**RNA-Seq reveals changes in human placental metabolism, transport and endocrinology across the first-second trimester transition**

**Running title: RNA-Seq of first and second trimester placenta**

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**Summary Statement**

The first-second trimester transition is associated with placental transcriptome changes of protein processing in the endoplasmic reticulum, hormone secretion, transport, extracellular matrix, vasculogenesis, and reactive oxygen species metabolism.

**Abstract**

The human placenta undergoes major changes at the end of the first trimester, associated with a three-fold rise in oxygen concentration, which coincides with the switch from histotrophic to hemotrophic nutrition. We evaluated the impact on trophoblastic development and function using RNA-Sequencing (RNA-Seq) and DNA methylation on the same chorionic villous samples at 7-8 (n=8) and 13-14 (n=6) weeks of gestation. Reads were adjusted for fetal sex. Most DEGs were associated with protein processing in the endoplasmic reticulum (ER), hormone secretion, transport, extracellular matrix, vasculogenesis, and reactive oxygen species metabolism. Transcripts higher in the first trimester were associated with synthesis and ER processing of peptide hormones, and glycolytic pathways. Transcripts encoding proteins mediating transport of oxygen, lipids, protein, glucose, and ions were significantly increased in the second trimester. The motifs of CBX3 and BCL6 were significantly overexpressed, indicating the involvement of these transcription factor networks in the regulation of trophoblast migration, proliferation and fusion. These findings are consistent with a high level of cell proliferation and hormone secretion by the early placenta to secure implantation in a physiologically low-oxygen environment.

**Introduction**

The placenta is essential to a successful pregnancy and the life-long health of the offspring. Impaired placental function has both immediate obstetric consequences (Brosens et al., 2011), including miscarriage, fetal growth restriction, pre-eclampsia and stillbirth, and long-term impact on the risk of chronic disease for the offspring through developmental programming (Burton et al., 2016). Recent advances in imaging and biomarker studies indicate that the pathophysiology of many non-communicable complications of pregnancy starts during early pregnancy (Smith, 2010). The human gestational sac from which the placenta and fetus develop undergoes a major transition towards the end of the first trimester with the switch from primarily histotrophic to hemotrophic nutrition (Burton et al., 2010). This transition, which involves the same placental structure being supplied with nutrients by contrasting pathways, is unique to the human and great apes, and may explain why conditions such as pre-eclampsia are virtually restricted to the human species.

During the first trimester, maternal arterial blood flow into the placental intervillous space is restricted by aggregates of endovascular trophoblast that migrate down the lumens of the endometrial spiral arteries. The trophoblast cells are loosely linked by desmosomes (Roberts et al., 2017), creating a network of intercellular channels through which plasma may seep. Consequently, the placental tissues develop in a relatively low-oxygen environment (Jauniaux et al., 2000), supported principally by carbohydrate- and lipid-rich secretions from the endometrial glands (Burton et al., 2002). These secretions are also a potential source of mitogenic growth factors, including epidermal growth factor that stimulates proliferation of cytotrophoblast cells when applied to explant cultures (Burton et al., 2020; Maruo et al., 1992). Metabolism of the placental tissues is heavily glycolytic, and the phylogenetically ancient polyol metabolic pathways are highly active (Burton et al., 2017).

With full onset of the maternal intraplacental circulation towards the end of the first trimester there is a three-fold rise in local oxygen concentration (Jauniaux et al., 2000). Oxygen has been implicated in the induction of many changes that take place at the transition between the first and second trimesters, including villous regression to form the definitive placenta and the membranes, and regulating trophoblast proliferation and invasion, hormone production and transporter expression (Caniggia et al., 2000; Graham et al., 2000; Pringle et al., 2010). The transition from histotrophic to hemotrophic nutrition involves other potential influences, such as the dilution of growth factor support and increased biomechanical forces, including shear stress at the villous surface. Previous studies have compared gene expression in the first trimester placenta as assessed by microarray analysis with that of term placentas (Mikheev et al., 2008; Sitras et al., 2012). In order to address the critical changes taking place during the first-second trimester transition, we performed RNA-Seq and array-based DNA methylation profiling on the same samples of placental villous tissue obtained from accurately dated and narrow windows of gestation; 7-8 weeks and 13-14 weeks. The samples were obtained under optimal conditions using an ultrasound guided chorionic villous sampling (CVS) technique that avoided the stress induced by curettage (Cindrova-Davies et al., 2015), and were frozen immediately. We focussed our analyses on transcripts encoding proteins involved in metabolism, hormonal activity, transport and cell proliferation. We also discuss transcripts with both differential expression and differentially methylated regions found in their promoters/gene-bodies.

**Results**

Villous samples separated clearly on the basis of gestational age with both principal component analysis (PCA) and hierarchical clustering (Fig. 1A, Suppl. Fig. 1). Differential expression analysis identified 3242 differentially expressed genes (DEG)(P*adj*. ≤ 0.05, absolute fold change ≥ 2; Fig. 1B, Suppl. Table 1). In view of the sex bias in sample numbers (Fig. 1A), we performed a sex-adjustment analysis. Sex of the samples was confirmed using sex specific genes, *Xist, Rps4y1, Ddx3y, Usp9y* and *Sry,* and was included in the design formula (~ sex + condition) as a blocking factor to account for variation in the data.

Using Kegg pathway analysis and Gene Ontology analysis (GO) we identified several classes of genes that change simultaneously between the first and second trimesters (Figs. 1C-F, Suppl. Tables 2-5). Notably, genes associated with protein processing in the endoplasmic reticulum (ER) were amongst the most differentially expressed, as were genes regulating cellular metabolism and hormone secretion, transport, and extracellular matrix (Figs. 1C-F). Changes for selected transcripts were validated at the protein level, using either western blotting or immunohistochemistry. To ascribe the DEGs to individual cell types the RNA-Seq results were compared with a published scRNA-Seq dataset (Liu et al., 2018), with first (8 weeks) and late second trimester (24 weeks) samples. The scRNA-Seq identified several cell types in 8-week placentas, including cytotrophoblast (CTB), syncytiotrophoblast (STB), extravillous trophoblast (EVT) and stromal cells (STR), and EVT in 24-week placentas.

**Metabolism**

Analysis of GO and Kegg pathway enrichment showed genes associated with protein processing in the endoplasmic reticulum (ER) to be amongst the most differentially expressed between the two time points. The Kegg pathway ‘Protein processing in ER’ was enriched in the first trimester (*P.adj* = 1.63 x 10-3) (Suppl. Fig. 2), as were the GO term ‘protein folding in ER’ (*P.adj* = 6.31 x 10-3) and ‘regulation of protein secretion’ (*P.adj* = 3.70 x 10-8) (Figs. 1C-F). These, and other related GO terms suggest that ER functional activity is greater during the first than the second trimester, despite the relatively low oxygen concentration.

