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Molecular mechanisms of placental pathophysiology

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Thèse de doctorat de BIOLOGIE CELLULAIRE ET MOLÉCULAIRE

Dirigée par Daniel VAIMAN

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PS: This page might be more for the writer than for the readers, a memento of the people that surrounded me at this moment in time, like a page of a diary, I am jotting down feelings, memories and wishes. PS2: In the likely event I have forgotten someone, please forgive me and pin it down on a deranged brain, high on caffeine and sugar, low on vitamins and water. PS3: I had to use a mixture of English, Italian and French because otherwise it would not have been honest and spontaneous. PS4: Thankfully it turned out shorter than my introduction... otherwise, it would have been embarrassing!

PS5: Arrivederci!! (cit., Camino Ruano San Martin)

Synthèse de travaux: résumé français

Pendant la grossesse, le placenta se développe quelques jours après la fécondation et assure la régulation des échanges de nutriments entre la mère et le bébé, une protection contre les infections, une immunotolérance et la sécrétion d'hormones clés de la grossesse. Le type cellulaire primaire du placenta est le trophoblaste d'origine fœtale. Les cytotrophoblastes mononucléés (CTB) prolifèrent et peuvent se différencier en trophoblastes extra-villeux (EVT) responsables de l'invasion placentaire et du remodelage des artères spiralées maternelles, ou en syncytiotrophoblastes multinucléés (STB) par fusion cellulaire. Le STB est en contact direct avec le sang maternel et à proximité immédiate de la circulation fœtale assurant les échanges entre les deux systèmes circulatoires. De plus, c'est le site principal de la sécrétion d'hormones dans le placenta.

Les pathologies de la grossesse ont été largement liées à des anomalies placentaires ; nous nous intéressons à la prééclampsie (PE), caractérisée par une hypertension de novo et une protéinurie après 20 semaines de gestation et un retard de croissance intra-utérin (RCIU). PE et RCIU entraînent des conséquences potentiellement dramatiques sur la santé maternelle (pendant et longtemps après la grossesse) et la santé fœtale pendant la période périnatale et plus tard dans la vie, la PE étant également l'un des facteurs de risque les plus élevés de décès du nouveau-né.

L'objectif de cette thèse de doctorat était d'aboutir à une meilleure compréhension de la physiologie du placenta au niveau moléculaire, en étudiant une grande variété de mécanismes. Si ce projet peut se diviser en quatre axes de recherche principaux développés dans quatre chapitres, il englobe deux thèmes principaux : la contribution de la génétique et de la transcriptomique (OMICS) du placenta dans la santé et la maladie, et la nécessité de mieux caractériser le syncytiotrophoblaste en raison de son rôle central dans la fonction placentaire. Nous avons commencé ce voyage d'exploration en examinant la modulation génétique de l'expression des gènes pour définir des loci influençant l'expression des gènes placentaires (eQTL). Un eQTL se définit comme un changement de séquence à un locus du génome (eSNP) corrélé avec des altérations des niveaux d'expression d'un ou plusieurs gènes (eGene). Nous avons élargi les connaissances actuelles sur les eQTL placentaires, ajoutant au nombre relativement limité d'études antérieures dans le domaine. Nous avons identifié 279 cis-eQTL

placentaires (False Discovery Rate $\leq 0,05$) dans 57 échantillons placentaires humains, en corrélation avec les changements d'expression de 43 gènes uniques, parmi lesquels 23 constituaient de nouveaux « eGenes ». Parmi les eSNPs les plus significatifs, 111 des 279 cis-eQTL sont associés à des eGenes placentaire décrit précédemment : une protéine Zinc-finger-like ZSCAN9, un pseudogène placentaire PSG7, un pseudogène du transducteur de ERBB2 (TOB2P1), une aminopeptidase du réticulum endoplasmique (ERAP2) et l'aquaporine 11 (AQP11), corroborant la validité de nos résultats. Pour la première fois nous décrivons dans le placenta des eSNPs agissant *in cis* qui sont en corrélation avec plusieurs eGenes et qui identifient des régions régulatrices potentielles du placenta. Sur le chromosome 11, un nouveau eGene LOC646029 et AQP11 partagent 10 eSNP. En aval, les 3 eSNP associés au niveau d'expression de CARD17 sont également retrouvés des eSNP pour le nouvellement décrit eGene CASP1P2. De même, 24 des 42 eSNP associés à ZSCAN9 sont corrélés avec des changements d'expression du transcrit en amont TOB2P1, sur le chromosome 6.

Un résultat intéressant des ensembles de données eQTL est l'identification de mécanismes putatifs de régulation génique qui influencent les eGenes, tels que la description d'enhancers, de facteurs de transcription, ou encore de complexes induisant des modification épigénétique. Pour identifier de tels mécanismes, il est nécessaire de descendre jusqu'aux régions génomiques qui abritent ces eSNP et eGenes, à la recherche de signatures d'activité amplificateur comme des marques de modification d'histone, de sites accepteurs d'épissage, de séquences consensus de facteurs de transcription. Compte tenu de leur rôle pléiotrope apparent, nous avons concentré notre attention sur les trois régions chromosomiques mentionnées précédemment. En examinant la position relative des eSNP avec les marques de modification des histones annotées à partir de la base de données ENCODE ainsi que les annotations fonctionnelles des SNP de la base de données dbSNP, nous avons pu identifier un réseau régulateur potentiel impliquant les facteurs de transcription putatifs Zinc-finger ZKSCAN4 et ZSCAN9 ainsi que TOB2P1. La fonction de ZSCAN9 est encore incertaine, alors que TOB2P1 est un pseudogène du transducteur de la tyrosine kinase ERBB2, la dernière impliquée dans la morphogenèse du cytosquelette et dérégulée dans le cancer. Il est intéressant de noter que ZKSCAN4 agit comme un répresseur de l'expression génique, en particulier dans la voie p53. Les régions régulatrices putatives que nous avons présentées ici sont cependant encore très spéculatives, et des validations *in vitro* de la présence des marques de modification des histones ainsi que de l'impact des niveaux de ZKSCAN4 sur les eGenes sont nécessaires. De plus, ayant accès à des échantillons placentaires provenant de pathologies de la grossesse, PE et RCIU, la prochaine

étape consiste à voir si la maladie a une interaction avec la séquence génétique en modulant l'expression des eGenes.

Dans la perspective de comprendre la contribution du placenta aux PE et RCIU, nous avons mené une seconde étude multi-OMICS. Cette fois, alors que nous nous concentrons toujours sur l'expression des gènes, nous avons plutôt cherché des changements au niveau de l'expression des exons, en étudiant la contribution de différentes isoformes et de l'épissage alternatif dans les pathologies de la grossesse par rapport au contrôle. Cet intérêt pour l'épissage alternatif découle de l'observation que certains des biomarqueurs clés qui caractérisent les grossesses pré-éclampsiques sont soumis à un épissage alternatif et que les isoformes sont exprimées différemment entre la maladie et les grossesses à bas risque. En effet, la tyrosine kinase-1 de type fms (FLT-1) et l'endogline (ENG) sont exprimées sous leur forme longue et sont liées à la membrane, médiant des cascades de signalisation pro-angiogéniques dans les placentas normaux. Dans la PE et RCIU, les isoformes plus courtes sont fortement exprimées et libérées dans la circulation sanguine sous forme solubles pour FLT-1 (sFLT-1) et ENG (sENG). Cela entraîne une déplétion des facteurs de croissance pro-angiogénique dans la circulation sanguine maternelle et une dysfonction endothéliale.

Notre approche peut être considérée comme pionnière, puisque nous avons cherché à savoir si la dérégulation de l'épissage alternatif pouvait constituer une caractéristique des pathologies placentaires, plutôt que d'être restreinte à quelques protéines ; une telle piste de recherche n'avait jamais été explorée en ces termes auparavant. En comparant les expressions des isoformes dans 17 échantillons témoins, 7 PE et 13 RCIU, nous avons en effet pu montrer comment l'épissage alternatif est profondément affecté dans les pathologies de la grossesse, et de fortes différences caractérisent PE et RCIU. 1060 et 1409 gènes se sont avérés être épissés de manière différentielle dans les comparaisons PE vs CTRL et IUGR vs CTRL (valeur de $p \leq 0,001$) et seulement 176 étaient communs entre les deux pathologies. Ces gènes sont associés à des mots clés ontologiques relatives aux « complications de la grossesse », aux « maladies fœtales », à « l'hypertension gestationnelle », à « l'hypoxie » et aux voies associées au cancer. Alors que le sous-ensemble distinct dérégulé dans le RCIU semblait être impliqué dans l'hypoxie et les voies métaboliques telles que la stéroïdogénèse, la synthèse hormonale et le transport des anions, dans la PE les termes « matrice extracellulaire », « système circulatoire », et « physiologie neuronale » étaient enrichis.

De nombreux gènes ont déjà été décrits comme étant fortement dérégulés dans les pathologies de la grossesse telles que la pappalysine 2 (PAPPA2), la sérine peptidase 4 (HTRA4) et la leptine (LEP) trouvées augmentée dans les placentas de grossesses compliquées par la PE. Nous avons pu montrer pour la première fois comment ces mêmes gènes semblent également fortement altérés au niveau de l'épissage, suggérant que différents répertoires d'isoformes sont exprimés en pathologie par rapport au contrôle. Cependant, nos résultats ont souligné l'importance d'examiner les changements dans les niveaux d'expression au niveau des différentes isoformes, car même si certains gènes ne semblaient pas être modifiés entre la maladie et le contrôle au niveau total, des différences d'épissage alternatives pourraient entraîner des différences fonctionnelles au niveau protéique. Compte tenu du manque d'informations concernant la régulation génétique de l'épissage dans le placenta, nous avons généré le premier ensemble de données préliminaires identifiant des loci influençant l'épissage des gènes placentaires (sQTL), 52 cis-sQTL ainsi que 52 trans-sQTL ($FDR \leq 0,05$).

Grâce à ces investigations OMICS, alors que nous avons identifié la régulation génétique de l'expression et de l'épissage dans le placenta, nous n'avons pas encore élucidé les mécanismes qui lient les changements alléliques dans les SNPs avec les changements dans les gènes cibles. Des expériences d'édition de gène visant à évaluer l'impact de chacun des allèles sur l'expression des gènes et les répertoires d'isoformes pourraient aider à démêler ces phénomènes.

Le deuxième thème principal de ce travail de thèse a été la caractérisation du processus de syncytialisation. Le STB se développe précocement au cours de la placentation par fusion de CTB, qui constituent la réserve de cellules trophoblastiques dans le tissu et assurent par prolifération leur auto-renouvellement. Ce flux continu de noyaux de CTBs dans le STB multinucléé permet l'expansion du STB nécessaire pour répondre aux besoins métaboliques croissants du fœtus en développement, ainsi que le remplacement des noyaux vieillissants. L'altération de cet équilibre, souvent observée sous la forme d'une prolifération accrue de CTBs et d'une syncytialisation réduite, entraîne un dysfonctionnement des STB caractérisé par un stress oxydatif et la libération de molécules de stress.

Les altérations morphologiques et fonctionnelles du STB sont une caractéristique des pathologies de la grossesse telles que la PE et le RCIU. Compte tenu de son rôle clé dans la fonction et la pathologie placentaires, le processus de syncytialisation fait l'objet d'efforts de

recherche constants visant à identifier de nouveaux gènes candidats. La majorité des études portent sur les protéines membranaires, les kinases et phosphatases impliquées dans les cascades de signalisation, et les facteurs de transcription. Au-delà de ces fonctions, l'attention portée aux mécanismes épigénétiques de régulation impliqués dans la syncytialisation est pratiquement inexistante, à l'exception de quelques études qui ont montré le rôle de divers microARN (miR).

En 2019, nous avons passé en revue les mécanismes épigénétiques de régulation dans le placenta sain et dans la prééclampsie, montrant leur rôle central dans l'établissement des modèles d'expression génique. Il est intéressant de noter que de nombreux longs ARN non codants (lncRNA) sont souvent dérégulés dans les placentas prééclampsiques. Néanmoins, ces lncRNA sont généralement étudiés dans des modèles cellulaires de trophoblastes extra-villeux (EVT), caractérisés par un potentiel prolifératif et migratoire élevé, mais pas dans des cellules de type syncytiotrophoblaste. Nous avons contribué de manière significative à ce domaine en clarifiant le rôle des ARN non-codants confirmés dérégulés dans la PE et le RCIU, en particulier le lncRNA Urothelial Cancer Associated 1 (UCA1) et de le miR-193b qui ont un rôle significatif dans la syncytialisation des trophoblastes. Les modifications transcriptionnelles de la lignée cellulaire BeWo lors de l'administration de forskoline (FSK), liées à la syncytialisation et par rapport aux ARN non codants ont été analysées.

Dans les cellules traitées par la FSK par rapport au contrôle, 800 et 1281 gènes totaux étaient respectivement régulés à la hausse et à la baisse, parmi lesquels 196 étaient des lncRNAs régulateurs, appartenant aux classes des lncARN, miR, ARN circulaires, petits ARN nucléaires et piARN. Nous avons ensuite étudié leurs cibles putatives et leur expression relative dans les placentas avec IUGR et PE dans notre ensemble de données transcriptomiques placentaires humaines ainsi que dans des ensembles de données précédemment publiés. miR-193b a été identifié comme un hub central pour la syncytialisation dans le modèle des cellules BeWo, avec 135 cibles prédites, dont 108 régulées à la baisse dans les cellules traitées par FSK. miR-193b est également régulé à la hausse dans les placentas malades, ce qui soutient l'hypothèse d'une dérégulation de la syncytialisation des trophoblastes dans ces pathologies de la grossesse. Cependant, les effets globaux de cette dérégulation sur les taux de syncytialisation ne sont pas encore clairs et d'autres études *in vitro* sont nécessaires.

Notre contribution majeure dans ce travail est relative au rôle d'UCA1 sur la syncytialisation. Cet ARN non codant avait été précédemment trouvé régulé à la hausse dans les placentas pré-éclamptiques et sa dérégulation dans le modèle EVT a montré son rôle de modulateur négatif de la prolifération et des capacités migratoires de ces cellules. Ici, nous avons confirmé les niveaux augmentés de UCA1 dans les placentas PE et IUGR et caractérisé sa fonction dans la différenciation du STB. Le knock-down d'UCA1 dans les cellules traitées par FSK a profondément modifié le profil transcriptomique de ces cellules, réduisant les changements associés à la syncytialisation. Cela suggère que UCA1 est nécessaire pour une syncytialisation réussie. En résumé, dans des conditions physiologiques, la régulation positive d'UCA1 a un impact négatif sur la prolifération et la migration des trophoblastes, en empêchant la différenciation de CTB en EVT, alors qu'elle favorise la syncytialisation du CTB et qu'elle est nécessaire pour les changements transcriptionnels qui se produisent au cours de ce processus. Dans notre quête pour accroître notre compréhension de la syncytialisation, nous avons entrepris de concevoir un criblage à l'échelle du génome pour identifier les gènes candidats avec une approche non ciblée. Nous avons utilisé la technologie CRISPR/Cas9 avec une bibliothèque d'ARN à guide unique optimisée ciblant plus de 19,000 gènes codant dans le génome humain. Nous avons testé une grande variété d'approches expérimentales afin d'optimiser ce criblage pour étudier la syncytialisation des trophoblastes dans le modèle cellulaire BeWo. Cependant, il est encore nécessaire de trouver des conditions expérimentales optimales pour distinguer au mieux les cellules en différenciation dans la population BeWo traitée au FSK, et les cellules non compétentes dans le même échantillon. Il semble que, selon la méthode expérimentale de détection des STB en différenciation, dans le modèle cellulaire BeWo, les taux de fusion et de différenciation varient selon les différentes études. Nous avons essayé de séparer les cellules de type CTB et de type STB sur la base de leurs niveaux d'expression nucléaire de marqueurs protéiques associés à l'un ou l'autre phénotype dans la littérature. Ces marqueurs nucléaires de l'état de type CTB et STB ont été évalués par cytométrie en flux ; respectivement, le marqueur de prolifération Ki67, le facteur de transcription de la lignée trophoblastique GATA3, et le facteur de transcription de fusion GCM1.

Alors qu'aux niveaux transcriptomique, nous pouvions détecter des changements profonds entre les cellules traitées par FSK et les cellules témoins (quoique pas spécifiquement pour le gène GATA3), comme en témoignent nos études, nous n'avons pas vu de changements étendus du taux de protéines dans la population BeWo traitée par FSK; étant limité à seulement ~ 10%

des cellules qui ont montré une réduction de GATA3. Néanmoins, avant de tirer des conclusions définitives, il sera nécessaire de répéter les expériences de cytométrie en flux que nous avons présentées ici, en testant des concentrations croissantes de FSK ainsi que des temps d'exposition plus longs (par exemple 5 jours). Alternativement, l'utilisation de cellules souches pluripotentes induites (iPSc) pourrait fournir une voie supplémentaire pour le criblage, étant donné la possibilité de les différencier spécifiquement en sous-types de trophoblastes et les ressources croissantes relatives à l'utilisation de CRISPR/Cas9 dans iPSc.

Ce projet a certainement contribué au progrès des connaissances en biologie du placenta, même si nous ne pouvons pas encore discerner l'ensemble du tableau. Avec ce travail nous avons posé quelques pièces de la fonction et de la régulation des gènes placentaires. En espérant que ces résultats fourniront une base pour d'autres études et caractérisations, et favoriseront un jour une meilleure compréhension des pathologies de la grossesse.

Abstract

The placenta is the temporary organ that develops during gestation to provide nutrients and respiratory gases to the developing embryo, from the maternal circulation; additionally, it promotes maternal immunotolerance and is the key site of endocrine secretion of pregnancy hormones. The primary cell type of the placenta is the trophoblast of foetal origins. Mononucleated cytotrophoblasts (CTB) proliferate and differentiate in extra-villous trophoblast (EVT) responsible of placental invasion and maternal spiral arteries remodelling, or in the multinucleated syncytiotrophoblast (STB) through cell fusion. The STB is in direct contact with maternal blood and in close vicinity to the foetal circulation ensuring the exchanges between the two circulatory systems. Additionally, it is the key endocrine site for hormone secretion in the placenta. The pathologies of pregnancy have been largely linked to placental abnormalities; we are interested in preeclampsia (PE), characterized by *de novo* hypertension and proteinuria after 20 weeks of gestation and intra-uterine growth restriction (IUGR). Both diseases have potentially dramatic consequences on maternal health for PE (during and long after pregnancy) and foetal health perinatally and later in life, PE being also one of the highest risk factors for death of the new-born. In this project we investigated four different aspects of placental physiology: **1)** We analysed the transcriptome and the genotype of 57 human placental samples and identified a set of 279 statistically significant (False Discovery Rate ≤ 0.05) *cis*-expression Quantitative Trait Loci (*cis*-eQTL), correlating with changes in expression of 43 unique genes. These eQTLs highlighted three distinct regions, one on chromosome 6 and two on chromosome 11, that could function as regulatory hotspots in the placenta **2)** We identified fundamental differences in regulation of alternative splicing between healthy, PE and IUGR placentas **3)** We investigated the role of non-coding RNAs in these pregnancy pathologies identifying functional differences in a repertoire of miRNAs and long non-coding RNA UCA1, which showed a critical role in syncytialisation **4)** We present the progress towards the design of a whole genome-screen with CRISPR/Cas9, aimed at identifying candidate genes involved in trophoblast fusion. This PhD project contributed to our understanding of a broad spectrum of molecular mechanisms involved in the regulation of placental function: genetic regulation of gene expression, disease-specific expression of RNA-isoforms by alternative splicing, non-coding molecules that are involved in epigenetic regulation of gene expression. These elements could pave the way to an improved integration of basic mechanisms of gene expression regulation in the human placenta.

Table of Abbreviations

AFR	African Ancestry
AMR	American Ancestry
AQP	Aquaporin
BeWoA	STOX1A-overexpressing BeWo
BeWoB	STOX1B-overexpressing BeWo
BeWo-IFI6	BeWo cell line carrying IFI6 promoter-Blasticidin
BeWo-IFI6-Cas9Hyg	BeWo-IFI6 cell line carrying Cas9-Hygromycin B vector
BeWoIFI6-Cas9Hyg-CL2	BeWo-IFI6-Cas9Hyg clone 2
BeWo-WT	BeWo wild type cells
cAMP	cyclic AMP
Cas9	CRISRP-associated protein 9
Cas9+BeWo	Cas9-expressing cell line
CASP1P2	Caspase 1 pseudogene 2
cCREs	Candidate Cis-Regulatory Elements
cis-eQTL	eQTL in cis
CL	Corpus Luteum
CO₂	Carbon dioxide
CRISPR	Clustered regularly interspaced short palindromic repeats
CTB	Cytotrophoblast
CTRL	Control
CVD	Cardiovascular Disease
DEGs	Differentially expressed genes
DMSO	Dimethyl Sulfoxide
DSB	dsDNA-break
dsDNA	double-stranded DNA
EAS	East Asian Ancestry
ECM	Extracellular Matrix
ED	Endothelial Dysfunction
eGene	eQTL-associated gene
EGF	Endothelial Growth Factor
ENCODE	Encyclopedia of DNA Elements
ENG	Endoglin
EO-PE	Early-onset preeclampsia
eQTL	expression-Quantitative Trait Loci
ERRγ	Estrogen-related receptor γ
ERVW-1	Endogenous Retrovirus W-1
eSNP	eQTL-associated SNPs
EUR	European Ancestry
EVT	Extra-Villous Trophoblast
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum

FDR	False Discovery Rate
FGF	Foetal Growth Factor
FLT-1	Fms Related Receptor Tyrosine Kinase 1
FSK	Forskolin
FSTL3	Follistatin Like 3
GC	Placental Bed Giant Cells
GCM1	Glial Cell-missing 1
GDM	Gestational Diabetes Mellitus
GLUT	Glucose Transporter
GM12878	Lymphoblastoid cell line
GTex	Genotype Tissue Expression
GWAS	Genome-Wide Association Studies
H1-hESC	Human embryonic stem cells
H3K27Ac	Acetylation of Lysine ²⁷ of histone H3
H3K4Me3	Tri-methylation of Lysine ⁴ on Histone 3
hCG	Human chorionic gonadotropin
hCGα	Human chorionic gonadotropin subunit α
hCGβ	Human chorionic gonadotropin subunit β
HELLP	Hemolysis, Elevated Liver enzymes and Low Platelets
HERV-FRD-1	Endogenous retrovirus group FRD member 1
HLA	Human Leukocyte Antigen
HLA-G	Human Leukocyte Antigen-G
hPGH	Human placental growth hormone
hPL	Human placental lactogen
HR	Homologous Recombination
HSMM	Human Skeletal Muscle Myoblasts
HTRA4	HtrA Serine Peptidase 4
HUVEC	Human Umbilical Vein Endothelial Cells
HW	Hardy-Weinberg
HIF-1α	Hypoxia-inducible Factors 1 α
HIF-2α	Hypoxia-inducible Factors 2 α
ICM	Inner Cell Mass
IGF	Insulin-like Growth Factor
IGFBP-1	Insulin-like Growth Factor-Binding Protein 1
IL-1	Interleukin-1
IL-11	Interleukin-11
IL-6	Interleukin-6
INDELS	Insertions and Deletions
INHA	Inhibin α
iPSCs	induced Pluripotent Stem Cells
IUGR	Intra-uterine growth restriction
JAK-STAT	Janus kinase–signal transducer and activator of transcription
K562	Erythroleukemia
LD	Linkage Disequilibrium

LEP	Leptin
LGALS	Galectin
LH	Luteinizing Hormone
LIF	Leukaemia inhibitory factor
lncRNA	long non-coding RNA
LO-PE	Late-onset preeclampsia
MAF	Minor Allele Frequency
MALAT-1	Metastasis Associated Lung Adenocarcinoma Transcript-1
mCherry-	mCherry negative
mCherry+	mCherry positive
MEG3	Maternally Expressed 3
MFSD2	Major Facilitator Superfamily Domain Containing 2
MHC	Major Histocompatibility Complex
miR	miR
MMP-2	Matrix metalloproteinase 2
MMP-9	Matrix metalloproteinase 9
MOI	Multiplicity of infection
ncRNA	non-coding RNA
NHEJ	Non-Homologous End Joining
NHEK	Normal Human Epidermal Keratinocyte
NHLF	Normal Human Lung Fibroblast
NK	Natural Killer
O₂	Oxygen
PAI	Plasminogen Activator Inhibitor
PAM	Protospacer Adjacent Motif
PAPPA2	Pregnancy-Associated Plasma Protein A2
PC	Principal Component
PCA	Principal Component Analysis
PCBP2	Poly(RC) Binding Protein 2
PCR	Polymerase Chain Reaction
PE	Preeclampsia
PGBD1	PiggyBac Transposable Element Derived 1
PIGA	Phosphatidylinositol Glycan Anchor Biosynthesis Class A
PKA	Protein Kinase A
PKC	Protein Kinase C
PIGF	Placental Growth Factor
PRL	Prolactin
PSG7	Pregnancy Specific Beta-1-Glycoprotein 7
Puro-	Medium puromycin-free
Puro+	Medium containing puromycin 4µg/ml
QC	Quality Control
q-value	FDR adjusted p-value
R²	R-squared
RMA	Robust Multi-Array

ROS	Reactive Oxygen Species
RPKM	Read Per Kilobase of exon model per Million mapped reads
rRNA	ribosomalRNA
RV	Residual Variance
SAS	South Asian Ancestry
sENG	soluble Endoglin
SERPINA3	Serine Protease Inhibitor Alpha-3
sFLT-1	soluble FLT-1
sgRNA	single-guide RNA
sgRNA-273	sgRNA targeting PIGA exonic region
sgRNA-274	sgRNA targeting PIGA intronic region
sgRNA-CTRL	sgRNA targeting a control gene
siRNA	small interfering RNA
SNP	Single Nucleotide Polymorphism
SpCas9	<i>S. pyogenes</i> Cas9
sQTLs	splicing-QTLs
STAT3	Signal Transducer And Activator Of Transcription-3
STB	Syncytiotrophoblast
TAC	Transcriptome Analysis Console
TF	Transcription Factor
TGFβ	Transforming Growth Factor B
TIMP3	TIMP Metalloproteinase Inhibitor 3
TIMPs	Tissue Inhibitors Of Matrix Metalloproteinases
TOB2P1	Transducer Of ERBB2 Pseudogene 2
tRNA	transfer-RNA
TSS	Transcription Start Site
UCA1	Urothelial Cancer Associated 1
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VEGFR-1	VEGF Receptor 1
VEGFR-2	VEGF Receptor 2
WP	Work Package
XIST	X-inactive Specific Transcript
ZP	Zona Pellucida
ZSCAN9	Zinc Finger And SCAN Domain Containing 9

Introduction

This PhD thesis consists of a multi-faceted study into the function of the placenta as part of the European Union Horizon 2020 Project “Innovation in modelling placenta for Maternal and Foetal Health” ([iPlacenta](#)) (Murdoch et al., 2021). The Project has the overarching aim of improving the understanding of placental physiology keeping an eye upon the burden of pregnancy pathologies, that have most generally found to have a causal link with placenta dysfunction. Essentially, pregnancy is a challenge for the maternal organism; placental function when optimal can admit a suboptimal vascular maternal system, while in maternal poor health (obesity, diabetes), a suboptimal placental function will lead to disease. The focus of the project is to better characterise preeclampsia (PE) and intra-uterine growth restriction (IUGR), both diseases being supreme compelling questions for the field of placentology.

The iPlacenta consortium includes fifteen Early-Stage Researchers, respective supervisors affiliated with eleven different institutions across Europe, and additional research partners. The consortium is focused on different research axes divided in three Work Packages (WP). WP1 has the aim of identifying and developing novel *in vitro* models to mimic placental function as well as pathology, mainly by mean of a placenta-on-a-chip technology and the use of induced pluripotent stem cells (iPSCs) lines to recapitulate the PE and IUGR phenotype. WP3 is focused on the development and characterisation of *in vivo* models of pathological pregnancies as well as developing accurate clinical assessment methods by imaging technologies for early prediction of pathological outcomes. My PhD project falls within the scope of WP2 that, by integrating different multi-dimensional datasets relative to human placental samples, has the goal of identifying genetic and epigenetic mechanisms important for the function of the placenta, with a particular attention to the PE and IUGR phenotypes, hunting for detectable signatures of disease.

The Placenta: an overview

During pregnancy, the placenta develops a few days after fertilisation of the egg and grants nutrient exchange regulation between mother and baby, protection from infection, immunotolerance and secretion of key pregnancy hormones. The placenta is one of the four extra-embryonic annexes, together with the amnios, yolk sac and allantois, which while being of foetal origins will not be part of the body of the developing foetus but will assist its growth during gestation (Carlson, 2014; Gude et al., 2004). The chorion comprises mesenchymal cells and trophoblast cells, trophoblasts will be the first foetal cell type to differentiate from the newly formed embryo. It constitutes the outermost extra-embryonic tissue, the only one that comes in direct contact with maternal tissues; it eventually will give rise to the placenta and the chorionic leave of the foetal membranes. The amnion arises from the epiblast cells of the blastocyst and grows to surround the developing embryo, creating a fluid-filled cavity. This amniotic sac provides a protective environment to the conceptus in which to grow and move freely, shielded from mechanical shocks. The liquid contains stem cells, water, growth factors and hormones, as well as protective immune cells and anti-inflammatory molecules. The yolk is a small ventral sac that serves as the first site for angiogenesis and vasculogenesis, while the allantois is a ventral diverticulum which in other vertebrates has the primordial role of favouring gas exchange and clearance of metabolic anabolites. In humans these functions are mediated by the placenta, however the allantois will develop into the circulatory system of the umbilical cord that dispenses foetal blood to the placenta (Carlson, 2014). **Figure 1a** gives a schematic representation of the foetus within the uterus, annotating maternal (*in cursive*) and foetal tissues and showing the organisational structure of the placenta.

