctional zone is largest in absolute terms. The *Tpbpa*-Cre-EGFP reporter was used to visualise the trophoblast endocrine cells, which were found to be enriched in the second layer of our Percoll gradient (between 1.028 and 1.050 g/ml; Figure 1A&B). Trophoblast cells from this layer were then cultured for up to 120h and the optimal time point for secretome analysis was identified to be 48h based on dynamics of trophoblast density (*Krt18* expression) and the levels of viability (XTT levels), necrosis (LDH levels) and apoptosis markers (*p53* and *Bax* expression) throughout the culturing (Figure 1C). As expected, the 48h primary cultures contained a high density of endocrine trophoblasts, as indicated by the high expression of *Tpbpa* and comparatively very low expression of the transport labyrinth zone marker, *Mct4* (Figure 1D). These cultures contained all three types of junctional zone cells, *i.e.* the endocrine spongiotrophoblasts, glycogen cells and giant cells, as evidenced by the expression of their unique gene markers *Prl8a8, Gjb3* and *Hand1*, respectively (Figure 1E&F).

**Peptidome and secretome analysis of primary cultured trophoblast cells from mouse placenta**

We then determined the secretome of our primary mouse trophoblast endocrine cell cultures. This involved performing LC–MS/MS on both the cells and conditioned medium from the cultures at 48h and then applying a bioinformatics pipeline (Figure 1G). We identified a total of 1,534 and 1,445 proteins in the cells and conditioned medium of the cultures, respectively. After considering only proteins that were detected in 4 out of 5 samples, protein lists were then converted to their corresponding gene ID and expression by the mouse placenta verified using publicly available RNA datasets (Table 1). As we wanted to ultimately translate our findings from the mouse to humans, we additionally overlaid our converted mouse gene lists with publicly available RNA datasets for the human placenta (Table 1) and performed systematic orthologue searches. To further refine our lists to secreted proteins, we applied SignalP[27] and gene ontology analysis[28] to capture proteins that employ both the "conventional", as well as "unconventional" secretion pathways[29] (see methods for details). This resulted in a refined list of 158 and 257 secreted proteins detected in the cultured cells and conditioned medium (110 were common between the sample types), respectively that are

expressed by both the mouse and human placenta. Reactome pathway analysis revealed that the proposed functions of secreted proteins in the cultured cells and conditioned medium were largely similar, with the highest scoring pathways including those involved in the immune system, neutrophil degranulation, homeostasis and insulin-like growth factor (IGF) regulation (Fig. 1H and G). All data outputs at each step of the pipeline, including the proteins/genes expressed in the mouse but not the human placenta can be found in GitHub (https://github.com/CTR-BFX/2020-Napso_Sferruzi-Perri).

**Peptidome and secretome analysis of sorted endocrine cells from the mouse placenta**

As a complementary approach to the primary trophoblast cultures, endocrine cells from the mouse placenta on day 16 of pregnancy were isolated using FACS. This was performed by mating the *Tpbpa*-Cre-EGFP mouse line to the double-fluorescent Cre reporter line, mTmG[30] (Figure 2A). As expected, placentas obtained from these matings showed EGFP in the junctional zone of the placenta, whereas the labyrinth, decidua and fetus were positive for tdTom (Figure 2B,C). Moreover, sorting the EGFP-positive cells provided us with highly enriched isolates of junctional zone cells (as indicated by the high expression of *Tpbpa*, with little to low detection of the *Mct4* gene; Figure 2D) containing the three major endocrine cell types of the mouse placenta (Figure 2E). Using LC-MS/MS we identified a total of 1,142 proteins in the sorted placental endocrine cells (Figure 2F). Applying a similar pipeline to the analysis of cultured placental endocrine cells, we narrowed down our list of proteins obtained in the sorted placental endocrine cells to 105 secreted proteins that are shared between the mouse and human placenta (Figure 2F). Gene ontology analysis indicated that these secreted placental proteins function in pathways related to the immune system, neutrophil degranulation and metabolism of proteins, among others (Figure 2G). All data outputs at each step of the pipeline, including the proteins/genes expressed in the mouse but not the human placenta can be found in GitHub (https://github.com/CTR-BFX/2020-Napso_Sferruzi-Perri). Proteins detected in the sorted placental endocrine cells that were not predicted to be secreted were analysed by gene ontology analysis. This revealed that many proteins detected are proposed to play roles in protein synthesis, translation and metabolism, amongst others and are in line with their possible regulatory role in modulating the function of placental endocrine cells (Supplementary Figure 1 and Supplementary Table 1).

**Creating a placental secretome map**

Given that the LC-MS/MS method is unbiased, but cannot exhaustively characterize the entire proteome of a given set of samples, we then combined the lists of secreted placental proteins

196 expressed by mouse and human placenta obtained using the two methods presented above, to
197 generate a more comprehensive placental secretome map (Figure 3A). This approach resulted in a
198 total of 319 secreted proteins that are expressed by both mouse and human placenta (Figure 3A) and
199 another 31 that are specific to the mouse (Supplementary Figure 2). We aligned our list of 319 secreted
200 placental proteins with data from single-cell RNA-Seq analysis of the human placenta at 8 and 24
201 weeks of gestation[31] and found that 94% of our proteins (299 out of 319) were expressed in the STB
202 (Figure 3B and F). We also aligned our list of 319 secreted placental proteins with data on the
203 conditioned media from trophoblast organoids prepared from first trimester human placenta[32] and
204 identified 56 secreted placental proteins in common (Figure 3B). Gene ontology analysis of the
205 complete list of 319 proteins/genes demonstrated that they play roles in the response to stimuli and
206 stress and regulation of organismal process (Figure 3C). Moreover, many placental proteins identified
207 are implicated in protein and signalling receptor binding and contain protein-interacting domains,
208 such as serpin, conserved sites and the EGF-like domains, observations which are overall consistent
209 with the notion that they are secreted (Figure 3C). Using gene expression enrichment analysis for
210 mouse and human tissues, we found that 20 of the proteins were highly expressed (>10 fold) in the
211 mouse placenta compared to other tissues (Figure 3D) and 4 secreted placental proteins, TFPI2,
212 SERPINE2, IGF2 and FLT1 were enriched in the human placenta compared to other tissues (Figure 3E).
213 Further alignment of our complete list of secreted placental proteins with single-cell RNA-Seq analysis
214 of the human placenta revealed that several proteins were enriched predominantly in the STB,
215 including FLT1, TFPI2 and ANGPT2 (Figure 3F). Moreover, all the proteins that we identified are
216 reported to be expressed by the syncytiotrophoblast (STB), extravillous cytotrophoblast (EVT) or
217 cytotrophoblast (CTB) of the human placenta.

218

219 **Placental secretome map is enriched in proteins that are differentially expressed in human**
220 **pregnancy complications**

221 We wanted to know whether our novel placental secretome map could help us to identify placental
222 proteins that may serve as circulating biomarkers/diagnostic indicators of maternal and fetal
223 wellbeing in human pregnancy. We collated publicly available RNA and protein expression datasets
224 for the human placenta from pregnancies complicated by PE, GDM, IUGR, small for gestational age
225 (SGA) and large of gestational age (LGA) (Table 2). We then overlaid our placental secretome map to
226 our collated database of placental RNA/protein expression for these pregnancy complications (Table
227 2). This identified 119 secreted proteins that were dysregulated in the human placenta in the
228 pregnancy complications studies (Figure 4A). There was some overlap in the expression of secreted

placental proteins between pregnancy complications and, aside from LGA, all complications showed an altered expression of ANGPT2, FLT1, IGF2 and TIMP2 (Figure 4A). Of note, FLT1 and IGF2 are particularly enriched in the human placenta compared to other tissues (see also Figure 3F). Furthermore, we found several secreted placental proteins that were uniquely altered in specific pregnancy complications. For instance, we found 18 secreted placental proteins that were only altered in the placenta of women with GDM, 47 specifically altered in PE and 5 uniquely altered in IUGR pregnancies (Figure 4A). Of those uniquely altered in PE and IUGR, secreted placental proteins TFPI2 and SERPINE2, respectively, are reported to be highly enriched in the human placenta compared to other tissues. GO analysis revealed that secreted placental proteins uniquely altered in GDM are involved in the metabolism of proteins and extracellular matrix organisation (Supplementary Table 2-A), those altered in PE largely function in the immune system and platelets (Supplementary Table 2-B) and those changed in IUGR are implicated in fibril, collagen and laminin formation (Supplementary Table 2-C).

**MIF/sFLT1 and ANGPT2/MIF ratios are altered in human GDM blood samples**

We wanted to know whether our secretome map may be useful in identifying placental biomarkers that could be measured in the circulation of women and aid in the detection of a pregnancy complication. To test this possibility, we analysed the abundance of secreted placental proteins in blood taken from women at booking (12 weeks of gestation) and after glucose tolerance testing (28 weeks of gestation) who were subsequently classified as normoglycemic or diagnosed with GDM. Maternal clinical characteristics and pregnancy outcomes for the women with normal glucose tolerance or GDM are shown in Table 3. We quantified the abundance of the following secreted placental proteins, sFLT1, ANGPT2, MIF and IGF2 as they were highly enriched in human placenta and/or differentially altered in several pregnancy complications (Figure 4A). We first visualised the cell specific expression of these proteins at the maternal–fetal interface in early human pregnancy using the CellxGene tool (https://maternal-fetal-interface.cellgeni.sanger.ac.uk/) based on findings from[33]. FLT1 was shown to be highly and mainly expressed in syncytiotrophoblast and extravillous trophoblasts (Figure 4B), whilst ANGPT2, MIF and IGF2 were more broadly expressed by trophoblast cell populations (Supplementary Figure 3). All four secreted placental proteins were detectable in the maternal circulation as early as 12 weeks of gestation (Figure 4C). Furthermore, several showed changes in abundance with gestational age and in those women who developed GDM. ANGPT2 and MIF declined in the maternal circulation between 12 and 28 weeks of gestation, in line with the reduction in placental expression indicating that the placenta is the main source (Fig 3F). However the

decline in MIF with gestational age was not observed in women who developed GDM (due, in part to non-significantly lower values in GDM versus healthy women at 12 weeks). Moreover, sFLT1 circulating levels were overall, significantly elevated in the circulation of women with GDM diagnosis ($p=0.05$). In contrast, IGF2 levels in the maternal circulation were not significantly different between 12 and 28 weeks of gestation or in women who developed GDM (Figure 4C and Supplementary Figure 4). As pregnancy complications can be caused by an alteration of several pathways and biological systems, it is common to evaluate the relationship between the abundance of different biomarkers. We found that the ratio of sFLT1 to MIF concentration was increased by 210% ($p=0.0003$) and ANGPT2 to MIF was increased by 97% ($p=0.02$) in women at 12 weeks of gestation who went on to develop GDM compared to the healthy pregnancies (Figure 4D).