The transcript profile observed provides further evidence that placental tissues are not energetically compromised during the first trimester. Glycolysis is the primary route to energy generation, supported by the polyol pathways that preserve carbon skeletons for synthesis of purines and other molecules required for rapid cell proliferation (Burton et al., 2017). Consistent with this metabolic profile is the finding that *HK2* and *PKLR*, which encode the key regulators of glycolysis - hexokinase and pyruvate kinase, are among the most differentially expressed genes (Figs. 2C-D). Whilst present at low levels in most normal adult cells, HK2 is highly expressed in embryonic tissues and in many cancer cells (Patra and Hay, 2013). We found HK2 expression to be significantly higher in the first trimester, with a switch to the HK3 isoform during the second trimester. HK3 is regulated by hypoxia, when it exerts protective effects against oxidative stress by increasing ATP levels, reducing oxidant-induced ROS production, preserving mitochondrial membrane potential, and increasing mitochondrial biogenesis (Wyatt et al., 2010). Our data thus support the notion that glycolysis is replaced by oxidative mitochondrial respiration as the primary method of energy generation. Indeed, this is underpinned by the upregulation of the transcriptional co-repressor, *CBFA2T3,* in the second trimester, which contributes to inhibition of glycolysis and stimulation of mitochondrial respiration (Kumar et al., 2013), once the utero-placental circulation is established. Conversely, β-oxidation of fatty acids appears to be suppressed in the first trimester. Transcripts encoding long-chain acyl-CoA dehydrogenase (*ACADL*) rise in the second trimester (Fig. 2B). These changes may serve to protect the placental tissues from excessive production of reactive oxygen species when oxygen availability is low (Burton et al., 2017; Huang et al., 2014).

The endoplasmic reticulum is a key intracellular organelle responsible for the synthesis and post-translational modification of secreted and membrane proteins, and for intracellular Ca2+ storage. Loss of homeostasis activates the ER stress response pathway, the unfolded protein response (UPR), which is mediated by three conserved signalling transducers: IRE-1 (inositol-requiring transmembrane kinase-endoribonuclease 1), PERK (PKR-like endoplasmic reticulum kinase) and ATF6 (activating transcription factor 6). Activity of these transducers is inhibited in the physiological state by binding of the ER chaperone protein BiP/GRP78/HSPA5. Transcripts encoding the sensor *IRE1*, known as *ERN1* (Fig. 2A), and its downstream X-box binding protein 1 (*XBP1*) were significantly higher in the first trimester (Figs. 2A, G-H), with no change in *ATF6* (Fig. 2H), and a modest increase in *PERK* (*EIF2AK3)* (Fig. 2A, and Suppl. Fig. 2). In addition, transcripts encoding several heat shock proteins (*DNAJB9, DNAJB11, DNAJC3, HSPA5, HSP90B1*), those involved in protein post-translational modifications (*LMAN1, MAN1C1, MAN1A2, PDIA6, PDIA3, ERO1A*) and protein quality control (*ERP44, HERPUD1, EDEM2*), were also higher in the first trimester (Fig. 2A). These are likely to be homeostatic responses to a high synthetic activity.

Heat shock proteins perform chaperone functions by stabilizing new polypeptides, while PDIAs, ERO1A and MAN1s assist in disulphide bond formation and glycosylation to ensure correct folding or refolding of proteins. Activation of IRE-1 and its downstream XBP1 are involved in the synthesis of lipoproteins essential for cell and organelle membranes. However, the actions of these pathways may be broader than just restoration of ER homeostasis and support of cell proliferation as activation of the IRE-1 pathway has been observed during the development of the labyrinth zone and knock-out of the gene leads to abnormal vascularisation (Iwawaki et al., 2009). We confirmed activation of the IRE-1/XBP1 pathway in the first trimester by immunostaining. XBP1 was strongly expressed by the villous and extravillous trophoblast of the cell columns and XBP1 expression coincided with that of IRE-1 and P-IRE-1 (Fig. 2H).

As expected, there was upregulation of genes associated with antioxidant defences during the second trimester, most notably *GPX1, CAT, SOD3, HIF3A, COX4I2, CYP1A1, CYP1A2* and *NOS1AP* (Figs. 2B, F), in agreement with our previous findings (Jauniaux et al., 2000). Several GO terms associated with metabolism of oxygen are also significantly different between the gestational ages, including ‘﻿reactive oxygen species metabolic process’ and ‘﻿reactive oxygen species biosynthetic process’ (see Suppl. Tables 3-5). Consistent with these terms, we observed increased phosphorylation of eIF2α in the second trimester (Fig. 2E).

**Hormonal activity**

Transcripts encoding peptide hormones showed considerable differential expression, with some being higher in the first trimester and others upregulated in the second trimester (Figs. 3A-B). The former included sub-units of hCG; *CGA* showed a 3.79-fold change while *CGB1*, *CGB2*, *CGB3*, *CGB5*, *CGB7* and *CGB8* showed fold changes of 3.68, 6.69, 3.84, 3.87, 4.94 and 3.59, respectively. These results confirmed previous findings that all six hCG genes are transcribed *in vivo* (Bo and Boime, 1992), and are consistent with secretion of hCG peaking at around 10 weeks of gestation, and then declining, its secretion likely stimulated by the epidermal growth factor receptor (EGFR) pathway (Wang et al., 2018). EGF is produced by the endometrial glands (histotroph pathway), and so this signalling loop may be part of the trophoblast-endometrial dialogue that stimulates early placental development (Burton et al., 2020). Also higher in the first trimester were transcripts encoding leptin (*LEP*) (fold change 3.16, Fig. 3A-B), relaxin (*RLN1*) (fold change 2.82) and insulin like 4 (*INSL4*) (fold change 3.15). Comparisons with the scRNA-Seq data indicated the transcripts were enriched in the syncytiotrophoblast (Fig. 3A). This corresponds to the known site of expression of hCG, supporting our analytical approach (Beck et al., 1986).

Of the transcripts upregulated in the second trimester, the greatest change was observed for spexin (*SPX*) (fold change 11.08) (Figs. 3A, C). Immunostaining showed it to be localised to the syncytiotrophoblast (Fig. 3C). The hypocretin receptor 2 (*HCRTR2*), also known as orexin receptor 2, is another pathway that regulates appetite and lipid metabolism (Burdakov and Alexopoulos, 2005), and we found that its mRNA increased 8.55 fold between the first and second trimester (Fig. 3A). By contrast, the mRNA encoding the hunger and satiety-maintaining hormone leptin was higher in the first trimester placenta (Fig. 3A-B).

Kegg pathway analysis showed that transcripts associated with ‘autoimmune thyroid disease’ and ‘parathyroid hormone synthesis, secretion and action’ were differentially expressed across the transition (Fig. 1C, Suppl. Table 2). Thyroid hormones are important for fetal development during the first trimester, in particular for the central nervous system, and must be transported across the placenta (Chan et al., 2009). The three major binding proteins, T4 binding globulin, transthyretin and albumin have all been identified in the mature placenta (McKinnon et al., 2005). Here, we show for the first time that transcripts of *CRYM*, which encodes crystalline mu, a T3 binding protein, are present in the placenta and higher during the first trimester (fold change 2.97). By contrast, *TTR* encoding transthyretin is more highly expressed in the second trimester (fold change 2.9) (Figs. 3A-B). These findings suggest novel regulatory pathways for the transfer of thyroid hormones across the placenta, which may change as pregnancy progresses.