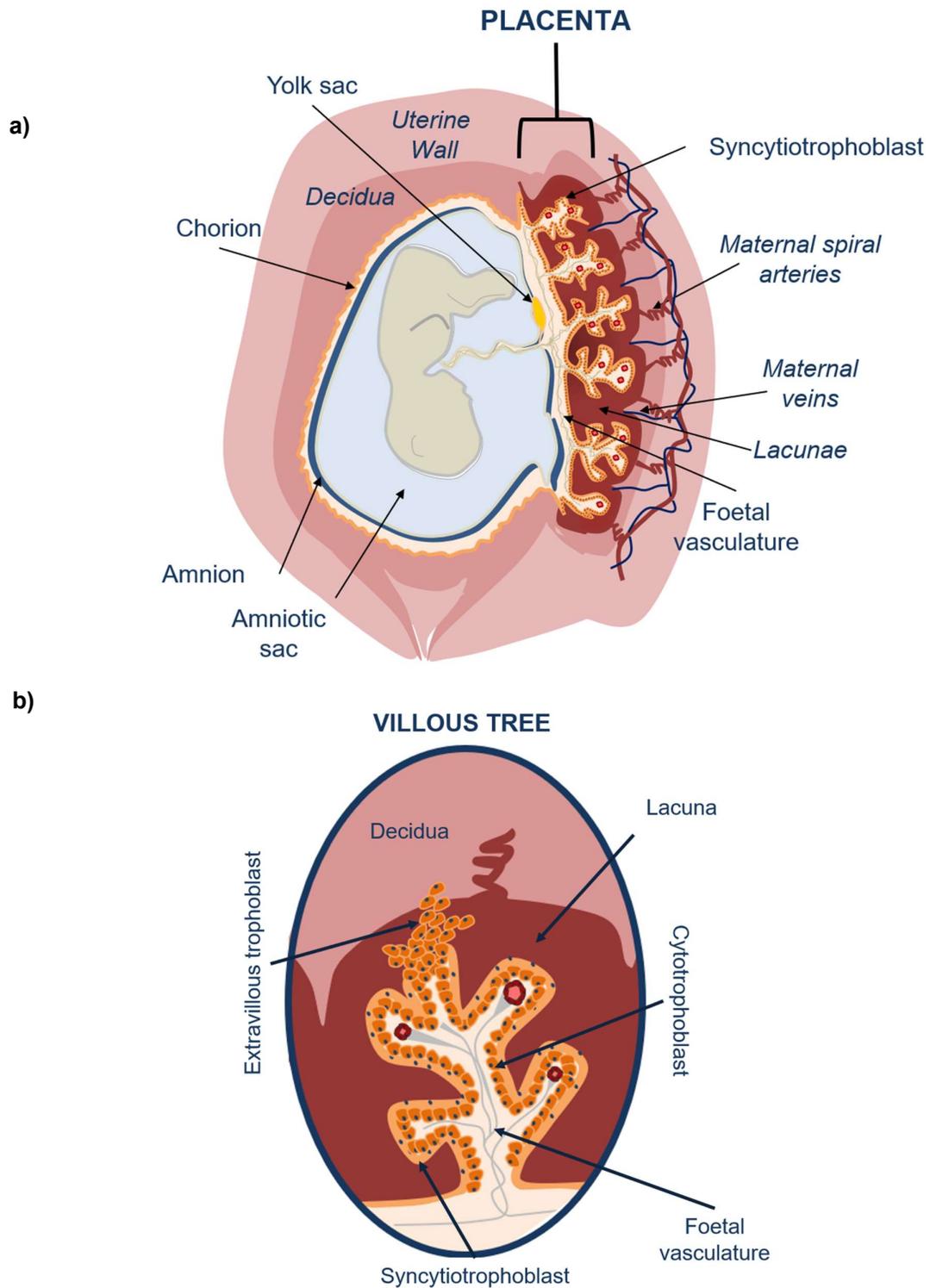


Figure 1. Schematic representation of the growing foetus in the maternal uterus in the second trimester. a) The placenta maternal and foetal sides, with respective cell types, as well the other extra-embryonic membranes, the yolk sac, amnion and amniotic sac, and chorion (at this stage, the allantois is embedded in the umbilical cord). Maternal tissues are indicated *in cursive*. **b) Placental Villous Tree.** Zoom-in view of the functional unit of the placenta.

The formation of the placenta necessitates the coordinated interaction between maternal and foetal cells. The maternal uterus is comprised of three layers, the endometrium faces the uterine lumen and is made of endometrial stromal cells with secretory function, the thickest layer is the myometrium consisting of smooth muscle, and finally the perimetrium is the outer lining of the uterus, in contact with internal organs (Jones and Lopez, 2013). During the menstrual cycle around 6 days post-ovulation, decidualisation begins by differentiation of the endometrial stromal cells into decidual cells characterised by rounded shape, larger cytoplasm, and specialised secretome. Decidual cells secrete hormones prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP-1) which stimulate trophoblast proliferation, growth, and invasion of the maternal uterine wall, fostered also by the extracellular matrix (ECM) remodelling mediated by the decidua itself. Decidual cells migrate deep into the uterine wall to modify maternal spiral arteries and promote angiogenesis to increase the blood supply to the uterus, which will be needed to establish the supply of nutrients in the placenta especially from the second trimester of gestation. Decidualisation is also a necessary process in establishing maternal immunotolerance of the foreign foetal cells, which would be otherwise target of the maternal immune system (Okada et al., 2018).

Approximately one-week post-fertilisation, the blastocyst makes the first contact with the uterine wall, implantation occurs thanks to rapid proliferation of the outer blastocyst layer comprised of cytotrophoblast (CTB) cells. At this stage, CTBs fuse to create a multinucleated layer called the syncytiotrophoblast (STB), which continuously expands and promotes the first invasion of maternal tissues. Mononucleated CTBs continuously fuse to the STB layer, promoting its expansion and advancement of the STB front deep into the uterine walls. Over-time, and at very early stages following the implantation (around 9 days post-coïtus), vacuoles form in the STB that will develop into the maternal lacunae. The proliferating CTBs form columns of mononucleated cells that traverse the invading STB, from these columns extravillous trophoblasts (EVT) differentiate by undergoing an epithelial-to-mesenchymal transition acquiring high migratory capacity. EVT proliferate rapidly and start invading the decidual tissue, to anchor the developing placenta deep within the uterine wall, migrate deeper in the decidua and secrete key regulatory hormones that promote invasion as well as immunotolerance (Benirschke and Kaufmann, 2000; Gude et al., 2004; Pijnenborg, 1990; Viganò et al., 2003). Between 8-10 weeks of gestation, EVT clusters reach the maternal spiral arteries and form trophoblast plugs, blocking the lumen and maintaining hypoxic conditions. EVT migrating within the placental bed are interstitial trophoblasts that expand the radius of

invasion of the placenta and anchor it to the endometrium. These cells differentiate into multinucleated placental bed giant cells (GC) that secrete growth factors and hormones to promote invasion and angiogenesis. By 10-to-12 weeks, EVT's further differentiate into endovascular trophoblasts which mediate spiral arteries remodelling; they migrate deep within the endometrium and replace maternal most smooth muscle cells as well as intercalating endothelial cells in the arterial linings. This process reduces vascular resistance, increasing the calibre of the vessels as well as rendering them not responsive to changes in blood flow and pressure, so that constant blood flow to the placenta is ensured (Benirschke and Kaufmann, 2000; Gude et al., 2004; Pijnenborg, 1990; Viganò et al., 2003; Wang and Zhao, 2010).

Entering the second trimester, on the foetal side, the STB is the outmost layer of foetal cells, at the base CTBs continue to proliferate ensuring self-renewal, while fusing with the expanding STB, providing additional functional nuclei and cellular factors. The decidua has been deeply modified to form lacunae, large cavities that are filled with oxygenated maternal blood coming from the spiral arteries in the endometrium. This blood comes in close contact with the foetal cells at the level of the STB layer, which is now organised in placental villi and villous trees, the functional unit of the placenta (**Figure 1b**). Each tree is a branched structure composed of placental villi, with an outer layer of STB in direct contact with the maternal blood, a basal layer of CTBs that provides continuous addition and renewal of the STB by syncytialisation and other cell types depending on the developmental stage of the villus. The branched nature of mature villi grants high surface-to-volume ratio, ensuring efficient nutrient exchange as well as potent endocrine secretion. Within the tree lumen, connective tissue supports the foetal vasculature, with ECM, stromal cells such as fibroblast and specialised immune cells (i.e. Hofbauer cells, which are considered the placental equivalent of macrophages). Branched foetal vascular capillaries coming from the umbilical cord permeate the villus tree, near the trophoblastic layer. This allows for proximity but no contact between maternal and foetal circulatory systems. Between maternal and foetal blood, at the beginning of gestation 4 layers of cells are found, from maternal to foetal side: STB, CTB, connective tissue, endothelial cells of foetal capillaries. However, as gestation progresses, to account for the increase in nutrient demand and exchange rate, the CTB layer is reduced and mostly extinguished in the majorities of villi by the end of term, concomitantly with a reduction in the width of connective tissue (Castellucci et al., 2000, 1990; Kingdom et al., 2000). The placenta reaches its complete maturation at 34 weeks of gestation, when the two sides have a very distinctive structure: the maternal side called basal plate and the foetal chorionic plate, in between we have the

intervillous space (**Figure 2**). From the foetal side, the branching of the foetal vasculature within the villous trees can be seen, radiating from the umbilical cord, the surface is smooth as it is covered by the amnion. The placental villi are organised in circular clusters, special compartments called cotyledons, that characterise the maternal side, covered by a layer of decidual cells (Wang and Zhao, 2010).

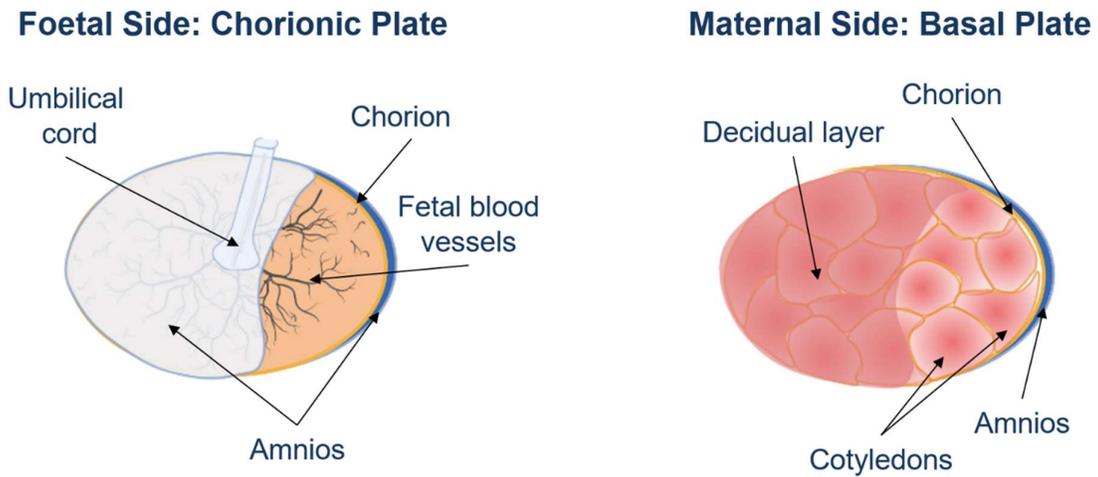


Figure 2. Placental foetal and maternal sides by 34 weeks of gestation. Figure based on (Ziadie, 2011), image created with [Biorender.com](https://www.biorender.com).

Placenta development

Fertilisation, Implantation, and Invasion

The journey of the zygote up to implantation and invasion in the maternal wall is recapitulated in **Figure 3**. The maternal oocyte is protected by an outer layer rich in glycoproteins, the zona pellucida (ZP), that mediates communication with the ovarian follicle cells, with the sperm and protects the early embryo. Once the sperm has successfully penetrated the ZP, it transfers its genetic material to the oocyte and the fusion of the two pro-nuclei gives rise to the diploid zygote. During the first 3 to 4 days after fertilisation, the zygote enters a series of rapid mitotic divisions that reduce the zygotic cytoplasm in smaller cells, without cell growth (Singh, 2018). At days 4-5, the 16-cell stage, the morula, is reached, cells continue to divide within the ZP layer, causing increasing mechanical stress. Between 32 and 64-cell stages, a portion of these totipotent cells will define the Inner Cell Mass (ICM), while a cavity, the blastocoel, starts to form due to liquid secretion from the cells. The magnitude of the mechanical stress in the morula is believed to have a role in cell fate determination during the formation of the blastocyst (Bissiere et al., 2018; Samarage et al., 2015) (**Figure 3a**). During blastocyst development, the first event of cell differentiation occurs, the outer layer becomes the trophoblast, which will give rise to the chorion and the placenta, while the ICM will later generate the tissues of the true embryo as well as the other foetal membranes (amnion, yolk sac and allantois) (Singh, 2018).

At day 6-7, the blastocyst hatches from its ZP and contacts the uterine wall, the CTBs on the point of contact start then to rapidly proliferate and to invade the decidual tissue. The adhesion between the blastocyst and the epithelial layer of maternal decidua is favoured by the secretion of cytokines from both maternal endometrial glands and trophoblast cells. Interleukin-11 (IL-11) and Leukaemia inhibitory factor (LIF) are secreted by decidualised cells and act on both the decidua and the trophoblast through specific receptors, activating downstream the Janus kinase–signal transducer and activator of transcription (JAK- STAT) signalling, through STAT3 (**Figure 3b**). This pathway is characteristic of the invasive phenotype of metastatic cancers (Fitzgerald et al., 2008; Kamran et al., 2013).

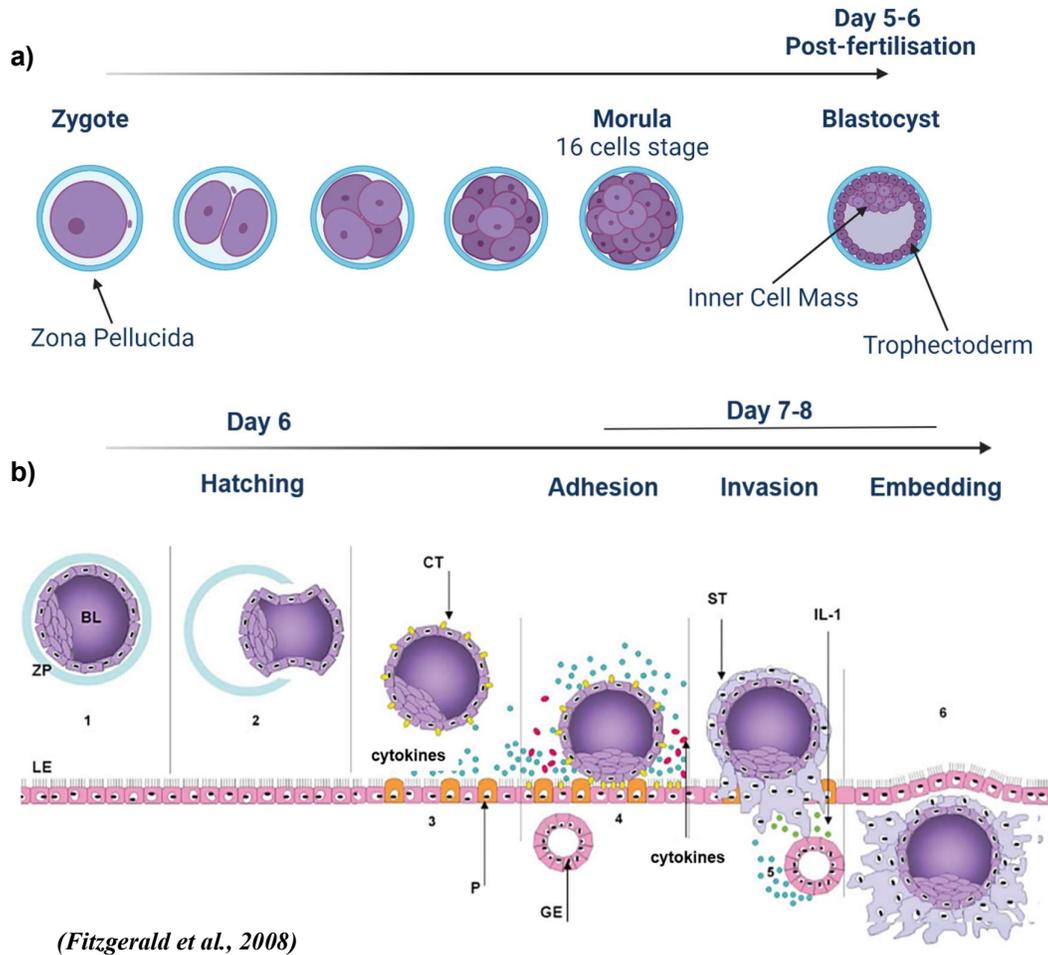


Figure 3. Embryo implantation and invasion into the maternal uterine wall. a) Blastocyst development. After fertilisation has occurred, the zygote undergoes a series of rapid mitotic divisions inside the Zona Pellucida (ZP), characterised by absence of cell growth, daughter cells are progressively smaller. The morula is defined starting from the 16-cell stage. The blastocyst is formed after the first event of differentiation in the embryo occurs with the formation of an inner cavity, the establishment of the trophoctoderm lineage on the surface, trophoblast progenitor cells. Inside the cavity the Inner Cell Mass comprises cells that will give rise to the true embryo as well as the other extra-embryonic tissues. **b) Stages of embryo implantation and invasion.** 6 days post-fertilisation the blastocyst hatches from the ZP and trophoblast as well as decidual modifications are activated by cytokines. Between Days 7-8, the blastocyst loosely adheres to the uterine wall and tight interactions with the decidual cells promote rapid proliferation and fusion of CTBs into STB. The expanding STB layer promotes invasion of the endometrial wall, deep inside the tissue. By Day 8 the blastocyst is completely embedded within the uterine wall. BL = Blastocyst; ZP = Zona Pellucida; LE= Luminal epithelium of the endometrium; CT= Cytotrophoblast, GE = Glandular epithelium, ST = syncytiotrophoblast, IL-1 = Interleukin-1, P= Pinopode. Adapted from (Fitzgerald et al., 2008), image created with [Biorender.com](https://www.biorender.com).

The CTBs start to fuse to create the STB front that invade the maternal uterine walls. Trophoblast cells secrete high levels of Interleukin-6 (IL6), a potent activator of STAT3, that interacts with receptors on the epithelial decidua and on trophoblasts themselves. These signalling cascades have the overall effect of promoting invasion, activating expression and secretion of proteases that digest the connective tissue and ECM of the decidua, such as matrix metalloproteinase 9 and 2 (MMP-9 and MMP-2 respectively), and collagenases. Meanwhile, trophoblasts can harness nutrients from the degraded decidual tissue (Fitzgerald et al., 2008).

The blastocyst sinks below the level of the uterine wall and the tissue overhead is repaired so that the whole blastocyst is now completely embedded in the maternal endometrium (**Figure 3b**). At this early stage, proliferating CTBs start to invade the STB to create protrusions, cytotrophoblast columns, that will then develop in primary villi. At the same time, CTBs at the distal end of the column differentiate in EVT_s that undergo an epithelial-to-mesenchymal transition and migrate over the STB front, deeper in the endometrium. Here they secrete key hormones and enzymes that will further promote invasion from the STB and CTBs, as well as digestive enzymes facilitating the invasion and the creation of lacunae, and factors that promote the maternal immunotolerance. Trophoblasts release Interleukin-1 (IL1) that increases LIF expression, high levels of LIF trigger negative feedback and dampen the inflammatory response. In EVT, LIF triggers specific expression of Human Leukocyte Antigen-G (HLA-G), member of the Major Histocompatibility Complex (MHC), Class I, that modulates maternal immunotolerance (Ferreira et al., 2017; Fitzgerald et al., 2008; Gude et al., 2004). Given the extensive damage to the maternal decidua, anti-inflammatory cytokines are released by the EVT_s to reduce maternal immune system activation, mainly transforming growth factor β (TGF β), as well as plasminogen activator inhibitor (PAI), tissue inhibitors of matrix metalloproteinases (TIMPs). Endovascular EVT_s deep in the decidua reach the maternal arterioles and arteries and in this first instance block the blood flow by obtruding the lumen, forming EVT plugs (Gude et al., 2004).

Placental villi

During placental development, the first villi develop by invagination of the STB, and proliferation of CTBs to form an underlayer that will continue to supply new STB nuclei to the syncytium ensuring renewal of this layer as well as increase in surface of the membrane during gestation. In the foetal side of these invaginations, foetal cells colonise the lumen and angiogenesis and vasculogenesis mediated by differentiation of foetal mesenchymal precursor cells form *de novo* foetal blood vessels, which start to occupy the villus lumen longitudinally. Vascularisation begins at 3 weeks post-fertilisation and is completed by week 6-8 (Demir et al., 1989).

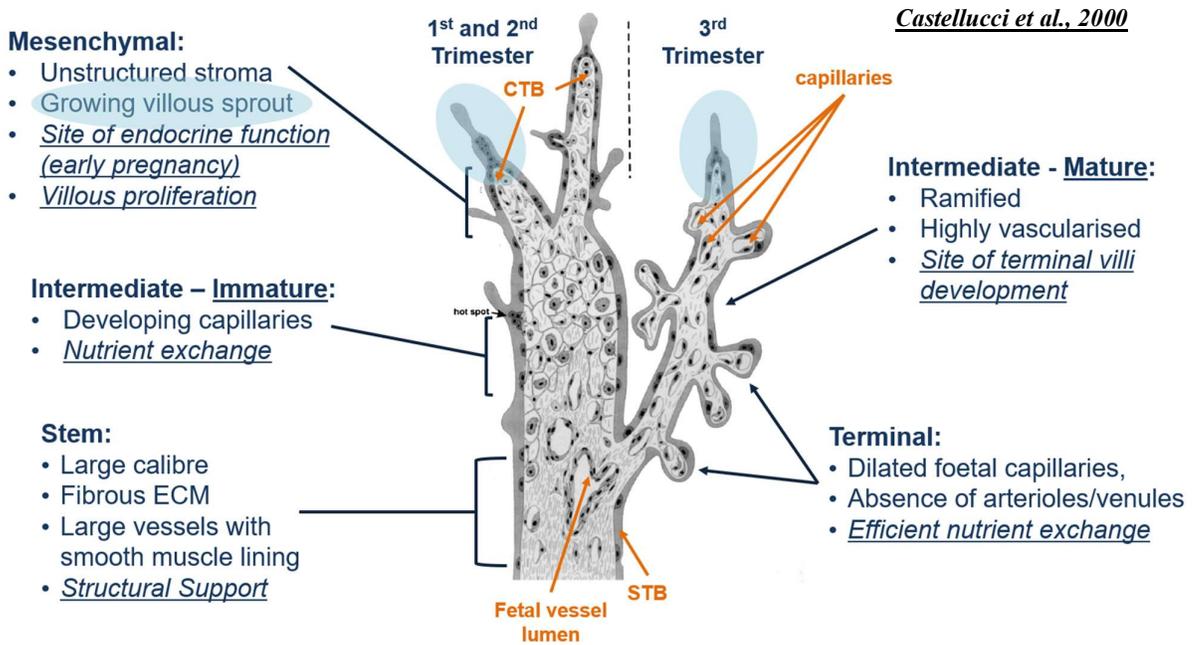
The villi in the placenta can be classified based on their level of vascularisation, complexity of the structure and cell types (**Figure 4a**). The features of each villous type in the developing placenta have been clarified by pioneering work based on scanning electron microscopy techniques from the team of Dr. Peter Kaufmann (Castellucci et al., 2000, 1990; Demir et al., 1989; Kaufmann et al., 1988). **Mesenchymal villi** are the first to develop and for this reason are the simplest in term of structure and composition. In these villi the stroma is unstructured, with few foetal capillaries that are not well developed and not branched. The STB layer at the surface is smooth and at the apex, the STB protrudes to form a growing villous sprout. The CTB layer below is continuous, with cells actively proliferating and fusing to the growing STB. This is the main villous type during the first stages of pregnancy, and the STB at this stage has key endocrine functions, while nutrient demand is low, for the developing embryo. With progression of pregnancy, these villi constitute the region of villous proliferation, but only a minimal portion of the mature placenta. These primitive villi develop and the region that connects to the chorionic plate undergoes morphological and functional changes, becoming the **villous stem**. Its function is to sustain and support the developing villous tree at the apical extremity, for this reason the structure becomes more fibrous thanks to foetal fibroblasts and stromal cells that deposit ECM. Additionally, the CTB layer becomes discontinuous since some are replaced by fibrin agglomerates. Foetal vasculature develops further inside the lumen, having endothelial cells as well as smooth muscle lining. However, the surface of the vessels in this section of the villous tree is limited. The developing villi transition into **immature intermediate villi**, when the overall surface is still ellipsoid and blood vessels are increasing in numbers and calibre. These villi are responsible for most of the nutrient exchange in the first

and second trimesters. **Mature intermediate villi** are ramified and highly vascularised, important site of differentiation of **terminal villi**. Terminal villi present numerous thin branches, filled with foetal capillaries, coiled, and twisted to fill the lumen. The CTB layer is discontinuous, the foetal vessel walls are simplified and lack the smooth muscle lining, so that the space between foetal and maternal circulation is reduced to the minimum, allowing for highly efficient exchange, meeting the demands of the foetus in the third trimester (Benirschke et al., 2012).

In terms of spatial organisation at term, each villous stem on the foetal side has developed in a full villous tree, originating from the villous stem on the chorionic plate, fully branched. Branches from each stem form a cotyledon on the maternal side. The villi that are through the whole depth of the decidual tissue and in which the STB contacts the maternal interface between decidua and myometrium are called anchoring villi, since they are anchored to the maternal tissue. The other villi float in the maternal blood that fills the lacunae, and the whole STB layer is in direct contact with maternal blood (Kaufmann et al., 1988; Wang and Zhao, 2010) (**Figure 4b**).

a)

Developmental Stages of Placental Villi



b)

The Villous Tree in Space

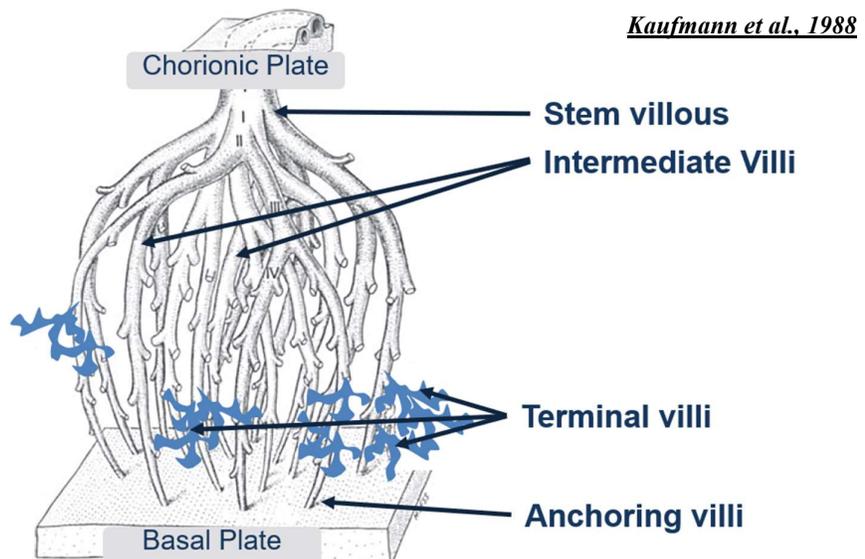


Figure 4. Developmental stages of placental villi (a) and their spatial organisation (b). Adapted from (Castellucci et al., 2000; Kaufmann et al., 1988). CTB = cytotrophoblast, STB = Syncytiotrophoblast, ECM = extracellular matrix.

Oxygen tension, maternal vascular remodelling, and placental vascularisation

During the first trimester of pregnancy, placenta develops in a state of hypoxia, where the average oxygen (O₂) tension is below 2.5%. This is due to maternal spiral arteries being temporarily plugged by endothelial EVT, impeding maternal blood to flood placental lacunae, allowing only plasma to pass through to the foetus (slice views of first trimester maternal intervillous space are empty of red blood cells, despite their small size). At the beginning of the second trimester, between 10-12 weeks of gestation, the plugs are progressively expelled, the arteries become functional and maternal blood fills the lacunae, increasing O₂ levels to tissue normoxia (~8.5%). Hypoxia in the first trimester is an important regulatory stimulus on trophoblast function and essential for the correct development of the placenta: it affects proliferation, fusion, and migration promoting invasion, as well as *de novo* vasculogenesis and angiogenesis (Genbacev et al., 1997; James et al., 2006). Between 16-18 weeks of gestation, once the maternal circulation has been established in the placenta, the increase in oxygen tension triggers further invasion of EVT in the decidua, to complete the spiral arteries remodelling that ensure a “high flow, low resistance phenotype”. By replacing endothelial and smooth muscle cells in the arteries with endothelial EVT cells, the responses of maternal arteries to changes in blood pressure are inhibited, probably also decreasing the shear stress at the surface of the STB, this stress being an important issue in the ageing of this tissue. These changes ensure a large blood supply to the developing foetus, apt at meeting the increasing nutritional demands, especially from the foetal brain, requesting up to 50% of the nutrients brought by the maternal blood. In addition to the morphological vascular remodelling of existing vessels, maternal arterioles and veins develop *de novo* to increase the number of vessels that supply the lacunae. The foetal side of the placenta needs to mirror the vascular development observed in the mother, this is achieved through vascular development within the villi. Pluripotent mesenchymal cells that migrated into the placenta from the developing embryo are the stem cell precursors of endothelial and erythrocytes that will later drive the process of vasculogenesis and angiogenesis, which begins approximately at 3 weeks of gestation in mesenchymal villi (C.-Y. Chen et al., 2015; Huppertz et al., 2014a; Wu et al., 2018).