**A transcriptional network controlling placental proteome highlights links with pregnancy complications**

We wanted to gain further information on the regulation of placental endocrine function and its significance for determining pregnancy outcome. To this aim, we searched for transcription factors (TFs) that had significant enrichment of binding sites at the promoters of genes encoding the 319 proteins in our placental secretome map (Figure 3A). We used two computational tools, Analysis of Motif Enrichment and Ingenuity Pathway Analysis and identified 33 common TFs expressed by trophoblast cells in human placenta and controlling the expression of a total of 96 proteins from our placental secretome map (Supplementary Table 3). All of these TFs were expressed by the STB at 8 weeks of gestation, when their placental secreted targets were too. Furthermore, the expression of 10 of these TFs has been reported to be perturbed in human pregnancy complications (Figure 5A). These 10 TFs control the expression of 36 members of the placental proteome map, 20 of which are reported to be differentially expressed in human pregnancy complications (Figure 5B). Of note was ARNT2, which is reported to be dysregulated in PE and IUGR and implicated in the control of 10 genes encoding proteins in the placental secretome, of which 5 were further reported to be altered in pregnancy complications (Supplementary figure 3). PLAG1 and CREB1 were specifically altered in the placenta from women with GDM and proposed to be key in regulating of placental secreted proteins like IGF2 and FLT1, which were also identified as altered in expression in such pregnancies. FOS, MYCN and NFYC were found to be altered in PE pregnancies and are predicted to modulate the gene expression of up to 11 proteins in our secretome list that are also reported to be differentially expressed in PE (Figure 5A and Supplementary Table 3).

## Discussion

Our study has established a comprehensive secretome map of the placenta. By utilising transgenic mouse lines for tracking placental endocrine cells, together with advanced molecular techniques and bioinformatics analysis, we have characterized a placental secretome map relevant for both mouse and human pregnancy. To achieve this, we performed mass spectrometry on three types of samples, i) primary cultures containing mouse placental endocrine cells, ii) conditioned media from primary cultures of mouse placental endocrine cells and iii) endocrine cells isolated from the mouse placenta by fluorescence-activated cell sorting. A robust bioinformatics pipeline was then used to integrate our proteins lists and to include in our analysis only proteins expressed by both the mouse and human placenta, as well as those destined to be secreted. By overlaying our secretome map to publicly available datasets for the human placenta, we were able to identify that several secreted placental proteins were altered in pregnancy complications including GDM, PE and IUGR. Moreover, in proof of concept experiments using blood samples from women who were healthy or developed GDM, our findings suggest that the relative abundance of secreted placental candidates identified using our secretome map, may be altered as early as week 12 of gestation, which predates traditional clinical diagnosis at 24 weeks. Lastly, we identified transcription factors that are likely to govern the expression of placental hormones with important implications for pregnancy outcome. Taken together, our data demonstrate that our methodology and placental secretome map may illuminate promising biomarker candidates as early diagnostic indicators and therapeutic targets for pregnancy complications linked to placental malfunction. Moreover, our methodology and findings may have relevance for understanding the significance of placental endocrine function in mammalian development and pregnancy physiology, more broadly.

Three parallel approaches were used to obtain lists of secreted placental proteins that could be integrated as a secretome map. This was fundamentally important, as we wanted to maximise our ability to detect secreted placental proteins without being limited by sample preparation, method sensitivity and specificity. We adapted published methods[34-36] and used the *Tpbpa*-Cre-EGFP mouse line[24] to obtain primary cell cultures containing a high density of mouse placental endocrine cells. By monitoring the behaviour of our cultured cells, we were able to show that at 48h of culture, cell viability was stable, with no increase in cell necrosis or cell death. Furthermore, at 48h, our primary cell cultures contained the three main endocrine cell types of the mature mouse placenta. We analysed the cultured cells and conditioned media separately, as proteins can be secreted at low concentrations into the culture media, resulting in recovery difficulties. Moreover, salts and other

328 compounds in the media may interfere with protein detection[37]. Furthermore, highly abundant
329 proteins can mask the detection of lowly expressed proteins, resulting in selective detection or a mis-
330 representation of the proteome when analysing cultured cells[38]. To further increase the sensitivity of
331 detecting secreted placental proteins, we concentrated the conditioned media of our primary cell
332 cultures prior to mass spectrometry and likely due to this, we obtained a larger list of secreted
333 placental proteins from the conditioned media (26 proteins in the mouse and 257 in mouse and
334 human) compared to the cultured cells (5 proteins in mouse and 158 in mouse and human). However,
335 our primary placental endocrine cell cultures were not pure, and cells may alter their protein
336 expression when cultured. As a complementary approach, we also identified the proteins in freshly
337 isolated mouse placental endocrine cells using the *Tpbpa*-Cre-EGFP and mTmG murine lines and
338 fluorescence-activated cell sorting. This approach delivered highly pure samples containing all three
339 placental endocrine lineages. However, some trophoblast giant cells in the mouse placenta may have
340 been lost due to size limitation of the nozzle for the fluorescence-activated cell sorting (100µm).
341 Moreover, both the preparation of primary cultures and fluorescence-activated sorting of mouse
342 placental endocrine cells resulted in a relatively lower abundance of glycogen cells than expected for
343 the mouse placenta at day 16 (normally the relative proportion of endocrine cell types is
344 spongiotrophoblast > glycogen cells > trophoblast giant cells). This is somewhat expected, as glycogen
345 cells are sensitive to lysis and may thus be particularly sensitive to the sample preparation technique[39].
346 Nevertheless, 112 secreted placental proteins (7 proteins in mouse and 105 in mouse and human)
347 were detected in the pure placental endocrine cell isolates, of which 82% were also found in cultured
348 cells. Reactome pathway analysis of individual protein lists revealed that the proposed functions of
349 secreted proteins in the cultured cells, conditioned culture media and placental endocrine cell isolates
350 were overall similar, with the highest scoring pathways including those related to the immune system,
351 homeostasis and insulin-like growth factor (IGF) regulation. However, several of the proteins detected
352 were specific to one approach/sample type, which reinforces our approach of combining different
353 sample types/methodologies to optimise and broaden the detection of proteins secreted by the
354 placenta. Indeed, the combination of the three methodologies enabled the creation of map of 319
355 secreted proteins expressed by both mouse and human placenta and another 31 specific to the
356 mouse.

357

358 Of the 319 secreted placental proteins in mouse and human, 56 were previously reported to be
359 secreted from first trimester human trophoblast organoids[32], including progranulin (GRN), insulin-like
360 growth factor II (IGF2), insulin-like growth factor-binding protein 2 (IBP2) and macrophage migration
361 inhibitory factor (MIF). Moreover, the majority of the proteins (300 out of 319) were localised to the

362 syncytiotrophoblast of the human placenta[31], which is in direct contact with maternal blood and the
363 primary site for hormone production in the human placenta. The predominate localisation of proteins
364 in our secretome map to the syncytiotrophoblast in humans validates our method and highlights how
365 data generated may improve our understanding of the role and regulation of human placental
366 endocrine function. More than 60% of the proteins in our placental secretome map were predicted to
367 function in "response to stimulus". This GO term includes secondary biological groups such as
368 regulation of signal transduction downstream of the interleukin-1 receptor type 2 (IL1R2),
369 macrophage metalloelastase (MMP12) and apolipoprotein A-I (APOA1) that participate in the
370 response to cytokines. It also included insulin-like growth factor-binding proteins (IGFBP2, IGFBP4 and
371 IGFBP6), which modulate the mitogenic and metabolic actions of insulin-like growth factors that play
372 important roles in pregnancy physiology[40]. Indeed, several of these proteins have been previously
373 shown to be secreted from the human placenta, such as the IL1R2[41], IGF2 and IGFBPs[42], MMP12[43] and
374 APOA1[44] and are consistent with our findings. Many of the proteins in our secretome map were also
375 identified to be hormone binding proteins, or proteins that regulate signalling downstream of
376 receptors. These included annexin A5 (ANXA5) an anticoagulant protein, inhibin beta A chain (INHBA),
377 which is a subunit of both inhibin and activin, transthyretin (TTHY) a thyroid hormone-binding protein,
378 insulin-degrading enzyme (IDE), which binds to insulin and leukaemia inhibitory factor receptor (LIFR).
379 Whilst proteins like ANXA5, inhibins/activins, TTHY and LIFR have been previously reported to be
380 secreted from the placenta[15,45-47], we are not aware of any studies describing the secretion of other
381 proteins, like IDE from the human placenta. Furthermore, other secreted proteins such as adipocyte
382 enhancer-binding protein 1 (AEBP1) and Y-box-binding protein 1 (YBOX1), which can regulate
383 transcription were also detected in the placental secretome. Again however, to date, there are no
384 studies related to the secretion of AEBP1 and YBOX1 from the human placenta. Collectively our
385 findings may suggest that the secretome map comprises known and novel secreted placental proteins.

386

387 Two main protein domains featured in our placental secretome map were the "Serpin conserved site"
388 and the "EGF-like domain" which are important in regulating inflammatory processes, growth factor
389 signalling and extracellular matrix and cytoskeletal remodelling[48,49]. Thus, in addition to identifying
390 proteins secreted by the placenta with systemic actions, proteins in our secretome map may also play
391 local autocrine and paracrine roles in modulating processes at the fetal-maternal interface, including
392 decidual remodelling/function, immune tolerance and placentation. Using tissue gene expression
393 enrichment analysis, we found that of the proteins in our placental secretome map, 20 were most
394 abundantly expressed by the placenta in mouse and 4 were enriched in the placenta in human. Of
395 note, TFPI2 a plasmin-mediated matrix remodelling, FLT1 a receptor for vascular endothelial growth

396 factor and proteins from the SERPIN family were enriched in both the mouse and human placenta.
397 This is consistent with the findings of others showing genes and proteins in the placenta overlap in the
398 two species[25,26]. Indeed, as a key aim of our work was to create a secretome map that would be
399 applicable for both human and mouse, we used orthologue searches in our initial steps in narrowing
400 our list of protein candidates. We found that the majority of the proteins we found in our mouse
401 placental endocrine cell protein lists were also expressed by the human placenta, which is higher than
402 reported previously for other placental datasets. However, of note, we found that there were an
403 additional 31 secreted proteins in our complete placental secretome map, which were only expressed
404 by the mouse and not the human placenta. These included 17 members of the PRL/PL gene family and
405 7 members of the cathepsin gene family, which have both undergone robust species-specific
406 expansion, particularly in rodents, and exhibit unique spatial expression patterns by endocrine
407 trophoblast lineages in mice[50-52]. Overall, PRL/PL and cathepsin family members are thought to play
408 divergent roles in driving physiological changes in the mother during pregnancy, including modulation
409 of insulin production and action, vascular remodelling and immune system regulation. However,
410 further work is required to understand the significance of individual family members to determining
411 pregnancy outcome.

412

413 Using a collection of published datasets, we identified that around 30-40% of proteins in our placental
414 secretome map (319 proteins expressed by human and mouse placenta) were reported to be
415 aberrantly expressed by the human placenta in pregnancy complications. Moreover, we found 4
416 secreted placental proteins to be differentially expressed in the placenta of women who developed
417 PE, GDM, IUGR and SGA. This suggests that there may be common pathways related to the production
418 of specific placental hormones, which may underlie or be reflective of the development of such
419 pregnancy complications. Alternatively, the common expression of secreted placental proteins
420 between these four pregnancy complications may reflect that there is overlap between these
421 pregnancy complications, as PE often leads to IUGR or SGA and GDM is linked to PE. Interestingly, two
422 of these proteins; FLT1 and IGF2 were highly enriched in the human placenta compared to other
423 tissues. Moreover, circulating FLT1 (sFLT1) has been previously reported as a suitable biomarker for
424 PE[53], IUGR[54] and SGA[55]. Whilst the expression of the FLT1 gene is reported to be altered in the placenta
425 of women with GDM, previous work has not evaluated whether sFLT1 levels are altered and could
426 serve as a biomarker for GDM pregnancies[55]. Similarly, IGF2 was also reported to be altered in the
427 placenta in PE[56], IUGR[57,58], GDM[9] and SGA pregnancies[59] however, less is known about changes in
428 circulating IGF2 in the mother. Aside from being altered in LGA pregnancies, ANGPT2 and TIMP3 in
429 our secretome map were also reported to be differentially expressed by the placenta in the pregnancy

complications assessed. ANGPT2 has previously been explored as predictive candidate biomarker for PE, however it was shown to be unsuccessful[60]. In the context of GDM, ANGPT2 has been reported be differentially secreted from cultured trophoblast of the term placenta in GDM pregnancy[61], but no maternal serum analysis was described. Similarly, altered placental expression of ANGPT2 were reported for SGA[62] and one study has found it to serve as a predictive biomarker for IUGR[63].