The corpus luteum, under the trophic stimulation by hCG, is the source of progesterone and estrogen during the first trimester. Luteal production of progesterone declines after 6-8 weeks of pregnancy and the placental syncytiotrophoblast takes over as the main source by the end of the first trimester (Aspillaga et al., 1983). We found that the transcripts for two enzymes that catalyse the conversion of cholesterol to progesterone (via pregnenolone), *CYP11A1* (P450scc) or *CYP17A1*, did not change significantly between the first and second trimesters. By contrast, the gene encoding cholesterol 25-hydroxylase (*CH25H*), which converts cholesterol to 25-hydroxycholesterol (25OHC), was significantly upregulated in the second trimester (Figs. 2B, 3A), and seems to be regulated through methylation (Fig. 5E). 25OHC and other oxysterols are substrates of P450scc, and 25OHC enhances the production of steroids by the ovary and testis (Risbridger et al., 1986; Toaff et al., 1982). The increase in *CH25H* may contribute to the increase in progesterone synthesis by the placenta at the end of the first trimester, and which is pivotal to maintain the pregnancy.

**Transport**

Our data show that the transcripts encoding proteins mediating oxygen, lipid, protein, glucose, and ion transport changed significantly between the first and second trimesters (Figs. 3D-E). Transcripts encoding the hemoglobin subunits epsilon 1 and zeta (*HBE1* and *HBZ*) were within the top 3 differentially expressed genes, and were 43.16 and 42.47-fold lower in the second trimester samples (Figs. 3D-E). Conversely, the mRNA for hemoglobin G2 and hemoglobin beta (*HBG2* and *HBB*) were 2.98 and 3.49-fold higher in the later samples. HBB is a component of adult hemoglobin and while HBG2 is a component of fetal haemoglobin, present at birth. HBE1 and HBZ make up embryonic hemoglobin that predominates during the first trimester. The yolk sac was not included in our CVS samples, and thus these transcripts are likely to arise from the hemangioblastic clusters within the villous stromal core (Aplin et al., 2015), as confirmed by the presence of both embryonic and fetal hemoglobin transcripts within villous trophoblast cells of purified CTB and EVT cells (Apps et al., 2011). Together, these findings show hemoglobin transcripts are expressed within villous stroma and trophoblast cells and subunit expression changes with the onset of blood flow, possibly protecting against oxidative stress (Nishi et al., 2008; Saha et al., 2014). Whether this is determined by changes in the oxygen concentration or is ontogenetic remains unknown.

We found that the pattern of expression of lipid transporters and apolipoproteins in villous samples was profoundly different between the first and second trimesters. Transcripts highly abundant during the first trimester included *APOA2* and *ABCB11,* whereas there seemed to be a switch in the expression of transporters, with many being upregulated in the second trimester, in particular *ABCA6, ABCC8* and *APOD* (Figs. 3E, Suppl. Fig. 3). ApoD has been shown to protect against ischemia-reperfusion injury in myocardial infarcts and has potent antioxidant activity, which may buffer the placenta once maternal blood flow is established (Tsukamoto et al., 2013). Cholesterol is essential in early embryonic metabolism, cell signalling and elaboration of cell and organelle membranes, and must be transported across the placenta into the chorionic cavity where the secondary yolk sac lies. From there it is transported into the embryonic circulation via the secondary yolk sac, which expresses mRNAs encoding multiple apolipoproteins, the cholesterol efflux transporter ABCA1, and lipoprotein receptors, including megalin and cubilin (Cindrova-Davies et al., 2017). The high abundance of transcripts in the yolk sac encoding apolipoproteins present in lipoprotein particles and chylomicrons (ApoB, ApoA1, ApoA2, and ApoA4) is matched by high levels of these proteins in the chorionic cavity. The placental villi, the chorionic cavity, and the secondary yolk sac thus function together as a physiological equivalent of the choriovitelline placenta during early gestation. At the end of the first trimester, the chorionic cavity becomes obliterated by the growing amniotic cavity and the secondary yolk sac degenerates. At the same time, onset of the maternal and fetal placental circulations permits transport of cholesterol and lipids across the villous membrane. Our data showing increased mRNAs encoding lipid transporters may reflect the changes in the route of lipid transport.

Transcripts encoding transporters of metal ions important for antioxidant defences were also higher in the first compared to the early second trimester (e.g. *SLC30A10,* *SLC30A2* (fold changes 9.56 and 3.14). This differential might reflect higher transport of manganese and zinc ions that are essential cofactors for the superoxide dismutase enzymes during early pregnancy (Fig. 3E) in preparation for the rapid rise in local PO2 when a continuous maternal arterial flow enters the intervillous space (Jauniaux et al., 2000). Genes involved in the transport of iron also changed, in particular *LTF* that encodes lactotransferrin and *HEPH* that encodes hephaestin (fold changes 3.68 and 4.07, respectively).

Transcripts encoding ion channels, for example *SCN1A* (fold change 31.37), *MCOLN2* (fold change 8.83), *TRPA1* (fold change 7.38) and *SCN7A* (fold change 3.42)(Fig. 3E) were among the most significantly raised in the early second trimester. This may reflect in part a switch in the way amino acids are transported across the placenta. In the first trimester, they are transported by uptake and subsequent breakdown of proteins in maternal histotroph (Burton et al., 2002) whereas in the second, there is active uptake of individual amino acids from the maternal circulation through accumulative and exchange transporters. Activity of the latter needs to be balanced by other ionic fluxes in order for them to function.

**Cell proliferation, differentiation and WNT signalling**

WNT signalling plays an important role in cell proliferation, differentiation and motility under normal and malignant conditions (Bienz and Clevers, 2000), and the WNT signalling pathway has been identified as essential for the growth of trophoblast organoids (Turco et al., 2018). Recent evidence suggests that WNT signalling may also be implicated in the regulation of placental development and human trophoblast differentiation (Haider et al., 2017; Nayeem et al., 2016). Our data show that *RSPO4*, *WNT10B*, as well as several other genes mediating the canonical WNT signalling, including *PORCN* (Galli et al., 2016), and *SDC1* were increased in the first trimester compared to the early second trimester. By contrast, *WNT3A, WNT10A, WNT2, LRRK2, RYR2, LRP6, CCND1,* and *RSPO3* transcripts were significantly upregulated in the second trimester (Figs. 4B). Canonical WNT signalling has been shown to be critical for invasive trophoblast differentiation (Pollheimer et al., 2006). In addition, several genes that regulate the non-canonical WNT pathway were upregulated in the second trimester, including *WNT5B* and *LEF1*. Negative regulators of the WNT signalling, *NKD2* and *DKK3*, were also upregulated in the second trimester. This suggests that paracrine mechanisms play a role in the regulation of trophoblast invasion in the second trimester. The majority of the WNT signalling transcripts upregulated in the second trimester were localised to EVT, whilst transcripts that were higher in the first trimester were localised to both EVT and villous syncytiotrophoblast (Fig. 4A).