The development of blood vessels in the lumen of developing villi is first initiated through *de novo* vascularisation. In the first trimester, foetal Hofbauer cells (placental macrophages) in the

villous stroma and CTBs secrete high levels of Foetal Growth Factor (FGF) proteins, primarily FGF1 and FGF2, that activate mesenchymal precursor stem cells to differentiate into endothelial cells, initiating vasculogenesis. FGF is also involved in angiogenesis by promoting endothelial cells proliferation; moreover, it stimulates CTB self-renewal in positive feedback. At term, FGF is mainly expressed by the STB. Members of the Vascular Endothelial Growth Factor (VEGF) family of proteins are potent pro-angiogenic factors. In the placenta, VEGFA, B, C and D are secreted by CTBs, Hofbauer, endothelial cells and pericytes. They bind to receptor tyrosine-kinases, that upon ligand-binding undergo activation through phosphorylation and dimerization, resulting in intracellular signalling cascades that promote proliferation, angiogenesis, causing formation of capillary networks and blood cells in their lumen. Among VEGF receptors, VEGF Receptor 1 (VEGFR-1) is also known as Fms Related Receptor Tyrosine Kinase 1 (FLT-1), VEGFR-2 known as Flk-1/KDR and FLT-4 (VEGFR-3). FLT1 and Flk-1/KDR are expressed on the surface of mesenchymal precursor cells, and once activated promote cell differentiation into vascular and lymphatic cells. Endothelial cells are the primary target of VEGF signalling, which promotes proliferation and endothelial cells activation resulting in blood vessel branching through sprouting of endothelial cells from an existing blood vessel (Demir et al., 2004; Wang and Zhao, 2010). VEGF signalling is potentiated by the action of placental growth factor (PlGF) that competes for the binding of FLT-1, increasing VEGF bioavailability and activation of VEGFR-2 associated with recruitment of myeloid cells and macrophages promoting vessel growth. Many different PlGF isoforms exist, secreted in the placenta by GC trophoblasts that promote vascularisation of the placenta as well as the decidua. VEGF is also the main target gene of Hypoxia-inducible Factors 1 α (HIF-1 α) and 2 α (HIF-2 α), which in the first trimester are stabilised by the hypoxic conditions in the placental bed; this results in activation of VEGF signalling in endothelial cells, secretion of pro-angiogenic factors from trophoblast and decidual cells, EVT invasion (Chiarello et al., 2020; Wang and Zhao, 2010; Zygmunt et al., 2003).

The functions of the placenta

Nutrient, gases and anabolites exchange

In a broad sense, and among other functions, the placenta performs the function of a lung by letting gas diffusion occur between maternal and foetal blood cells, of an intestine by facilitating nutrient exchange from the maternal circulation to the foetus, and as a kidney carrying out the clearance of metabolic wastes from foetal circulation.

Respiratory gases O₂ and carbon dioxide (CO₂) freely diffuse from maternal to foetal circulation and *vice versa*, through the permeable membrane of the STB which is in direct contact with maternal blood in the lacunae. Depending on the stage of gestation, the thickness of the cell layers between maternal and foetal blood varies. At the beginning of gestation, multiple layers of CTBs, ECM and endothelial cells separate the foetal blood stream from the STB and the maternal blood. However, with the progression of gestation, the foetal capillaries in terminal villi are fully dilated and come in close proximity to the STB, while CTBs and ECM were progressively reduced. In the third trimester, < 5 µm separate the two circulatory systems, fostering efficient diffusion rates (Benirschke et al., 2012; Demir et al., 1989). The directionality of oxygen transfer from maternal erythrocytes to foetal cells is favoured by specific expression of the foetal Hemoglobin which has a higher affinity for molecular oxygen, favouring release of CO₂ from foetal erythrocytes and O₂ uptake (Engel et al., 1969; Nye et al., 2018).

Nutrient and waste exchange occurs primarily at the STB membrane facing the maternal blood in the lacunae. Since the STB is a unique structure that covers the surface of all villi in the placenta, it acts as a barrier making it possible to regulate the transfer of specific molecules. Depending on the nature of the molecules, the transfer will occur either from mother-to-foetus or from foetus-to-mother, for nutrients and waste respectively. In the instance of nutrients for example, once the molecules have crossed the STB membrane, they need to cross the STB cytoplasm and the foetal-facing membrane, as well as the stroma of placental villi, endothelial lining of foetal blood vessels, to then be carried to foetal tissues by the foetal circulatory system. The primary energy source of foetal metabolism is glucose which is preferentially uptaken from maternal blood over other carbon sources, mainly through glucose transporter proteins

(GLUTs). GLUTs are 12 transmembrane-pass proteins that facilitate glucose transfer through the plasma membrane following diffusion, resulting in a flux of glucose from the maternal blood to the foetal glucose-depleted blood stream. Different GLUTs are expressed by the placenta, depending on the trimester and cell types; GLUT1 is ubiquitous and equally expressed throughout gestation, other transporters are GLUT3, 4 and 12 respectively expressed by endothelial, stromal, smooth muscle and stromal cells at term (Baumann et al., 2002; Gude et al., 2004; Xing et al., 1998). However, in the STB, glucose molecules are also used for the metabolic needs of the STB itself through anaerobic glycolysis pathways and aerobic respiration in the mitochondria. High metabolic rates have been registered in CTB as well, and placental anaerobic metabolism results in production of high volumes of lactate (Deng et al., 2014; Kolahi et al., 2017; Suidan et al., 1984).

Other key nutritional molecules include water, amino acids, lipids and inorganic ions. Water simply diffuses through the placental cells between mother and foetus, guided by the osmotic pressure; this diffusion is aided by the expression of aquaporins (AQP), transmembrane proteins that create pores in the plasma membrane for the free diffusion of water molecules through the hydrophobic bilayer (Verkman and Mitra, 2000). Different AQP genes are expressed by trophoblast cells in the placenta as well as by foetal membranes, and the flux of water has been linked with volume changes in amniotic fluid (Beall et al., 2007; Liu et al., 2004; Mann et al., 2002; Wang et al., 2001).

Foetal blood stream is enriched in amino acids compared to maternal blood levels in the placenta, this is achieved by active transport of amino acids to the foetal blood stream mediated by sodium-dependent as well as sodium-independent transporters. These transporters are transmembrane protein complexes specialised in the transfer of different classes of amino acids, based on their charge at physiological pH (Jansson, 2001). Similarly, lipid transport can occur by direct diffusion of lipids from the maternal blood to the foetus, thanks to the ability of lipid molecules to diffuse through the lipid bilayer. However, active lipid transporters make possible the lipid enrichment in foetal blood. In the maternal blood stream, lipids are transported as lipoproteins, bound to specific transporters such as albumin; once they reach the STB layer, lipid molecules are released by action of lipases expressed on the cell surface (Herrera et al., 2006). Finally, ionic pumps use ATP to actively transfer inorganic ions such as potassium, sodium, calcium and phosphate (Gude et al., 2004).

To better characterise placental physiology, mathematical modelling has recently been applied to *in vitro* placental perfusion models of isolated cotyledons as well as *in vivo* magnetic resonance imaging (MRI) of the placenta during gestation. These approaches have allowed to better characterise the impact of nutrient transport, blood flow, placental and foetal metabolism on the overall placental perfusion, as well as clarified the role and function of specific molecular transport systems such in the case of cortisol (Byrne et al., 2021; Lewis et al., 2020; Stirrat et al., 2018).

Immunotolerance

Immunology of pregnancy is a vast topic, beyond the subject of my thesis, therefore I will not go into details on this subject but just mention a few key elements.

The term immunotolerance indicates the unresponsiveness of the immune system to a subset of antigens, usually associated with the identification of cells as “self”. The determination of “self” and “non-self” is carried out by immune cells, such as lymphocytes T, B and Natural Killer (NK) cells, that recognise antigens expressed on the surface of cells in the body. Each lymphocyte expresses unique antigen-specific receptors. During lymphocyte development in lymphatic organs such as the thymus, antigen-presenting cells expressing self-antigens MHC complexes train the immune system so that lymphocytes that can bind self-antigens without mounting an immune response are positively selected, while self-reacting lymphocytes are eliminated (von Boehmer and Kisielow, 1990). Self-antigen MHC I complexes are expressed on the surface of cells in the tissues and define the “self”. MHC genes in human are also known as the Human Leukocyte Antigen (HLA), encompassing genes located in a cluster on human chromosome 6. Each HLA locus presents multiple alleles which increase the spectrum of inter-individual differences at the level of “self” (Cruz-Tapias et al., 2013).

During pregnancy, a series of immunological mechanisms act at the level of the maternal-foetal interface to reduce the reactivity of the maternal immune system to the foreign cells that belong to the foetus, which indeed carries a distinct genetic material and therefore should be otherwise recognised as “non-self”. One of the key mechanisms to promote maternal immunotolerance towards the foetus is linked to the specific expression of HLA-G by trophoblast cells. Among the other HLA genes, HLA-G has the least number of alleles, when compared to highly polymorphic HLA-A and B loci, reducing the chances of foetal placental cells to be recognised as “non-self” by the maternal immune system. Invading EVT_s secrete HLA-G-derived peptides in the maternal decidua that bind to receptors on lymphocyte T and NK cells, effectively inhibiting the immune response (Hunt et al., 2005). Other mechanisms include the expression of immunomodulators galectins (LGALS genes) by the STB during the first trimester when the STB invades deep into the maternal decidua, negatively regulating T cell populations and altering their cell surface protein profiles (Balogh et al., 2019).

Endocrine function

The STB is the primary site of endocrine secretion in the placenta. Hormones synthesised by the placenta (together with the network of placental-borne imprinted genes, or under their controls (Fauque et al., 2010; Senner and Hemberger, 2010) strictly regulate the progression of the gestation by monitoring and adjusting foetal growth and trophoblast cell differentiation to ensure that placental function mirrors the needs of the developing foetus. The hormone of excellence for pregnancy is the human chorionic gonadotropin (hCG), a protein dimer of hCG α and hCG β subunits. It can be found in maternal blood as well as in urine, where it is detected by commercial pregnancy tests (Wang and Zhao, 2010). Before implantation, trophoblast cells in the blastocyst start to secrete hCG to promote progesterone synthesis by the cells of the corpus luteum (CL) inside the ovary, the degenerated oocyte follicle left behind after ovulation. hCG promotes vascularisation of the CL and progesterone release that stimulates decidualisation of the uterine wall, inhibiting the shedding of the decidua and maintaining an optimal uterine environment for implantation and pregnancy, progesterone also having anti-inflammatory properties (Jarvela et al., 2008) (**Figure 5**).

While CTB can secrete hCG, the major volumes are later secreted by the expanding STB, and hCG directly stimulates CTB syncytialisation promoting placental development and growth. After 4 weeks of gestation, the STB can directly release enough levels of progesterone and estrogens to regulate the uterine environment, without the need for the CL. However, high levels of hCG are continuously produced throughout gestation to stimulate other key functions: angiogenesis of the uterine vasculature, CTB differentiation into STB, inhibition of myometrium contractions during pregnancy, CTB release of metalloproteinases to favour invasion, placental growth and expansion (e.g. cell proliferation, vasculogenesis and angiogenesis) (Cole, 2010). Additional endocrine products of the STB include growth hormones, human placental growth hormone (hPGH) and human placental lactogen (hPL) that are similar to the maternal growth hormone secreted by the pituitary gland and regulate glucose and lipid metabolism in the placenta to promote foetal growth. Insulin-like growth factors (IGF) target CTBs activating proliferation and differentiation pathways (Forbes and Westwood, 2008; Handwerger and Freemark, 2000). As mentioned before, STB is also a key site for PlGF and VEGF production involved in angiogenesis in placental villi as well as in the maternal decidua (Shiraishi et al., 1996; Shore et al., 1997).

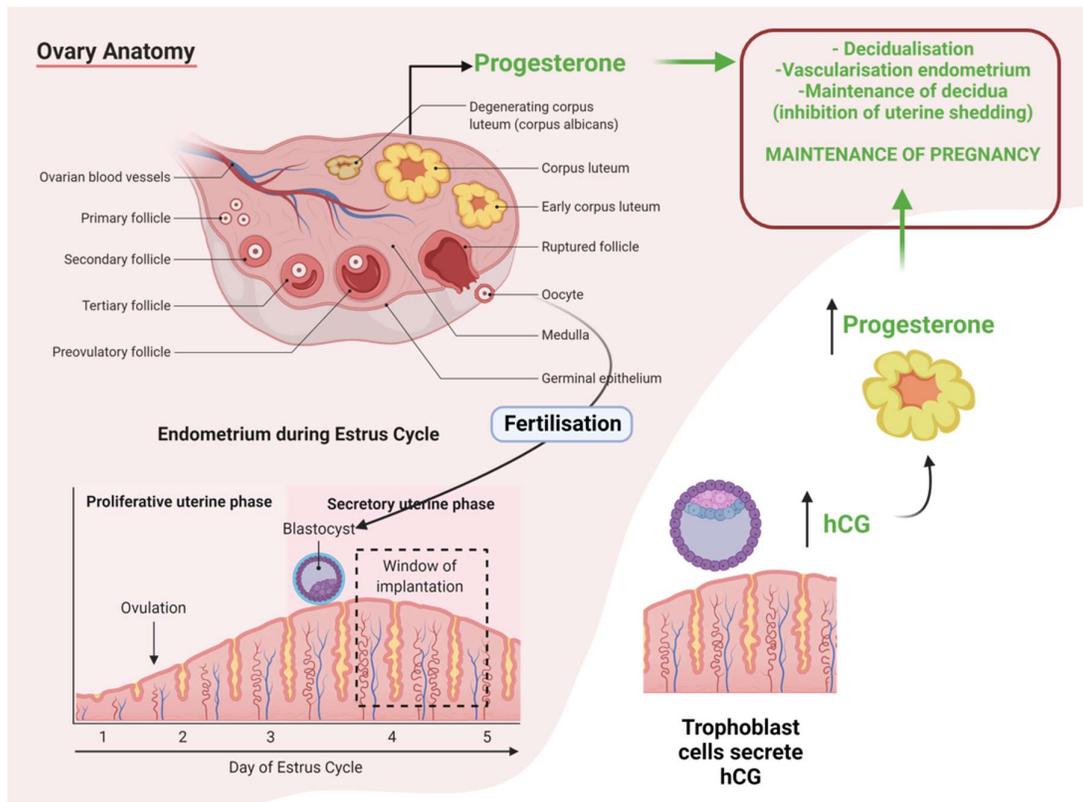


Figure 5. Overview of hCG effects on progesterone production by the CL. During the menstrual cycle, after ovulation the ruptured follicle deteriorates in the CL that produces progesterone, stimulated by maternal luteinizing hormone (LH). This results in enhanced decidualisation and vascularisation of the endometrium, primed to receive a potential embryo. Once fertilisation has occurred, the zygote develops into the blastocyst and early trophoblast cells secrete hCG which binds to the LH receptors on the CL, stimulating progesterone production and maintenance of maternal decidua and pregnancy. Image created with [Biorender.com](https://www.biorender.com)

Given this key endocrine function of the placenta, the effects of endocrine disruptors on placental function and pregnancy, as well as on foetal health, are a pressing matter; these environmental pollutants and pharmaceutical agents can in fact cross the placental barrier and alter the normal course of pregnancy (Gingrich et al., 2020; Tang et al., 2020). For instance, the plastic derivative bisphenol S has been found to significantly reduce the hormonal output of the sheep placenta, explained by an abnormal trophoblast differentiation resulting in reduced syncytialisation (Gingrich et al., 2018).

Pregnancy pathologies

During gestation, pre-existing maternal health conditions, genetic predispositions, inflammatory and developmental complications can result in the development of pregnancy pathologies that put mother and baby at risk.

Some pathologies are directly linked with the site of placental development and the degree of invasion within the uterine walls. Pregnancies with placenta previa are characterised by the development of the placenta at the level of the cervix, which results in vaginal bleeding during gestation and can cause heavy bleeding during labour (Anderson-Bagga and Sze, 2021; Rosenberg et al., 2011). Most severe conditions include the range of placenta accreta, increta and percreta. In patients affected by these pathologies, the placenta invades the uterine wall too deeply, reaching the myometrium in accreta, invading the myometrium for more than 50% of its width in increta, and crossing the myometrium to reach internal organs such as the bladder and the intestine in placenta percreta (**Figure 6**). These conditions complicate placental detachment after labour, often resulting in incomplete removal of the placenta and heavy bleeding, requiring to perform Caesarean delivery before term and often necessitating the removal of the uterus by hysterectomy (Ali and Chandraharan, 2021).

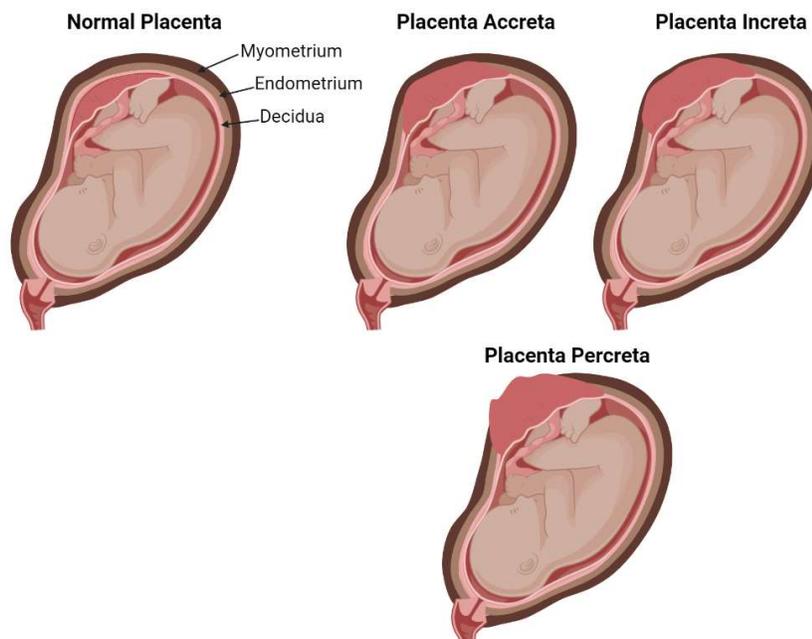


Figure 6. Degree of placental invasion in normal placenta, placenta accreta, increta and percreta.

Image created with [Biorender.com](https://biorender.com)

Abnormal placental development in relation to reduced placental invasion and incomplete vascular remodelling has been implicated in the aetiology of both PE and IUGR. PE is defined as *de novo* hypertension and elevated proteinuria after 20 weeks of gestation, in these pregnancies the placenta shows extensive oxidative damage, accompanied by abnormal release of placental factors that cause endothelial dysfunction (ED), culminating in hypertension and systemic organ damage such as in maternal kidney, heart and brain (Ives et al., 2020; Wang et al., 2021). Similarly, impaired placental development causes reduced blood flow to the foetus in IUGR pregnancies, characterised by metabolic insufficiencies and hypoxia that hinder foetal growth causing an *in utero* growth deceleration, with detectable halt in the growth curve. IUGR foetuses often are born small for gestational age (SGA), when birth weight falls below the 10th percentile for equivalent gestational age. IUGR is a complex multifactorial condition, resulting from a combination of a wide range of risk factors coming from the mother, the baby and the placenta (Fleiss et al., 2019; Sharma et al., 2016).

When the endocrine function of the placenta is altered and accompanied by an incomplete adaptation of the maternal hormonal response, Gestational Diabetes Mellitus (GDM) can develop during gestation. This condition is a metabolic disorder of pregnancy characterised by reduced glucose tolerance diagnosed in the second and third trimesters. The most common outcomes include overgrown foetuses, that can develop obesity and metabolic syndromes later in life, while for the mother hypoglycaemia and type-2 diabetes post-partum (Carrasco-Wong et al., 2020; Desoye and Hauguel-de Mouzon, 2007). GDM and PE appear to be closely interlinked since women with GDM show elevated risks of developing PE during gestation (Nerenberg et al., 2013), while women that have undergone a preeclamptic pregnancy have increased risks of GDM in subsequent pregnancies (Lee et al., 2017); this is probably due to the role of anomalies linked with the cardiovascular systems that can play a role in the aetiology of both conditions (e.g. cardiovascular disease, obesity, weight gain during pregnancy) (Ottanelli et al., 2020).

The presence of PE, IUGR and GDM during pregnancy dramatically increases the risks of intra-uterine fetal demise (IUFD) and stillbirth (Fu et al., 2019; Harmon et al., 2015; Stacey et al., 2019). The developmental defects of the placenta observed in PE and IUGR resulting in reduced placental perfusion, as well as the placental lesions characteristic of GDM are among the placental abnormalities causative of stillbirth and IUFD. The placental abnormalities conducive to incomplete maternal artery remodelling are amongst the most common causes of

IUFD and stillbirth, observed in ~20-35% of these pregnancies, depending on term (Man et al., 2016; Manocha et al., 2019). Given the central role of the placenta in granting oxygen and nutrients to the fetus, the fact that placenta lesions can dramatically affect the neonatal survival should come to no surprise.

The causes of the incomplete vascular remodelling observed in PE and IUGR have not been completely elucidated. However, a reduced immunotolerance of the uterine environment has been implicated in shallow placentation: invading foetal EVT are attacked by the maternal immune system and unable to migrate deep into the maternal uterine wall (Geldenhuys et al., 2018; Robertson et al., 2018). As previously mentioned, maternal immunotolerance is achieved through a series of adaptive mechanisms that include the expression of specific surface markers by foetal cells and the secretion of anti-inflammatory cytokines to dampen the maternal immune response by both the placenta and the maternal decidua. Immunological imbalance is also the pivotal cause of recurrent pregnancy loss, defined as the occurrence of three consecutive miscarriages or spontaneous pregnancy loss before 20 weeks of gestation (Ford and Schust, 2009). If the trophoblast cells are unable to successfully establish an initial immunotolerance (e.g. insufficient cytokine secretion, reduced HLA-G expression, reduced trophoblast autophagy), and the maternal decidua cannot further promote this immunosuppressive environment, the maternal immune response can result in rejection of the conceptus and spontaneous pregnancy loss. The maternal antigens developed during this first pregnancy have the potential to mount an even stronger immune response to the paternal antigens expressed by the foetus during a consecutive pregnancy (Deshmukh and Way, 2019; Guerrero et al., 2020; Peterson et al., 2020; Tan et al., 2020).

To improve maternal and foetal survival, put in jeopardy by pregnancy pathologies during gestation, programmed or emergency Caesarean delivery is often performed, and it usually results in preterm birth (< 37 weeks of gestation) (Soltanifar and Russell, 2012). Programmed delivery and removal of the placenta is still the only clinical solution to treat preeclamptic symptoms. However, prematurity itself is considered the highest risk factor for foetal demise and has a huge impact on hospitalisation and long-term care of premature infants, due to the resulting developmental abnormalities (Glass et al., 2015; Soleimani et al., 2014).

In my project, for their impact on maternal and foetal survival, as well as invalidating long-term effects later in life, I have focussed on pregnancy pathologies PE and IUGR which are discussed in the following sections.

Preeclampsia

PE is traditionally estimated to affect 2-10% of pregnancies world-wide (Duley, 2009; Osungbade and Ige, 2011). However, more recent estimates have found a small decrease also in developing countries with a global incidence of 4.6%, varying from 1-2.8% in Europe and Asia, up to 5.6% in Africa (Wang et al., 2021; Yang et al., 2021).

The preeclamptic phenotype includes *de novo* hypertension and elevated proteinuria after 20 weeks of gestation. If left untreated, preeclamptic patients develop systemic side effects such as heart and kidney lesions, HELLP (Hemolysis, Elevated Liver enzymes and Low Platelets) syndrome that affects liver function and platelet homeostasis. Moreover, preeclampsia can degenerate into eclampsia where the appearance of brain oedema causes seizures and often results in maternal and foetal demise (Ghulmiyyah and Sibai, 2012). The only effective treatment of PE is the delivery of placenta, resulting in the termination of pregnancy. Preeclampsia may require induced labour and delivery for the sake of the mother, and be therefore a cause of premature birth, induced by medical decisions. Delivery by Caesarean-section may be necessary if there are clinical or obstetric conditions that require a speedy delivery (Ananth and Vintzileos, 2006; Kim et al., 2010). Therapeutic treatments aimed at the managing of the hypertensive symptoms are also administered (e.g. aspirin and α -microglobulin), but their effectiveness is strongly dependent on the early diagnosis of PE, at least for aspirin (Anderson et al., 2015; Gunnarsson et al., 2017).

ED is a hallmark of PE, with this term we indicate the presence of anti-angiogenic, pro-inflammatory and cytotoxic molecules in the maternal blood, which led to the previous name of preeclampsia as “gravid toxemia”. These molecules are believed to be released by the placenta and/or by maternal tissues, the primary target being endothelial cells of the maternal cardiovascular system which are abnormally activated to produce vasoconstriction factors, and ultimately undergo cellular senescence. The overall outcome is systemic hypertension, followed soon by damage to the structural integrity of capillaries, especially in the kidney at

the level of the delicate structure, which is the glomerulus, causing proteinuria (Raymond and Peterson, 2011).

Two major issues about the necessity of a clinical treatment of PE cases are relative to the current limitations in early detection of women that will eventually develop PE (pre-symptomatic), and to the fact that a preeclamptic pregnancy has shown to significantly increase risks associated with the development of cardiovascular disease (CVD) later in life, as well as nephropathies and alterations of healthy brain ageing (Basit et al., 2018; Covella et al., 2019; Turbeville and Sasser, 2020).

Current hypotheses on the aetiology of PE

Different hypotheses have been brought forward over the years, and to this date there is no definitive answer on the mechanisms behind PE aetiology. However, a consensus in the scientific community has been reached regarding key features of the PE phenotype at the physiological and molecular level, as summarised in **Figure 7**, and the existence of two distinct populations of PE patients.

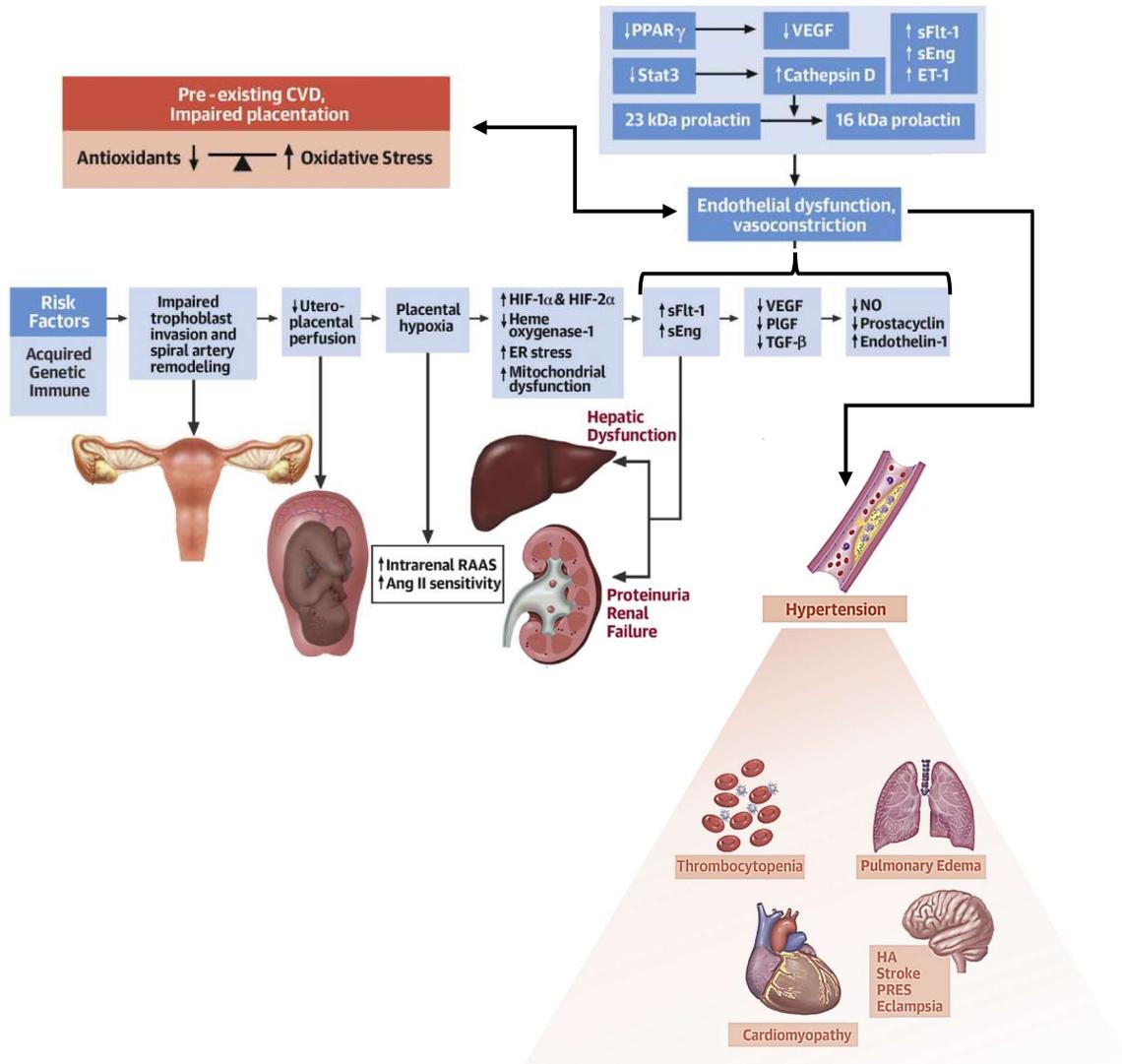


Figure 7. Overview of the causative steps of PE and its systemic effects. Impaired placentation is a result of genetic and immune predisposition, in the mother as well as in the fetus, which results in abnormal placental development and reduced perfusion. Oxidative stress of the placenta results in the deregulated release of bioactive molecules and the development of ED which is causative of CVD as well as aggravated by pre-existing CVD, resulting in hypertension. Systemic effects include thrombocytopenia, pulmonary edema, cardiomyopathy, and neurological conditions such as stroke, headache (HA), posterior reversible encephalopathy syndrome (PRES) and eclampsia. Figure adapted from (Ives et al., 2020). Ang II = angiotensin II, ER = endoplasmic reticulum, ET-1 = endothelin 1, HIF = hypoxia-inducible factor, NO = nitric oxide, PIGF = placental growth factor, PPAR γ = peroxisome proliferator-activated receptor γ coactivator, RAAS = renin-angiotensin-aldosterone system, sEng = soluble endoglin, sFLT = soluble fms-like tyrosine kinase, Stat3 = signal transducer and activator of transcription3, TGF = transforming growth factor, VEGF = vascular endothelial growth factor.