We identified several secreted placental proteins altered specifically in some of the pregnancy complications we studied. Analysing the gene ontology terms of these unique groups of proteins revealed that for those uniquely altered in PE, they were implicated in the control of immune processes, platelet aggregation and leukocytes like neutrophils, which is in line with previous work focussed on understanding the pathogenesis of PE in women[64,65]. Similarly, GO analysis of the secreted placental proteins specifically altered in GDM featured terms including protein metabolism, extracellular matrix remodelling and the control of the unfolded protein response and is consistent with studies exploring the aetiology of GDM[66,67]. Secreted placental proteins changed in IUGR are proposed to be involved in fibril, collagen and laminin interactions, however the functional relevance of this finding requires further study.

As a proof of concept that our methodology and placental secretome map may be beneficial for illuminating potential circulating biomarkers with clinical relevance, four secreted placental protein (sFLT1, MIF, ANGPT2 and IGF2) were assessed in the serum of women who had normal or GDM pregnancies. All four proteins were detectable as early as 12 weeks of gestation. We found that across the two gestational time-points, sFLT1 was overall higher in the circulation of women who developed GDM. This was despite the fact that the women with GDM were normotensive. Studies previously exploring changes in sFLT1 in GDM pregnancies have yielded inconsistent results. There was no difference in circulating sFLT1 concentration in lean women with and without GDM in the third trimester of pregnancy[68], and no difference in the secretion of sFLT1 by term placental explant from women who developed GDM[69]. However, sFLT1 has been reported to be elevated in the early second trimester (16-20 weeks of gestation) in women who went on to develop GDM in later pregnancy[70], which is in line with our findings. The relationship between placental sFLT1 production and the development of GDM warrants further study.

We found that the circulating levels of ANGPT2 and IGF2 were not different between women with normal glucose tolerance or GDM at either 12 or 28 weeks of gestation. However, circulating levels of MIF in the mother failed to decline in women with GDM between these two gestational time points. Moreover, the ratios / relationships between the concentrations of MIF to sFLT1 and ANGPT2 to MIF were altered in women at week 12 of pregnancy, which is at least 10 weeks before diagnosis of GDM, suggesting that they may serve as potential early biomarkers for GDM. MIF promotes the secretion of insulin from beta cells and also increases glucose uptake by skeletal muscle[71], thus its significance in development of GDM should be explored further. The value of assessing the ratio / relationship between the concentration of different biomarkers in the maternal circulation for early detection of pregnancy complications is supported by previous work showing a change in the ratio of sFLT1 to PIGF for PE, as well as IUGR in women[4,72]. The number of women with normal glucose tolerance and GDM in our study was small and both groups had an elevated body mass index. Further work is required to validate our findings of a change in the ratios / relationships between MIF to sFLT1 and ANGPT2 concentrations in larger cohorts and in women who have a normal body mass index. Moreover, work is required to further explore the clinical value of generating a secretome map of the placenta utilising our approach.

We identified 33 transcription factors that are expressed by the STB of the human placenta and are predicted to control the gene expression of approximately 30% of the proteins in our placental secretome map (and are also expressed by the STB at the same time). Of these, ten were previously linked with pregnancy complications. These included ARNT2, which is dysregulated in the placenta of women with PE and IUGR and modulates the expression of genes that are also reported to be altered in human pregnancy complications, such as ANGPT2[22,73]. ARNT2 is implicated in mediating cellular responses to stimuli including hypoxia[74] and our findings are consistent with recent work applying novel sequencing strategies and an integrated systems biology approach[75,76]. We also identified transcription factors, PLAG1 and CREB1 that were altered in the placenta of GDM pregnancies and were predicted to modulate the expression of numerous proteins in our secretome map including those that were additionally differentially expressed in GDM, such as IGF2 and FLT1[73,77,78]. Consistent with this, CREB1 is regulated by metabolic stimuli like glucose[79] and PLAG1 was pinpointed as a critical factor altered in women who developed a complicated pregnancy[80]. In addition, both PLAG1 and CREB1 have been reported to be dysregulated in mouse genetic models showing placental dysfunction and/or poor pregnancy outcome[81,82]. Thus, numerous transcription factors likely govern the endocrine function of the placenta and may have significance for understanding the pathogenesis of human

pregnancy complications. Hence, future work should centre on testing the significance and upstream regulators of transcription factors identified as putative regulators of placental hormone production.

In summary, we have generated a comprehensive secretome map of the placenta. This map was proven to be suitable for gaining further information on the significance and regulation of placental endocrine function in mice and humans. Furthermore, we have uncovered different types of secreted placental proteins, which function in the endocrine and paracrine regulation of maternal physiology, but also possibly in an autocrine manner to modulate placental biology. Whether secreted placental proteins may reach the fetal circulation to modulate fetal growth requires further exploration. However, our placental secretome map revealed that more than 100 proteins may be differentially secreted from the placenta in complicated human pregnancies. Further work is required to extend our findings, including by employing the same methodology and bioinformatics pipeline however, using placental endocrine cells taken from other gestational ages and pregnant mice exposed to environmental conditions, such as maternal obesity, which is a risk factor for complications like PE, GDM and abnormal birthweight. This will further build knowledge on the role and control of the endocrine placenta during pregnancy and may pave the way for the discovery of novel or improved biomarkers for early detection and prevention of pregnancy complications.

**Methods:**

**Animals**

All experiments were performed under the UK Home Office Animals (Scientific Procedures) Act 1986. All mice used (wild type and transgenic) were on a C57BL/6 genetic background and housed under 12h dark-light conditions with free access to water and the standard diet (RM3, special dietary services) used in the University of Cambridge Animal Facility. For the preparation of primary cultures of junctional zone trophoblast endocrine cells, wild type females were mated with males homozygous for *Tpbpa*-Cre-EGFP transgene[24]. For the fluorescence-activated cell sorting of junctional zone trophoblast endocrine cells, *Tpbpa*-Cre-EGFP homozygote males were mated to females homozygous for the double fluorescent Cre reporter construct, mTmG, which expresses membrane-targeted tdTomato prior to, and membrane-targeted EGFP following, Cre-mediated excision (kind gift from Dr Marika Charalambous, King's College London; [30]). The day a copulatory plug was found was denoted as day 1 of pregnancy (term is 20.5 days). Placentas were harvested from mouse dams that were schedule 1 killed by cervical dislocation on day 16 of pregnancy.

526

527 **Preparation of primary cultures of junctional zone trophoblast endocrine cells**

528 Placentas (average of 6-8 per mouse dam) were enzymatically dissociated using a buffer (Medium 199
529 with Hank's salts, 20mM HEPES, 10mM NaHCO, 50U penicillin/ml, and 50pg streptomycin/ml, pH 7.4)
530 containing 0.1% collagenase and 0.002% DNase at 37˚C for 1h, as described previously[36]. Dissociated
531 samples were passed through a 200µM nylon filter to remove tissue debris and cells were centrifuged
532 at 500g for 5 minutes. Cell pellets were resuspended with Medium 199 X1 and cells subsequently
533 separated using a three layer Percoll density gradient (1.028, 1.05 and 1.088 g/ml) according to
534 manufacturer instructions (Percoll plus, GE Healthcare Life Sciences) and as described previously[83] by
535 centrifuging at 600g for 30 minutes with controlled acceleration and braking. Layers from the density
536 gradient were recovered into medium199 X1 to dissolve the Percoll solution. Cells were then
537 centrifuged for 5 minutes at 500g and further washed with PBSX1 prior to counting using
538 Haemocytometer. Cells isolated from each layer of the Percoll gradient were fixed with 4% PFA for 20
539 min and subjected to 5µ/ml of Hoechst solution for 10 min at 37˚C. Cells were then visualised using
540 fluorescence microscopy (Leica TCS SP8 Confocal laser scanning microscope) and the second layer was
541 found to contain the greatest density of junctional zone trophoblast endocrine cells (EGFP positive
542 cells due to Tpbpa-Cre-EGFP) and therefore used for further analysis. Namely, cells in the second
543 Percoll density layer were seeded in 96 well plates, 8 chamber-slides or in 6 well plates ($10^5$ cells/ml)
544 and time of seeding was defined as time 0. Cells were grown in NTCT-135 medium containing 10%
545 fetal bovine serum, 50 IU/ml ampicillin, 50 µg/ml streptomycin, 2mM l-glutamine, 20 mM HEPES and
546 10 mM NaHCO and maintained in a humidified atmosphere of 5% $CO_2$ at 37˚C. Cell medium was
547 replaced every 24h. Cells were washed three times in PBSX1 and serum-free medium applied 24h prior
548 to any downstream analysis.

549

550 **Viability assay**

551 Cell viability was determined using an XTT cell proliferation assay kit (Abcam- ab232856) according to
552 manufacturer's instructions. Values were calculated as % of values at time 0h and each experiment
553 was performed in triplicate in 8-10 independent assays.

554

555 **Cell death assay (LDH release assay)**

Cell death was determined by measuring the activity of lactate dehydrogenase (LDH) in the conditioned media of primary cell cultures using a LDH cytotoxicity assay kit, according to the manufacturer's instructions (Thermo scientific). Cell-free medium and cells treated with medium containing Triton-X were used as negative and positive controls, respectively. Each experiment was performed in triplicate in 6 independent assays.

**In situ hybridisation**

In situ hybridization was performed on primary cell cultures seeded on chamber slides at 90% confluency ($10^6$ cells/well) (Thermofisher Scientific, UK). Cells were fixed in 4% PFA for 30 min, washed twice with PBSX1, dehydrated in increasing concentrations of ethanol and stored in 100% ethanol at -20°C. In situ hybridization was performed using the RNAScope 2.5 RED chromogenic assay kit following the manufacturer's instructions (Bio-Techne, UK). Briefly, slides were allowed to equilibrate to room temperature and rehydrated in PBSX1. RNAscope® Hydrogen Peroxide was applied to the slides for 10 min at RT, followed by RNAscope® Protease Plus in RT for 10 min. Slides were then incubated with the target or control probes at 40°C for 2h (negative control probe (310043), positive control probe (313911), Tpbpa-probe (405511), Prl8a8-probe (528641), Gjb3-probe (508841) and Hand1-probe (429651) in a HybEZ oven for 2h at 40˚C. Next, slides were washed twice with wash buffer and were subjected to 6 rounds of amplification and the probe signal was developed via a reaction with fast red. Slides were then counterstained with Haematoxylin and mounted in EcoMount. Slides were scanned on a NanoZoomer 2.0-RS slide scanner (Hamamatsu, Hamamatsu City, JP) at 40x magnification.