**Extracellular matrix and angiogenesis**

Significant differences were found between the first and second trimester in placental expression of transcripts regulating the extracellular matrix (ECM) remodelling (Fig. 4B). The expression of laminins and of cell-matrix adhesion molecules was differentially regulated in the first and second trimester, in particular laminin *LAMA1* was highly expressed in the first trimester whereas *LAMB3, LAMA2, LAMC3* were upregulated in the second trimester. Laminins have an important role during implantation (Haouzi et al., 2011), maintenance of trophoblast stemness (Kiyozumi et al., 2020) and EVT migration (Zhang et al., 2018b).

Shortly after implantation, extravillous trophoblast cells migrate from the placenta into the endometrium where they are involved in the remodeling of the maternal spiral arteries that ultimately supply the placenta. The invasive properties of these cells are widely attributed to the matrix metalloproteinases 2 and 9 (Isaka et al., 2003; Staun-Ram et al., 2004). We found an upregulation of the mRNAs for *MMP9* (3.14-fold), *MMP1* (7.99-fold) and *MMP28* (3.77-fold) in the second trimester. *MEP1A* encodes for meprin, a member of the astacin family of metalloproteinases, it was 2.46-fold higher in second trimester. Meprins can be secreted, and thus may assist in matrix digestion, or membrane-bound, where they may be involved in the extracellular cleavage of proteins (Sterchi et al., 2008). Meprins are abundantly expressed by epithelial cells of the intestine, kidney and skin, and we show for the first time that are located in the villous syncytiotrophoblast (Fig. 4D). We speculate they might be involved in remodeling the basement membrane during villous growth.

Vasculogenesis and angiogenesis are critical for successful placental exchange, and it is suggested that low oxygen conditions during the first trimester stimulate these processes. However, we did not find the classical hypoxia-regulated factors, such as *VEGF,* to be differentially expressed. This supports our previous findings showing that HIF protein is not stabilised during early pregnancy (Cindrova-Davies et al., 2015). By contrast, transcripts encoding other potent regulators of angiogenesis, angiogenin (*ANG*) and endoglin (*ENG*), were found to be higher during the first trimester (fold change 5.72 and 3.23, respectively). Angiogenin mRNA and protein have been localised in the trophoblast and endothelial cells of the fetal placental vessels (Pavlov et al., 2014). In contrast, markers of vascularisation such as *PECAM1 (CD31), VWF, ICAM1* were significantly upregulated in the second trimester compared to the first trimester (Figs. 4B, E), indicating that the vascular components of the villi, which are essential for materno-fetal exchange, develop rapidly during the second half of pregnancy.

During pregnancy, the maternal immune system is modulated by signals from the placenta, with evidence of increased activation of innate cells in the systemic circulation. Regulatory CD4+CD25+Foxp3+ T cells (Tregs) expand during the second and third trimesters of pregnancy in the peripheral blood and in the decidua, believed to be induced by paternal antigens and contributing to the local control of fetus-specific maternal immune responses (Kahn and Baltimore, 2010; Rowe et al., 2012). The transcription factor musculin (MSC) is critical for the development of induced Treg cells by repression of the T helper type 2 transcriptional program (Wu et al., 2017). The transcript for MSC was significantly upregulated in the second trimester (Figs. 4B, F). This is the first demonstration of the presence of this immune-regulator in the syncytiotrophoblast. It is likely to have a different function in the placenta to its role in Tregs (Fig. 4F).

**Transcription factors**

Rapid cell proliferation and differentiation occurs during the first trimester to establish the placenta. Transcripts encoding the transcriptional regulator high-mobility group AT-hook 2 protein (*HMGA2*) were 7.55-fold higher in the first compared to the early second trimester (Fig. 4C). HMGA2 is known to play a role in proliferation and differentiation, homozygous mutations in the *Hmga2* gene result in the *pygmy* phenotype in mice (Zhou et al., 1995). We found that expression of *HAND1*, a transcription factor which regulates differentiation of trophoblast sub-types in the mouse (Scott et al., 2000), was also higher in the first trimester (6.63-fold) compared to early second trimester (Fig. 4C), but its function during human placental development is unknown. Many of the transcription factors upregulated in the second trimester regulate mammalian development and differentiation processes. These include *KLF2*, *SOX14,* *SOX18, LHX6, MEF2C, SOX7, HEYL*, *TFAP2E, MYT1*, *BNC2,* and *STAT4* (Fig. 4C).

To investigate potential regulatory networks of the placenta, we performed a motif rankings analysis. DEGs were scanned for the motifs, and DNA motifs significantly over-represented in a gene-set were identified. The CBX3 motif was the only common transcription factor motif that was significantly overrepresented in both searches (235 DEGs with CBX3 motif at 500UP TSS and 691 genes with this motif in 10kb centered around TSS). We have compared our DEGs enriched with CBX3 motif with the targetome of CBX3 transcription factor, based on ENCODE Transcription Factor Target Database (Consortium, 2011; Rouillard et al., 2016). We found that 68 out of 237 DEGs with CBX3 motif within 500bp US of TSS were already identified as CBX3 targets within that database (CBX3 ChIP-seq experiments from 3 human cell lines: K562, GM12878and HCT116).  Some of the unidentified CBX3-enriched DEGs may be unique for the placenta, as many TF binding patterns are tissue-type specific.

The *CBX3* gene was slightly downregulated in the second trimester (fold change -1.28, padj 0.0029). CBX3 is a repressor. It plays a role in transcriptional silencing in heterochromatin-like complexes, and may contribute to the association of the heterochromatin with the inner nuclear membrane through its interaction with lamin B receptor. In mouse, Cbx3 inhibits vascular smooth muscle cell proliferation, migration, and neointima formation (Zhang et al., 2018a). DEGs with the CBX3 motif (500bp US TSS) were extracted and pathway analysis with enrichR was performed (R package enrichR, v. 1.0). The top enriched term for WikiPathways\_2019 and Kegg\_2019 was 'Wnt Signaling' (adj.P.Value 0.00049 and  2.794813e-03, respectively) (Fig. 4G), while the term 'Ion Channels and Their Functional Role in Vascular Endothelium' (Adj.P.al = 0.042) (Fig. 4G) was enriched from BioCarta\_2016. These findings indicate that CBX3 could be the transcription factor, which links at least some of the differential changes taking place in the two trimesters, such as the differential regulation of the WNT signalling pathway which mediates EVT migration and trophoblast function, and endothelial function and ion channel transport.

In addition to the CBX3 motifs and transcription networks, the motif for BCL6 was significantly overrepresented for tss-centered-10kb of genesX3 (lower confidence motifs).  The pathway analysis for DEGs enriched in BCL6 motif (within their 10kb around TSS) has shown similar terms to these for CBX3, i.e. 'Ion Channels and Their Functional Role in Vascular Endothelium' from BioCarta (Adj.P.al =0.0026) and `Vascular smooth muscle contraction` (Adj.P.al =0.00765) from Kegg (Suppl. Fig. 4). The BCL6 gene expression  is significantly lower in the second trimester (fold change -2.175, padj 0.018) and it has been previously shown to be required for proliferation of villous cytotrophoblast cells (Muschol-Steinmetz et al., 2016), whilst BCL6 overexpression reduces trophoblast fusion, and it is increased in pre-eclamptic placentas (Jasmer et al., 2017).