In ~15% of PE pregnancies symptoms appear before 34 weeks of gestation and histological analysis of the placenta shows shallow invasion in the maternal uterine wall, which is accompanied by incomplete spiral arteries remodelling as detected by Doppler assessment (Burton et al., 2009; Matijevic and Johnston, 1999; Mayrink et al., 2019; Palei et al., 2013). This patient group is defined as early-onset PE (EO-PE) and IUGR is usually a common complication, due to the reduced vascular perfusion of the placenta that results in reduced nutrients flux to the foetus and drastically impaired foetal growth. Because of the severity of the maternal and/or foetal symptoms, these pregnancies often result in premature birth. EO-PE is therefore suggested as the result of faulty placental development, with impaired placental invasion and dysfunctional EVT which fails to complete spiral arteries remodelling. EVT inability to migrate deep within the maternal decidua is believed to be intimately linked with defects in the establishment of immunotolerance, leading to a placental development being held back by the maternal decidual immune system. In this patient group, placenta has a key role in PE aetiology, since the reduced blood flow to the villi results in metabolic dysfunction, oxidative stress, tissue damage and abnormal release of pro-inflammatory, anti-angiogenic molecules in the maternal circulation which correlate with ED (Raymond and Peterson, 2011; Veerbeek et al., 2015).

In contrast, late-onset PE (LO-PE) occurs after 34 weeks of gestation and corresponds to ~85% of total PE pregnancies. In these patients, normal placentation is observed usually without concomitant IUGR (Mayrink et al., 2019). In this case, since the placental development and morphology appear normal, the ED phenotype and hypertension seem to originate from the inability of the maternal cardiovascular system to sustain the toll of pregnancy, sometimes as a result of pre-existing syndromes and pathological conditions such as CVD risk factors, obesity and diabetes. Once the preeclamptic symptoms develop however, systemic damage is sustained by the placenta as well as the other organs (e.g. heart, kidney ...), and therefore molecular alterations detectable in PE placentas from EO-PE as well as LO-PE have a strong overlap (Melchiorre et al., 2021; Raymond and Peterson, 2011; Veerbeek et al., 2015). Even though the severity of LO-PE can be extreme, EO-PE is generally the severest form, with symptoms exacerbating systematically through time (Lisonkova and Joseph, 2013).

One controversial issue in the characterisation of PE is the detection of oxidative stress markers in placental extracts, such as increased antioxidant molecules (i.e. glutathione), these findings being not always consistent across studies, but are usually interpreted as a symptom of hypoxia

and reperfusion injury. Hypoxia would be explained by the reduced blood flow in maternal lacunae, not sufficient to meet foetal demands, which stimulates physiological reactive oxygen species (ROS) involved in signalling cascades apt at improving cellular fitness under hypoxia. However, under chronic hypoxia, due to sustained impaired placental morphology for the duration of gestation, excessive ROS result in cellular damage and release of necrotic, pro-inflammatory and anti-angiogenic factors (Aouache et al., 2018; Sánchez-Aranguren et al., 2014). Alternative hypotheses have suggested that oxidative stress could be rather caused by hyperoxia, since higher O₂ pressure has been recorded in placental villi from preeclamptic pregnancies (Huppertz et al., 2014b; Kakogawa et al., 2010). In the presence of excess O₂, accelerated mitochondrial respiration can result in release of free electrons from the electron-transport chain and generation of potent free-radicals, resulting in structural damage to cell membranes, DNA and organelles (Zhao et al., 2019). Therefore, both hypoxia and hyperoxia would cause similar outcomes, resulting in placental tissue damage and ED. In cellular systems, a frequent model is the use of hypoxia/reoxygenation cycles to induce ROS production; this could indeed mimic the actual *in vivo* exposure of these cells in preeclampsia (Pennington et al., 2012; Sagrillo-Fagundes et al., 2018).

Clinical, morphological, and molecular alterations in PE

From a clinical and diagnostic standpoint, the identification of blood biomarkers is a powerful tool for easy and direct diagnosis of disease predisposition and phenotype. Key biomarkers of PE have been established in the maternal blood levels of soluble form of VEGF receptor FLT1, sFLT-1, as well as PlGF. The ratio of sFLT-1/PlGF has shown promising predictive power over the development of LO-PE (Maynard et al., 2003; Souders et al., 2015). However, prompt diagnosis of EO-PE is still challenging. sFLT-1 is the product of alternative splicing of the precursor mRNA which encodes for more than 40 exons. In its native form, FLT1 is membrane-bound and as discussed in previous sections it promotes angiogenesis downstream of VEGF signalling in endothelial cells. However, the short isoform sFLT-1 encodes for the soluble extracellular domain alone and once released in the blood stream binds to free VEGF reducing its bioavailability (Palmer et al., 2015; Roberts and Rajakumar, 2009; Thomas et al., 2009). This is believed to be the primary cause of ED in PE pregnancies. Other similar patterns have been observed for angiogenesis factors such as Endoglin (ENG), for which similarly soluble

short isoforms (sENG) are found upregulated in maternal blood and correlate with ED phenotype (Blanco and Bernabeu, 2011).

In terms of morphological alteration of the placenta, other than the reduced decidual invasion and incomplete maternal vascular remodelling, morphological aberrations have been found at the level of the STB which often shows signs of damage, insufficient development, and early aging. This has been proposed to be a consequence of abnormal CTB differentiation (Bastida-Ruiz et al., 2019; Colson et al., 2020; Mariana A Costa, 2016; Roland et al., 2016; Wang et al., 2019). This topic will be further developed and discussed in **Chapter 4**.

To better understand the differences between PE placentas and placentas from control patients, at low risk of complications, extensive OMICS approaches have been followed. The analysis of the placental transcriptome has identified hallmark PE genes which are consistently deregulated in PE placentas. Among these, there are protein-coding genes vascular proteins FLT1 and ENG, metabolic regulators such as leptin (LEP), TGF β signalling effectors inhibin α (INHA) and glycoprotein Follistatin Like 3 (FSTL3), Pregnancy-associated plasma protein A2 (PAPPA2) a metalloprotease that regulates IGF bioavailability by cleaving IGF receptors, serine protease HTRA1 involved in invasion and immune response (Leavey et al., 2016; Saei et al., 2021; Söber et al., 2015; Vaiman and Miralles, 2016; Vennou et al., 2020).

Key regulatory non-coding RNAs (ncRNAs) are also deregulated in PE and have been recently reviewed in (Sun et al., 2021). miR-210 and miR155 are microRNAs (miRs) respectively involved in regulation of hypoxic response and blood pressure through VEGF signalling, both molecules have been found upregulated in PE placentas as well as maternal blood and are implicated in endothelial and trophoblast function (Pineles et al., 2007; Shen et al., 2018; Yang et al., 2017; Zhao et al., 2014). Among long non-coding RNAs (lncRNAs) deregulated in PE we consistently find Maternally Expressed 3 (MEG3) and Metastasis associated lung adenocarcinoma transcript-1 (MALAT-1). MALAT-1 is found downregulated in PE placentas and has been associated with EVT migratory and proliferative phenotypes, thought to be involved in promoting spiral artery remodelling mediated by EVTs, as well as regulating angiogenesis and vascularisation during placentation (H. Chen et al., 2015; Li et al., 2017; Tseng et al., 2009). MEG3 instead is downregulated in PE and has been shown to have anti-apoptotic and pro-migratory effects in trophoblast cell models (Song et al., 2017; Zhang et al., 2015). Recent efforts have focused on integrating deregulation in regulatory non-coding RNAs

and protein-coding genes to clarify the regulatory landscape of PE (Inno et al., 2021; Medina-Bastidas et al., 2020).

Epigenetic regulation such as DNA methylation can dynamically alter gene expression in response to external stimuli, while simultaneously conferring hereditary patterns to such modifications, given the fact that DNA methylation signatures can be copied in daughter DNA strands during cell mitosis. These phenomena have nurtured the frightening concept that *in utero* environment defined by pregnancy pathologies will deeply alter the transcriptomic profile of the foetus and result in the development of pathologies in the adult (Gluckman et al., 2008). Analysis of DNA methylation changes in PE placentas have identified genes whose promoter is under strong epigenetic regulation. For example, the promoters of TIMP Metalloproteinase Inhibitor 3 (TIMP3) and serine protease inhibitor alpha-3 (SERPINA3) genes are strongly hypomethylated in PE placentas (Chelbi et al., 2007; Yuen et al., 2010). These and similar findings sparked hope for the identification of DNA methylation signatures associated with disease.

The changes detected in the preeclamptic placenta at the transcriptomic and epigenetic level are a snapshot of the gene expression and regulatory landscape of the placenta at the moment of collection of the samples. The differences observed in respect to control samples of similar gestational age are therefore a complex combination of genes and regulatory elements that when altered contribute to the aetiology of the disease, as well as genes and epigenetic signatures that have been altered because as a consequence of the diseased phenotype itself. We know that PE placentas are characterised by signatures of Oxidative Stress and ED, which are believed to develop early on as a result of faulty placental development (Ferreira et al., 2020; Granger et al., 2001; Valencia-Ortega et al., 2019). Which in turn will cause the PE phenotype and subsequent transcriptional changes. For instance, the PAPP2 gene consistently found upregulated in PE placentas has been found to be deregulated as a consequence of the oxidative stress that characterises the PE placenta (Lamale-Smith et al., 2020; Macintire et al., 2014; Wagner et al., 2011).

The use of *in vivo* and *in vitro* models allows to evaluate the direct role of specific genes and regulatory elements on the transcriptome and epigenome. These models offer the unique possibility to assess the changes in a time-course framework making it possible to elucidate the temporal sequence of changes that occur in the PE placenta. In particular, the recent

development of placental organoids will greatly contribute to understanding how specific candidate genes are involved in regulating placental function at the level of the different cell types, cell-cell interaction as well as on paracrine and autocrine signalling mechanisms (Alzamil et al., 2021; Sheridan et al., 2020; Turco et al., 2018).

Looking for genetic markers of predisposition

Given the challenges of early diagnosis associated with blood biomarkers, which often manifest once the disease has already caused systemic damage, genetic studies have been carried out to identify predisposing genetic variants associated with PE. Some studies have indeed managed to expand the list of candidate genes which could have a central role in PE aetiology, such is the case for transcription factor STOX1 (van Dijk et al., 2005). Genome-Wide Association Studies (GWAS), making use of large sample sizes (in the tens of hundreds), try to look for enrichment of variant alleles in the disease group compared to healthy controls. GWAS conducted in the placenta have identified Single Nucleotide Polymorphisms (SNPs) upstream of the FLT1 locus, rs4769613; the risk allele (T) is associated with PE (McGinnis et al., 2017). The major pitfall of GWAS is in fact the absence of functional insights into the potential mode-of-action of such risk alleles on disease phenotype (Ishigaki, 2021; Laivuori, 2013; Tam et al., 2019). However, the use of combinatorial approaches that exploit knowledge from different types of datasets helps with drawing these functional conclusions. Recently, the rs4769613 locus was genotyped in a cohort of placental samples including 57 PE and 277 control; the presence of the risk allele correlated with changes in placental gene expression level of FLT1 only in the PE group, defining rs4769613 as a conditional expression-Quantitative Trait Locus (eQTL) (Kikas et al., 2020). This suggests that the risk variant, when present in placentas from mothers with preeclampsia, favours the increase in expression of the FLT1 gene and therefore could be located in a regulatory region for the gene itself, targeted by regulatory factors that are expressed specifically in the PE placenta alone.

In this project we are interested in genome-wide expression-Quantitative Trait Loci (eQTL) analysis, as investigative approach that combines transcriptomic with genetic data to look for mechanisms of genetic influence on gene expression. Current state-of-the art of eQTL datasets in human placenta as well as our own experimental design and results are presented in **Chapter 1**.

Intra-uterine growth restriction

IUGR is clinically diagnosed when a deceleration in *in utero*-growth rate is registered for the given infant; additionally, IUGR pregnancy often leads to premature birth and SGA babies. This dip in the growth distribution indicates the presence of nutritional imbalance during gestation. About 80% of IUGR pregnancies are detected in the third trimester when this halt in growth rate is observed. Along with maternal genetic predisposition, maternal age (< 16 years old, > 35), maternal malnutrition and anaemia, and *in vitro* fertilisation, PE is a major risk factor for the development of IUGR with ~1/3 of PE cases leading to IUGR. The IUGR phenotype is ultimately determined by the combination of genetic, placental, foetal, and maternal factors that define the foetal microenvironment during pregnancy, which culminate in defective placental function (Fleiss et al., 2019; Roberts and Post, 2008; Sharma et al., 2016). Given, however, its wide definition, IUGR is probably an imprecise term gathering a continuum of various causes, linked to vascular dysfunction, placental developmental gene defects, or external factors as evoked previously (malnutrition, disease, etc.) (**Figure 8**).

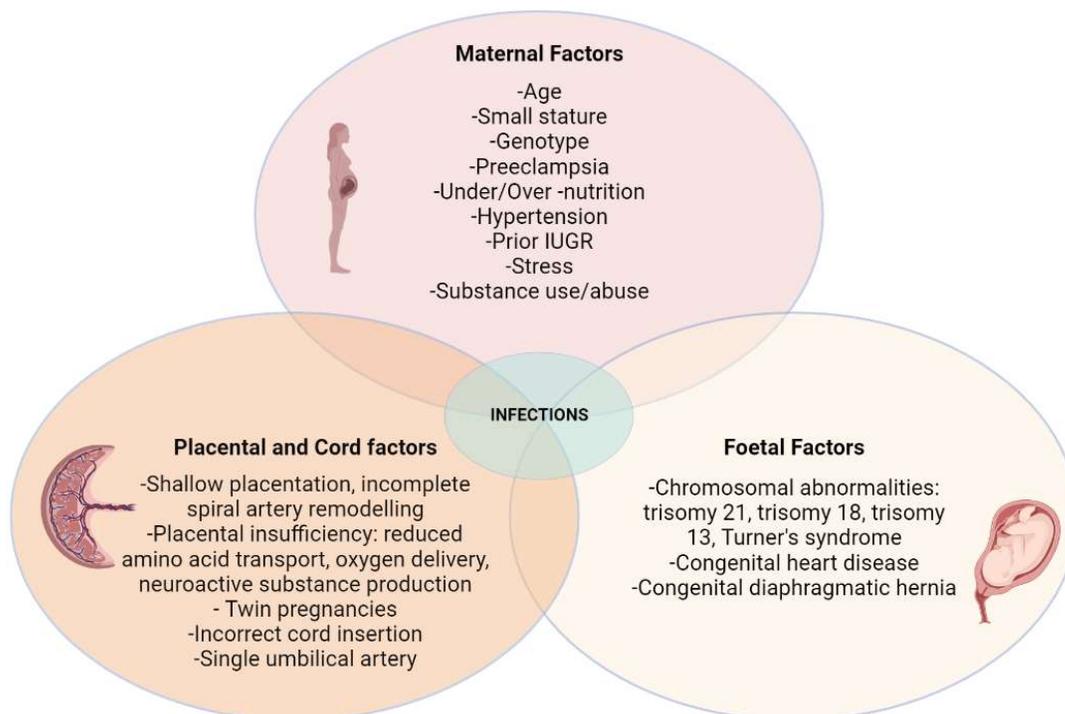


Figure 8. Overview of the principal causes of IUGR. Based on Figure from (Fleiss et al., 2019).

Image created with [Biorender.com](https://www.biorender.com)

The causal phenomenon of IUGR is an insufficient nutritional apport, in term of carbohydrates, lipids, amino acids as well as oxygen, to the developing foetus. This is consistent with numerous rodent models of IUGR where protein depletion of the mother leads to this phenotype in the litter (Buffat et al., 2007). Nutrient shortage leads to incomplete placental development, and to a strong association of IUGR with reduced placental weight at birth, when compared to the control group. These placentas (especially the cases of vascular IUGR) are characterised by shallow invasion, with incomplete spiral arteries remodelling that result in reduced blood flow to the placental lacunae and reduced exchange rate at the STB interface (Sharma et al., 2016). Placental villi development is also impaired, showing reduced proportion of terminal villi in the third trimester; while in a healthy placenta 50% of the total STB surface is organised in terminal villi as these are the primary site of nutrient exchange at this demanding stage of pregnancy (Benirschke et al., 2012). A recent study showed an intimate connection between alteration of nutritional metabolites levels in maternal and foetal circulation, with risen circulating levels of amino acids, glucose and lipids in maternal blood corresponding to a decrease in umbilical cord blood levels. These observation are explained by dysfunctional and insufficient transport at the level of the STB in placental villi (Moros et al., 2021).

Given the prominent role of placental dysfunction in IUGR and its strong correlation with EO-PE, we have decided to focus our molecular analyses in both disease groups, PE and IUGR pregnancies, to find common as well as distinct traits that could potentially be used to better understand the aetiology of these conditions.

Project Objectives

In this project, we wanted to increase our understanding of the placental physiology with the goal of gathering insights into mechanisms behind the development of PE and IUGR. To this aim, we set out the following four different objectives:

- 1) To identify human placental eQTLs by combining placental genotype and transcriptomic datasets: an eQTL describes the correlation between a change in sequence at a specific locus on the genome and levels of expression of a target gene, giving insights into its regulation, identifying potential regulatory elements (e.g. transcription factor binding sites, enhancer region).
- 2) Given the evidence that alternative splicing is implicated in deregulation of protein isoforms associated with such an emblematic feature of PE such as ED, we wish to assess the role of alternative splicing genome-wide in pathological placentas.
- 3) To investigate the process of trophoblast fusion in the context of placental diseases, looking for key ncRNAs involved in syncytialisation and that could be relevant for the aetiology and phenotype of disease.
- 4) To identify candidate genes involved in trophoblast fusion and might be implicated in faulty placental development, by developing a whole genome screen using technologies based on the CRISPR/Cas9 system in an *in vitro* model of trophoblast. This is based on the presence in the genomes of clustered regularly interspaced short palindromic repeats (CRISPR) that can be recognised by the endonuclease CRISPR-associated protein 9 (Cas9), this system has been harnessed to mediate targeted gene function inactivation.

For these investigative axes, the results are presented in each of four chapters.

Chapter 1. eQTL analysis in human placenta

“The beginning of knowledge is the discovery of something we do not understand.”

God Emperor of Dune,

Frank Herbert.

« élément sous droit, diffusion non autorisée »

Chapter 2. Alternative splicing in placental pathologies

"Find a group of people who challenge and inspire you, spend a lot of time with them, and it will change your life."

Amy Poehler

PE is a pregnancy pathology characterised by *de novo* hypertension and proteinuria after 20 weeks of gestation, that affects 4.6% of pregnancies world-wide, associated with systemic complications impacting the brain, the heart, the kidneys and the survival of both mother and foetus (Wang et al., 2021). It is often associated with IUGR defined as the inability of the foetus to reach a normal growth rate for its potential; however IUGR can occur independently of PE and it is believed to be strongly multifactorial (Fleiss et al., 2019; Mandruzzato et al., 2008; Sharma et al., 2016). Extensive consensus exists on the pivotal role of the placenta in the origins of preeclampsia, even though more recent hypotheses stress the role of the maternal environment and maternal cardiovascular risk factors (Huppertz, 2008; Melchiorre et al., 2021; Rana et al., 2019). One of the features of preeclampsia is ED, characterised by abnormal secretion in the maternal blood of anti-angiogenic molecules, resulting in vasoconstriction and hypertension (Granger et al., 2001).

Amongst these anti-angiogenic factors that characterise the maternal serum profile and are partially originating from the placenta itself, sFLT-1 is a well-established biomarker of PE, firstly identified by the group of Prof. Karumanchi (Maynard et al., 2003; Souders et al., 2015). sFLT-1 is a short form of FLT-1, also known as VEGFR1; in its full form, FLT-1 is membrane-bound and contributes to pro-angiogenic signalling, downstream of VEGF, stimulating vasodilation, angiogenesis, and *de novo* vascularisation. sFLT-1 instead is generated through alternative splicing, different short isoforms have been identified; once translated into protein, these isoforms are released in the blood stream, where can bind free VEGF, reducing its bioavailability and contributing to the ED phenotype (Palmer et al., 2015; Roberts and Rajakumar, 2009; Thomas et al., 2009). Other anti-angiogenic factors involved in PE have been identified as short forms of their membrane-bound counterparts, such as sENG which has been found to be the product of alternative splicing during endothelial senescence (Blanco and Bernabeu, 2011; Venkatesha et al., 2006).

Even though dysregulation of alternative splicing appears to be critical for the PE phenotype, the idea that alternative splicing could be altered at a global level in preeclamptic pregnancies had not been previously investigated, and it is the focus of our work “Alternative splicing in normal and pathological human placentas is correlated to genetic variants” (Ruano & Apicella et al., 2021) included in this chapter.

Our main aims were:

- To establish if alternative splicing deregulation can be detected on a global level in PE and IUGR placentas, compared to control.
- To clarify the molecular and cellular functions of the genes deregulated at the splicing levels in PE and IUGR and the degree of overlap between the two conditions.
- To clarify which isoforms are specifically expressed in CTRL, PE and IUGR samples for a subset of genes key to placental function (e.g. FLT1, PAPP2, TXK, etc.).
- To perform a preliminary assessment of the contribution of genetic variation on the regulation of expression of splicing isoforms in the placenta (sQTL analysis).

In this study, we investigated patterns of alternative splicing in preeclamptic placentas compared to healthy controls, as well as placental samples from PE+IUGR and IUGR, we also presented preliminary data on genetic regulation of splicing mediated by a series of SNPs in the placental genome. At this stage in the project, we had access to only a limited number of placental samples, restricted to the Cochin and Angers cohorts described in **Chapter 1**. After quality control, the sample set included 17 CTRL samples, 7 PE and 13 IUGR. We obtained transcriptomic data using the Affymetrix microarray ClariomD that has been optimised to provide gene expression levels both at the gene-level and at the exon-level, providing information relative to alternative isoforms of each gene, as annotated in ENSEMBL database. To ascertain global differences in alternative splicing between healthy and diseased placentas, we used the “splicing index” unit, which is a measure of how much differently expressed isoforms are between the tested groups (ThermoFisher Scientific, 2019). We found 1060 and 1409 genes differentially spliced between PE and CTRL, and between IUGR and CTRL, respectively, with a statistical significance threshold set at $p\text{-value} \leq 0.001$. 176 genes were found in common between PE and IUGR. Interestingly, we showed how the changes that we observed in splicing index, indicating that different isoforms are expressed by healthy and diseased placenta, did not generally correlate with total changes at the expression level of the

gene. However, genes that have been previously described as deeply upregulated in pathological pregnancies such as PAPP2, HtrA Serine Peptidase 4 (HTRA4) and LEP (Vaiman et al., 2013) were found to be also significantly differentially spliced.

To better understand the role of these genes sensitive to alternative splicing regulation in both diseases, IUGR and PE, we performed an enrichment analysis on the common 176 alternatively spliced genes with disease terms, we found a significant enrichment for “pregnancy complications”, “fetal diseases”, “gestational hypertension”, “hypoxia” and cancer pathways. In terms of biological functions, the genes alternatively spliced in IUGR and PE appeared to be involved in distinct pathways; hypoxia and metabolic pathways such as steroidogenesis, hormonal synthesis and anion transport were enriched in IUGR, while in PE extracellular matrix, circulatory system and neuronal physiology terms were enriched.

We attempted to better elucidate the repertoire of isoforms that are differentially expressed in the disease placentas, by experimentally validating expression levels of different transcripts for a subset of strongly modified genes (FLT1, CLDN1, LEP, FSTL3, TXK, CAP2, CA10, TNFRSF1B, ACOXL, TIE1, LAGLS14, CPXM2). We showed that, while the short isoform of FLT1 is expressed by control tissues as well, it is increased in PE and PE+IUGR, and the whole FLT1 gene is upregulated in all disease groups, including IUGR. This finding is in accordance with the existing literature and validated our experimental approach.

It is known that genetic changes can affect alternative splicing, by either directly changing the consensus sequences recognised by splicing factors, as well as splicing acceptor sites or by altering indirectly the function of the splicing factors and regulatory proteins themselves. By comparing the genotype of samples and the relative levels of gene isoforms it is therefore possible to identify sQTLs which have been found to have a critical role in gene regulation, and can help unravel the functional role of disease variants (Li et al., 2016). In this study, we selected 48 genes that appeared to be strongly subjected to splicing regulation, since a strong difference in splicing index was found between sample groups; then, we tested for correlation between splicing changes in these genes and the genotype dataset, correcting for the disease variable. This allowed to identify 52 *cis*-sQTLs as well as 52 *trans*-sQTLs with a FDR ≤ 0.05 .

As acknowledged in the paper, the sQTL analysis is to be considered only exploratory due to the small sample size, and the results have yet to be validated in an independent cohort;

nonetheless, this analysis has value in showing a suggestive role for genetic variation on splicing regulation independent of disease in the placenta. However, in retrospective, in addition to the small number of samples that limits our statistical power to detect true correlations, we also chose an analytical workflow in which we approximated population stratification, during QTL analysis, by assigning to each sample a categorical value of “Ethnicity” (i.e. African, European). As explained in **Chapter 1**, however, the more appropriate strategy is to perform a principal component analysis on the genotype dataset, and to use the first PCs to summarise the global variability linked to population stratification (Listgarten et al., 2010). It follows, that we could have better approximated global genetic changes by the latter approach, and therefore some findings could be spurious.

The core outcomes of the paper are listed below:

- Alternative splicing deregulation in the placenta is a feature of pregnancy pathologies IUGR and PE, with 1060 and 1409 genes differentially spliced respectively between PE and CTRL, and IUGR and CTRL (p-value ≤ 0.001). Of these, 176 genes were deregulated in both conditions.
- Key placental genes previously associated with PE such as PAPP2, HTRA4 and LEP, are also altered at the splicing level stressing the importance of assessing isoform-specific expression when investigating pathology.
- Distinct biological functions are associated with the genes altered in PE and IUGR: hypoxia, steroidogenesis, hormonal synthesis and anion transport were significantly enriched in IUGR, while extracellular matrix, circulatory system and neuronal physiology terms were enriched in PE. This underlies key functional differences in the two pathologies.
- Different repertoires of isoforms, experimentally validated, are expressed by PE and IUGR when compared to control for genes important for placental function. FLT1 was used as control, and novel insights into placental pathology specific isoform expression is presented for CLDN1, LEP, FSTL3, TXK, CAP2, CA10, ACOXL, TIE1, TNFRSF1B. LGALS14 and CPXM2 were found not significantly differentially spliced between sample groups by qPCR.
- Preliminary evidence suggests that genetic sequence influences expression levels of alternative splicing isoforms in the placenta.

Since this publication, only the work of (Georgiadou et al., 2021) further investigated the role of alternative splicing regulation in the placenta. In this study however, they took a more targeted approach by assessing how the experimental knock-down of gene Poly(RC) Binding Protein 2 (PCBP2), a factor involved in the splicing complex, affects trophoblast differentiation. Using an *in vitro* model of EVT, they showed how the invasive phenotype of EVTs is impaired upon PCBP2 downregulation. This is accompanied by global changes in expression levels of alternative isoforms for many genes involved in cell cycle, proliferation, and growth as well as cell-to-cell interaction. This is an additional proof of the pivotal role of alternative splicing regulation in placental physiology, and marks our work presented here as ground-breaking in giving a global overview of this process for the first time in the placenta.

Alternative splicing in normal and pathological human placentas is correlated to genetic variants.

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Alternative splicing in normal and pathological human placentas is correlated to genetic variants

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Abstract

Two major obstetric diseases, preeclampsia (PE), a pregnancy-induced endothelial dysfunction leading to hypertension and proteinuria, and intra-uterine growth-restriction (IUGR), a failure of the fetus to acquire its normal growth, are generally triggered by placental dysfunction. Many studies have evaluated gene expression deregulations in these diseases, but none has tackled systematically the role of alternative splicing. In the present study, we show that alternative splicing is an essential feature of placental diseases, affecting 1060 and 1409 genes in PE vs controls and IUGR vs controls, respectively, many of those involved in placental function. While in IUGR placentas, alternative splicing affects genes specifically related to pregnancy, in preeclamptic placentas, it impacts a mix of genes related to pregnancy and brain diseases. Also, alternative splicing variations can be detected at the individual level as sharp splicing differences between different placentas. We correlate these variations with genetic variants to define splicing Quantitative Trait Loci (sQTL) in the subset of the 48 genes the most strongly alternatively spliced in placental diseases. We show that alternative splicing is at least partly piloted by genetic variants located either in cis (52 QTL identified) or in trans (52 QTL identified). In particular, we found four chromosomal regions that impact the splicing of genes in the placenta. The present work provides a new vision of placental gene expression regulation that warrants further studies.

Camino S. M. Ruano and Clara Apicella contributed equally to this study.

Céline Méhats and Daniel Vaiman supervised this work equally.