**Conditioned medium preparation for mass spectrometry**

Conditioned medium from cultured cells was collected at 48h of culture. At 24h prior to medium collection, cells were washed three times with PSBX1 and cultured in serum-free medium. The conditioned medium was centrifuged at 1000g for 10 min and total protein concentration measured using a Bradford assay. Proteins in the conditioned medium were concentrated up to 1.2ug/ul of total protein using cellulose membrane Ultra-4 Centrifugal Filter Unit of 3KDa cut-off (Merck) as per the manufacturer instructions.

**RNA extraction and quantitative real time PCR (qPCR)**

Total RNA was extracted from cultured cells using RNeasy Plus Mini Kit (QIAGEN) and 0.5 µg of RNA was reverse transcribed with random hexamer primers using a High-Capacity cDNA RT kit (Applied Biosystems) according to the manufacturer's instructions. qPCR was performed using MESA BLUE qPCR Master Mix (Eurogentec) on a Bio-Rad T100 thermocycler using gene-specific intron-flanking primers (Table 4). Gene expression was analysed in triplicate and the Ct values were normalised to the expression of internal housekeeping genes (*Gapdh, RpII* and *Hprt*). Results are presented as mean ± SEM and relative to time 0h.

**Fluorescence-activated cell sorting (FACS) of live cells for mass spectrometry**

Single cell suspensions were prepared from whole placentas digested with the dissociation buffer containing 0.1% collagenase and 0.002% DNase at 37˚C for 1h, as described for the preparation of primary cell cultures. Cells were filtered through 100µM nylon filter and centrifuged at 500g for 5 minutes. Cell pellets were resuspended with PBSX1 and 7AAD (Life Technologies, A1310) added prior sorting using a FACSJazz machine (BD Biosciences, Singapore). Live cells were gated and cells positive for EGFP or tdTomato were sorted and lysed directly into 80% acetonitrile (ACN) in water (v/v) in a Protein LoBind Eppendorf tube.

**Mass spectrometry**

**LC-MS/MS analysis of conditioned media**

Conditioned media from primary cultures of trophoblast cells were standardised to a final concentration of 2µg/µl in 4% SDS loading buffer with 100mM dithiothreitol (DTT). Samples were denatured at 95°C for 5 min and 10µg of total protein per sample was loaded onto a 12% SDS PAGE gel and run at 120 Volts. The gel was then stained with colloidal coomassie stain and washed with water. Protein bands in each lane were cut into $1mm^2$ pieces, de-stained, reduced (using DTT) and alkylated (using iodoacetamide) and subjected to enzymatic digestion with sequencing grade Trypsin (Promega, Madison, WI, USA) overnight at 37°C. After digestion, the supernatant was pipetted into a sample vial and loaded onto an autosampler for automated LC-MS/MS analysis. All LC-MS/MS experiments were performed using a Dionex Ultimate 3000 RSLC nanoUPLC (Thermo Fisher Scientific Inc, Waltham, MA, USA) system and a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA) as described previously [84].

For medium generated spectra, all MS/MS data were converted to mgf files and the files were then submitted to the Mascot search algorithm (Matrix Science, London UK, version 2.6.0) and searched against the UniProt mouse database (61295 sequences; 27622875 residues) and a common contaminant sequences containing non-specific proteins such as keratins and trypsin (115 sequences, 38274 residues). Variable modifications of oxidation (M) and deamidation (NQ) were applied, as well as a fixed modification of carbamidomethyl (C). The peptide and fragment mass tolerances were set to 20ppm and 0.1 Da, respectively. A significance threshold value of $p<0.05$ and a peptide cut-off score of 20 were also applied. All data (DAT files) were then imported into the Scaffold program (Version 4.5.4, Proteome Software Inc, Portland, OR).

**LC-MS/MS analysis of primary cultured cells and sorted cells**

Trophoblast from both primary cell cultures and fluorescence activating cell sorting were treated with 800µL of 80% ACN in water and centrifuged for 5 min at 10,000g. The supernatant was removed and the pellet was reduced and alkylated using 50mM ammonium bicarbonate and 10mM DTT at 60˚C for 1h, followed by the addition of 100mM iodoacetamide in the dark for 30min. Enzymatic digestion was performed using Trypsin at 10µg/mL in 50mM ammonium bicarbonate overnight at 37˚C (enzymatic digestion was halted by the addition of 1% formic acid). Samples were analysed by LC-MS using a Thermo Fisher Ultimate 3000 nano LC system coupled to a Q Exactive Plus Orbitrap mass spectrometer (ThermoScientific, San Jose, CA, USA) as described previously[85]. For generated spectra, all LC-MS files were searched using PEAKS 8.5 (Bioinformatics Solutions Inc) software against the Swissprot database (downloaded 26-Oct-2017) with a *Mus musculus* filter. For the proteins, a tryptic digestion approach was used with a semi-specific setting and up to a maximum of 3 missed cleavages. The search outputs had a 1% FDR setting applied, along with a unique peptide setting of at least 1 peptide. The precursor and product ion tolerances were 10 ppm and 0.05 Da, respectively.

**Bioinformatics analysis**

Protein/peptide annotations in LC-MS datasets were converted to their gene accession ID via UniProt (https://www.uniprot.org/uploadlists/). Gene lists were then overlaid with publicly available datasets for the mouse and human placenta, which are detailed in Table 1 (3 from mouse placenta and 8 for human placenta). Mouse-human ortholog searches were also undertaken using three sources of data, MGI (http://www.informatics.jax.org/), NCBI (https://www.ncbi.nlm.nih.gov/homologene) and Ensembl (biomaRt_2.42.1 and homologene_1.4.68 in R version 3.6.2; https://www.R-project.org/).

649 Then using R, a combined ortholog list for Mouse-Human was generated (details of the list and Rscript
650 can be found in GitHub, https://github.com/CTR-BFX/2020-Napso_Sferruzi-Perri). Mouse-human
651 ortholog results were classified as one-to-one when a gene/protein from mouse was found at the end
652 of a node in human. Any results classified as one-to-many were excluded. Gene ontology analyses
653 were performed using both STRING and Panther tools[86]. Gene enrichment analyses were conducted
654 using TissueEnrich (tissue-specific gene enrichment analysis; [87]), which utilises datasets available in
655 the Human Protein Atlas compiling RNAseq datasets from 35 human tissues[88,89] and the Mouse
656 ENCODE database comprised of RNAseq datasets of 17 mouse tissues[90]. Refined gene/protein lists
657 were overlaid with publicly available RNA and protein expression datasets for human pregnancy
658 complications (Table 2) and aided by searches in Pubmed and the OMIM repository
659 (http://www.ncbi.nlm.nih.gov).

660

661 To further refine our lists to secreted proteins, we applied SignalP (Signal Peptide Prediction, version
662 4.1; [27]) and gene ontology analysis using four different gene ontology (GO) terms: extracellular region
663 (GO: 0005615), extracellular exosome (GO: 0070062), extracellular region parts (GO: 0005615) and
664 signal IP (excluding signals detected for ER lumen proteins)[28]. This was undertaken because SignalP
665 can only detect the signal peptide for proteins secreted via the canonical route, which is also known
666 as the "classic" or "conventional" secretion pathway. However, eukaryotic cells also utilize an
667 unconventional protein secretion route for protein sorting and delivery, an "unconventional"
668 secretion pathway, including leaderless proteins that are secreted into the extracellular space[29]. This
669 approach allowed us to capture proteins that employ the "conventional", as well as "unconventional"
670 secretion pathways. All data outputs at each step of the pipeline, including the proteins/genes
671 expressed in the mouse but not the human placenta and the refinement of our list to secreted proteins
672 can be found in GitHub (https://github.com/CTR-BFX/2020-Napso_Sferruzi-Perri).

673

674 To search for enrichment of transcription factor (TF) binding motifs at the promoters of the genes
675 encoding the 319 proteins that are part of placental secretome, we first used EPD (Eukaryotic
676 Promoter Database - https://epd.vital-it.ch/index.php) to retrieve the DNA sequences from 1,000bp
677 upstream to 100bp downstream of the transcriptional start site (TSS). These sequences were then
678 analysed using AME (Analysis of Motif Enrichment v4.12.0 - http://meme-suite.org/tools/ame) by
679 selecting *Mus musculus* and HOCOMOCO Mouse (v11 FULL) as motif database. An additional search
680 for upstream regulators was performed using the Ingenuity Pathway Analysis (IPA, Qiagen), and only
681 TFs predicted by both tools (n=77) were selected for further analysis. Next, we filtered for TFs with

682 enriched expression in STB cells from human placenta at 8 weeks of gestation that had at least one
683 common target gene encoding one of the 319 placental secretome proteins (n=33). Literature search
684 led to identification of ten of those TFs that were linked with pregnancy complications. Transcriptional
685 network visualization for the 10 TFs and the corresponding targets was performed using IPA.

686

687 **Human study population and sampling**

688 Peripheral blood samples were retrospectively selected for this study from women recruited via the
689 Ophelia study (REC number 18/LO/0477 approved 5/4/2018). Inclusion criteria included (1) singleton
690 pregnancy, (2) no evidence of severe congenital anomaly and (3) a referral for an oral glucose
691 tolerance test (OGTT) for clinical reasons, according to NICE guidelines
692 (https://www.nice.org.uk/guidance/ng3). Exclusion criteria for this study were (1) multiple pregnancy
693 (2) severe congenital anomaly on ultrasound, (3) severe anaemia on previous blood tests, (4) previous
694 diagnosis of diabetes outside of pregnancy and (5) medications at the time of the OGTT, which may
695 interfere with the results of the OGTT. Screening for GDM was performed at 24 weeks of gestation
696 using a 75 g OGTT and diagnosis of GDM was made in accordance with the IADPSG glycaemic cut-off
697 values (fasting value ≥ 92 mg/dL (5.1 mmol/L), 1 h post-glucose load ≥ 180 mg/dL (10 mmol/L), 2 h
698 post-glucose load ≥ 153 mg/dL (8.5 mmol/L))[91]). Blood samples were collected from pregnant women
699 in the 1$^{st}$ trimester (12 weeks of gestation) and 2$^{nd}$ trimester (28 weeks of gestation) and were analysed
700 for HbA1c concentration. Plasma was recovered by 2500 rpm for 10min and samples were stored at -
701 80c. Blood pressure measurements were taken at various times in pregnancy using a calibrated
702 automatic oscillometric sphygmomanometer (Dinamap, machine) and systolic and diastolic blood
703 pressure recorded. For inclusion in this study, women with GDM were selected and a control group of
704 age- and BMI-matched participants with normal glucose tolerance were included for comparison.
705 Further details about the OPHELIA study are provided elsewhere (Research Registry number 5528)[92].

706

707 **Human plasma analysis of protein candidates by ELISA**

708 Candidate secreted placental proteins were quantified in maternal plasma samples from healthy
709 women and those diagnosed with GDM, using commercially available ELISAs for sFLT1 (K15190D,
710 MSD), MIF (K151XJK-1, MSD), ANGPT2 (F21YR-3, MSD) and IGF2 (DG200, R&D) by the Core
711 Biochemical Assay Laboratory, Cambridge and according to the manufacturer's instructions.