**DNA Methylation**

Placental DNA methylation increases over gestation, and *in utero* exposures alter methylation and impact placental function and fetal health (Vlahos et al., 2019). Previous studies have compared first and second trimester methylation; however, these studies were not designed specifically to study the impact of onset of the maternal circulation inside the placenta. Thus, Novakovic *et al*. compared 8-12 week *vs*. 17-24 wk samples using a 27K methylation array focusing on gene promoters, but the first trimester samples overlap the first-second trimester transition (Novakovic et al., 2011). Nordor *et al.* compared 8-10 week to 12-14 week samples using a 450K methylation array, with the later time points overlapping the onset of maternal blood flow (Nordor et al., 2017). In both studies, gene expression data were not taken from the same samples, and standard bisulfite-treatment was used, which also include a confounding 5-hydroxymethylcytosine (hmC) signal. In the present study, we extracted DNA from the same patient samples which we used for the RNA-Seq analysis. We have used an oxidative bisulfite treatment on samples assaying only 5-methylcytosine (mC). We also performed the methylation analysis using an EPIC array assaying over 850K CpG sites with higher coverage to previous studies. Our study is therefore uniquely placed to study the gene expression and methylation differences immediately before and after the onset of maternal blood flow on the same samples (see Fig. 5A for a comparison of related datasets). To remove any sex-specific effects we performed batch correction using sex as a variable and excluded X and Y chromosome CpG sites from the analysis.

Samples clustered separately by gestational age in a PCA using the most variably methylated CpG positions (n=500)(Fig. 5B). Mean CpG methylation across all positions assayed on the EPIC arrays showed that there is a globally higher level of methylation during the second trimester (Fig. 5C). Previous studies have indicated the importance of promoter methylation (Novakovic et al., 2011), and its positive correlation with gestational age. Our study compared the first and second trimester methylation levels across specific genomic features associated with gene regulation: promoters (2 kb upstream of TSS), gene bodies and CpG Islands. We found the largest difference in methylation within gene bodies (Fig. 5D). We performed differential methylation analysis between the first and second trimester samples and found 329 DMRs, with 233 overlapping at least one gene promoter/body. We then correlated the differentially expressed genes from the RNA-Seq with differentially methylated CpGs in the same first and second trimester samples. After applying minimum thresholds of a fold change of 2 (log2 fold change 1) for gene expression and a 20% methylation difference for DMRs, we found 49 DMRs overlapped DEGs. In the majority (36/49), increased gene expression was associated with a corresponding decrease in methylation at the promoter/gene body. Overall, a correlation of increased methylation with decreased expression was observed (R2 0.04, Pearson’s correlation p = 0.0025) (Fig. 5E, Suppl. Table 6). For example, increased *WNT2* expression correlated with reduced methylation in the second trimester (Fig. 5E), and may reflect the important role of canonical WNT signalling for the differentiation of invasive extravillous trophoblast (Pollheimer et al., 2006).

A GO term analysis suggests the differentially methylated genes are enriched (adjusted p.value < 0.05) for molecular function terms related to in transcriptional regulation (Suppl Table 7). The GO analysis for biological processes are enriched for terms associated with cell fate commitment/specification, embryonic development/morphogenesis, regulation of metabolic processes and regulation of transcription (Suppl. Table 8).

In the first trimester samples, reduced methylation correlated with higher expression of transcripts encoding the EGFR ligand, epiregulin (EREG), and *EREG* expression decreased after the onset of maternal blood flow. EGF is abundantly secreted from the endometrial glands in early gestation when it stimulates cytotrophoblast proliferation and maintains their stemness (Burton, 2018; Maruo et al., 1992). Epiregulin promotes the cytotrophoblast-EVT transition through O-fucosylation on urokinase-type plasminogen catalysed by protein O-fucosyltransferase 1 (poFUT1) (Cui et al., 2019). This role seems critical for the pregnancy viability as both epiregulin and poFUT1 were reduced in placentas of patients suffering early pregnancy failure (Cui et al., 2019).

By contrast, expression of *CYP2R1* and *RBP7* was increased in the second trimester, with a corresponding decrease in methylation at this time. These genes regulate vitamin D and A metabolism; CYP2R1 converts vitamin D into its active form, whilst RBP7 affects vitamin A stability and metabolism. Adequate vitamin D function is essential for fetal skeletal development, tooth enamel formation and general fetal growth and development (Brooke et al., 1980), and vitamin A (retinoic acid) is essential for the development of heart, embryonal circulatory and central nervous systems and the regulation of heart asymmetry (Zile, 1998).

Decreased methylation levels were also correlated with the upregulation of genes activated in response to oxidative stress in the second trimester (*FAS, WFDC1, AOX1, CH25H*). Transcripts encoding several transporters mediating the uptake of sodium/potassium (*SLC24A4*), choline and thiamine pyrophosphate (B1 homeostasis; *SLC44A4*), organic anions and bile acids (*ABCC3*), and drugs (*ABCC9*) were increased in the second trimester, whereas magnesium and zinc transporter *SLC39A8* were increased in the first trimester. Methylation levels were differentially regulated in all these genes.

The inverse correlation of DNA methylation with expression suggests an association for a subset of genes in response to the onset of blood flow. GO pathways in this subset of genes include signal transduction, anatomical structure development, cellular protein modification process, cell differentiation, small molecule metabolic process, response to stress, transport and immune system process (Suppl. Table 8).

Interestingly, there was an overlap between the CBX3 motif targets and the differentially methylated regions of 22 genes, including WNT2, EREG, ABCC3, TLL1 and LY75 (Suppl. Fig. 5). These findings elude to the potential role of CBX3 in transcriptional silencing of some of its targets.

**Discussion**

In this study we sought to examine the effects of the changes in intrauterine environment that occur across the first-second trimester transition on the placenta at the levels of the transcriptome and methylome. The major changes center around onset of the full maternal arterial circulation into the intervillous space, with the switch from histotrophic to hemotrophic nutrition. They include a three-fold rise in oxygen concentration, together with increases in shear stress and other biomechanical stimuli at the villous surface. These constitute an environmental stress to which the placenta must successfully adapt (Jauniaux et al. 2000). Our data reveal that expression of transcripts involved in the synthesis and ER processing of peptide hormones, including hCG, is high during the first trimester. These findings may initially appear paradoxical since protein synthesis is energy demanding, yet despite the low-oxygen early environment there are no significant differences in placental concentrations of the main energy metabolites (ATP/ADP, NAD+, glucose and lactate) across gestation (Cindrova-Davies et al., 2015). This constancy in energy supply is due to high activity of glycolytic pathways during the first trimester, as shown by the transcriptome profile here and supported by a plentiful supply of glycogen in the histotroph from the endometrial glands (Burton et al., 2017). A high output of hCG by the syncytiotrophoblast soon after implantation is essential to maintain the coprpus luteum and prevent onset of the next menses.