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Introduction

Preeclampsia (PE) is a major disease of pregnancy, marked by hypertension, proteinuria, and a generalized endothelial maternal disorder (Sibai et al. 2005; Rana et al. 2019). PE induces 70,000 maternal deaths and ~1,000,000 fetal deaths worldwide (1.5–10% of pregnancies according to the country, with a high prevalence in sub-Saharan Africa). IUGR, a failure of the fetus to reach its normal growth potential, affects up to 10% of the fetuses, and is a major cause of the birth of small babies, that are at risk for acute diseases of the retina (Gupta et al. 2008), the lung (Arroyas et al. 2020; Fandino et al. 2019; Zana-Taieb et al. 2015), the heart (Crispi et al. 2008) (in the most severe cases) and long term increased risk of cardiovascular diseases (less severe cases) (Jansson and Powell 2007; Savage et al. 2020). These two gestational pathologies are strongly connected since one third of the preeclampsia cases results in IUGR. PE and IUGR are accompanied by epigenetic alterations at the placental level and have been strongly associated with an imbalance of the oxidative and nitrosative stresses (Myatt 2010; Aouache and Biquard 2018; Doridot et al. 2014). One of the

earliest markers of preeclampsia is an excessive increase of the soluble form of the VEGF receptor, sFLT1, in the maternal plasma (Souders et al. 2015). This form is generated by an alternative exon splicing event that will generate a protein encoded by the first 13–15 exons of the FLT1 gene (Szalai et al. 2015).

This soluble receptor is classically thought to perform as a trap for the VEGF and PGF angiogenesis factors (Phipps et al. 2019). Thus, it is classically assumed that an exon-splicing event of FLT1 is a major causative factor in the onset of PE. The idea that alternative splicing mechanisms are more general in the pathophysiology of pregnancy, including IUGR, has not been systematically analyzed so far.

Exon splicing events on a global scale are currently theoretically measurable by two technical approaches: exon-specific microarrays and RNA sequencing. Nevertheless, splicing events are rarely mentioned in the RNA-seq experiments carried out in normal and pathological placental samples, with a recent exception (Majewska et al. 2019). To address alternative splicing, RNA-seq need very high depth of reads (60–100X), and paired-end reads. These requirements are seldom achieved and studies using RNA-seq approaches on preeclamptic placental or decidual samples do not dive into details on splicing profile (Tong et al. 2018; Sober et al. 2015). This is also true for other defective pregnancy outcomes, such as recurrent miscarriage (Sober et al. 2016). Notwithstanding, genes involved in splicing and mRNA processing are highly expressed both in normal and pathological placentas (Aouache and Biquard 2018).

In the present study, we decided to thoroughly analyze alternative splicing events in the placenta in the context of pathological pregnancies. We profiled placental RNA levels from controls, preeclamptic patients and IUGR patients at the exon level using exon-microarrays. In parallel, the same patients and controls were genotyped using SNP-microarray technology. This design allowed us to discover extensive alterations of splicing throughout the placental genome in pathological situations. In addition, we identified genetic variants that are associated to splicing modifications, located in *cis* and *trans*, with or without connection with the disease status. These splicing Quantitative Trait Loci (sQTLs) may thus reflect individual-specific regulations of placental functions.

Results

A genomic search for alternative splicing in the human placenta

Placental alternative splicing is a prominent feature of gestational diseases

To determine whether there are differences in mature transcripts for a given gene between healthy and diseased placentas, gene expression at the exon level was measured in placentas from two distinct cohorts of patients: a cohort of PE-affected women and matched controls (Institut Cochin-PE cohort, $n=7$ and 9, respectively) and another of IUGR-affected women and matched controls (Angers Hospital-IUGR cohort, $n=13$ and 8, respectively). Patient characteristics are presented in Supplementary Table S1. As expected, both gestational age and birth weight are significantly different between disease and control groups. This difference is a recurrent and expected feature of transcriptome analysis comparing normal and pathological placentas. In this case, since most of the samples (including the pathological ones) are from third-trimester placentas, the effects linked to placental age are probably meek compared to the ones associated with the disease per se. To more thoroughly ascertain this, we proceeded to identify and estimate surrogate variables for known and unknown sources of variation in our dataset using the SVA package (Leek et al. 2012). In the IUGR vs CTRL comparison, 6.5% of the probes differentially expressed according to disease, were also associated to gestational age, 12.5% to birth weight, and 10.9% to sex. In the PE vs CTRL comparison, 7.5% of the genes significantly changed due to PE effects were significantly associated to gestational age, 6.8% to birthweight and 10% to the sex of the baby. Considering these data, we assumed that the gene expression and splicing alterations detected are strongly influenced by the disease status. These data are summarized as Supplemental Table S2a, S2b and S2c for the comparison between CTLs on the one hand, vs IUGRs, all PEs, and isolated PEs, respectively.

The comparison of gene expression at the exon level was carried out using Affymetrix ClariomD microarray, an attractive solution for tackling the complex question of alternative splicing regulation, compared to other more computation-intensive approaches depending upon RNA-seq. Analyses through the Affymetrix Transcriptome Analysis Console software lead to the definition of a splicing index associated with a relative p value for each gene and each comparison (Gardina et al. 2006), allowing to rank the genes from the most differentially spliced, to the least. The complete dataset is available with the accession number E-MTAB-9416 (EMBL-EBI ArrayExpress).

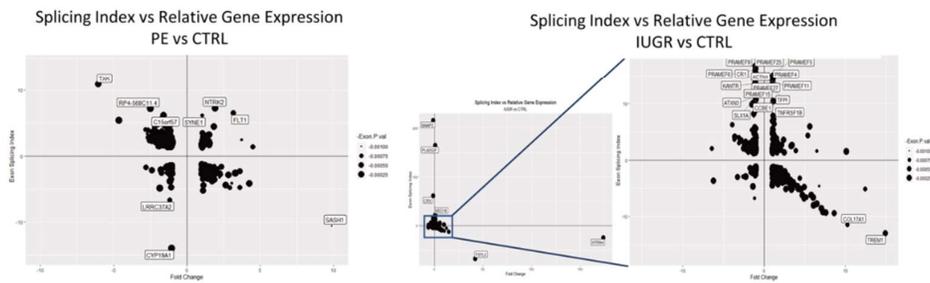
When using a *p* value threshold of 0.001, 1060 and 1409 genes had a significant splicing index value in PE vs controls and IUGR vs controls, respectively. Splicing index alterations were not correlated with gene expression deregulation between preeclamptic and control samples (Fig. 1a).

For instance, *CYP19A1* or *BAIAP2* were not strongly modified at the expression levels in PE placental samples and IUGR samples, respectively, while the splicing index alteration in these genes was amongst the highest (Table 1).

Alternative splicing concerns specific gene categories in PE and IUGR

Since *p* value allows to pinpoint significant but also small variations in splicing (that may not be relevant), we decided to focus on transcripts with $SI > |3|$, a list marginally different from the one given with the $p < 0.001$ threshold. We identified a list of 1456 transcripts in PE vs control placentas, and 725 transcripts in IUGR vs control placentas at the same threshold. Among those, 1071 and 575 have an official gene symbol (Supplementary Tables S3 and S4) and 176 were found alternatively spliced in both diseases (Fig. 1b). These

A



B

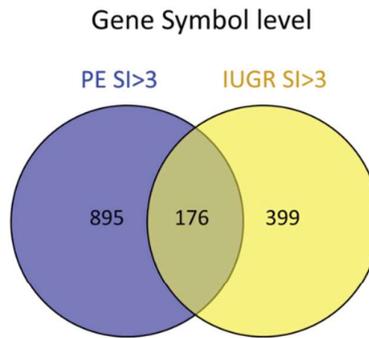


Fig. 1 a Splicing index represented in function of the fold change of gene expression in PE vs CTRL (left) or IUGR vs CTRL, right. A zoom of the cloud in the blue square is presented. For preeclampsia, gene labels are shown for genes with $SI > |6|$ and for IUGR $> |10|$.

Overall, there was no marked correlation between splicing and gene expression level (see text). **b** A part of the genes only is found both in preeclampsia and IUGR

Table 1. 48 genes sorted by Splicing Index as modified in pregnancy diseases

Gene	Max SI PE	Max SI PE + IUGR	Max SI IUGR	RT-qPCR validation	Institut Cochin Cohort		Anger Hospital Cohort		Expression 3rd trimester (Pique-Regi et al. 2020)	
					Ctrl expression	PE expression	Ctrl expression	IUGR expression		
BALAP2	51.89	35.61	215.91	No	6.87	6.6	6.59	6.78	6.73	STB
PLA2G2F	29.19	22.57	164.47	No	6.1	5.86	6.01	6.14	6.15	
FNDC3B	19.23	18.27	1.69	No	19.31	19.31	19.44	19.16	19.15	EVT
RP11-113122.1	18.85	16.99	-1.46	No	4.3	4.22	3.95	4.38	4.26	
CLDN1	15.51	9.17	4.25	Yes	15.54	11.55	11.79	15.67	13.4	Endometrial
HMHA1	11.65	4.31	7.84	No	3.46	3.61	3.82	3.45	3.58	
FLT1	11.27	5.43	-6.24	Yes	17.8	19.47	19.7	17.36	19.89	EVT
TXK	10.9	16.7	4.67	Yes	11.17	8.57	8.55	11.54	10.24	
CA10	10.84	7.1	3.14	Yes	3.88	3.7	3.41	10.3	8.94	
CPXM2	10.64	10.81	4.11	Yes	12.48	9.7	10.54	12.13	10.82	
CDY7P	9.14	-1.64	-1.07	No	4.27	4.32	4.43	4.17	4.03	
FLT4	9.05	8.72	6.42	No	10.09	9.27	9.65	9.83	10.16	LED (Lymphoid Endothelial Decidual cells)
ACER3	8.51	-9.21	5.99	No	13.58	14	13.19	13.3	14.01	
STAT4	8.37	6.48	2.63	No	7.37	6.34	6.18	7.46	6.79	
PSMD13	8.35	3.39	3.31	No	12.96	12.96	12.49	12.81	12.6	
FAM47E; STBD1; STBD1	8	7.05	1.86	No	5.56	5.14	5.22	5.56	5.43	
SPX	8	-7.01	7.15	No	8.14	5.92	6.41	8.16	5.52	
NCKAP1	7.86	2.85	-2.5	No	13.81	13.63	13.84	13.57	13.5	EVT
NTRK2	7.26	4.1	-4.44	Yes	5.14	6.06	5.51	4.62	6.3	
SPP1	7.16	2.19	-3.48	No	15.54	13.15	15.73	15.86	15.22	Macrophage
TNFRSF1B; MIR4632; MIR7846	7.03	5.39	10.42	Yes	8.37	8.83	9.03	8.96	9.14	
EIF1AY	6.93	-11.93	5.64	No	8.16	6.46	10.75	8.05	7.35	VCT
CYP19A1	-68.93	-5.02	3.56	No	14.08	13.08	13.42	19.92	19.92	CTB
PSG4	-31.84	-28.56	3.8	No	19.91	19.85	19.88	19.89	19.93	STB
XIST	-24.17	298.39	-24.55	No	13.95	16.16	6.71	12.71	17.02	
FSTL3	-21.76	-16.73	-67.97	No	8.03	11.87	11.72	7.06	12.45	EVT

Table 1. (continued)

Gene	Max SI PE	Max SI PE+IUGR	Max SI IUGR	RT-qPCR validation	Institut Cochin Cohort		Anger Hospital Cohort		Expression 3rd trimester (Pique-Regi et al. 2020)
					Ctrl expression	PE expression	Ctrl expression	IUGR expression	
RPSA; SNORA62; SNORA6	-20.35	2.81	-1.67	No	15.46	15.39	15.19	15.33	mpiCTB (non-proliferative interstitial cytotrophoblast) CTB
GABRE; MIR224; MIR452	-20.35	-2.44	-8.66	No	16.08	15.37	16.49	15.99	CTB
SH3BP5	-14.23	-2.01	-4.91	No	8.95	8.88	8.09	10.15	EVT
PDXDC2P	-13.05	-5.63	2.27	No	12.29	11.82	13.08	12.76	EVT
BIN2	-10.91	-11.55	-8.01	No	6.42	7.17	8.84	11.45	EVT
SASH1	-10.59	-16.6	-9.28	No	7.96	11.27	8.01	10.93	LED
LGALS8	-9.52	-5.48	2.31	No	14.39	13.58	14.77	14.47	LED
ST18	-9.36	3.05	-3.69	No	3.37	3.47	3.25	3.19	EVT
HTRA4	-9.29	-12.66	-24.88	No	10.91	15.72	10.23	17.67	EVT
ATG2B	-9.11	-2.92	1.87	No	8.33	8.06	8.34	8.39	EVT
DDX17	-8.9	-1.77	2.02	No	16.78	16.58	16.82	16.84	EVT
CAP2	-8.58	-5.82	1.92	No	14.36	13.62	14.59	14.14	STB
BCAP29	-6.6	-2.59	2.67	No	13.53	13.4	12.98	13.38	LED
ORC4	-6.17	-3.95	1.62	No	11.53	10.81	11.13	11.14	LED
SLC6A10P	-6.11	-4.13	-8.59	No	8.96	11.13	8.56	11.39	EVT
P4HAI	-6.08	-6.88	-4.86	No	8.83	10.65	8.79	10.47	EVT
LYRM5	-6.06	-7.47	-1.84	No	8.5	8.26	6.13	5.02	EVT
DPP4	-5.94	1	-1.01	No	5.95	6.37	6.42	6.42	CTB
ACOXL	4.46	6.24	3.64	Yes	9.12	7.47	9.35	8.1	CTB
TIE1	4.01	5.13	-1.66	Yes	6.19	6.49	6.08	6.29	LED
LGALS14	-2.22	-2.42	-2.02	Yes	19.9	19.7	19.92	19.58	STB
LEP	-1.91	-2.54	-1.56	Yes	11.85	12.79	7.42	15.28	STB

latter 176 genes encoded proteins that constituted a network of Protein–Protein Interactions ($p = 0.00097$, Supplemental figure S1, String database, <https://string-db.org/cgi> (Szklarczyk et al. 2019)), showing that the splicing alteration does not occur randomly in the placental genome in pathological situations. We then performed an over-representation analysis using WebGestalt (Wang et al. 2017; Liao et al. 2019) on the 176 genes found alternatively spliced in both disease conditions. We compared our gene list with the GLAD4U database (Jourquin et al. 2012), that collects gene sets associated with diseases. We found a significant enrichment for Pregnancy complications, fetal diseases, gestational hypertension, hypoxia and several cancer pathways (Fig. 2a). The exhaustive values and genes involved are presented as Fig. 2b for ‘Gestational Hypertension’, ‘Pregnancy complications’, ‘Anoxia’, and ‘Pregnancy’.

Functional clustering of alternatively spliced genes partly differs between PE and IUGR

Then we proceed to separately analyze the genes spliced in either IUGR or PE. We found that while the two pathologies shared biological processes related to secretion, exocytosis and vesicle metabolism, substantial clustering differences were observed.

In IUGR (Table 2), KEGG and Reactome pathways related to hypoxia, steroidogenesis, hormone metabolism and anion transport were enriched. Against disease database (GLAD4U and OMIM), the alternatively spliced genes in IUGR were widely associated to ‘pregnancy complications’, ‘Nitric Oxide metabolism’ (known as intimately linked to placental pathology) (Aouache and Biquard 2018; Motta-Mejia et al. 2017; Dymara-Konopka and Laskowska 2019), ‘preeclampsia’ and ‘eclampsia’, but also to autoimmune diseases, especially ‘lupus erythematosus’ (SLE), which is consistent with the increased risk of IUGR and other defective pregnancy outcomes documented in autoimmune patients, especially those affected by SLE (Do and Druzin 2019).

The same type of analysis applied to the PE-alternatively spliced genes yielded quite different results (Table 3). In terms of Biological processes, extracellular matrix, circulatory system and several neuron/axon ontology terms were found enriched specifically in PE. KEGG and Reactome pathways consistently pointed out to extracellular matrix organization pathways. In terms of diseases databases (GLAD4U and OMIM), ‘pregnancy’ and ‘pregnancy complications’ appeared enriched, but also, interestingly, numerous pathological pathways implicated in neurodevelopmental diseases emerged with GLAD4U (‘Brain diseases’, ‘CNS diseases’, ‘Dementia’, ‘Cri-du-Chat syndrome’), while OMIM pointed out to SLE (Qing et al. 2011) and Malaria susceptibility, consistently with the IUGR OMIM terms. In this case, nevertheless, an enrichment in genes involved in

Alzheimer’s disease was also found, consistently with the GLAD4U keywords.

In summary, this comprehensive description of abnormal splicing in IUGR and PE reveals quite different enrichment of alternatively spliced genes between two important placental diseases and normal placentas. Notably, a connection with neurological disease genes was exclusive to PE. Comparing our sQTLs results, with placental eQTLs identified in previous studies (Peng et al. 2017), we found four genes in common amongst the cis-eQTLs (ACER3, CLDN1, PSG4, LGALS8). A contingency chi2 revealed a marginally significant enrichment (12.8% expected versus 33% observed, $p = 0.034$).

Individual validation of alternative splicing on an enlarged collection of placental samples

We next validated some of these alterations through a targeted approach, based upon exon-specific primers and RT-qPCR. For this validation, we selected 12 genes based upon their known involvement in preeclampsia and/or their very high splicing index differences between healthy and preeclamptic placentas: FLT1, CLDN1, LEP, FSTL3, TXK, CAP2, CA10, TNFRSF1B, ACOXL, TIE1, LAGLS14, CPXM2. Maximal splicing indexes (p values) were respectively: FLT1 11.27 ($p = 0.0016$), CLDN1 15.51 ($p = 0.0063$), LEP 2 (0.2957), FSTL3 – 21.76 ($p = 0.005$), TXK 10.9 ($p = 8.55E^{-05}$), CAP2 – 8.58 ($p = 0.0331$), CA10 10.84 ($p = 0.0557$), TNFRSF1B 7.03 ($p = 0.0035$), ACOXL 4.46 (0.0241), TIE1 4.01 ($p = 0.0034$), LGALS14 – 2.22 ($p = 0.5275$), CPXM2 10.64 ($p = 0.0301$). Additional new samples were included along with the samples used in the microarray analysis, for a final total of 13 controls, 15 isolated PE, 5 PE+IUGR and 10 isolated IUGR samples. Overall, we confirmed splicing alterations in diseased placentas for 10 out of 12 genes (except LGALS14 and CPXM2). An example of analysis is given for five genes (FLT1, CLDN1, LEP, FSTL3 and TXK) in Fig. 3, and the remaining genes are presented as Supplementary figures S2.

Genetic regulation of alternative splicing in the human placenta—determination of sQTLs

We next analyzed the genetic basis of splicing alterations in the different individual placentas, focusing upon a sample of 48 genes with the most differential splicing index between normal and preeclamptic placentas (Table 1). Several of these genes are well known from the literature to be modified at the expression level between PE and normal placentas such as FLT1, LEP, PAPP2, HTRA4 (Vaiman et al. 2013). Nineteen out of the 48 genes were also significantly modified at the expression level between controls and IUGR, as assessed by the analysis of the Angers Hospital-IUGR

cohort. By PCA analysis, we could show that there was no major effect of the batch (Supplementary Figure S3).

Splicing is influenced by variants located in the vicinity of genes (cis-sQTL) and at far locations (trans-sQTL)

Each placental DNA was genotyped using a SNP genotyping array that encompasses ~710,000 SNPs and MatixEQTL R package was used to investigate the impact of SNP variants on individual splicing indexes (Shabalin 2012). Placental Individual Splicing Indexes (ISIs) were computed along with the genotype data. A QQ-plot (Fig. 4) was obtained and allowed the identification of 180 cis-sQTLs (1 Mb around the gene under scrutiny) with $p < 0.01$ and 52 with $FDR < 0.05$ and 199,884 trans-sQTLs (> 1 Mb) with $p < 0.01$ and 52 with $FDR < 0.05$. The detailed list of cis-sQTLs identified is given as Supplementary Table S5. To add stringency to the approach, we re-ran the program focusing only on the control samples (Tong et al. 2018). Despite the loss of power due to this reduced size, we were still able to detect 5 cis-sQTLs with a $FDR < 0.05$ that are all common to the complete list and presented in bold in Table S5.

Cis-sQTLs have additive effects on splicing

A selection of highly significant cis-sQTLs are presented in Fig. 5. It is interesting to note that in these cases, the splicing effect is overall linear in function of the occurrence of one of the alleles (additive effect). For instance, in the first example (CYP19A1-rs12907866), the splicing index is the lowest in AA genotypes, intermediate in AB and higher in BB. The same type of profile is visible in the other examples presented. These influences on SI do not appear to be strictly connected to the disease status. Cis-sQTLs may induce alternative splicing by influencing the binding of splicing factors, by modifying the secondary structure of mRNAs, or any other local influence. However, they are not giving an image of the alternative splicing regulation at the genome level. Thus, to identify variants that influence splicing distantly from their location, we studied in more detail trans-sQTLs.

Trans s-QTL analysis reveals major loci associated to splicing in the placenta

The conventional significance threshold for a genome-wide analysis ($p < 10^{-8}$) identified 52 significant SNP-gene couples for trans-sQTL (Supplementary Table S6). At $p < 0.001$, 24,867 QTLs were found, while 3,639 QTLs passed the $p < 0.0001$ threshold. As reported in many studies, keeping an $FDR < 0.05$ or a $p < 10^{-8}$ will miss QTL with biological relevance (Morrow et al. 2018). Therefore, we developed a novel approach not to lose this relevant biological information; in particular, we were interested in identifying

bandmaster loci that would control the splicing of a large part of the 48 genes that we identified as the most strongly spliced.

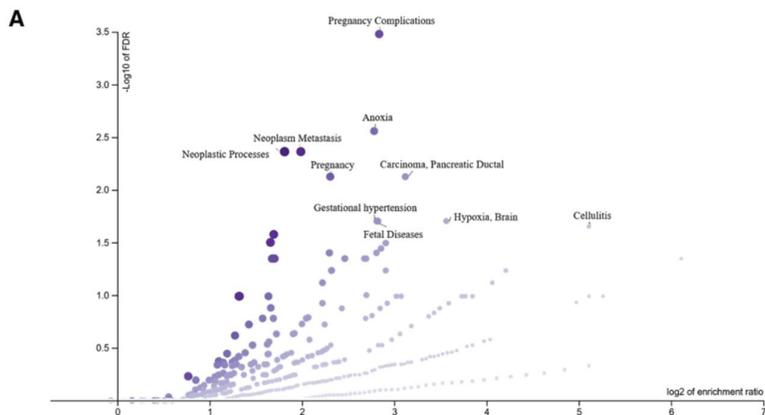
To perform this, we organized the dataset by sorting the SNPs along the chromosomes to find isolated SNPs (or windows of SNPs separated by less than 2000 bp—a distance in the range of minimal chromosomal block sizes of markers in linkage disequilibrium (Pritchard and Przeworski 2001)) that were significantly associated to alternative splicing of more than one gene.

A Monte-Carlo statistical analysis was performed to evaluate the longest possible windows of consecutive SNP-gene couples obtained from a random organization of the SNPs (simulated windows). At a threshold of 0.001, the maximal window length was of 19 consecutive gene-SNP couples, while random cases lead to a maximal size of 10 gene-SNP couples. Performing the same operation at the 0.0001 threshold we identified a maximum window size of 6 in the randomized dataset (one occurrence), while nine windows of more than 6 consecutive SNP-gene couples (Fig. 6a) were identified from the real dataset. The nine windows identified are located on chromosomes 2, 5, 7, 8, 10, 13, 14 (at two locations) and 21. To note, four of these windows were also detected at the threshold of 0.001: on chromosomes 5, 7, 8, and the second region on chromosome 14. Also, two of these windows (on chromosome 2 and 14) encompassed gene-SNP couples that were significant at a genome-wide threshold ($p < 10^{-8}$, rs13006826-SLC6A10P and rs9323491-CYP19A1). We decided to focus on these four regions, presented in Fig. 6b as the blue, green, orange and purple fountains of a circus Plot (Gu et al. 2014).

The Chromosome 5 region contains two SNPs separated by 702 bp, rs13185255 and rs12520828. These SNPs are located inside the ARHGAP26 gene, or its antisense and are associated with increased splicing alterations for FLT4, CLDN1, PLA2G2F, SH3BP5, P4HA1 and SLC6A10P.

The Chromosome 7 region spans 314 base pairs and encompasses 2 SNPs (rs6964915 and rs6965391). These SNPs are located inside the LOC105375161 non-coding RNA, which harbors the highest level of expression in the placenta (<https://www.ncbi.nlm.nih.gov/gene/105375161>) and (Fagerberg et al. 2014). RT-qPCR results interrogating two distinct regions of LOC105375161 showed no difference in expression between the sample groups (Supplementary Fig. S5). The splicing alterations of 3 genes were found associated with variants in this Chr7 region (FLT1, TXK and NTRK2).

The Chromosome 8 region encompasses a unique SNP (rs1431647) located inside the non-coding RNA LOC105375897, which is expressed at a low level in the placenta. Eight genes were potentially affected by this variant at the splicing level (FSTL3, FLT1, P4HA1, NTRK2, FLT4, BAIAP2, CLDN1, ST18). By RT-qPCR, we could



B

Select an enriched gene set...

PA165108364: Gestational hypertension

Gene set: PA165108364 Gestational hypertension

FDR: 0.021387 P Value: 0.00005911

Gene Set Size: 171 Expected Value: 0.98174 Overlap: 7 Enrichment Ratio: 7.1302

User ID	Gene Symbol	Gene Name	Entrez Gene ID
DDAH1	DDAH1	dimethylarginine dimethylaminohydrolase 1	23576
FLT1	FLT1	fms related tyrosine kinase 1	2321
FSTL3	FSTL3	folliculin like 3	10272
HTRA4	HTRA4	HtrA serine peptidase 4	203100
PAP9A2	PAP9A2	pappalysin 2	60676
PLAC1	PLAC1	placenta specific 1	10761
PLAC3	PLAC3	placenta specific 3	51316

Select an enriched gene set...

PA445403: Pregnancy Complications

Gene set: PA445403 Pregnancy Complications

FDR: 0.0024792 P Value: 9.1182e-7

Gene Set Size: 290 Expected Value: 1.6649 Overlap: 11 Enrichment Ratio: 6.6069

User ID	Gene Symbol	Gene Name	Entrez Gene ID
CCBE1	CCBE1	collagen and calcium binding EGF domain 1	147372
DUS3L2	DUS3L2	DUS3 like 7-5' nucleotidase 2	129563
FLT1	FLT1	fms related tyrosine kinase 1	2321
FLT4	FLT4	fms related tyrosine kinase 4	2324
FERMD4A	FERMD4A	FERM domain containing 4A	55691
FSTL3	FSTL3	folliculin like 3	10272
HTRA4	HTRA4	HtrA serine peptidase 4	203100
IL13R2	IL13R2	interleukin 1 receptor type 2	7850
PAP9A2	PAP9A2	pappalysin 2	60676
PLAC1	PLAC1	placenta specific 1	10761

Select an enriched gene set...

PA443382: Anoxia

Gene set: PA443382 Anoxia

FDR: 0.0023609 P Value: 0.00000188

Gene Set Size: 251 Expected Value: 1.4410 Overlap: 10 Enrichment Ratio: 6.9395

User ID	Gene Symbol	Gene Name	Entrez Gene ID
BHLHE40	BHLHE40	basic helix-loop-helix family member 40	8553
EROC1A	EROC1A	endoplasmic reticulum oxidoreductase 1 alpha	30001
ESRRG	ESRRG	estrogen related receptor gamma	2104
FLT1	FLT1	fms related tyrosine kinase 1	2321
LDAHA	LDAHA	lactate dehydrogenase A	3939
NDRG1	NDRG1	N-myc downstream regulated 1	10397
PHN1	PHN1	profilin 4-hydroxylase subunit alpha 1	5033
PXM	PXM	pyruvate kinase M1/2	5315
PLD2	PLD2	phosphatidylinositol-3-OH transferase 5-delta	5352
THBS1	THBS1	thrombospondin 1	7057

Select an enriched gene set...

PA166048714: Pregnancy

Gene set: PA166048714 Pregnancy

FDR: 0.0216991 P Value: 0.00007462

Gene Set Size: 384 Expected Value: 2.2046 Overlap: 10 Enrichment Ratio: 4.5360

User ID	Gene Symbol	Gene Name	Entrez Gene ID
FLT1	FLT1	fms related tyrosine kinase 1	2321
FERMD4A	FERMD4A	FERM domain containing 4A	55691
FSTL3	FSTL3	folliculin like 3	10272
HTRA4	HTRA4	HtrA serine peptidase 4	203100
IL13R2	IL13R2	interleukin 1 receptor type 2	7850
PAP9A2	PAP9A2	pappalysin 2	60676
PLAC1	PLAC1	placenta specific 1	10761
PRG2	PRG2	proteoglycan 2, pro-vostigallil major basic protein	5553
PSG4	PSG4	pregnancy specific beta-1-glycoprotein 4	5672
PSG7	PSG7	pregnancy specific beta-1-glycoprotein 7 (gene/neurotigin)	5676

Fig. 2 WebGestalt analysis of the genes that are alternatively spliced in common in the two diseases under scrutiny, in comparison with the disease database GLAD_4U. The database recognized 116 genes among the 176 submitted. **a** The enrichment in genes in the group is represented on the X-axis, while the FDR is represented on the Y-axis. The most significant pathways (with $FDR < 0.05$) are identified. **b** Highly relevant gene sets are represented. As an example of how to read the figure, the first on the upper left means that 178 genes are in the geneset ‘Gestational Hypertension’, 7 of which are in common with the 116 that constitute the input (the 116 that were identified out of the initial input of 176). This enrichment is highly significant (7.1 more genes than expected, $FDR = 0.0214$)

show that LOC105375897 is expressed at similar levels between control and preeclamptic placentas, while is over-expressed significantly in IUGR ~ 30 fold (p value < 0.01) (Supplementary Fig. S5).