712

## Statistical analysis

Data for viability, cell death and qRT-PCR are presented as mean ± SEM. Two-way ANOVAs (with Tukey correction for multiple comparisons) or t-tests were used for determining significant differences. All analyses were performed using GraphPad Prism version 7.00 (GraphPad Software). $P<0.05$ was considered to indicate a statistically significant difference between the groups analysed.

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## Conflict of Interest

743 Authors do not have any competing financial interests in relation to the work described.

744

745 **Table 1: RNA expression database for mouse and human placenta.**

| Species | RNA-Seq Annotation | N | Reference |
|---|---|---|---|
| **Mouse** | GSE79121 | n=1 pooled litter (day 20) | [19] |
| | GSE11224 | n=2-3 pooled litters (day 8.5 – day 20) | [20] |
| | GSE11224 | n=2-3 pooled litters (day 8.5 - day 20) | [20] |
| **Human** | GSE9984 | n=4 first trimester<br>n=4 second trimester<br>n=4 term placentas | [93] |
| | GSE28551 | n=16 first trimester<br>n=21 third trimester | [21] |
| | GSE10588<br>GSE25906<br>GSE4707<br>GSE30186<br>GSE24129<br>GSE44711 | n=26 (term placenta)<br>n=37 (term placenta)<br>n=4 (term placenta)<br>n=6 (term placenta)<br>n=8 (term placenta)<br>n=8 (term placenta) | [22] |

746

747

748 **Table 2: Compilation of publicly available RNA and protein expression datasets for the human**
749 **placenta from complicated pregnancies.**

| Complication | Diagnosis of complication | Data sets available | RNA or Protein | Number of samples per sample type | Reference |
|---|---|---|---|---|---|
| **PE** | Hypertension (systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥ 110 mmHg) after week 20 with at least one of the following symptoms; thrombocytopenia, impaired liver function, new development of renal insufficiency, proteinuria >5 g in 24 hours, new-onset cerebral or visual disturbances | 4 | RNA<br>RNA<br>RNA<br><br><br><br><br>RNA | n=8 (term placenta)<br>n=12 (term placenta)<br>n=4 (syncytiotrophoblasts*)<br>n=3 (invasive cytotrophoblasts*)<br>n=4 (endovascular cytotrophoblasts*)<br><br>n=77 (7 microarray studies combined^) | 73<br>94<br>95<br><br><br><br><br>22 |
| **GDM** | Glucose intolerance determined using an oral glucose tolerance test (75g) at 24-28 weeks of gestation and revealed by either a fasting venous plasma glucose level of >5.1 mmol/l, and/or >10 mmol/l and > 8.5 mmol/l at 1h and 2h, respectively | 6 | RNA<br>RNA<br>RNA<br>RNA<br>RNA<br>Protein | n=8 (term placenta)<br>n=12 (term placenta)<br>n=19 (term placenta)<br>n=7 (term placenta)<br>n=4 (term placenta)<br>n=135 (first trimester serum) | 73<br>94<br>96<br>97<br>98<br>99 |
| **SGA** | Birth weight < 10th centile | 2 | RNA<br>RNA | n=8 (term placenta)<br>n=12 (term placenta) | 73<br>94 |
| **LGA** | Birth weight > 90th centile | 2 | RNA<br>RNA | n=8 (term placenta)<br>n=12 (term placenta) | 73<br>94 |
| **IUGR** | Abnormal Doppler waveforms in the umbilical or middle cerebral artery (both <10$^{th}$ centile) and/or fetal weight and/or abdominal circumference (both <10$^{th}$ centile) and/or head circumference (>10$^{th}$ centile) | 3 | RNA<br>RNA<br><br><br>RNA | n=5 (term placenta)<br>n=12 (third trimester, placental RNA from blood<br><br><br>n=5 (term placenta) | 100<br>80<br><br><br>73 |

750 * From cell populations isolated by laser capture microdissection on human placenta from pre-term
751 pregnancies. ^ Compilation of 7 microarray datasets for the human placenta of PE pregnancies. Note
752 there were inconsistencies between studies in the diagnosis and inclusion criteria for these 7
753 microarray datasets for PE.

754

755

**Table 3: Clinical characteristics of women used for the analysis of circulating placental hormone abundance in human pregnancy.**

| Characteristics | Healthy pregnancy (n=10) | GDM pregnancy (n=6) | *p* (t-test) |
|---|---|---|---|
| **Parity** | 1.14 ± 0.26 | 1.29 ± 0.52 | 0.78 |
| **Early pregnancy BMI** | 33.45 ± 1.78 | 35.37 ± 2.42 | 0.52 |
| **GA at OGTT** | 28.513 ± 0.75 | 26.55 ± 1.83 | 0.28 |
| **OGTT 0h (mmol/L)** | 4.46 ± 0.07 | 5.07 ± 0.2 | **0.002** |
| **OGTT 2h (mmol/L)** | 5.65 ± 0.23 | 7.66 ± 0.94 | **0.014** |
| **Systolic BP (mm Hg) 1st trimester** | 116.8 ± 0.8 | 112.5 ± 1.8 | 0.386 |
| **Diastolic BP (mm Hg) 1st trimester** | 71.09 ± 0.84 | 68.0 ± 2.1 | 0.576 |
| **Systolic BP (mm Hg) 2nd trimester** | 117 ± 1.75 | 122.5 ± 4.26 | 0.541 |
| **Diastolic BP (mm Hg) 2nd trimester** | 70.66 ± 1.6 | 71.75 ± 3.43 | 0.855 |
| **HBA1C (mmol/mol)** | 33.42 ± 0.58 | 36.0 ± 0.53 | **0.01** |
| **GA at delivery** | 39.47 ± 0.41 | 38.76 ± 0.57 | 0.30 |
| **Birthweight (g)** | 3556.36 ± 126.24 | 3392.1 ± 170.00 | 0.44 |

GA = gestational age, BP= Blood pressure

761 **Table 4: The sequences of qRT-PCR primers used to analyse mouse placental preparations.**

| Gene | Primers |
|---|---|
| *Krt18* | F: 5'-CAAGACCTGAACGATCGCCT-3' |
| | R: 5'-ATTCGCAAAGATCTGAGCCCT-3' |
| *Tbpba* | F: 5'- TGAAGAGCTGAACCACTGGA -3' |
| | R: 5'- CTTGCAGTTCAGCATCCAAC -3' |
| *Gjb3* | F: 5'- GGGGCTCTCC TCAGACATA -3' |
| | R: 5'- ACCTGCTAGCCACACTTGCT-3' |
| *Prl8a8* | F: 5'- TCAGAGCTGCA TCTCACTGC -3' |
| | R: 5'- GGGACA TCTTTCA TGGCACT-3' |
| *Hand1* | F: 5'- GGAGACGCACAGAGAGCATT -3' |
| | R: 5'- CACGTCCATCAAGTAGGCGA -3' |
| *Mct4* | F: 5'- GGCTGGCGGTAACAGAGTA -3' |
| | R: 5'- CGGCCTCGGACCTGAGTATT -3' |
| *p53* | F: 5'-GTATTTCACCCTCAAGATCCGC-3' |
| | R: 5'-GGAGCTAGCAGTTTGGGCTT-3' |
| *Bax* | F: 5'-ACCAGGGTGGCTGGGAAG-3' |
| | R: 5'-CCTTTCCCCTTCCCCCATTC-3' |
| *RPII* | F: 5'-AGATGTATGACGCCGACGAG-3' |
| | R: 5'-AATCGGTGGTGCATCTTCCA-3' |
| *Gapdh* | F: 5'- GGGAAATGAGAGAGGCCCAG-'3 |
| | R: 5'- GAACAGGGAGGAGCAGAGAG-'3 |
| *Hprt2* | F: 5'- CAGGCCAGACTTTGTTGGAT-3' |
| | R: 5'- TTGCGCTCATCTTAGGCTTT-3' |

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764

765 **Figure legends:**

766 **Figure 1: Detection of secreted proteins in primary cultures of mouse trophoblast.**

767 **A)** Workflow for the detection of secreted proteins in primary cultures of trophoblast from mice at
768 day 16 of pregnancy. **B)** Visualisation of EGFP (*Tpbpa*-Cre-EGFP reporter) by fluorescence microscopy
769 to identify the Percoll gradient layer containing trophoblast endocrine cells. Cells in layers were
770 counterstained with Hoechst dye (blue) and photographed at magnification 10X (scale bar-0.6mm). **C)**
771 Primary cell culture viability (determined by XTT), cell death (LDH release levels), trophoblast density
772 (*Krt18* gene expression) and apoptosis (*p53* and *Bax* gene expression) from time 0 to 120h. **D)**
773 Proportion of junctional zone (*Tpbpa* gene expression) and labyrinthine zone (*Mct4* gene expression)
774 trophoblast at 0h and 48h. **E)** Relative abundance of the three junctional zone endocrine cell types,
775 spongiotrophoblast, glycogen cells and giant cells (gene expression of *Prl8a8, Gjb3* and *Hand1*,
776 respectively) at 0h and 48h of culture. **F)** Representative images of cells stained in situ using RNAscope
777 probes against; *Tpbpa, Prl8a8, Gjb3* and *Hand1* to visualise trophoblast endocrine cells,
778 spongiotrophoblast (SpT), glycogen cells (Gly) and giant cells (TGC), respectively. **G)** Pipeline and
779 results of the analysis of proteins detected by mass spectrometry in cultured trophoblast and their
780 conditioned medium including conversion to RNA sequences and overlay with published RNA data for
781 the mouse and human placenta. Secreted proteins identified using SignalP and combined gene
782 ontology (GO) terms: extracellular region, extracellular exosome, extracellular region parts and signal
783 IP. **H and I)** Pathway over-representation analysis using Reactome pathway by STRING V.11 for the
784 158 and 257 secreted placental proteins in cultured trophoblast and their conditioned medium,
785 respectively that are expressed by both mouse and human placenta. XTT, LDH and gene expression
786 data relative to geometric mean of three housekeeping genes: *Gapdh, RPII* and *Hprt* are presented as
787 mean ± SEM and expressed relative to expression at time 0h. Asterisks denote statistical significance
788 versus time 0h, using Two-way ANOVA (B) or t-test (C-D), **P<0.01, ****P<0.001, n=6-10 of 4 pooled
789 litters.

790

791 **Figure 2. Detection of secreted proteins in sorted mouse trophoblast endocrine cells.**

792 **A)** Workflow for the cell sorting and protein expression analysis of mouse trophoblast endocrine cells
793 from mice at day 16 of pregnancy. **B)** Fluorescent image of placenta showing EGFP in *Tpbpa* positive
794 cells (junctional zone of the placenta) and tdTom for *Tpbpa* negative cells. **C)** Representative image of
795 cell sorting of EGFP/tdTom cells by fluorescence activated cell sorting. **D)** Expression of junctional zone
796 and labyrinth zone markers, *Tpbpa* and *Mct4*, respectively in the EGFP and TdTom sorted cells. **E)**
797 Expression of markers for junctional zone cell types, spongiotrophoblast cell (*Prl8a8*), glycogen cells
798 (*Gjb3*) and giant cells (*Hand1*). **F)** Pipeline and results of the analysis of proteins detected by mass
799 spectrometry in sorted *Tpbpa*+/EGFP cells. **G)** Pathway over-representation analysis using Reactome
800 pathway by STRING V.11 for the 105 secreted placental proteins expressed by both mouse and human
801 placenta. Data presented as mean ± SEM and genes expressed relative to geometric mean of two
802 housekeeping genes: *RPII* and *Hprt*. Asterisks denote statistical significance to the *Tpbpa*+/EGFP sorted
803 cells, using t-test, *P<0.05, **P<0.01, n=5 for each group.