The transcript pattern encoding proteins mediating transport of oxygen, lipid, protein, glucose, and ions changed significantly between the first and early second trimesters, reflecting increased oxidative stress and the onset of exchange between the maternal and fetal circulations. There were also increases in transcripts encoding steroidogenic enzymes, consistent with the placenta taking over from the corpus luteum, and in transcripts driving differentiation of the invasive extravillous trophoblast cells that anchor the placenta and are involved in remodelling of the spiral arteries that supply the placenta.

We identified the hormone spexin for the first time in the syncytiotrophoblast, and found it to be upregulated in the second trimester. Spexin is known to be involved in the regulation of body weight and metabolism, and inhibits the uptake of long-chain fatty acids by adipocytes and hepatocytes (Kolodziejski et al., 2018). Synthesis of spexin by the villous trophoblast suggest that this protein may play a role in regulating maternal lipid metabolism during pregnancy, possibly making more fatty acids available for transport to the fetus. A recent study measured circulating spexin (SPX) during the course of pregnancy in women with gestational diabetes mellitus (GDM) *vs*. healthy controls (Al-Daghri, et al., 2019). The study does not provide direct comparison with ours, as the starting measurements were taken at 10.3 (± 4.9) weeks and thus spanned both the first and second trimesters. However, the authors reported a significant increase in circulating levels of spexin in patients who developed GDM, with values correlating positively with glucose levels. Hence, the increase in placental spexin in the second trimester might influence maternal glucose utilisation and availability during pregnancy. Our finding that transcripts encoding the hypocretin receptor 2 also increased during the second trimester suggests the placenta may play an important role in modulating maternal appetite and energy intake during pregnancy. Interestingly, an inverse correlation between circulating spexin and leptin levels was reported in adolescents with obesity (Kumar et al., 2018). Leptin mRNA and protein have been found to colocalize to the syncytiotrophoblast and fetal endothelial cells in human placentas, suggesting that the placenta is a source of both fetal and maternal leptin (Lea et al., 2000). In addition, human fetal adipose tissue is capable of producing leptin at the beginning of lipogenesis and differentiation (Atanassova and Popova, 2000). Placental leptin is transported bidirectionally at both fetal and maternal interfaces (Hoggard et al., 2001; Wyrwoll et al., 2005). However, leptin may play other roles at the maternal-fetal interface. Its receptor is expressed on invading extravillous trophoblast cells, and the addition of leptin to isolated cytotrophoblast cells in culture increases the production of matrix metalloproteinases (Castellucci et al., 2000). Hence, the hormone may stimulate trophoblast invasion, particularly during the first trimester. In addition, research in animal models has demonstrated that leptin is involved in the development and maturation of a number of organs, including the heart, brain, kidneys, and pancreas (Briffa et al., 2015). Placental leptin may, therefore, also play a role in organogenesis during the first trimester.

In addition, the motifs of two transcription factors, CBX3 and BCL6, were significantly overexpressed, indicating the involvement of these transcription factor networks in the differential regulation of trophoblast migration, proliferation and fusion following the onset of maternal blood. Aberrant expression of both transcription factors has been reported in many types of cancer (Polo et al., 2007; Zhao et al., 2019). It is possible there is an interaction between the two transcription factor networks, which regulates trophoblast migration, proliferation and fusion following the onset of maternal blood flow. Supporting this notion is the fact that CBX3 is one of the target genes of the repressor gene BCL6 (Polo et al., 2007). In addition, cyclin dependent kinase inhibitor 1A (CDKN1A) is a target of CBX3 (Zhao et al., 2019) and it is downregulated in the second trimester. CBX3 is known to be involved in transcriptional silencing in heterochromatin-like complexes. There are several differentially regulated growth regulating factors with CBX3 motif, including SCML4 (protein coding gene, downregulated in the second trimester), PRDM6 and 8 (histone methyltransferases), ZBTB16 (transcription repressor) and PRKCB (all upregulated in the second trimester).

Our study has a number of key strengths that limit potential confounding factors. Firstly, the placental samples were obtained using a CVS technique, avoiding the rapid changes in the transcriptome documented in villous tissue collected using other commonly used methods, such as curettage (Cindrova-Davies et al., 2015). Secondly, the gestational age of the pregnancies was accurately dated with ultrasound. Thirdly, the samples were taken from narrow windows of gestation prior to and following onset of the maternal circulation. The precise timing of the onset in an individual pregnancy is not known, but the intraplacental oxygen concentration appears stable before 10 weeks and after 12 weeks of gestation (Jauniaux et al., 2000). Onset is also a progressive phenomenon, starting in the peripheral region of the placenta where it stimulates villous regression and moving centripetally (Jauniaux et al., 2003b). Tissue effects are likely to be heterogeneous during the transition period, but our samples were all obtained from a consistent site, the central region of the placenta identified under ultrasound guidance, limiting regional variability. Finally, the RNA-Seq and methylation analyses were performed on the same samples.

The rigorous collection protocol meant, however, that sample sizes were relatively small. Nonetheless, some of the most DEGs identified, such as those for the various hCG loci, were consistent with previous reports. In addition, we confirmed many of the novel finding, such as for spexin and the mephrins, at the protein level using immunohistochemistry. Our sample groups were also biased towards samples from male fetuses. Hence we performed a sex-adjustment analysis at the outset. The differentially expressed genes identified in this study did not intersect with the genes identified by Gong *et al*. (2018b), indicating that sex-specific effects were unlikely to drive the first-second trimester gene expression differences.

The transcriptome can only provide an indication of potential protein levels and function due to differential translation and post-translational modifications. Nonetheless, it is clear that the transition between the first and second trimesters is associated with major physiological and morphological changes in the placenta. Villous regression over the superficial pole of the gestational sac leads to formation of the membranes and the definitive placenta, and there is a loss of stemness and proliferation of the villous cytotrophoblast cells (Burton et al., 2020). Premature onset of blood flow occurs in many patients with first trimester miscarriage, suggesting that a failure to adapt successfully may trigger subsequent pregnancy complications (Jauniaux et al., 2003a). Our findings indicate that DNA methylation changes are part of the placental response to these major environmental changes and may explain the altered DNA methylation profiles seen in placentas from pregnancies complicated by fetal growth restriction (Hillman et al., 2015). Whether these changes are purely ontological or responsive to the change in oxygenation, the loss of growth factor support from the histotroph, or to increases in biomechanical stimuli requires further research.

The unique nature of the first-second trimester transition during human pregnancy may explain the high incidence of complications of pregnancy in our species. In other mammals histotrophic and hemotrophic exchange occur in separate areas of the extra-embryonic membranes, such as the inverted yolk sac and the chorioallantoic placenta of the mouse respectively, often in parallel. In these species the placenta develops in a more constant oxygen environment, and the switch in nutrient pathways within the same tissue does not occur. The situation in the human reflects our invasive interstitial form of implantation, which is restricted to the great apes and for which the evolutionary advantage has yet to be identified.