The Chromosome 14 region spans 828 bp and encompasses two SNPs, rs7145295 and rs7151086 that are located inside the GPHN gene (harboring the non-coding RNA LOC105370538, which was not detectable in our samples by RT-qPCR). Six genes were potentially affected at the splicing level: CYP19A1, FLT1, P4HA1, FLT4, SH3BP5, and NTRK2).

The association between these SNPs and splicing levels is represented in Fig. 7. The two SNPs identified for chromosome 5 as well as for chromosome 7 had the same profile, and thus only one SNP of each region was represented. For rs13185255 it was the heterozygous genotype that was different from the homozygous genotypes (Fig. 7a). There were no obvious association between the levels of splicing, the genotypes of the QTL and the status of the patient. For rs6965391 the AA genotype was characterized by a higher splicing index for the three target genes (Fig. 7b), with a possible association with preeclampsia but that should be confirmed on a larger sample since the AA genotype was the rarest. For rs1431647, it was also the heterozygous genotype that was different from the others, without obvious connections with the disease status (Fig. 7c). In Fig. 7d are represented the two SNPs characterizing the fourth region studied. One of the SNPs (rs7145295) presents an additive behavior in terms of splicing levels for CYP19A1 ($r = 0.85$, $p < 10^{-4}$). AA was marginally associated with preeclampsia (Chi-Square, $p = 0.022$, Log-Likelihood, $p = 0.029$), and the data are consistent with the second SNP nearby rs7151086. In both cases, a higher splicing index characterizes the BB genotype.

Methods

Ethical statements and sample collection

The placenta collections have already been described elsewhere (Gascoin-Lachambre et al. 2010; Ducat et al. 2019).

Cochin Hospital cohort: placentas were collected from two maternity wards (Hôpital Cochin and Institut de Puériculture, Paris, France). This study was approved by the Ethics Committee and CCPPRB (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale) of Paris Cochin. Placentas were obtained from cesarean sections before the onset of labor. Exclusion criteria were diabetes, chromosomal and fetal malformations, maternal infections, and addictions. The inclusion criteria used for pre-eclampsia were systolic pressure above 140 mmHg, diastolic pressure above 90 mmHg and proteinuria above 0.3 g per day. Women who underwent a cesarean section without disease during pregnancy formed the control group. All patients gave their written consent for the use of their placenta and blood samples.

Angers University Hospital cohort: collection and use for research purpose (including genetic analyses) of placentas from pregnancies complicated with IUGR or healthy pregnancy have been approved by the Ethics Committee of Angers. The inclusion criteria used for IUGR was weight at birth < 10 th percentile. Vascular IUGR was defined by a reduction of fetal growth during gestation, with a notch observed by Echo-Doppler in at least one uterine artery and with Doppler abnormalities on umbilical Doppler and/or cerebral Doppler and/or ductus venosus, and with a birth weight below the 10th percentile according to Audipog growth curves. As for the Cochin cohort, women who exhibited any of the following criteria were excluded from the study: diabetes, pre-eclampsia, chromosomal and fetal malformations, maternal infections, aspirin treatment, and addictions (such as tobacco or drug usage). This collection is registered to the French Ministry of Research under number DC-2011-1467. The processing of personal data implemented as part of the project has been authorized by the French Data Protection Authority (CNIL, no. pWP03752UL). All participants provided written informed consent prior to inclusion. The study was conducted in accordance with the declaration of Helsinki (Chabrun et al. 2019).

The placental sampling was systematically done in placental regions that appeared visually normal.

RNA and DNA extraction from human placentas

RNA isolation followed classical protocols. The placentas were processed in the 30 min post cesarean section. The fetal membranes were discarded, and then, from two cotyledons to three cotyledons, villous trees were washed thoroughly in PBS and placed in TriZol™. Then the tissue was ground with a metal bead in a tissue grinder in 1.5 ml tubes. Then, 200 μ l of chloroform were added, the tubes were centrifuged at 10,000g RT, and the supernatant collected, reprecipitated in isopropanol by centrifugation. The pellet was then washed in 70% ethanol, dried under a fume hood, and resuspended

Table 2 Enrichment analysis of genes differentially spliced in IUGR

Gene set	Description	Expect	Ratio	p value	FDR
Gene ontology biological processes					
GO: 0,046,903	Secretion	33.803	1.8638	8.58E-07	0.006826
GO: 0,009,628	Response to abiotic stimulus	23.651	2.0295	2.16E-06	0.006826
GO: 0,032,940	Secretion by cell	31.002	1.8709	2.25E-06	0.006826
GO: 0,045,055	Regulated exocytosis	16.491	2.1831	9.5E-06	0.017593
GO: 0,071,363	Cellular response to growth factor stimulus	13.9	2.3021	1.07E-05	0.017593
GO: 0,009,719	Response to endogenous stimulus	33.592	1.7564	1.28E-05	0.017593
GO: 0,009,725	Response to hormone	20.261	2.0236	1.37E-05	0.017593
GO: 0,002,576	Platelet degranulation	2.6747	4.4864	1.55E-05	0.017593
GO: 0,006,820	Anion transport	12.321	2.3538	1.86E-05	0.018812
GO: 0,070,848	Response to growth factor	14.532	2.202	2.57E-05	0.023392
KEGG pathways					
hsa04913	Ovarian steroidogenesis	1.1546	6.9286	1.6E-05	0.003405
hsa04066	HIF-1 signaling pathway	2.3564	4.6681	2.09E-05	0.003405
hsa04923	Regulation of lipolysis in adipocytes	1.2725	5.5012	0.000249	0.027062
hsa04014	Ras signaling pathway	5.4669	2.7438	0.000359	0.029234
Reactome pathways					
R-HSA-114608	Platelet degranulation	3.1046	3.8652	6.28E-05	0.048135
R-HSA-76005	Response to elevated platelet cytosolic Ca ²⁺	3.2249	3.721	9.1E-05	0.048135
R-HSA-202733	Cell surface interactions at the vascular wall	3.2971	3.6395	0.000113	0.048135
R-HSA-879518	Transport of organic anions	0.2888	13.85	0.000139	0.048135
R-HSA-209822	Glycoprotein hormones	0.2888	13.85	0.000139	0.048135
Disease_GLAD4U					
PA445403	Pregnancy complications	5.0666	5.5264	2.53E-13	3.80E-10
PA166048714	Pregnancy	6.7088	4.7698	2.79E-13	3.80E-10
PA165108364	Gestational hypertension	2.9875	6.3598	1.73E-10	1.57E-07
PA445398	Pre-Eclampsia	2.8128	5.6882	2.44E-08	1.45E-05
PA443984	Eclampsia	2.8303	5.6531	2.67E-08	1.45E-05
PA445058	Neoplasm metastasis	11.409	2.8049	1.67E-07	7.58E-05
PA166048749	Pregnancy third trimester	1.1531	8.6724	2.07E-07	8.02E-05
PA165108943	Retained placenta NOS	0.47171	14.839	3.08E-07	0.000105
PA446021	Vascular diseases	8.5782	3.0309	5.96E-07	0.00018
PA166048848	Menopause	2.7429	5.104	7.00E-07	0.00019
Disease_OMIM					
611,162	Malaria, susceptibility to malaria, resistance to, included	0.076176	52.51	6.48E-07	4.28E-05
152,700	Systemic lupus erythematosus	0.035848	55.792	0.000523	0.017249
609,423	Human immunodeficiency virus type 1, susceptibility to	0.067214	29.756	0.001924	0.028948
601,665	Obesity, leanness included	0.067214	29.756	0.001924	0.028948
415,000	Spermatogenic failure, y-linked, 2	0.071695	27.896	0.002193	0.028948

in RNase-free water. The quality of the RNA was evaluated by 2100 bioanalyzer (Agilent). Samples with RIN > 7 were used for microarray analysis and RT-qPCR. DNA was prepared from the same sample by standard lysing protocols (Proteinase K treatment overnight at 55 °C), spooling of the DNA, washing with ethanol 70%, resuspension I TE (1–0.1) at ~ 200 ng/μl.

Transcriptomics and genotype datasets

One hundred ng of RNA from each placental sample were analyzed by ClariomD (Affymetrix) microarray assay. This tool interrogates 134,748 probes, including coding genes (18,858), non-coding genes (66,845) and genes deduced by bioinformatics analysis (10,001), in addition to pseudo-genes, small RNA and ribosomal RNAs. Overall, more than 540,000 transcripts are analyzed with this tool. With the

Table 3 Enrichment analysis of genes differentially spliced in PE

Gene Set	Description	Expect	Ratio	<i>p</i> value	FDR
Gene ontology biological processes					
GO: 0,016,192	Vesicle-mediated transport	70.148	1.6394	4.34E-08	0.000199
GO: 0,030,198	Extracellular matrix organization	12.534	2.7924	4.37E-08	0.000199
GO: 0,043,062	Extracellular structure organization	14.449	2.4916	4.80E-07	0.001455
GO: 0,006,887	Exocytosis	32.293	1.889	1.25E-06	0.002845
GO: 0,072,359	Circulatory system development	37.061	1.8078	1.7E-06	0.003095
GO: 0,031,102	Neuron projection regeneration	1.8783	5.3239	1.41E-05	0.021369
GO: 0,031,103	Axon regeneration	1.5532	5.7944	1.85E-05	0.024045
GO: 0,048,678	Response to axon injury	2.4201	4.5452	2.55E-05	0.028951
GO: 0,035,295	Tube development	35.832	1.7024	3.19E-05	0.030548
GO: 0,045,055	Regulated exocytosis	28.283	1.8032	3.38E-05	0.030548
KEGG pathways					
hsa04510	Focal adhesion	7.8332	2.6809	3.33E-05	0.010845
hsa04512	ECM-receptor interaction	3.2277	3.408	0.000337	0.05495
hsa04960	Aldosterone-regulated sodium reabsorption	1.4564	4.8063	0.000507	0.055129
Reactome pathways					
R-HSA-1474244	Extracellular matrix organization	11.893	2.8589	2.91E-08	5.03E-05
R-HSA-216083	Integrin cell surface interactions	3.3584	4.7641	1.70E-07	0.000147
R-HSA-114608	Platelet degranulation	5.0969	2.9429	0.000168	0.078328
Disease_GLAD4U					
PA443275	Adhesion	22.33	2.4183	2.04E-09	5.55E-06
PA443553	Brain diseases	16.179	2.2251	7.22E-06	0.008322
PA166048714	Pregnancy	11.194	2.5013	9.18E-06	0.008322
PA443657	Central nervous system diseases	16.674	2.159	1.39E-05	0.009476
PA444447	Carcinoma, hepatocellular	11.077	2.4374	2.1E-05	0.011403
PA443853	Dementia	8.1915	2.6857	2.83E-05	0.012844
PA445403	Pregnancy complications	8.4538	2.6024	4.57E-05	0.016622
PA166123006	interstitial fibrosis	2.128	4.6992	4.89E-05	0.016622
PA443812	Cri-du-chat syndrome	0.46642	10.72	6.92E-05	0.020562
PA445080	Neovascularization, pathologic	6.3841	2.8195	8E-05	0.020562
Disease_OMIM					
146,110	Hypogonadotropic hypogonadism 7 with or without anosmia	0.11153	35.866	3.14E-06	0.000207
611,162	Malaria, susceptibility to malaria, resistance to, included	0.13542	29.537	7.35E-06	0.000242
152,700	Systemic lupus erythematosus	0.063729	31.383	0.001671	0.036762
104,300	Alzheimer disease	0.087628	22.824	0.003234	0.053355

TAC tool, a splicing index can be calculated and a statistical value associated to it. The splicing index was calculated by the Transcriptome Analysis Console (TAC) from Affymetrix, using the following formula:

$$\text{Log}_2 \left[\frac{\left(\frac{\text{probeset1 intensity}}{\text{Gene1 intensity}} \right)}{\left(\frac{\text{probeset1 control intensity}}{\text{Gene1 control intensity}} \right)} \right] \quad (1)$$

The probeset is defined by the fluorescence of a given probe (either a junction probe, or an exon-specific probe),

present in the array. More details can be obtained in (Jimeno-Gonzalez et al. 2015).

To be able to evaluate the splicing variation on an individual basis, we first defined an individual splicing index for each sample (Individual splicing index—ISI). Inside a given gene and for a given placental sample, we obtained the different fluorescent measures for each of the probes spanning the gene. We then selected the probe with the highest splicing index and divided its fluorescent signal level by the geometric mean of the complete group of probes. For a given placental sample, the relative splicing index for Gene A is therefore given by

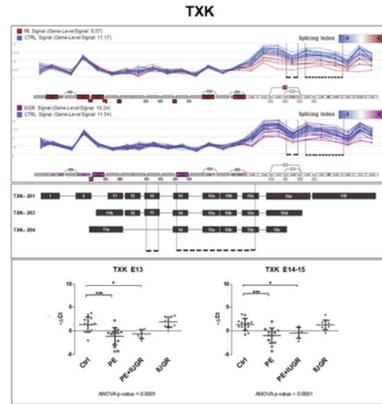
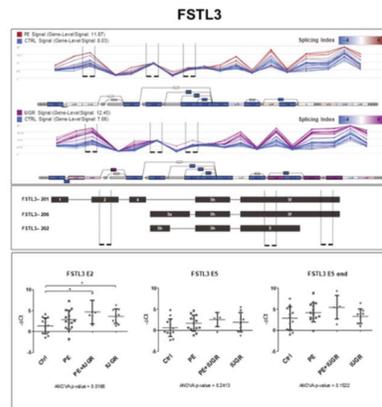
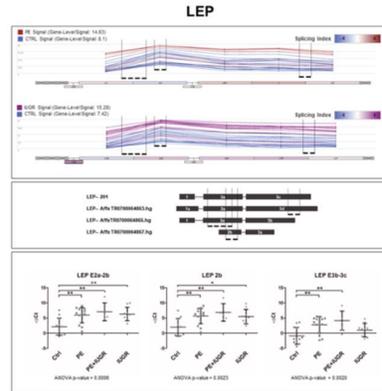
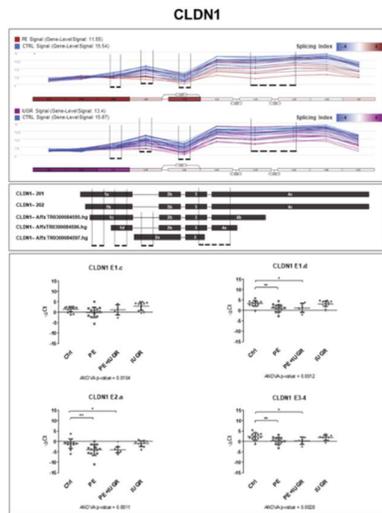
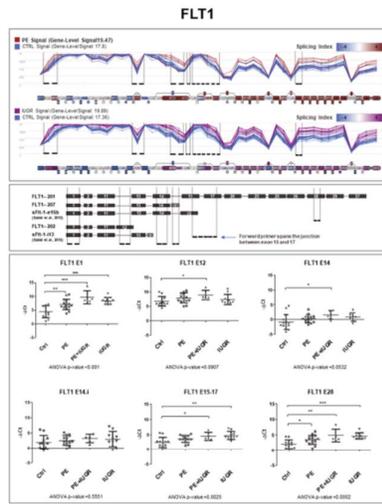


Fig. 3 Locus-specific RT-qPCR analysis of genes that were found modified in pathological placentas. Each panel represents the splicing profile of a gene. For instance, for FSTL3 there is an increased splicing index in the 5' and 3' regions of the gene, in preeclampsia versus controls and IUGR vs controls (two upper graphs), drawn with the positions of the primer couples that were used for the RT-qPCR. Significant alterations are in this case essentially detected in the E2 tested region, while the others are not significant, consistently with the microarray graph

$$\frac{\text{MAX}(\text{FprobeGAI}_i)}{\sqrt{\prod_1^n \text{FprobeGAI}_i}} \quad (2)$$

where 'FprobeGAI' refers to the \log_2 (fluorescence level) of a given i probe in gene A. An example of the calculation is given as Supplementary Fig. S4. In this example, for the long non-coding RNA RP11-113I22.1, the strongest splicing index is given for the probe PSR0500147825.hg.1. It appears that the abnormal splicing compared to the rest of the probes occurs clearly for some placentas but not for all.

Library preparation, hybridization and data acquisition were performed by GENOMIC platform according to manufacturer's instructions. ClariomD microarray data were extracted using the Transcriptomic Analysis Console provided by Affymetrix. The data from the control subjects of the two cohorts were merged to generate the control group data. When compared against the PE or the IUGR data, a batch correction was applied using the built-in *Batch* parameter in TAC.

Genomic DNA samples were genotyped using the Infinium OmniExpress-24 kit (Illumina) array, in collaboration with Plateforme P3S, Sorbonne Université – site Pitié Salpêtrière, Paris, France. Data were extracted using GenomeStudio 2.0.

Real time-qPCR

Four μg of RNAs from each placental sample were pre-treated with DNase-I to remove genomic DNA contamination with RQ1 RNase Free DNase (Promega) according to manufacturer's instructions. The DNase-I treated RNA samples were then retrotranscribed to cDNA, using the M-MLV kit (Invitrogen) according to manufacturer's instructions. Placental cDNAs were analyzed by RT-qPCR with SensiFAST SYBR No-ROX One-Step Kit (Bioline). To calculate probe expression levels, $-\Delta\text{Ct}$ was calculated by normalizing individual Ct values for each probe of interest, to house-keeping gene (SDHA) Ct. Primers are listed Supplemental Table S7.

Statistical analyses and computational details

Institut Cochin Cohort full cohort and Angers Control Samples were used for sQTL interrogation. SNPs whose three genotypes (AA, AB, BB) were not represented in at least two samples were discarded (corresponding to Minor Allele Frequency > 20%). A total of 331,204 SNPs was assessed against 48 individual splicing indexes using MatrxQTL (R package) under the ANOVA model and accounting for gestational age and sample sex as covariates. This means that a full genotypic model was used, surmising independent effects of the three possible genotypes, and not always additive effects of one given allele.

MatrxQTL generates a matrix correlating gene expression and SNP variants using an accelerated bioinformatics procedure basically dividing the initial matrix in smaller blocks. For cis-sQTL, the distance parameter was fixed to 1 Mb while for trans-sQTL all the genome was interrogated. Only cis-sQTLs and trans-sQTLs with an FDR < 0.05 were used for downstream analyses.

For the surrogate variable analyses (SVA), we used the SVA package available in R (Leek et al. 2012) on R version 3.6.3 (2020–02-29), to estimate latent sources of variations (surrogate variables) in our datasets. We considered three different subsets of samples: (1) CTRL + PEall (PE only + PE_IUGR), (2) CTRL + PEonly, and (3) CTRL + IUGR. The analysis was performed stratified by cases and controls thanks to the inclusion of the disease status variable, which described whether the sample had disease (= 1) or if it was a control sample (= 0). The *mod* and *mod0* parameters in the SVA package allow to build a linear model that describes the gene expression dataset according to the included variables with the function *model.matrix*. The two models were built as follow, in this case disease status is the Variable of Interest:

```
mod = model.matrix(Disease.status + Gestational.age + Birth.weight + Sex)
mod0 = model.matrix(Gestational.age + Birth.weight + Sex)
```

Then the *num.sv* function was used to see whether differences between the two linear models can be explained only by the presence of the variable of interest (disease status) or if any other surrogate variables can be identified, followed by the *sva* function to test whether these surrogate variables are significant:

```
n.sv = num.sv(edata,mod,method="leek")
svobj = sva(edata,mod,mod0, n.sv=n.sv)
```

No additional surrogate variables were found to play a role on gene expression differences in any of the three

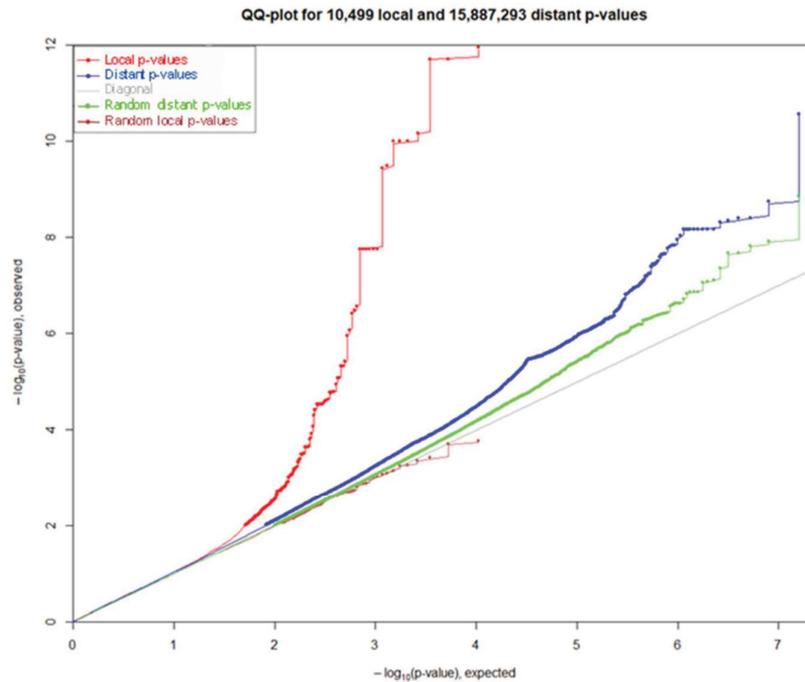


Fig. 4 QQ-plot measuring the discrepancy between real data and expected data from random (grey line)

datasets analyzed. At this stage we wanted to identify genes that could be significantly associated with either of the variables: disease status, gestational age, birth weight, Sex. In order to do so the Variable Of Interest was assigned in turn to each of the variables i.e. disease status, gestational age, birth weight, sex, followed by *p value* function that performs a F-statistic test to assign p-value to each gene in relation to the influence of the Variable of Interest under scrutiny (significance threshold at p val < 0.05). For example, to identify genes potentially influenced by “gestational age” the following code was used:

```
#Creating the full model
mod= model.matrix (~as.factor(Disease.status)
+Gestational.age+Birth.weight
+Sex, data=pheno)

#Creating the null model (mod)
- only adjustment variables
mod0= model.matrix (~as.factor(Disease.status)
+Birth.weight+Sex, data=pheno)

#identifying latent factors – no factors identified
n.sv = num.sv(edata,mod,method="leek")
svobj = sva(edata,mod,mod0, n.sv=n.sv)

#Testing for significant association between
Variable of Interest and genes
pValues = f.pvalue(edata,mod,mod0)
```

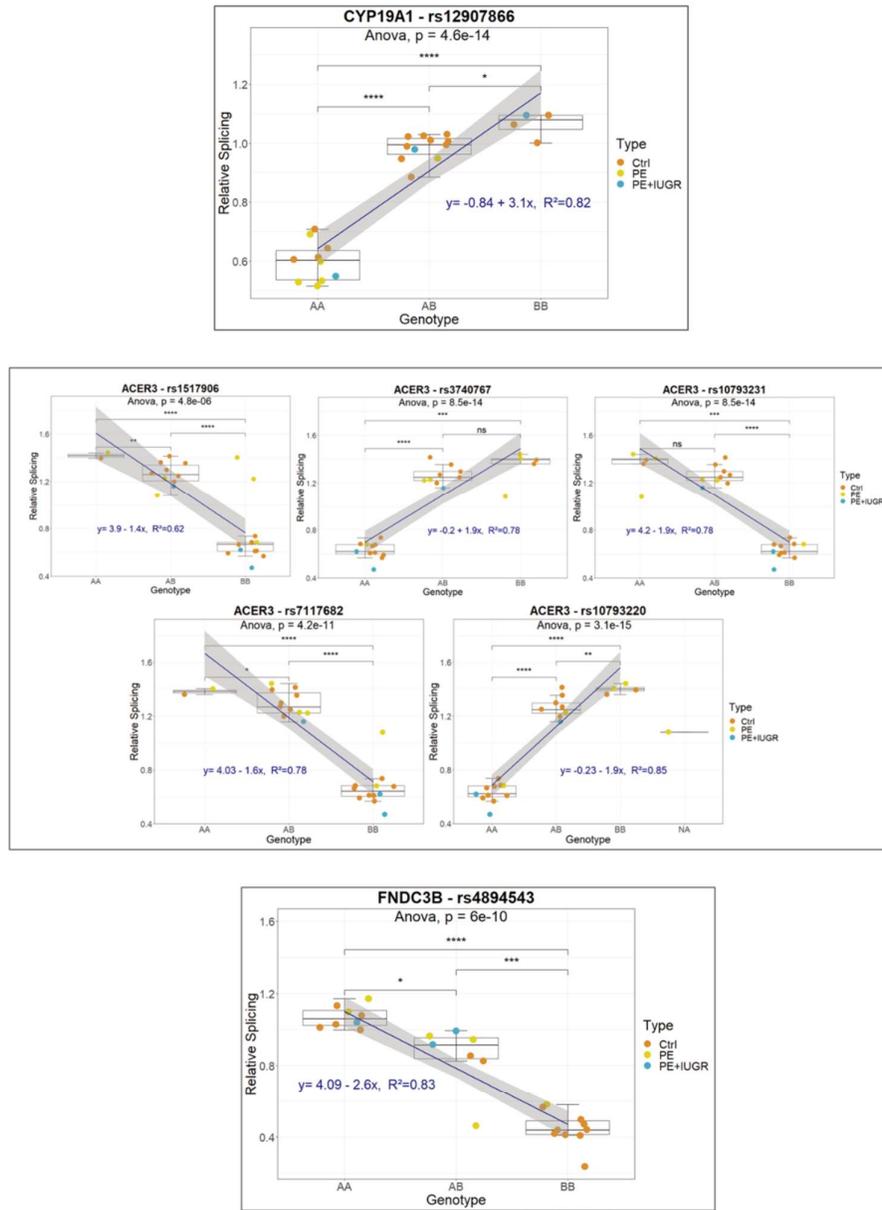


Fig. 5 Analysis of genotype versus splicing at the individual level for cis sQTLs in the placenta

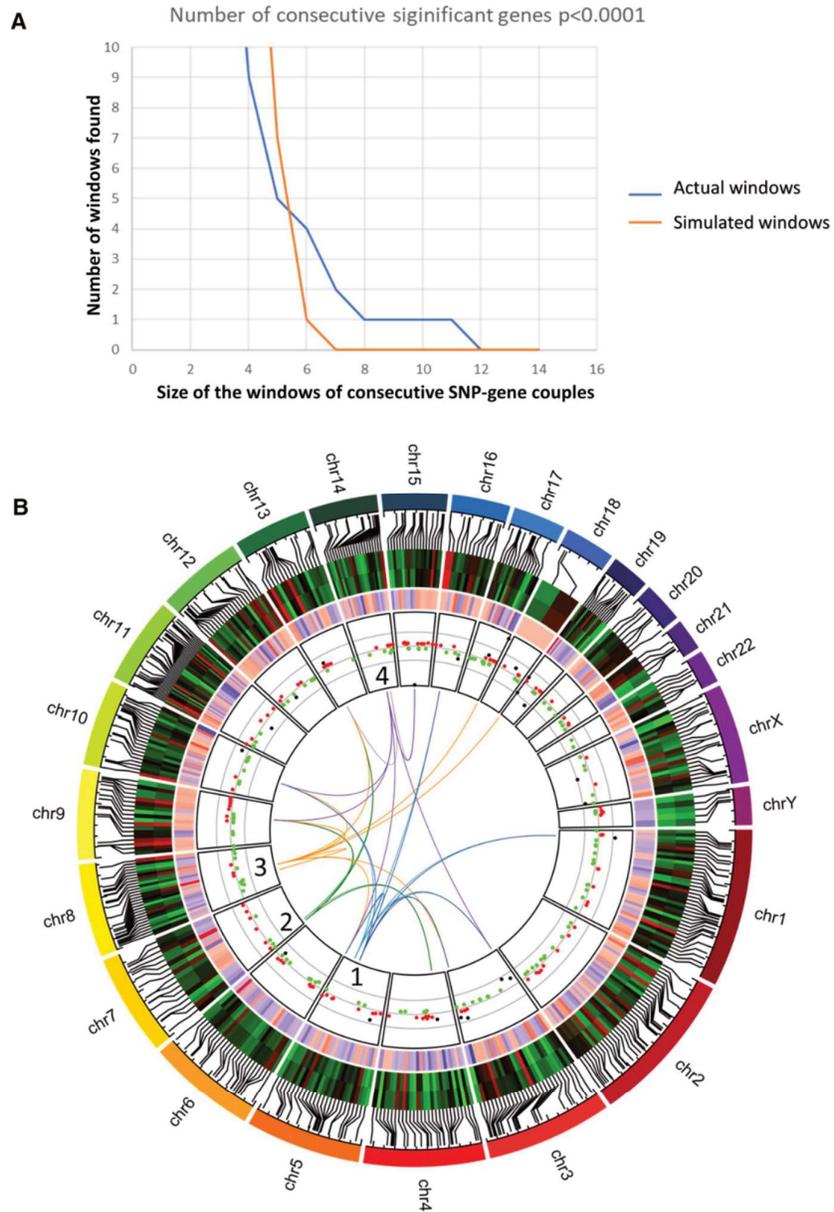


Fig. 6 Presentation of trans-sQTL. **a** Monte-Carlo analysis of windows of consecutive trans sQTL in the placentas. Trans sQTL were defined at a threshold of $p < 0.0001$ (see text). In the windows computed from the actual dataset (blue line) the longest found was of 11 consecutive SNP-genes. In the simulated windows, the longest found was composed of 6 consecutive SNP-genes (4 such in the actual dataset). Overall, while one > 5 window was found in the simulated data, 9 were found in the actual dataset. **b** circus-plot presenting the four trans-sQTL regions analyzed further in the study. Tracks are numbered from the outer to the inner part of the plot. 1st track: representation of each chromosome. 2nd track: location of the 467 genes used in the 3rd track. 3rd track: heatmap of the splicing indexes in PE and Ctrl samples for the 467 genes presenting a standard deviation equal or higher than 0.5: (Red = 19.42, Green = 3.82). 4th track: ratio of downregulation/upregulation of the 467 genes between PE and Ctrl (Red: > 3 (increased in PE), Blue < -3 (increased in controls)). 5th track: Splicing index of the 286 most differentially spliced genes observed in TAC comparing Ctrl vs PE (red dots = gene with SI between 10 and 3.99, green dots = genes with SI between -3.99 and -10 , black dots = genes with SI higher than 10 or lower than -10). 6th track: constant trans-eQTLs. Blue (1) SNPs located in ARHGAP26/AS; Green (2) SNPs located in LOC105375161; Orange (3) SNPs located in LOC105375897; Purple (4) SNPs located in GPHN/LOC105370538. The other side of the line marks the location of the gene in which the relative splicing index is affected by the corresponding SNP

In the boxplots, correlation of Individual Splicing Index (ISI) of the gene with the locus genotype was assessed by one-way ANOVA. Group–group comparison of means was executed by *T* test ($*p = 0.05$, $**p = 0.01$, $***p = 0.001$, $****p = 0.0001$). Linear regression was performed to determine the effect of genotype on ISI in each cis-QTL represented.