804

805 **Figure 3. Secretome map of the placenta**

806 **A)** Integrating the lists of secreted placental proteins expressed by mouse and human placenta and
807 obtained using our different methods to generate a comprehensive placental secretome map.

Proteins expressed by mouse but not human placenta shown in Supplementary data figure 1. **B)** Venn diagram showing the overlay of placental secretome map with first trimester trophoblast organoid secretome (Turco, 2018) and single cell RNAseq analysis for human placenta at 8 weeks of gestation (Liu, 2018). **C)** Gene ontology (GO) analysis for the 319 secreted proteins detected in placental secretome map using STRING V.11. **D and E)** Proteins in the placental secretome map that are highly enriched in the placenta of mouse **(D)** and human **(E)** compared to other tissues using TissueEnrich. **F)** Cell specific expression of the top 30 most highly expressed genes in the placental secretome map using single cell RNAseq data for the human placenta (Lui, 2018). CTB: cytotrophoblasts, STB: syncytiotrophoblast, EVT: extravillous trophoblasts, w: weeks.

**Figure 4. Applying the secretome map of the placenta to data available for human pregnancy complications.**

**A)** Venn diagram showing the number of placental proteins in the secretome map that are differentially expressed in the placenta of women with a pregnancy complication. **B)** FLT1 expression at the maternal–fetal interface of early human pregnancy via the CellxGene tool (https://maternal-fetal-interface.cellgeni.sanger.ac.uk/). **C)** Serum levels of FLT1, MIF, ANGP2 and IGF2 in pregnant women at 12 and 28 weeks of gestation who went on to have normoglycemic (healthy) pregnancies or developed GDM. Data are expressed as multiple for the median; MoM. **D)** Ratio of the levels of FLT1 to MIF and ANGPT2 to MIF at week 12 of pregnancy in healthy women and those who went on to develop GDM. Data are presented as mean ± SEM of MoM. Raw data for proteins levels are shown in supplementary figure 4. Asterisks denote statistical significance to the GDM pregnancies, using two way-ANOVA for C and t-test for D, *P<0.05, **P<0.01, n=10-11 for healthy pregnancies, n=6 for GDM. GDM: gestational diabetes mellitus, IUGR: intrauterine growth restriction, LGA: large for gestational age, PE: preeclampsia, SGA small for gestational age.

**Figure 5. Transcription factors disregulated in pregnancy complications identified as possible regulators of genes encoding secreted placental proteins.**

A) Table showing the ten transcription factors with altered expression in pregnancy complications and with binding sites enriched at the promoters of genes encoding proteins in the placental secretome map. B) Regulatory network built with the ten transcription factors listed in panel A. The ten transcription factors and their targets that are differentially expressed in pregnancy complications are highlighted in purple. Location of the target proteins is according to their main cellular/extracellular compartment indicated by IPA. The graph was generated using BioRender.

**Supplementary figures and tables**

**Figure S1: Placental enrichment expression of non-secreted proteins detected from sorted Jz cells.**

**Figure S2: Analysis of the 31 secreted proteins in the placental secretome map reported to be expressed by the mouse but not human placenta.** A) The expression of the 28 out of the 31 mouse-specific secreted placental proteins that were enriched (>10-fold) in the placenta compared to other tissues using TissueEnrich. B) Gene ontology (GO) analysis for the 31 mouse-specific secreted placental proteins using STRING V.11.

**Figure S3: Expression of ANGPT2, MIF and IGF2 at the maternal-fetal interface in early human pregnancy.** A-C) ANGPT2, MIF and IGF2 expression at the maternal–fetal interface of early human pregnancy via the CellxGene tool (https://maternal-fetal-interface.cellgeni.sanger.ac.uk/).

**Figure S4: Concentrations of placenta proteins in human pregnancy samples**. A-D) sFLT1, MIF, ANGPT2 and IGF2 concentrations in healthy and GDM pregnancies at week 12 and 28 of gestation. Data are from n=6-10 pregnancies and shown as mean ± SEM. Asterisks denote statistical significance between week of pregnancy, using Two-way ANOVA and **** P<0.001 (there was no significant difference between control and GDM).

**Table S1: Gene ontology and protein domain analysis for non-secreted proteins detected in sorted mouse placental endocrine cells (Jz +*Tpbpa* sorted cells). A)** Gene ontology performed by Panther analysis, **B)** Gene ontology performed using Reactome pathways performed by STRING V.11.

**Table S2: Placental secreted proteins differentially altered in human pregnancy complications. A)** Gene ontology by Reactome analysis for the 18 secreted proteins specifically altered in GDM pregnancies. **B)** Gene ontology by Reactome analysis for the 47 secreted proteins specifically altered in PE pregnancies. **C)** Gene ontology by Reactome analysis for 7 secreted proteins specifically altered in IUGR pregnancies.

**Table S3: Putative transcription factors (TFs) regulating the endocrine function of the placenta.** The 33 TFs regulating the expression of genes encoding proteins in the placental secretome map. The target genes differentially expressed in the placenta in pregnancy complications are indicated.

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Figure 1.

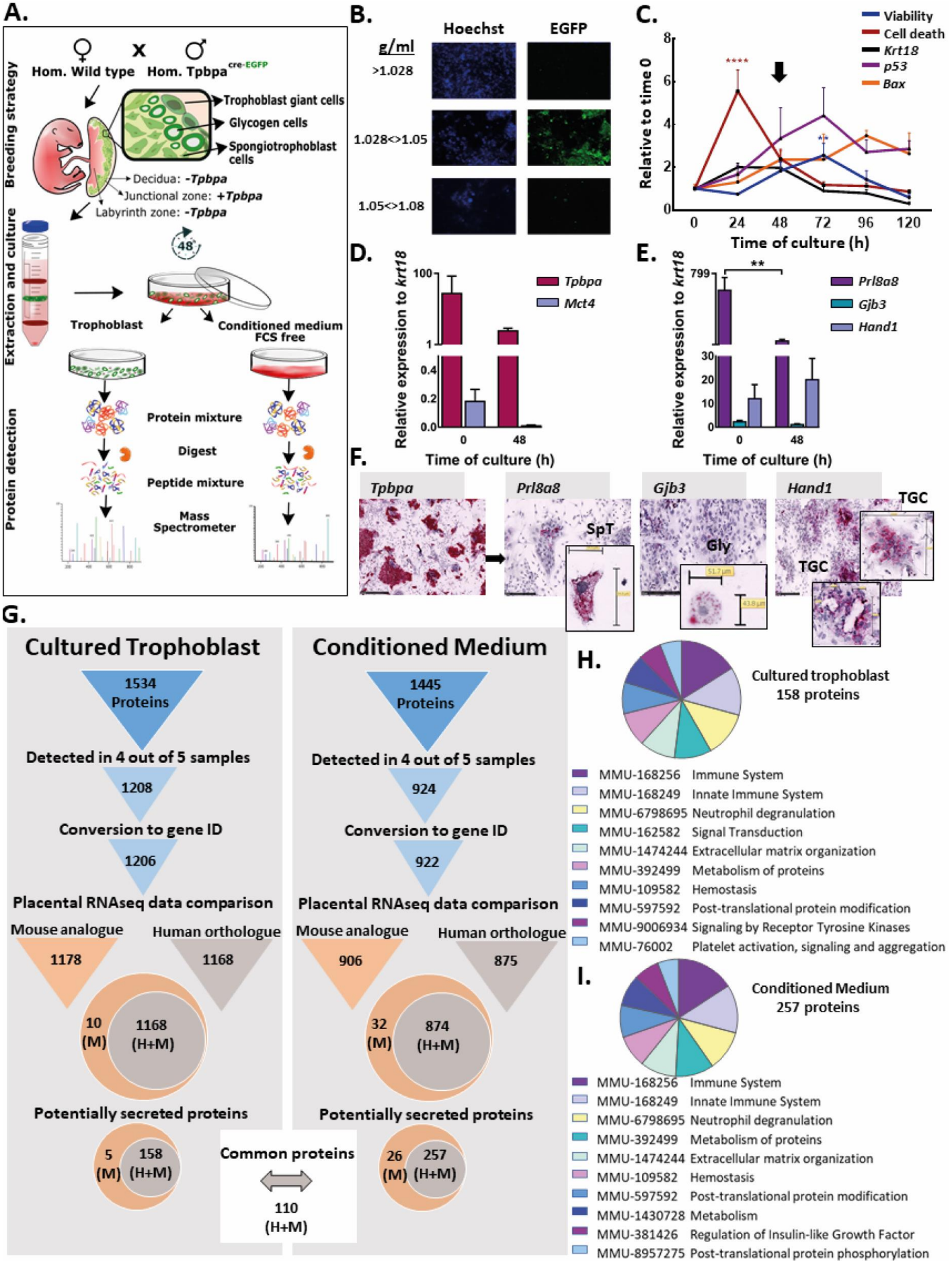

A.
Breeding strategy
Hom. Wild type
Hom. Tpbpa cre-EGFP
Trophoblast giant cells
Glycogen cells
Spongiotrophoblast cells
Decidua: -Tpbpa
Junctional zone: +Tpbpa
Labyrinth zone: -Tpbpa
Extraction and culture
48
Trophoblast
Conditioned medium FCS free
Protein detection
Protein mixture
Digest
Peptide mixture
Mass Spectrometer
B.
Hoechst
EGFP
g/ml
>1.028
1.028<>1.05
1.05<>1.08
C.
Viability
Cell death
Krt18
p53
Bax
Relative to time 0
Time of culture (h)
D.
Relative expression to krt18
Tpbpa
Mct4
Time of culture (h)
E.
Relative expression to krt18
Prl8a8
Gjb3
Hand1
Time of culture (h)
F.
Tpbpa
Prl8a8
Gjb3
Hand1
TGC
SpT
Gly
TGC
G.
Cultured Trophoblast
1534 Proteins
Detected in 4 out of 5 samples
1208
Conversion to gene ID
1206
Placental RNAseq data comparison
Mouse analogue
Human orthologue
1178
1168
10 (M)
1168 (H+M)
Potentially secreted proteins
5 (M)
158 (H+M)
Conditioned Medium
1445 Proteins
Detected in 4 out of 5 samples
924
Conversion to gene ID
922
Placental RNAseq data comparison
Mouse analogue
Human orthologue
906
875
32 (M)
874 (H+M)
Potentially secreted proteins
26 (M)
257 (H+M)
Common proteins
110 (H+M)
H.
Cultured trophoblast
158 proteins
MMU-168256 Immune System
MMU-168249 Innate Immune System
MMU-6798695 Neutrophil degranulation
MMU-162582 Signal Transduction
MMU-1474244 Extracellular matrix organization
MMU-392499 Metabolism of proteins
MMU-109582 Hemostasis
MMU-597592 Post-translational protein modification
MMU-9006934 Signaling by Receptor Tyrosine Kinases
MMU-76002 Platelet activation, signaling and aggregation
I.
Conditioned Medium
257 proteins
MMU-168256 Immune System
MMU-168249 Innate Immune System
MMU-6798695 Neutrophil degranulation
MMU-392499 Metabolism of proteins
MMU-1474244 Extracellular matrix organization
MMU-109582 Hemostasis
MMU-597592 Post-translational protein modification
MMU-1430728 Metabolism
MMU-381426 Regulation of Insulin-like Growth Factor
MMU-8957275 Post-translational protein phosphorylation

Figure 2.