**Materials and Methods**

**Human Tissue Collection**

First and second trimester tissue samples were collected with informed written patient consent and approval of the Joint University College London/University College London Hospital Committees on the Ethics of Human Research (05/Q0505/82) from uncomplicated pregnancies at 7-8 (n=8) and 13-14 weeks of gestation (n=6). Gestational age was confirmed by ultrasound measurement of the crown-rump length (CRL) immediately before the procedure. All samples were collected from patients undergoing surgical pregnancy termination under general anaesthesia for psycho-social reasons. Villous samples were obtained under transabdominal ultrasound guidance from the central region of the placenta using a chorionic villus sampling (CVS) technique. All samples were snap-frozen immediately in liquid nitrogen and stored at −80 °C until analysis.

**RNA extraction and RNA-Seq**

RNA was extracted from human first-trimester placental villi using the RNeasy Plus Universal Mini Kit (catalog no. 73404; Qiagen). Libraries were made using the Illumina TruSeq Stranded mRNA Library Kit according to the manufacturer’s instructions. Libraries were quantified (kappa qPCR), and equimolar pools were sequenced (paired end 100 base reads, PE100) in several lanes of the Illumina NextSeq.

**Bioinformatics**

**RNA-Seq Analysis**

Paired-end sequencing was performed on Illumina NextSeq Direct High Output with read lengths of 100 bp. QC of sequencing was assessed using FastQC (v 0.11.5), fastq\_screen (v0.9.3) and Picard Tools (v 2.9.0) and summarised with MultiQC (v1.8dev). Reads were trimmed to remove adapters and low quality bases with TrimGalore! (v0.6.4) and aligned to the human genome (GRCh38) with STAR aligner (v2.5.1b)), with a mean of 90.4% reads uniquely mapping and mean of 56M paired reads/sample. Gene quantification was determined with HTSeq-Counts (v0.6.1p1). Additional quality control was performed with a custom rRNA and mtRNA counts script (provided on GitHub). Counts extracted with htseq-counts were used to perform differential gene analysis in R (version 3.5.2) using package DESeq2 (v.1.22.2). Sex of the samples was assigned using sex specific gene expression *Xist, Rps4y1, Ddx3y, Usp9y* and *Sry* and was included in the design formula (~ sex + condition) as a blocking factor to account for variation in the data. Additionally, DEG list was compared with the list of genes expressed differentially in placenta depending on fetal sex (Gong et al., 2018a). Among our list of 3242 genes (with fold change > 2), there were none overlapping with the list (101 genes). Read counts were normalised on estimated size factors. Principal component analysis (PCA) was performed on rlog-transformed count data for all genes. Gene Ontology analysis (GO) and Kegg pathway analysis were performed using clusterProfiler package (v.3.10.1) on DEGs with absolute log2 fold change > 1 and adjusted *P* value < 0.05 were used. Kegg pathway analysis was performed for upregulated and downregulated genes separately. The data matrix for scRNA-seq data were obtained from the Wang lab (16) (GEO accession number GSE89497). Regularised log transformation function (from DESeq2 package) was applied to counts for heatmaps. Heatmaps were generated with ‘ComplexHeatmap' R package (v 1.20.0). Selection of differentially expressed genes on heatmaps was based on highest significance (lowest adjusted *P* value) and highest absolute log2 fold changes. To investigate potential regulatory networks of the placenta, RcisTarget R package was used (v.1.2.1). Motif rankings for tss-centered-10kb and 500bp upstream of TSS were used in the analysis (human motif collection version 9, ‘mc9nr', with 24453 motifs). DEGs were scanned for the motifs and DNA motifs significantly over-represented in a gene-set were identified. DEGs with enriched motifs were analysed with enrichR r package (v.1.0) for pathway enrichment.

**Integration of RNA-Seq with single cell RNA-Seq (scRNA-Seq)**

The GSE89497 scRNA-Seq dataset downloaded as a matrix processed as described in Liu *et al*. (Liu et al., 2018). The matrix was normalized and scaled in Seurat (Stuart et al., 2019) for use in the heatmaps.

**DNA Methylation Analysis**

**Infinium MethylationEPIC array**

In order to compare the sequencing and methylation changes in the placenta, we extracted the DNA from the same patient samples, as those used for RNA extraction. Genomic DNA was isolated by QIAamp DNA mini kit (Qiagen, cat. no. 51304) following the manufacturer’s instructions. Buffer AL (200 μl) was added to the sample, mixed by pulse-vortexing for 15 sec, before incubating at 70oC for 10 min. Absolute Ethanol (200 μl) was then added to the sample, and mixed by pulse-vortexing for 15 sec before transferring to the QIAamp Mini spin column and centrifuged at 6000 g for 1 min. The Mini spin column was washed once with Buffer AW1 (500 μl) following by Buffer AW2 (500 μl) before centrifuging at full speed for 1 min. For elution of genomic DNA, DNase-free water (100 μl) was added and incubated for 1 min before centrifuging at 6000 g for 1 min. The step repeated one more time with another 100 μl DNase-free water. DNA concentration of the samples were quantified by NanoDrop and the DNA quality was checked by resolving in 0.8% agarose gel, in which there was a major band visualized at around 10 kbp without obvious smear below, indicating good quality DNA.

Genomic DNA oxidative bisulfite (oxBS) conversion was performed using the CEGX TrueMethyl kit (Cambridge Epigenetix/NuGEN, cat. no. CEGXTMS) and used for microarray-based DNA methylation analysis, performed at GenomeScan (GenomeScan B.V., Leiden, The Netherlands) on the HumanMethylation850 BeadChip (Illumina, Inc., San Diego, CA, U.S.A). The EPIC array interrogates approximately 865,000 CpG sites representing about 99% of the RefSeq genes. The resulting iDAT files were imported and analysed using ChAMP (v2.9.10) (Aryee et al., 2014; Morris et al., 2014). Samples were processed filtering for a probe detection *P* value ≤ 0.01, probes with a beadcount <3 in at least 5% of samples, no CpG and known SNPs (Zhou et al., 2017) at probe starts, probes aligning to multiple locations, and QC using the on array control probes. In total, 750150 probes on the array passed the filtering and QC steps. The BMIQ (Teschendorff et al., 2013) method was used to normalise the two probe types (I and II) present on the array. Beta methylation values from the EPIC array range from 0 (unmethylated) to 1 (methylated) and are equivalent of percentage methylation.