Gene enrichment analysis was performed using the Gene Set Enrichment Analysis module available in WebGestalt. The principle of enrichment relative to any database used as reference is the following. The list of ranked genes of the study is compared to a given set of genes specific of a biological function, a gene ontology term, or a given disease, for instance. The rank of occurrence of each gene is recorded and compared to the average expected density (for instance 100 genes out of 10,000 are expected to occur at a frequency of one every 100 genes. When the density is higher than expected, the enrichment score increases. It will reach a maximum or a minimum that will be compared with 1000 random rankings, and the actual value obtained will be compared with the random values yielding a *p*-value and an enrichment score. The reference databases analyzed are KEGG (<https://www.genome.jp/kegg/>), Reactome (<https://reactome.org/>), OMIM (omim.org), GO (<http://geneontology.org/>).

For RT-qPCR analyses, statistical significance of differences in expression levels of each tested region was assessed by analyzing the $-\Delta\text{Ct}$ distributions between groups (Ctrl ($n = 13$), PE ($n = 15$), PE + IUGR ($n = 5$) and IUGR ($n = 10$)) by 1-way ANOVA followed by Dunnett's

multiple comparison test between diseased groups and control ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

The Monte-Carlo analysis was carried out using a home-made program in VisualBasic accompanying an Excel sheet entitled trans_sQTL_Relative splicing ANOVA, provided as a supplementary material.

The circus plot graph was generated according to the following documentation: https://jokergoo.github.io/circulize_book/book/.

Discussion

In this study, we present for the first time, to the best of our knowledge, a systematic analysis of alternative splicing alterations in the pathological human placenta. After describing splicing in IUGR and PE compared to control placentas, we validated the finding at the exon level through targeted quantitative RT-qPCR. The splicing index alterations that we observed may be a consequence of the disease, or maybe driving the disease (although in some cases, they can be indirectly triggered by birth weight or shorter gestational age). In fact, the two mechanisms can be operative differently for some specific genes. The large published GWAS analysis of preeclampsia (McGinnis et al. 2017), revealed a SNP in the vicinity of the gene FLT1 (rs4769613), not in the coding region, possibly influencing splicing, that we did not find significant in our study probably due to the limited size of our sample. Indeed, our current sample size qualify our study as exploratory; with a larger sample size, other sQTL with milder effects could be found. In the specific case of sFLT1, one can imagine that a variant may influence splicing and result in being disease-prone. Currently, the cause or consequence question cannot be resolved directly in our study, but is an interesting open question that could be addressed in cell and animal models.

Another interesting question is the relation between placenta eQTL (Peng et al. 2017; Kikas et al. 2019) and the splicing QTL that we identified here. Peng and coworkers surmised that the genes affected in the placenta may mark future risks for the patients (cardiovascular, neurologic). We compared our list of 48 top cisQTL corresponding to 12 different genes with the cis eQTL (3218 cis eQTL were found as associated to SNP variant at the expression level). Four genes were in common between the two lists (33%). A contingency Chi-square calculated between the two datasets revealed a marginally significant enrichment ($p = 0.034$), while none was detected for the trans eQTL. This suggests that there may be a link between expression and splicing regulation in the placenta.

Amongst the genes that we found alternatively spliced in disease, FLT1 was identified amongst the genes that are up-regulated as well as highly differentially spliced in both

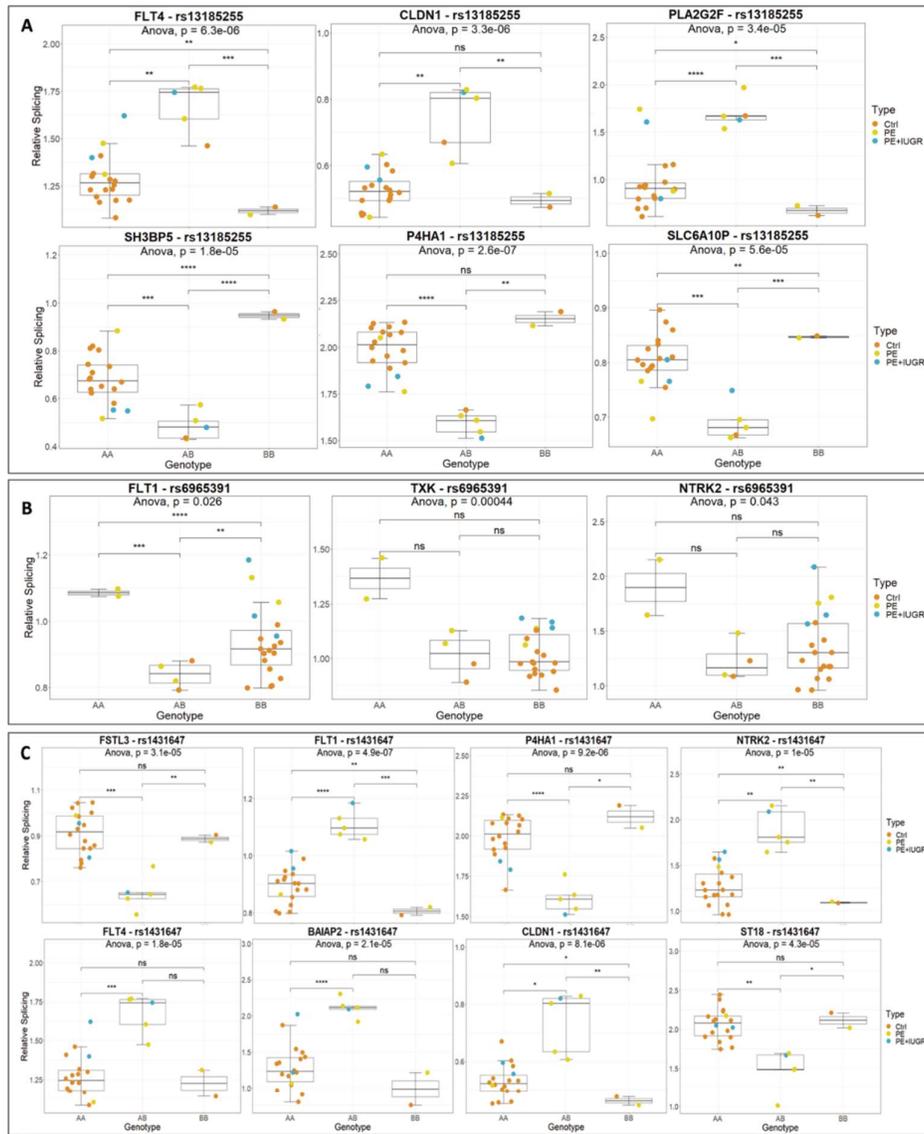


Fig. 7 Presentation of trans-sQTLs data for three SNPs found in the regions presented in Fig. 6. **a** rs13185255 from the chromosome 5 trans-sQTLs, **b** rs6965391 from the chromosome 7 trans-sQTLs, **c** rs1431647 from chromosome 8. **d** rs7145295 and rs7151086 from chromosome 14

pathological states, which is a good confirmation of the approach, given the abundant literature positing *FLT1* alternative splicing as a hallmark of preeclampsia. The quantitative analysis at the exon level revealed large heterogeneities along the mRNA molecules. We chose primer pairs located in the 1st exon, the 12th exon, the 14th exon, the 28th and between the 15th and the 17th exon. There were significant differences in expression in the first exon that is common to the different isoforms, confirming the well-known induction of this gene in the disease state. The significance weakened for exon 12 and 14, indicating that this increase in expression was untrue for this part of the gene, suggesting a relative decrease of long isoforms encompassing exons 12 and 14 in pathological placentas. When the farthest exon was analyzed (E28), the difference is also significant but to a lesser extent than for Exon 1. The induction ratio in disease compared to control was ~11-fold in exon 1, and ~ fivefold in exon 28. This indicates that short isoforms are increased in the pathological state. The data are consistent with an overall induction of the gene in disease, with a more pronounced overexpression of the shorter isoforms.

CLDN1 profile was opposite to *FLT1*, with a trend towards decrease in pathological state in the 3' region of the gene. The differential expression does not concern the region located in the 5' region of the first exon (E1c). However, at the 3' region of the same exon (E1d), a decreased expression in PE, but not in IUGR is observed. The same trend applies to the two more 3' regions of exon 2, 3 and 4. The amount of the protein coded by *CLDN1* is significantly reduced in PE placentas (Lievano et al. 2006). Here, we show that the two *CLDN1* shortest transcripts are differentially expressed in PE, but not the longest one.

Another example of a gene where differentially expressed transcripts can be identified in disease is the one coding for leptin (*LEP*). As expected, *LEP* was highly upregulated at the gene level in disease compared to control, confirming numerous previous observations, including from our group (Madeleneau et al. 2015; Biesiada et al. 2019). The alternative splicing profile of *LEP* showed that the first part of the gene (exons 1–2a) has a higher expression in diseased placentas as compared to controls. We designed primers to interrogate the expression of exon 2a, found only in three out of the four isoforms putatively expressed in the tissue (*LEP*-201, *LEP*—Afx TR0700064863.hg, *LEP*—AfxTR0700064865.hg), exon 2b common to all isoforms, and finally of the boundaries between Exons 3b and 3c (encompassed in *LEP*-201 and *LEP*—Afx TR0700064863.hg). The RT-qPCR results showed a significant upregulation of all three tested regions in PE compared to control, whilst only the 2a–2b region showed a higher expression in IUGR samples. Our results clearly indicate that IUGR and PE have different repertoires of *LEP* isoforms.

FSTL3 is also known as increased in preeclampsia (Founds et al. 2009; Gormley 2017). In this case, the increase of the gene was observable all along the molecule, but only significantly in 5' exons. In summary, the middle region of the gene is not deregulated while there is an increase in isoforms were this section could be spliced out. We also present the profile of *TXK* kinase. While the 5' part of the gene was not different according to the microarray data, the 3' appeared selectively deregulated in PE with or without IUGR. This deregulation was confirmed by RT-qPCR as shown by analysis of exons 13 and 14–15 that behave very similarly. This tyrosine kinase has not previously been associated with preeclampsia in the literature, but the mRNA is highly expressed in the placenta (trophoblast and endothelial cells), and the protein participates in immune regulation.

Pathology-driven alterations of alternative splicing require specific mechanisms and a specific molecular machinery, incorporating in particular small nuclear ribonucleoproteins, such as U1, U2, U4, U5 and U6, and spliceosome proteins, as nicely reviewed in (Scotti and Swanson 2016). To date, alternative splicing in complex diseases has essentially been considered in neurological and neuromuscular disorders, such as the abnormal splicing of Tau proteins encoding genes responsible for tauopathies (Bartsch et al. 2016). A paradigmatic example of alternative splicing in disease is the *LaminA* gene (*LMNA*), whose different dominant mutations induce respectively, and independently, muscular dystrophy, progeria, or dilated cardiomyopathy. In these cases, a monogenic variant induces a complex disease. However, understanding altered splicing in the context of polygenic and highly multifactorial diseases and processes is trickier, but starts to be addressed. For instance, in the aging brain, alternative splicing was recently studied in 44 mouse brain areas (Li et al. 2019). Interestingly, premature aging of the placenta is thought as a putative general feature of preeclampsia (Manna et al. 2019).

Any evaluation of the splicing species for complex diseases or conditions requires an accurate description of the spliced species found in the relevant organ. In obstetric diseases, this pivotal organ is the placenta. Therefore, a first effort should be carried out to evaluate the diseased placenta, which is the exact purpose of the present article. Here, we demonstrate that genes that harbor differential splicing are indeed a component in the pathogenesis of PE and IUGR. These associations with splicing are not systematically associated with a difference in the overall level change of a given mRNA, and are partly piloted by genomic variants.

Our sample size is relatively small, and our results have to be considered as a proof-of-concept warranting other targeted studies. Nevertheless, the simulation data presented in (Huang et al. 2018), indicate that a QTL with an effect of > 1 Standard Deviation is detected with sample of the size

of ours, if the Minor Allele Frequency is above 0.2 (which is the case here, since we selected SNPs for which each genotype was carried by at least two individual samples). Also for individual probes, we have a QTL effect > 1 SD in many cases, due to our stringent selection of the largest splicing indexes. Independently of variations in the expression level, we found that the subset of genes that are alternatively spliced have a disease-specific profile, with marked differences between preeclampsia and IUGR, showing in particular that the first condition but not the second, correlates with brain diseases. This fascinating observation is in accordance with many recent studies showing strong links between preeclampsia and brain vascular diseases (Andolf et al. 2020; Patel et al. 2020; Miller 2019; Basit et al. 2018; Cheng and Sharma 2016). For example, deregulation of alternative splicing of the gene *STOX1*, involved in preeclampsia and Alzheimer's disease (Dijk et al. 2005, 2010) induces a disequilibrium of its two isoforms and increases the risk of disease (Vaiman and Miralles 2016; Ducat 2020).

Focusing our work on 48 genes that were found strongly alternatively spliced between healthy samples and disease, we showed that genetic variants (SNP) impact these splicing differences. On the one hand, the cis-sQTLs had an additive effect, which is consistent with an impact of the genetic variant either on the mRNA tridimensional structure, or on the binding of splicing factors, that would affect the splicing of the RNA molecule carrying the variant. On the other hand, trans-sQTLs could give insights on how specific loci act on splicing at various genome locations. After a stringent screen, we show an allele-specific impact of four trans-sQTL loci on splicing.

The influence of the cis and trans-sQTL on splicing was generally disconnected from the disease state. This issue is not surprising since, albeit we initially restrict our genes of interest to genes that are alternatively spliced in disease, this screen was preferably used to detect genes that are highly splicing-prone. The genetic determination of the splicing is driven by specific sQTLs that may not be a strong determinant of the disease status, compared to other regulators modulating, for instance, gene expression levels. One exception could be rs1431647. This SNP located in the long non-coding RNA LOC105375897 is systematically associated with the disease when the SNP is heterozygous, associated systematically in this case to divergent splicing for *FLT1*, *FSTL3*, *P4HA1*, *NTRK2*, *FLT4*, *BAIAP2*, *CLDN1* and *ST18*. The ways these variants play their function remains an open mechanistic question.

Splicing in the placenta was observed previously for collagen 1 (Type XIII collagen) in the early human placenta (Juvonen et al. 1993). A more systematic approach was used by Kim and coworkers (Miyagawa et al. 2012), revealing that compared to other tissues, the splicing in the placenta presents specific aspects; interestingly, they found

that enrichment of genes involved in placental anomalies and pre-term birth are selectively enriched in the set of placental alternatively spliced genes. One of the most studied gene in terms of pathological alternative splicing is *FLT1*, recently shown to be alternatively spliced through the action of *U2AF65* and *JMJD6* (Eddy et al. 2020).

In summary, these analyses de novo re-identified a known factor of preeclampsia and IUGR, alternative splicing of *FLT1*, which validates the approach, and found hundreds of other disease-associated genes that are affected by splicing. We also show that these alterations are potentially genetically based. This present study paves the way towards a systematic exploration of the mechanisms and consequences of alternative splicing in normal and pathological placental function.

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Author contributions All the authors made a significant contribution to the present work.

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Availability of the data The datasets supporting the conclusions of this article are available in the EMBL-EBI repository, E-MTAB-9416.

Code availability Monte-Carlo program associated to an Excel file provided as supplementary material.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics approval Listed in Material and methods section.

Consent to participate Listed in Material and methods section.

Consent for publication All the authors validated the document.

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Chapter 3. Non-coding RNAs involved in syncytialisation are deregulated in pregnancy pathologies

"At times, our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us."

Albert Schweitzer

Non-coding RNAs include all those RNA molecules that are not translated into protein, from housekeeping ncRNAs such as transfer-RNAs (tRNA) and ribosomal RNAs (rRNA) involved in protein translation, as well as regulatory ncRNAs miR, small interfering RNAs (siRNA) and lncRNA which are usually involved in regulation of gene expression (Hombach and Kretz, 2016). This regulation occurs transcriptionally, post-transcriptionally, and by interacting with chromatin modifier complexes, inducing inheritable modifications. Additionally, ncRNAs can be stored long-term in the cell and can be inherited by daughter cells after cell division, allowing the patterns of gene expression regulation to be passed on; therefore these mechanisms are a form of epigenetic regulation (Costa, 2008; Kaikkonen et al., 2011).

In our review “The Role of Epigenetics in Placental Development and the Etiology of Preeclampsia” (Apicella & Ruano et al., 2019) (**Annex 2**), we showed how extensive literature exists to support the evidence that different classes of ncRNAs, focussing on lncRNAs and miRs, play a key role in the pathophysiology of the placenta. For instance, a cluster of 58 miRs on chromosome 19 (C19MC) is specifically expressed by trophoblast cells in the placenta and is believed to be crucial for trophoblast identity and differentiation (Donker et al., 2012; Xie et al., 2014). lncRNAs instead are usually identified when looking at gene expression alterations in placental pathologies; often lncRNAs are found to be downregulated in placentas from preeclamptic pregnancies and this correlates with altered proliferation, cell growth, invasion and apoptosis of trophoblast-like cell models. This was in fact observed for exemplary placental lncRNAs MEG3, MALAT1 and TUG1 (Li et al., 2017; Xu et al., 2017; Zhang et al., 2015).

The CTBs are the primary cell type of the placenta; these differentiate into EVT's which migrate into the maternal uterine walls to promote immunotolerance and spiral arteries remodelling, and syncytiotrophoblast STB a multinucleated unique layer formed by the fusion of CTBs,

which mediate the exchange of nutrients between mother and foetus as well as function as endocrine organ (Wang and Zhao, 2010). As discussed in our review, when ncRNAs are found to be deregulated in placental pathologies (i.e. PE), to investigate their function in placental physiology, EVT-like cell models are used and the effects of ncRNAs down/up-regulation are assessed on proliferation and apoptotic rates, as well as on the EVT characteristic migratory phenotype (Apicella & Ruano et al., 2019). However, the impact of such deregulations on the STB is understudied.

The focus of this chapter is our work “Urothelial Cancer Associated 1 (UCA1) and miR-193 Are Two Non-coding RNAs Involved in Trophoblast Fusion and Placental Diseases” (Apicella & Ruano et al., 2021). Here, we wanted to exploit even further the transcriptomic dataset we had previously produced in (Ruano & Apicella et al., 2021) (**Chapter 2**), to investigate whether key ncRNAs deregulated in pregnancy pathologies are implicated in the development of the placenta, specifically in the phenomenon of trophoblast fusion. This is further justified by the fact that morphological and functional changes in the STB were found in placentas from pregnancies with PE and IUGR phenotypes (Mariana A Costa, 2016; Roland et al., 2016).

Main aims of this study:

- Identify the set of regulatory ncRNAs differentially expressed during syncytialisation in an *in vitro* model of trophoblast which could act as master regulator of expression in this process.
- Identify ncRNAs consistently deregulated in pathological placentas (IUGR and PE).
- Investigate the role of syncytialisation regulation in pregnancy pathologies: find ncRNAs that are deregulated in pathology and are also involved in trophoblast syncytialisation.

To study the role of ncRNAs in syncytialisation, we used the choriocarcinoma cell line BeWo, which is usually mononucleated but can fuse to form small syncytia upon forskolin (FSK) administration (Orendi et al., 2010). BeWo control cells and cells treated with FSK for 72 hours were harvested for transcriptomic analysis, by microarray ClariomD, which provides expression levels of genes as well as alternative splicing isoforms. Analysis of the differentially expressed genes (DEGs), identified 800 upregulated and 1281 downregulated genes in FSK-treated cells, associated therefore with trophoblast syncytialisation in this model; of these

DEGs, 196 were regulatory ncRNAs. Through database interrogation (e.g. starBase, DianaTools LncBase), we identified putative targets of these 196 regulatory ncRNAs: 278 putative targets associated with TNF-signalling and hypoxia response were found upregulated in FSK-treated BeWo, while 572 putative targets were downregulated and appeared to be involved in cell-cycle progression. The analysis of ncRNAs with these respective putative targets by Cytoscape allowed to identify predicted transcript-transcript interactions and regulatory nodes, highlighting miR193b as key regulatory hub in trophoblast syncytialisation. Upregulated in FSK-treated BeWo, miR193b has 135 predicted targets amongst the differentially expressed genes, mostly downregulated (108 out of the 135) and interestingly was found upregulated in PE and IUGR placentas, consistently in our dataset as well as previously published studies. This stresses the functional role of miR193b as regulatory molecule for placental function.

Other miRs were found to be consistently associated with trophoblast fusion as well as placental pathologies, but only one lncRNA: Urothelial Cancer Associated 1 (UCA1). This gene had been previously localised to the STB specifically, and found upregulated in PE placentas, as well as in primary trophoblasts from PE and IUGR tissues exposed to hypoxia in culture (Gormley et al., 2017). To better understand the role of UCA1 in placental physiology and address its implication in syncytialisation, we assessed the impact of UCA1 downregulation on BeWo fusion, by siRNA-mediated knock-down, defined as UCA1-KD cells. We chose a set of genes selected for being profoundly altered between FSK-treated BeWo and control to be used as transcriptional markers of syncytialisation in this model. These included proliferative marker Ki67, STB hallmark genes hCG α , Syncytin and INHA, apoptosis genes BCL2 and BAX, and ECM protease MMP9. We compared the expression levels of these genes in FSK-treated BeWo expressing UCA1 and UCA1-KD. UCA1-KD caused a strong shift in the expression of these syncytialisation markers, pushing FSK-treated BeWo to express a transcriptomic profile closer to untreated cells, suggesting a pivotal role of UCA1 for successful CTB differentiation into STB in this cell model.

We had previously identified STOX1 as an important TF involved in syncytialisation of BeWo cells, where BeWo cells overexpressing the longer isoform STOX1A (BeWoA) showed increased syncytialisation, while overexpression of the shorter isoform lacking the transactivation domain STOX1B (BeWoB cells) correlated with impaired fusion (Ducat et al., 2020). Variants in this TF have been found associated with the PE phenotype in Dutch families

(van Dijk et al., 2005), STOX1-A overexpression in the murine placenta caused the development of PE-like symptoms during gestation as well as long-term effects on the cardiovascular system, as seen in PE women (Doridot et al., 2013; Miralles et al., 2019). Therefore, we also assessed potential interaction effects between UCA1 and STOX1 during syncytialisation; however, while both factors appear to be involved in cell fusion, UCA1 KD did not cause cumulative effects in BeWoA and BeWoB cells, treated or untreated.

The main findings of this work are summarised below:

- We identified 800 upregulated and 1281 downregulated genes as a result of FSK treatment in BeWo cells, of which 196 were regulatory ncRNAs.
- miR193b is upregulated upon FSK treatment in BeWo cells and it is a putative key regulatory hub for trophoblast syncytialisation with 135 predicted targets amongst the differentially expressed genes, 108 downregulated.
- miR193b is consistently upregulated in PE and IUGR placentas, in our dataset as well as previously published studies.
- lncRNA UCA1 is upregulated in FSK-treated cells and consistently deregulated in PE placentas in our dataset as well as existing literature, where it is found upregulated.
- UCA1 has a pivotal role for successful CTB differentiation into STB in the BeWo cell model, since UCA1-KD caused a strong shift in gene expression levels of STB-like markers tested *in vitro* (hCG α , Syncytin, INHA, BCL2, BAX, MMP9) towards the BeWo-untreated profile.
- The data relative to UCA1-mediated regulation shows how a regulatory ncRNA previously identified for its negative influence on the EVT phenotype, when upregulated, is also involved in regulating STB differentiation. This suggests a key role of UCA1 for the establishment of different trophoblast lineages.

In this study, we identified key regulatory ncRNAs that are involved in trophoblast fusion and are also found to be deregulated in placental pathologies. This supports the hypothesis that the impairment of syncytialisation is a feature of disease and should be more thoroughly investigated. For instance, having previously been found upregulated in preeclamptic placentas, the lncRNA UCA1 had been investigated for a potential role on proliferation, migration and apoptosis in trophoblast cell model HTR-8/SVneo, associated with EVT phenotype. In this model UCA1 upregulation caused inhibition of migratory and proliferative

potential of EVT-like cells (Liu et al., 2020; Msheik et al., 2020). However, in our study we showed strong evidence of its key role in syncytialisation, since UCA1 downregulation causes a shift in transcriptomic profile, associated with an impaired differentiation of FSK-treated Bewo. This evidence suggests that UCA1 might be an important factor in trophoblast differentiation pathways and the clarification of the set of target genes that are differentially regulated by UCA1 in the two trophoblast lineages, STB and EVT, will increase our understanding of mechanisms of placental development, and could help identifying other regulatory nodes involved in pregnancy pathologies.

The ncRNAs identified here as key regulatory hubs in trophoblast fusion, are found to be upregulated both during the stimulation of fusion in BeWo cells, and in preeclamptic and IUGR placentas compared to control. This could therefore suggest that syncytialisation processes driven by these molecules are enhanced in pathological placentas, and as a result the balance between CTB renewal and terminal differentiation would be skewed. Since the CTBs represent the pool of trophoblast cells that are responsible for continuous renewal of the STB layer, an improper, excessive activation of the fusion rates could result in CTB depletion, supporting the hypothesis that early placental aging is at the centre of the aetiology of PE and IUGR (Manna et al., 2019; Roland et al., 2016).

Urothelial Cancer Associated 1 (UCA1) and miR-193 Are Two Non-coding RNAs Involved in Trophoblast Fusion and Placental Diseases.

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Urothelial Cancer Associated 1 (UCA1) and miR-193 Are Two Non-coding RNAs Involved in Trophoblast Fusion and Placental Diseases

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A bioinformatics screen for non-coding genes was performed from microarrays analyzing on the one hand trophoblast fusion in the BeWo cell model, and on the other hand, placental diseases (preeclampsia and Intra-Uterine Growth Restriction). Intersecting the deregulated genes allowed to identify two miRNA (mir193b and miR365a) and one long non-coding RNA (UCA1) that are pivotal for trophoblast fusion, and deregulated in placental diseases. We show that miR-193b is a hub for the down-regulation of 135 cell targets mainly involved in cell cycle progression and energy usage/nutrient transport. UCA1 was explored by siRNA knock-down in the BeWo cell model. We show that its down-regulation is associated with the deregulation of important trophoblast physiology genes, involved in differentiation, proliferation, oxidative stress, vacuolization, membrane repair and endocrine production. Overall, UCA1 knockdown leads to an incomplete gene expression profile modification of trophoblast cells when they are induced to fuse into syncytiotrophoblast. Then we performed the same type of analysis in cells overexpressing one of the two major isoforms of the STOX1 transcription factor, STOX1A and STOX1B (associated previously to impaired trophoblast fusion). We could show that when STOX1B is abundant, the effects of UCA1 down-regulation on forskolin response are alleviated.

Keywords: trophoblast, placenta, preeclampsia, intra uterine growth restriction, syncytialisation, non-coding RNAs

INTRODUCTION

In humans, abnormal placental development is associated with two major pregnancy diseases: preeclampsia (PE) and intrauterine growth restriction (IUGR).

PE occurs in a range of 2–5% pregnancies, and it is characterized by hypertension and proteinuria, surging from the mid-gestation at the earliest (Stegers et al., 2010). Despite a certain degree of heterogeneity in its pathogenesis, a consensus exists that abnormal placentation or placenta development could be at the origin of the disease. Notably, placental ischemia would

cause intermittent hypoxia, oxidative stress, cell death, and the release to the maternal circulation of anti-angiogenic factors and debris that promote inflammation and a systemic endothelial dysfunction (Rana et al., 2019). In some cases, the disease poses a real threat to the survival of the mother requiring the delivery of the foeto-placental unit. Thus, PE is one of the major causes of premature births (before 37 completed weeks of pregnancy), with their cortege of neonate complications (Goldenberg et al., 2008). The symptoms of the disease disappear after delivery. However, epidemiological studies have shown that the women who have suffered a preeclamptic pregnancy have an increased risk of developing a cardiovascular disease (CVD) later in life (Newstead et al., 2007; Brouwers et al., 2018), as well as other diseases affecting strongly vascularized tissues, such as the brain (Basit et al., 2018).

IUGR refers to a somehow loosely defined condition in which the unborn baby is smaller than expected for his or her gestational age (Nardoza et al., 2017). IUGR babies typically have an estimated weight that falls below that of 90% of unborn babies of the same gestational age. In addition, IUGR babies are sometimes born prematurely. Babies with IUGR are at increased risk of health problems before, and after birth. These problems include low oxygen levels while in the womb and high levels of distress during labor and delivery. In the long term, IUGR increases the risk of developing a metabolic disease such as type 2 diabetes and CVD (Darendeliler, 2019).