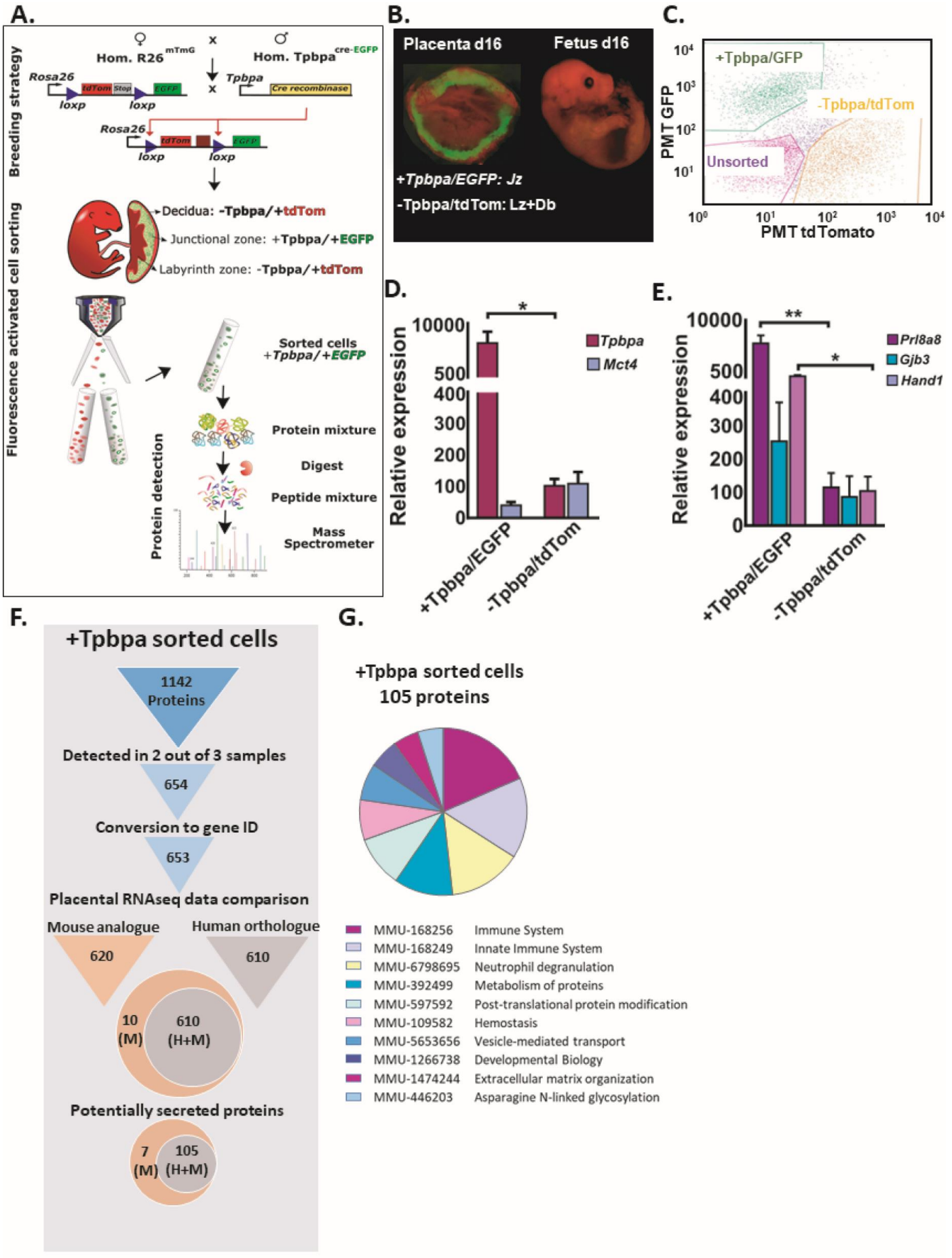
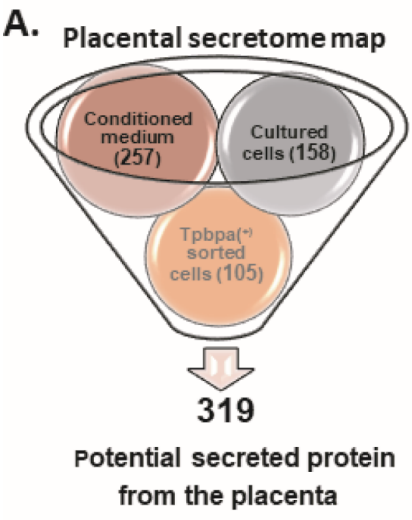
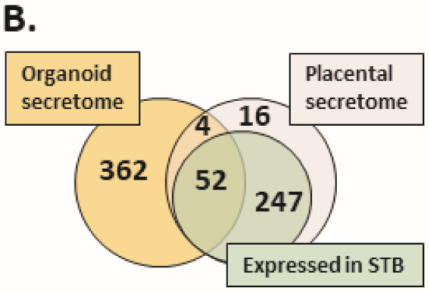

A.
Breeding strategy
Hom. R26 mTmG
Hom. Tpbpa cre-EGFP
Rosa26
tdTom Stop EGFP
loxp loxp
Tpbpa
Cre recombinase
Rosa26
tdTom EGFP
loxp loxp
Fluorescence activated cell sorting
Decidua: -Tpbpa/+tdTom
Junctional zone: +Tpbpa/+EGFP
Labyrinth zone: -Tpbpa/+tdTom
Sorted cells +Tpbpa/+EGFP
Protein detection
Protein mixture
Digest
Peptide mixture
Mass Spectrometer
B.
Placenta d16
Fetus d16
+Tpbpa/EGFP: Jz
-Tpbpa/tdTom: Lz+Db
C.
+Tpbpa/GFP
-Tpbpa/tdTom
Unsorted
PMT GFP
PMT tdTomato
D.
Relative expression
Tpbpa
Mct4
+Tpbpa/EGFP
-Tpbpa/tdTom
E.
Relative expression
Prl8a8
Gjb3
Hand1
+Tpbpa/EGFP
-Tpbpa/tdTom
F.
+Tpbpa sorted cells
1142 Proteins
Detected in 2 out of 3 samples
654
Conversion to gene ID
653
Placental RNAseq data comparison
Mouse analogue
620
Human orthologue
610
10 (M)
610 (H+M)
Potentially secreted proteins
7 (M)
105 (H+M)
G.
+Tpbpa sorted cells
105 proteins
MMU-168256 Immune System
MMU-168249 Innate Immune System
MMU-6798695 Neutrophil degranulation
MMU-392499 Metabolism of proteins
MMU-597592 Post-translational protein modification
MMU-109582 Hemostasis
MMU-5653656 Vesicle-mediated transport
MMU-1266738 Developmental Biology
MMU-1474244 Extracellular matrix organization
MMU-446203 Asparagine N-linked glycosylation

Figure 3.




C.

Biological Process

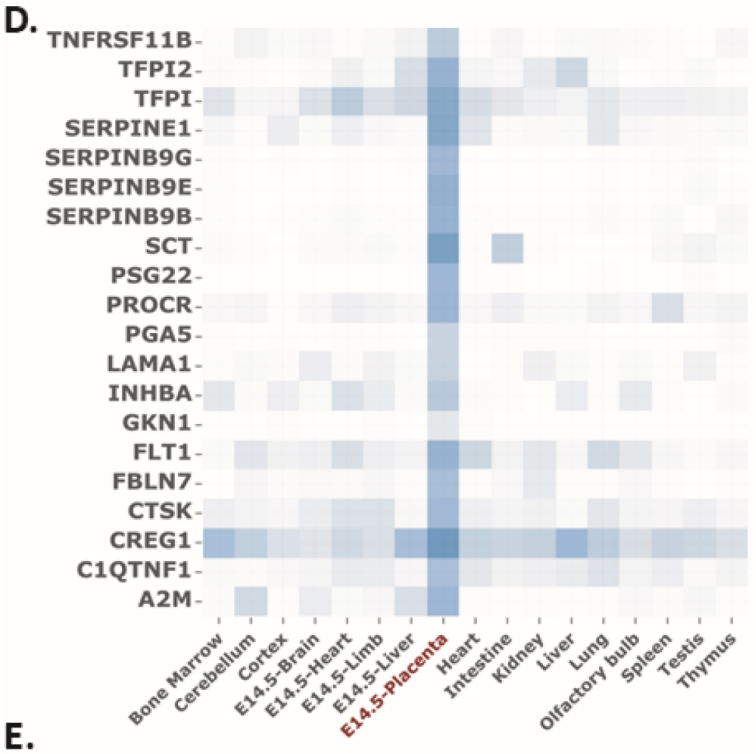
| #Term ID | Term description | # obs. | FDR |
|---|---|---|---|
| GO:0050896 | Response to stimulus | 204 | 2.44E-31 |
| GO:0051239 | Regulation of multicellular organismal process | 128 | 1.46E-29 |
| GO:0065008 | Regulation of biological quality | 138 | 3.54E-28 |
| GO:0006950 | Response to stress | 126 | 5.19E-28 |
| GO:0032501 | Multicellular organismal process | 181 | 6.5E-26 |
| GO:0050793 | Regulation of developmental process | 110 | 5.53E-24 |
| GO:0048519 | Negative regulation of biological process | 157 | 7.33E-24 |

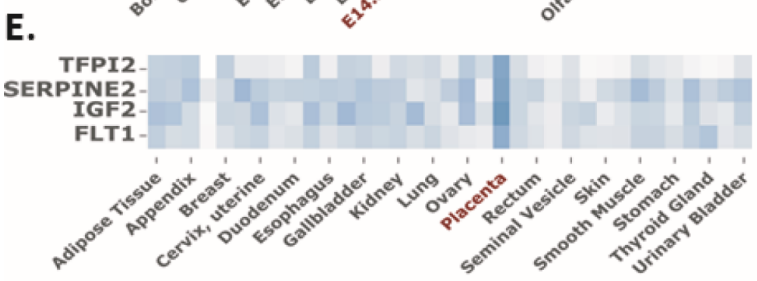
Molecular Function

| #Term ID | Term description | # obs. | FDR |
|---|---|---|---|
| GO:0005515 | Protein binding | 199 | 9.02E-31 |
| GO:0005488 | Binding | 258 | 5.2E-28 |
| GO:0005102 | Signaling receptor binding | 85 | 1.31E-24 |
| GO:0044877 | Protein-containing complex binding | 61 | 8.93E-17 |
| GO:0005509 | Calcium ion binding | 44 | 6.79E-16 |
| GO:0050840 | Extracellular matrix binding | 18 | 1.66E-15 |
| GO:0002020 | Protease binding | 24 | 2.04E-15 |

Protein Domains

| #Term ID | Term description | # obs. | FDR |
|---|---|---|---|
| IPR023795 | Serpin, conserved site | 13 | 3.04E-09 |
| IPR000742 | EGF-like domain | 22 | 5.60E-09 |
| IPR008160 | Collagen triple helix repeat | 14 | 7.34E-09 |
| IPR000215 | Serpin family | 13 | 1.11E-08 |
| IPR009030 | Growth factor receptor cysteine | 16 | 1.57E-08 |
| IPR001464 | Annexin | 7 | 2.37E-07 |
| IPR000867 | Insulin-like growth factor-binding protein | 7 | 3.88E-06 |