To account for sex specific differences we performed batch correction with champ.runCombat, using sex as the variable for correction. The PCA plot was generated for the top 500 most variably methylated CpGs. DMRs were calculated using the bumpHunter methods in ChAMP, and a methylation difference of 0.2 and adjusted p.value of 0.05 between first and second trimesters was used for filtering DMRs. Probes on the X and Y chromosomes were excluded to minimise sex specific in differential methylation calculations. Pearson’s correlation (R function cor.test) was used to calculate p values between DMR methylation and differential gene expression was calculated Pearson’s. Bedtools (Quinlan and Hall, 2010) was used to determine DMRs overlapping gene bodies and promoters (bedtools closest -D b -d) -a DMRs.bed -b GRCh37.87.gtf.bed and 1.5Kb upstream of the TSS used to define promoters). GO analysis on for DMRs in gene bodies and promoters was performed with ‘goregions’ from missMethyl (Phipson et al., 2016). Multiple DMRs per gene were manually checked and in all cases the methylation change was in the same direction. Where a DMR overlapped two genes, both were included in the correlation with gene expression. A list of the top 100 DMRs associated with sex-specific methylation in human placentas are from Gong et al. (2018a). An intersection of sex-specific DMRs with the identified DMRs with associated expression changes above thresholds (log2 FC 1, methylation difference 0.2) show no common genes, suggesting the identified DMRs are not sex-specific.

**Data and Code Availability**

RNA-Seq

Scripts used can be found on GitHub (<https://github.com/CTR-BFX/2020_Prater_Cindrova>). RNA-sequencing data is accessible through the EMBL-EBI ArrayExpress accession number: E-MTAB-9203. http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9203.

DNA Methylation (EPIC Array)

Code used to analyse the EPIC array samples is available on GitHub (<https://github.com/CTR-BFX/First-Second-Trimester-Methylation>). EPIC methylation array data have been deposited at EMBL-EBI ArrayExpress under accession number E-MTAB-9312 (<https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9312>).

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (Cindrova-Davies et al., 2007) using the following primary antibodies: anti-XBP1 (ab109621, Abcam), anti-phospho-IRE1 (ab48187, Abcam), anti-IRE1 (ab37073, Abcam), anti-ATF6 (ab37149, Abcam), anti-pyruvate kinase L/R (PB9499, Boster), anti-spexin (H-023-81, Phoenix Pharmaceuticals), anti-MEP1α (ab232892, Abcam), anti-CD31 (M0823, Dako), anti-musculin (ab64954, Abcam). Negative controls were performed by replacing primary antibodies with the blocking serum.

**Western Blotting**

Placental lysates were processed and run on western blots, as previously described (Cindrova-Davies et al., 2007), using the following antibodies: anti-TTR (PA5-80197, Thermo Fisher Scientific), anti-leptin (ab2125, Abcam), anti-ApoA1 (PA5-78798, Thermo Fisher Scientific), anti-eIF2α (#3398, Cell Signaling), anti-GRP78 (610978, Transduction Laboratories), anti-GPX1 (ab167989, Abcam).

**Competing Interest**

The authors have no conflict of interest.

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**Figure Legends**

**Figure 1. RNA-Seq identification of DEGs, functions and pathways distinct to first and second trimester placenta.** A) PCA separation of first and second trimester samples. B) Volcano plot of DEGs, with genes higher in the first trimester in red, and those higher in the second trimester in blue. C) Selected Kegg pathways, which were differentially regulated during the first and second trimester. D-F) Barplots showing selected differentially regulated GO terms related to Biological Processes (D), Molecular Functions (E) and Cellular Components (F). Each barplot shows how many genes within each term are expressed more in the first (red) or second trimester (blue). Transparency is used to show the most significant (*P.adj*) terms as least transparent. Terms were ordered by q-value.

**Figure 2. DEGs associated with ER processing, and oxidative stress and glycolytic processes.** A-C) Heatmaps of top DEGs in the first *vs*. second trimester samples, were compared to a previously published scRNA-Seq dataset with first and late second trimester placenta samples (see methods). A) Heatmap showing top DEGs involved in ER processing and stress. B) Heatmap of top DEGs related to oxidative stress. C) Heatmap of DEGs involved in the glycolytic pathway. D) Immunostaining of first and second trimester sections with anti-pyruvate kinase antibody. E-F) Western blots depicting GRP78 and phospho-eIF2α (E) or catalase (CAT) and glutathione peroxidate (GPX) (F) in first and second trimester placental lysates. Ponceau S (Ponc S) staining was used to normalise the protein loading. Data are expressed as mean ± SD. Comparisons were made using a two-tailed Student’s *t*-test. Differences were considered to be significant at *P* ≤ 0.05. G-H) Immunolocalisation of XBP1, ATF6, P-IRE1 and IRE1 in first and second trimester placental sections, stained with anti-XBP1, -ATF6, -IRE1 or P-IRE1 antibodies. Arrows denote cell columns (CC). Scale bars are 25 µm in D and G and 200 µm in H.

**Figure 3. Overview of hormonal activity and transport related placental genes differentially expressed in the first and second trimester of pregnancy.** A) Heatmap showing top DEGs related to hormone activity and compared to the scRNA-Seq data. B) Western blots depicting transthyretin (TTR) and leptin levels in first and second trimester placental lysates. Ponceau S (Ponc S) staining was used to normalise the protein loading. Data are expressed as mean ± SD. Comparisons were made using a two-tailed Student’s *t*-test. Differences were considered to be significant at *P* ≤ 0.05. C) Immunostaining of first and second trimester sections with anti-spexin antibody. D) Volcano plot of transport-related genes, with DEGs coloured red were higher in the first trimester, and those in blue higher in the second trimester. E) Heatmap of top transport related genes, split by the type of transport. Scale bars are 25 µm.

**Figure 4. Overview of DEGs related to transcription factors, WNT signalling and extracellular matrix related genes, differentially expressed between the first and second trimester.** A) Heatmap of DEGs associated with WNT signalling. Heatmap of differentially expressed transcription factors. B) Heatmap of extracellular matrix related genes differentially expression in the first and second trimester. C) Heatmap of DEGs associated with WNT signalling. D-F) Immunolocalisation of meprin (D), CD31 (E) and musculin (MSC) (F) in first and second trimester placental sections (n=4). Scale bars are 25 µm in D and F and 50 µm in E. G) CBX3 motif is enriched in proportion of DEGs. In the panel are: sequence motif for CBX3, incidence matrix of highly ranked DEGs with CBX3 motif, and pathway enrichment analysis for these genes.

**Figure 5. DNA methylation changes in first vs. second trimester.** A) Comparison of sample gestational ages between this and related studies. B) PCA shows a clear separation between first and second trimesters. C) Global methylation levels are higher in the second trimester than the first as indicated by a shift in points towards the top left. Methylation difference thresholds of 0.2 (20%) and 0.4 (40%) are shown as dashed lines. D) At specific genomic features, such as promoters, gene bodies and CpG islands there is the largest difference at gene bodies with higher methylation during the second trimester, as indicated with increased numbers of probes with a methylation difference of over 0.2 (dashed lines). E) A scatter plot comparing RNA expression and DNA methylation at promoters and gene bodies shows a correlation of reduced expression with increased methylation from first to second trimester for 36 genes (purple). Thresholds of log2 fold change of 1 and a methylation difference of 0.75 are indicated (red line). Further classifications of the direction of methylation and expression from first to second trimester are presented; Increased methylation with increased expression (8 genes, green), decreased methylation with decreased expression (3 genes, blue) and decreased methylation with increased expression (2 genes, red).