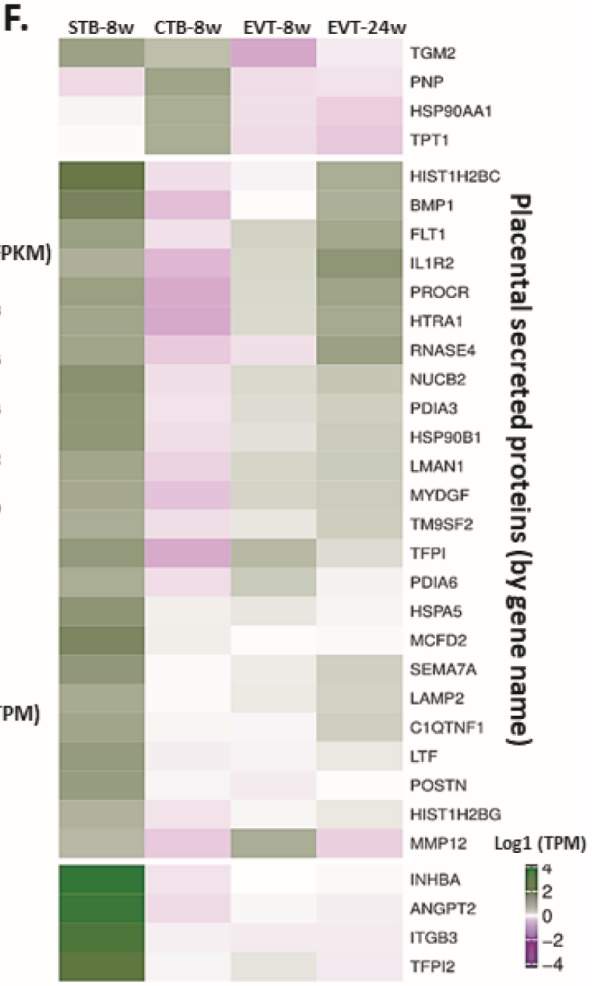



Figure 4.

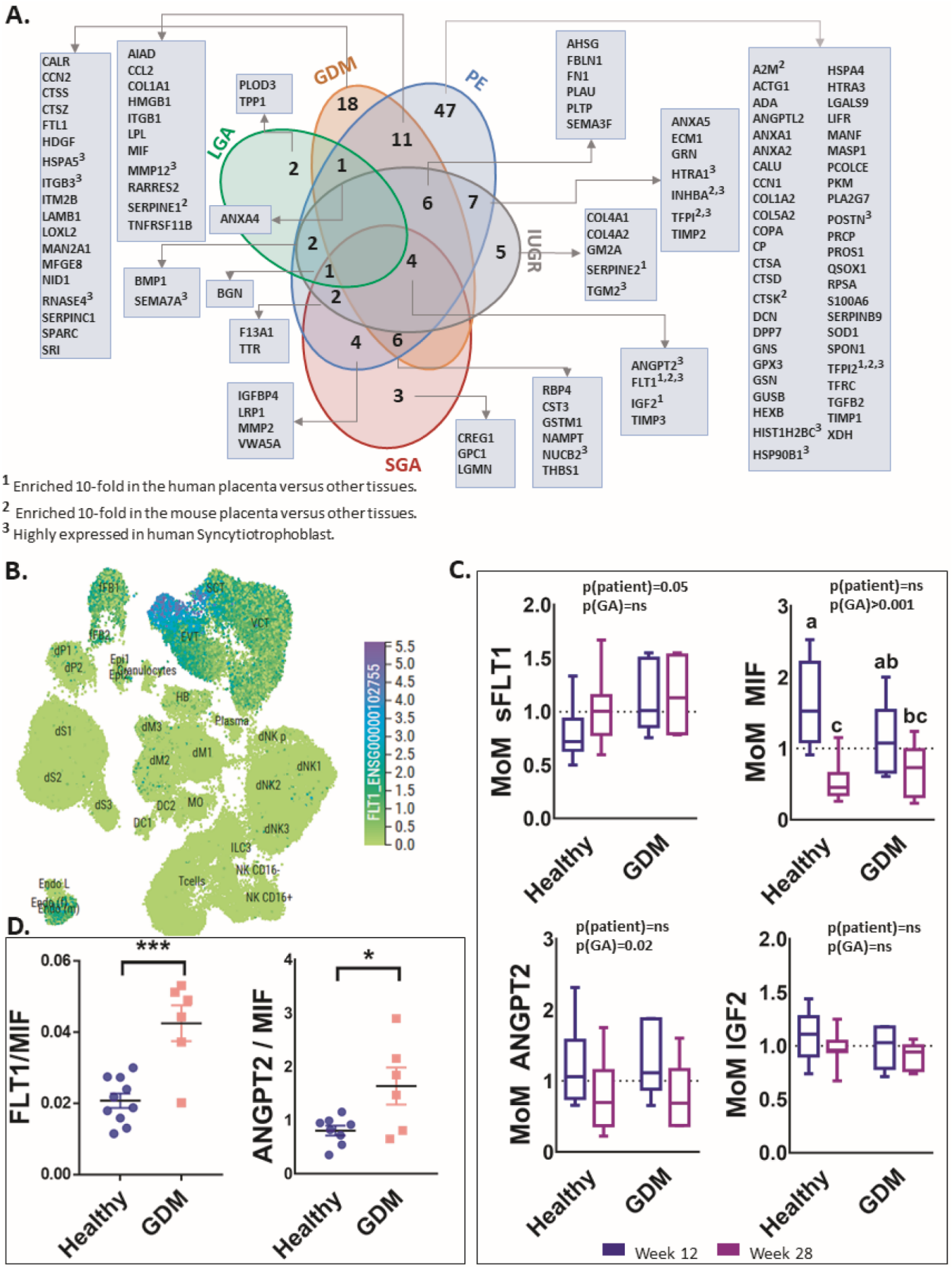
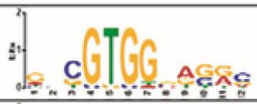
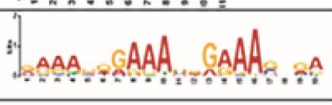

A.
CALR
CCN2
CTSS
CTSZ
FTL1
HDGF
HSPA5[3]
ITGB3[3]
ITM2B
LAMB1
LOXL2
MAN2A1
MFGE8
NID1
RNASE4[3]
SERPINC1
SPARC
SRI
AIAD
CCL2
COL1A1
HMGB1
ITGB1
LPL
MIF
MMP12[3]
RARRES2
SERPINE1[2]
TNFRSF11B
PLOD3
TPP1
ANXA4
BMP1
SEMA7A[3]
BGN
F13A1
TTR
IGFBP4
LRP1
MMP2
VWA5A
CREG1
GPC1
LGMN
RBP4
CST3
GSTM1
NAMPT
NUCB2[3]
THBS1
ANGPT2[3]
FLT1[1,2,3]
IGF2[1]
TIMP3
AHSG
FBLN1
FN1
PLAU
PLTP
SEMA3F
ANXA5
ECM1
GRN
HTRA1[3]
INHBA[2,3]
TFPI[2,3]
TIMP2
COL4A1
COL4A2
GM2A
SERPINE2[1]
TGM2[3]
A2M[2]
ACTG1
ADA
ANGPTL2
ANXA1
ANXA2
CALU
CCN1
COL1A2
COL5A2
COPA
CP
CTSA
CTSD
CTSK[2]
DCN
DPP7
GNS
GPX3
GSN
GUSB
HEXB
HIST1H2BC[3]
HSP90B1[3]
HSPA4
HTRA3
LGALS9
LIFR
MANF
MASP1
PCOLCE
PKM
PLA2G7
POSTN[3]
PRCP
PROS1
QSOX1
RPSA
S100A6
SERPINB9
SOD1
SPON1
TFPI2[1,2,3]
TFRC
TGFB2
TIMP1
XDH
GDM
PE
LGA
IUGR
SGA
18
47
11
2
1
6
7
2
1
5
4
2
4
6
3
[1] Enriched 10-fold in the human placenta versus other tissues.
[2] Enriched 10-fold in the mouse placenta versus other tissues.
[3] Highly expressed in human Syncytiotrophoblast.
B.
FB1
SCT
VCT
FB2
EVT
dP1
dP2
Epi1
Epi2
Granulocytes
HB
Plasma
dS1
dM3
dNK p
dM2
dM1
dNK1
dS2
dNK2
dS3
DC2
MO
DC1
dNK3
ILC3
NK CD16-
Tcells
NK CD16+
Endo L
Endo (f)
Endo (m)
FLT1_ENSG00000102755
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C.
p(patient)=0.05
p(GA)=ns
MoM sFLT1
2.0
1.5
1.0
0.5
0.0
Healthy
GDM
p(patient)=ns
p(GA)>0.001
MoM MIF
3
2
1
0
a
ab
c
bc
Healthy
GDM
p(patient)=ns
p(GA)=0.02
MoM ANGPT2
3
2
1
0
Healthy
GDM
p(patient)=ns
p(GA)=ns
MoM IGF2
2.0
1.5
1.0
0.5
0.0
Healthy
GDM
Week 12
Week 28
D.
FLT1/MIF
***
0.06
0.04
0.02
0.00
Healthy
GDM
ANGPT2 / MIF
*
4
3
2
1
0
Healthy
GDM

Figure 5.

A.

| TF | Motif | IPA (p value) | AME (adjusted p value) | Common (DEG in pathology complications) | Complications (TF as DEG) | Reference |
|---|---|---|---|---|---|---|
| ARNT2 |  | 14/319 (1.71E-06) | 174/319 (1.30E-13) | 10 (5) | PE SGA | Leavey et al. 2015 Sober et al. 2015 |
| ELF3 |  | 3/319 (1.04E-02) | 161/319 (5.07E-08) | 2 (2) | PE IUGR | Sober et al. 2015 |
| PLAG1 |  | 3/319 (4.34E-02) | 185/319 (1.82E-04) | 2 (1) | GDM | Sober et al. 2015 |
| SP2 |  | 2/319 (1.87E-02) | 166/319 (2.45E-51) | 2 (1) | IUGR LGA PE | Sober et al. 2015 |
| MEF2D |  | 6/319 (5.59E-04) | 124/319 (3.84E-23) | 4 (3) | IUGR PE | Sober et al. 2015 |
| MYCN |  | 25/319 (5.51E-13) | 71/319 (1.40E-08) | 2 (2) | PE | Leavey et al. 2015 |
| FOS |  | 49/319 (4.45E-24) | 41/319 (1.37E-02) | 7 (3) | PE | Gormley et al. 2017 |
| NFYC |  | 4/319 (2.93E-03) | 38/319 (7.54E-10) | 2 (1) | PE | Leavey et al. 2015 |
| CREB1 |  | 22/319 (2.69E-05) | 10/319 (1.83E-02) | 2 (2) | GDM | Bari et al. 2016 |
| IRF3 |  | 9/319 (8.50E-03) | 162/319 (5.83E-54) | 6 (3) | GDM PE | Sober et al. 2015 Leavey et al. 2015 |

B.