In about one third of the cases, PE is complicated with IUGR, suggesting that there could be an overlap in the etiology of both diseases. The considerable similarity in histopathology and gene expression in the placentas has been recently reported between both diseases (Awamleh et al., 2019; Gibbs et al., 2019; Medina-Bastidas et al., 2020).

In the human placenta, the maternal blood is in direct contact with a continuous multinucleated layer, the syncytiotrophoblast (STB). This polarized interface releases hormones and mediates the exchange of nutrients, gases and waste between mother and the developing fetus (Turco and Moffett, 2019). The STB is mitotically inactive, formation and constant renewal of the syncytium depends on the underlying mononuclear cytotrophoblasts (CTB). Throughout gestation, CTBs proliferate, differentiate and eventually fuse with the STB via cell-syncytial fusion. This process is balanced by a concomitant release of apoptotic material as syncytial knots from the STB to the maternal circulation. Hence, the process of syncytialization is critical to the integrity of the STB and in maintaining the essential functions of the placenta. Several *in vitro* and *in vivo* studies have demonstrated a close, if not a causal, relationship between structural/functional deficiency of the syncytium and the development of PE and IUGR (Guller et al., 2008; Roland et al., 2016; Costa, 2016).

Genome-wide transcriptomic and epigenomic studies have greatly contributed to the understanding of the molecular mechanisms involved in either normal or pathological placenta development. Thus, numerous studies have revealed altered placental expression of various genes in PE and IUGR (Cox et al., 2015; Deyssenroth et al., 2017; Chabrun et al., 2019; Majewska et al., 2019; Benny et al., 2020). A particular category concerns

those genes encoding for non-protein coding RNAs (ncRNAs). Classes of ncRNAs include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small RNAs, such as microRNAs (miRNAs), siRNAs, piRNAs, snoRNAs, snRNAs, exRNAs, scaRNAs and the long ncRNAs (Hombach and Kretz, 2016). The ncRNAs display a great variety of mechanisms of action including: post-transcriptional gene regulation through controlling processes like protein synthesis, RNA maturation, transport and decay, but also, transcriptional gene regulation through the modification of chromatin structure (Fernandes et al., 2019). They are an important basis of epigenetic regulation in the human placenta, in normal and pathological situations (Hayder et al., 2018; Apicella et al., 2019). Structurally different ncRNAs engage diverse mechanisms that lead to different regulatory outcomes. The discovery of the diversity of functions played by the ncRNAs in the cell physiology has boosted the exploration of their role in placental development, physiology and pathology.

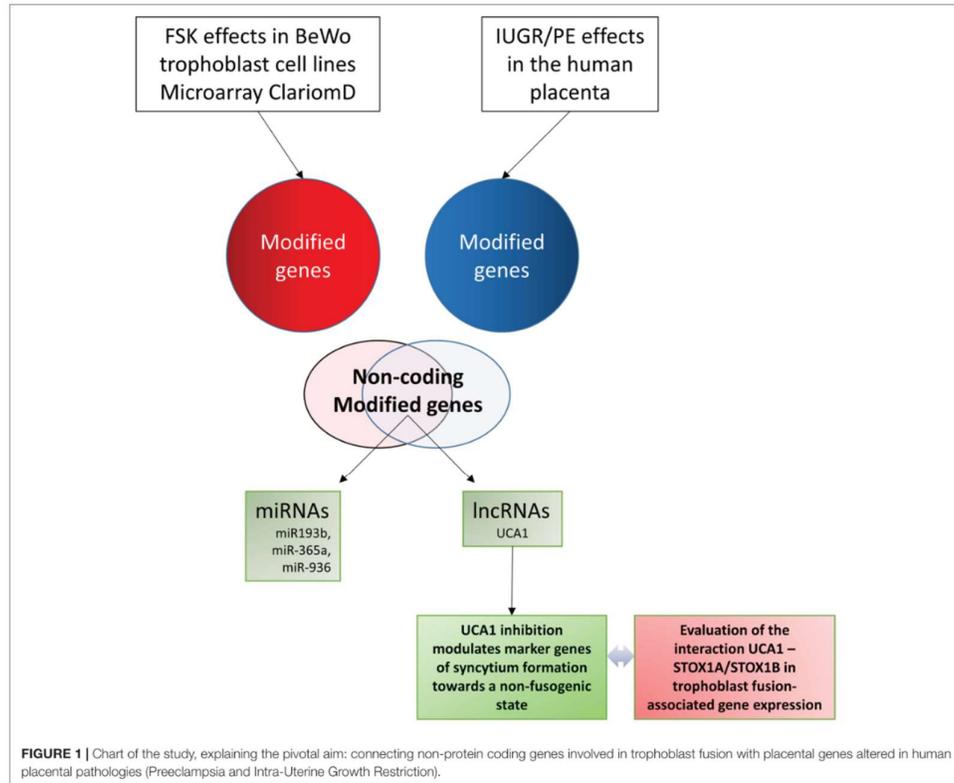
The central role of the STB in the physiology of the placenta, suggests that deregulation of ncRNAs specifically required for its formation and/or maintenance could be potentially involved in placental diseases. Here we combined two microarray analyses, one carried out on the classical fusogenic trophoblast model BeWo (under the accession number GSE148088) (Kudo et al., 2004; Ramos et al., 2008; Orendi et al., 2010; Shankar et al., 2015; Zheng et al., 2016), and one carried out on total human placentas with normal controls, PE and IUGR placentas (under the accession number E-MTAB-9416). A cross-analysis was carried out with a drastic filtering in order to identify ncRNA that are associated to disease (in the placentas) and to fusion (induced by forskolin treatment in the BeWo cells), in parallel.

This cross-analysis allowed the identification of a small subset of ncRNAs which are consistently modified both during fusion of trophoblast cells, and in the pathological placentas. We then carried on our analysis focusing upon the miRNA miR-193b (by a bioinformatic approach) and the lncRNAUCA1 (through knock-down (KD) experiments). In addition, we analyzed the effects of this KD in BeWo cells, overexpressing specifically one of the two major isoforms of the STOX1 transcription factor (STOX1A and STOX1B), previously identified as a key player in preeclampsia (George and Bidwell, 2013), and recently shown to modulate fusion through a specific equilibrium between its two isoforms (Vaiman and Miralles, 2016; Ducat et al., 2020). The chart of the present study is shown as Figure 1.

RESULTS

Transcriptional Modifications in BeWo Cells Following Forskolin Treatment

BeWo cells were cultured in the presence of 20 μ M forskolin to induce cell fusion (BeWo-FSK). Control cells were grown with the vehicle, DMSO (BeWo-CO). After 72 h, the total RNA was extracted, and global gene-expression profiles were analyzed with microarrays. Comparison of BeWo-FSK relative to BeWo-CO detected 2109 genes differentially expressed (DEGs) with a fold change (FC) either ≤ -2 or ≥ 2 , and

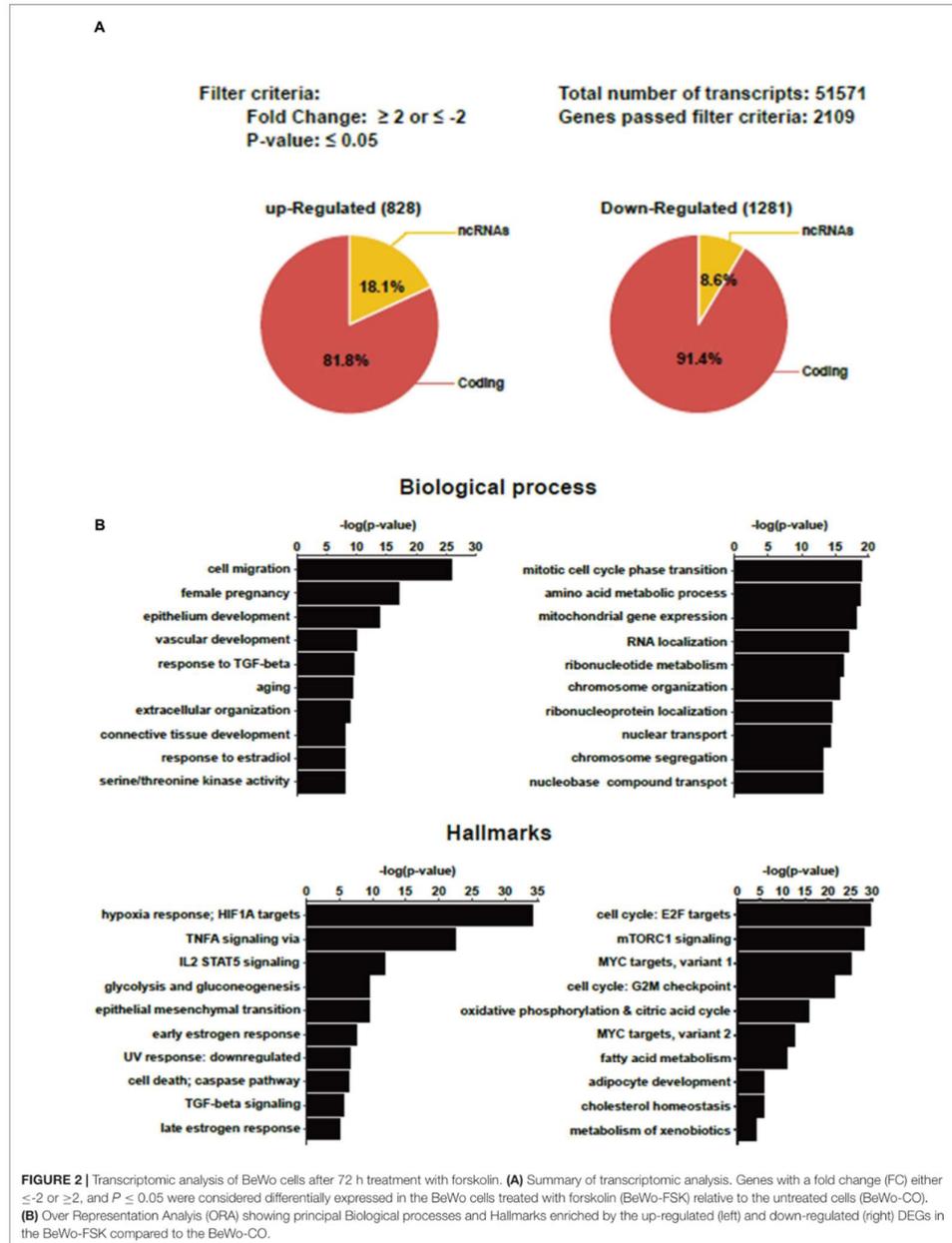


P -value ≤ 0.05 (Figure 2A and Supplementary Table 1). Of these, 828 genes were up-regulated and 1,281 down-regulated (Supplementary Table 1). Gene set enrichment analysis (GSEA), and over representation analysis (ORA) confirmed that our results were accurately consistent with previous reports having analyzed transcriptomic changes in BeWo cells after forskolin treatment (Supplementary Figure 1). In the BeWo-FSK we detected substantially increased expression of key markers of syncytialization such as CGA ($FC = 10.1$; $P = 2.1 \times 10^{-11}$), CGB1 ($FC = 20.5$; $P = 2.81 \times 10^{-12}$) or ERVFRD-1 (aka Syncytin2, $FC = 10.6$; $P = 6.23 \times 10^{-12}$). Up-regulated DEGs were associated with biological processes such as cell migration, vascular development, response to TGF-Beta and response to hypoxia. Down-regulated DEGs are mainly involved in cell cycle progression, amino-acid metabolism, and mitochondrial gene expression (Figure 2B). In terms of hallmarks present in the GSEA Broad database, inflammation and hypoxia pathways were particularly enriched in up-regulated genes, while cell cycle, nutrient sensing via mTORC1 pathway, were strongly enriched

in down-regulated genes, suggesting a whole silencing of basic pathways of cell physiology and energy expenditure slow down, accompanying the differentiation of the trophoblast cells into a syncytial structure.

Differentially Expressed Non-coding RNAs in Forskolin-Treated BeWo Cells

Three hundred and seven (307) of the DEGs detected in the BeWo-FSK relative to BeWo-CO, encode annotated ncRNAs. They belong to different categories including: sense-intronic RNA, antisense RNA, long non-coding RNA (lncRNA), circular (circRNA), microRNA (miRNA) and housekeeping ncRNAs (Y-RNAs, ribosomal RNAs, Small nucleolar RNAs and transfer RNAs) (Figure 3). The category of housekeeping ncRNAs was the most represented (111 genes). Nonetheless, we focused our study on the categories corresponding to regulatory ncRNAs. A selection of the most significantly modified regulatory ncRNAs detected



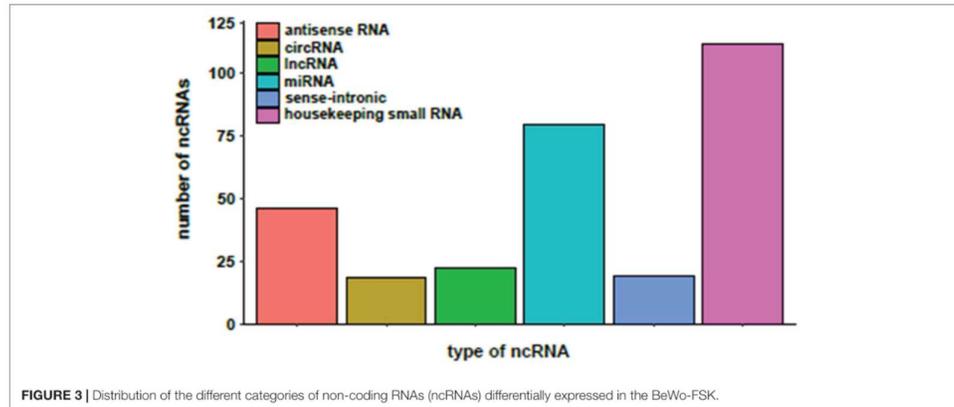


FIGURE 3 | Distribution of the different categories of non-coding RNAs (ncRNAs) differentially expressed in the BeWo-FSK.

TABLE 1 | Top 25 differentially expressed regulatory ncRNAs in the BeWo cells after 72 h of forskolin treatment.

Gene symbol	ncRNA class	Fold change	P-value	FDR P-value
hsa-miR-147b	miRNA	28.94	8.33E-11	3.53E-07
LINC01511	lncRNA	14.88	8.43E-13	1.63E-08
hsa-miR-4632	miRNA	8.93	6.38E-09	7.80E-06
RP11-420L9.5	antisenseRNA	7.6	1.61E-08	1.48E-05
LINC01237	lncRNA	4.45	2.70E-07	1.00E-04
MYCNUT	lncRNA	4.31	3.72E-09	5.15E-06
LINC01164	lncRNA	3.98	1.37E-07	8.14E-05
UCA1	lncRNA	3.76	2.19E-06	7.00E-04
CTB-60B18.12	antisenseRNA	3.46	2.06E-06	7.00E-04
hsa-miR-6810	miRNA	3.36	3.13E-06	9.00E-04
hsa-miR-936	miRNA	3.35	5.99E-06	1.50E-03
SLC2A1-AS1	antisenseRNA	3.23	6.17E-06	1.50E-03
IL10RB-AS1	antisenseRNA	3.14	5.32E-06	1.30E-03
hsa-miR-193b	miRNA	2.91	8.00E-04	4.54E-02
hsa-miR-365a	miRNA	2.73	3.90E-06	1.10E-03
hsa-miR-6888	miRNA	2.54	8.24E-05	9.80E-03
hsa-miR-3941	miRNA	2.16	3.18E-05	5.10E-03
Hsa-miR-636	miRNA	-2.16	4.69E-05	6.70E-03
hsa-miR-301a	miRNA	-2.27	2.92E-05	4.90E-03
RP11-884K10.7	antisenseRNA	-2.84	4.00E-04	2.68E-02
COX10-AS1	antisenseRNA	-3.12	5.63E-05	7.60E-03
OLMALINC	lncRNA	-3.33	9.98E-05	1.12E-02
hsa-miR-1908	miRNA	-3.45	6.12E-07	3.00E-04
DLEU2	lncRNA	-3.75	8.74E-05	1.03E-02
hsa-miR-6758	miRNA	-4.31	8.16E-07	3.00E-04

following forskolin treatment in the BeWo cells is shown in **Table 1**. These include the lncRNAs, antisenseRNAs and miRNAs. The list of the totality of ncRNAs (classified by category) detected as differentially expressed is provided as **Supplementary Table 2**.

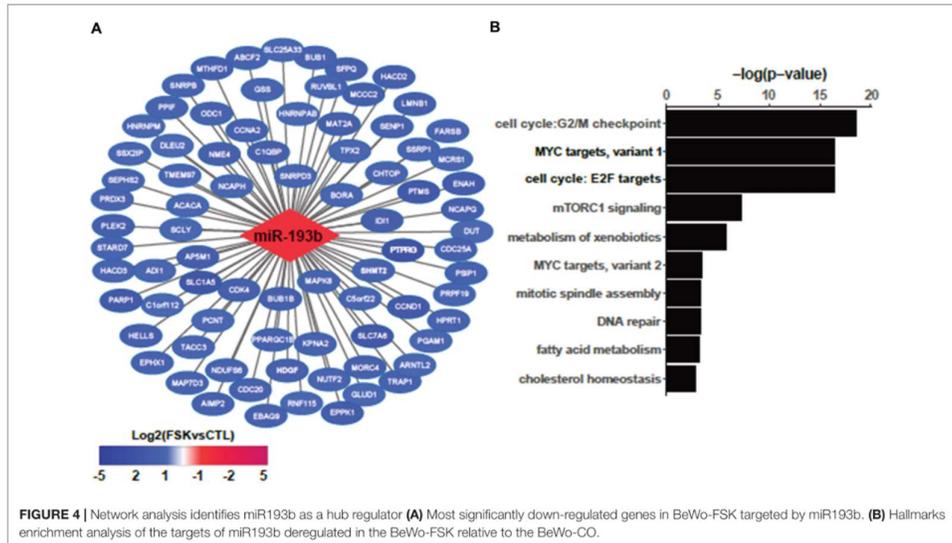
Identification of Regulatory ncRNAs Targets Reveals Their Potential Roles in Syncytialization

To investigate the role of these ncRNAs in the process of BeWo fusion we identified their putative targets (when known) using appropriate databases (miRBase, starBase v2.0. and DianaTools LncBase v.2). Since miRBase tends to provide a more limited list of putative target genes (~10% of the others) for a given miRNA, and since these were generally largely covered in the other databases, this constituted the major basis for the establishment of our lists of targets. Next, we selected among these targets those which are indeed detected as DEGs in the BeWo-FSK relative to the BeWo-CO cells. These resulted in a list of 278 up-regulated and 572 down-regulated DEGs. Out of the deregulated DEG list (**Supplementary Table 1**), this represents 33.6% for the up-regulated and 44.6% for the down-regulated DEGs. Assuming a total of 50,000 genes including the non-protein coding ones, the expected proportions are 1.6 and 1.1%, respectively, thus we observed a significant enrichment of putative targets in both cases (χ^2 contingency test, $p < 10^{-20}$).

Hallmark enrichment analysis using the ncRNAs targeted-DEGs revealed that the most significantly impacted functions are related to the cell cycle progression for the down-regulated DEGs, while TNF-signaling and hypoxia response are the most enriched processes for the up-regulated DEGs. Strikingly, these enriched pathways are quite similar to those obtained with the total DEG gene set, suggesting that many DEGs contributing to the definition of the hallmarks are targeted by the ncRNAs.

Network Analysis of ncRNAs and Their Targets Identifies miR-193b as a Hub

To further analyze the role of the differentially expressed ncRNAs in the process of BeWo fusion we used the Cytoscape tool to construct a regulatory network integrating these ncRNAs and their targets. This resulted in a network composed of



985 nodes (representing ncRNAs and targets) and 1,775 edges (representing interactions). Next, we submitted our network to topological analysis. MiR-193b was identified as the principal hub of our network, with 135 predicted targets (Figure 4A). Other lesser hubs corresponding to miR-16, miR-455, and miR-365 are presented as Supplementary Figure 2. Most of the targets of miR-193b were down-regulated (108 out of 135) in the BeWo-FSK relative to BeWo-CO, while miR-193b was significantly up-regulated ($FC = 2.91$; $P = 0.0008$). Hallmarks enrichment analysis shows that the down-regulated genes targeted by miR-193b are mainly involved in the control of cell cycle progression and energy usage/nutrient metabolism (Figure 4B).

A Small Subset of ncRNAs Involved in Syncytialization Is Also Associated With Preeclampsia and Intrauterine Growth Restriction

To identify ncRNAs associated with syncytialization in BeWo which could be also involved in PE and/or IUGR we compared the list of ncRNAs identified in BeWo with those identified in a list from our study on total human placentas with normal controls, PE and IUGR placentas (E-MTAB-9416) and other published datasets (GSE114349, GSE114691, GSE75010, GSE93839, and GSE66273). Comparison of all differentially expressed ncRNAs, revealed that two miRNAs (miR-193b and miR-365a) and one lncRNA (UCA1) were found most consistently up-regulated in both PE and IUGR. Therefore, miR-193b is associated to trophoblast fusion, together with pathological placentation, suggesting its overall implication in

normal placental function. A few additional miRNAs were also found simultaneously differentially expressed in preeclamptic placentas and in the BeWo-FSK (miR-936; miR-6886; miR-7110; miR-518A1; miR-4454, and miR-1283-2), but those were not studied further in the present paper.

We next focused our attention on the lncRNA UCA1. This was justified by the following: (i) UCA1 is up-regulated in PE and IUGR but also in primary cultures of human syncytiotrophoblasts exposed for 24 h to 1% oxygen as compared to the same cells exposed to 20% oxygen (Table 2), (ii) also, a study coupling laser microdissection to isolate specific trophoblast subpopulations and microarray analysis, identified UCA1 as the most differentially expressed ncRNA in STBs isolated from the placentas of pregnancies with severe PE relative to controls (Gormley et al., 2017), (iii) *in situ* hybridization confirmed up-regulation of UCA1 in the STBs compartment of the placenta. Alternative splicing isoforms have been described for UCA1 (Xue et al., 2016). We found that UCA1 was increased

TABLE 2 | Genome-wide transcriptomic studies showing up-regulation of UCA1 either in preeclampsia or intrauterine growth retardation.

Data set	Study	Fold change	P-value
E-MTAB-9416	IUGR vs. CO	8.91	3.20×10^{-03}
GSE75010	PE vs. CO	2.04	2.81×10^{-10}
GSE93839	PE STB vs. CO STB	5.93	1.55×10^{-03}
GSE66273	PE vs. CO	6.19	3.33×10^{-04}
GSE147776	IUGR vs. CO	2.01	3.58×10^{-02}
GSE41331	CTB 1%O2 vs. 20%O2	1.86	1.61×10^{-02}

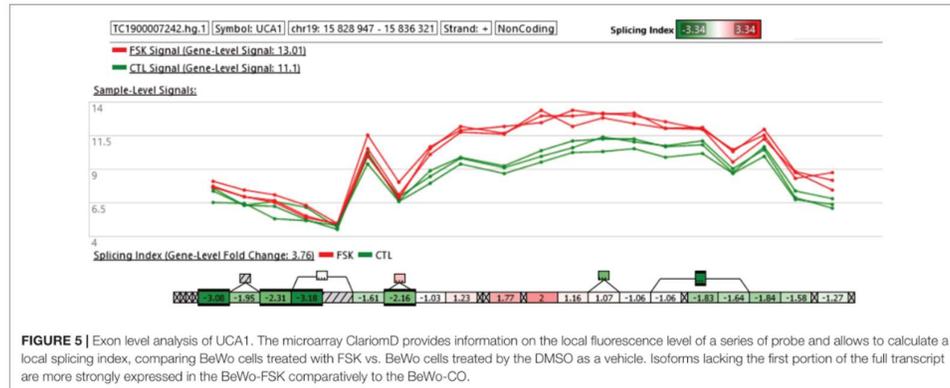


FIGURE 5 | Exon level analysis of UCA1. The microarray ClariomD provides information on the local fluorescence level of a series of probe and allows to calculate a local splicing index, comparing BeWo cells treated with FSK vs. BeWo cells treated by the DMSO as a vehicle. Isoforms lacking the first portion of the full transcript are more strongly expressed in the BeWo-FSK comparatively to the BeWo-CO.

in expression by 3.76-fold following FSK treatment. Exon level analysis of UCA1 (accessible through the ClariomD array used in this study) shows that probes located in the terminal part of the gene have a higher level of fluorescence in the forskolin-treated compared to the control cells (Figure 5). Thus, it shows that in the BeWo-FSK there is a specific increase in the production of the shorter isoforms (ENST00000600160.2, ENST00000589333.2) of UCA1, while the level of expression of the most complete isoform (ENST00000397381.4) is apparently unaffected.

UCA1 siRNA Knockdown Lead to Altered Regulation of Genes Involved in Fusion Mechanisms in BeWo Cells

The effect of the si-RNAs was evaluated by qRT-PCR. UCA1 levels were drastically affected (reduction ranging from 90 to 98% compared to the si-SCR, according to the experiment, Figure 6A). In a first characterization, we analyzed in BeWoC cells the expression of genes involved in cell proliferation (*ki67*, *ITIH5*), oxidative stress (*NOS3*, *GCLM* and *CAV1*-also involved in exosome physiology), membrane repair (*ANXA1*, *ANXA2*, *CAV1*), trophoblast fusion (*Syngctin1*, *Syngctin2*), endocrine differentiation of trophoblast (*CGA/CGB*), syncytiotrophoblast stabilization (*TGM2*) cell migration (*MMP9*), oxygen sensing (*INHA*), and apoptosis (*BAX*, *BCL2*, *DAPK1* and *BAD*). The KD of UCA1 led to significant alterations of almost all the genes involved in these pathways (Figure 6). A principal component analysis was carried out on the qPCR data and showed a clear separation of the cell replicates (Figure 7). The first axis (79% of the variation) is driven by the FSK treatment, while the second axis (10%) contrasts markers of differentiation vs. markers of proliferation. The analysis reveals that in terms of gene expression, the KD of UCA1 reduced the differences between FSK treated cells and control group in the center of the graph. This means that when levels of UCA1 are strongly reduced, the expression profile remains closer to that of untreated control cells. These

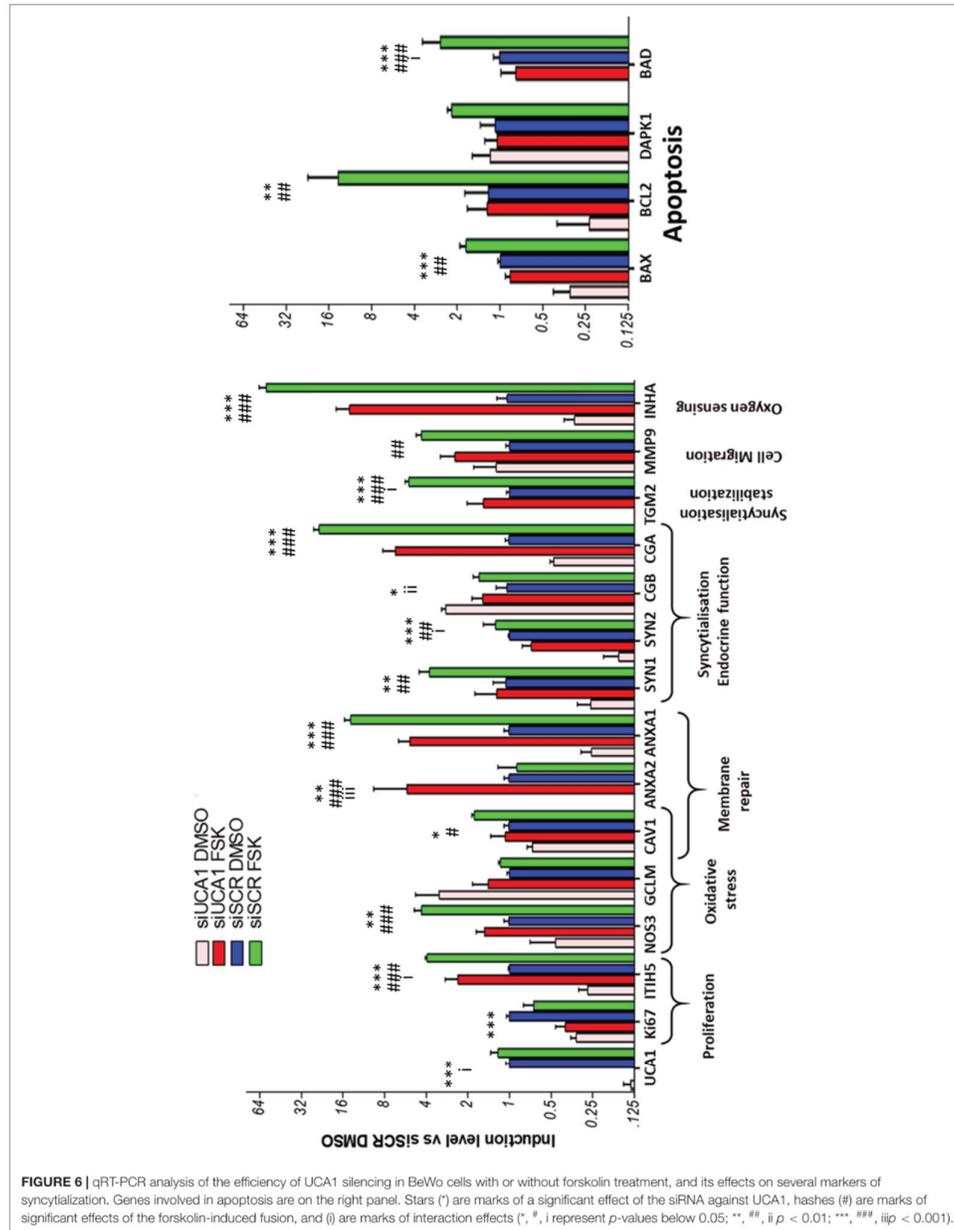
observations suggest that UCA1 is important for a successful syncytialisation process.

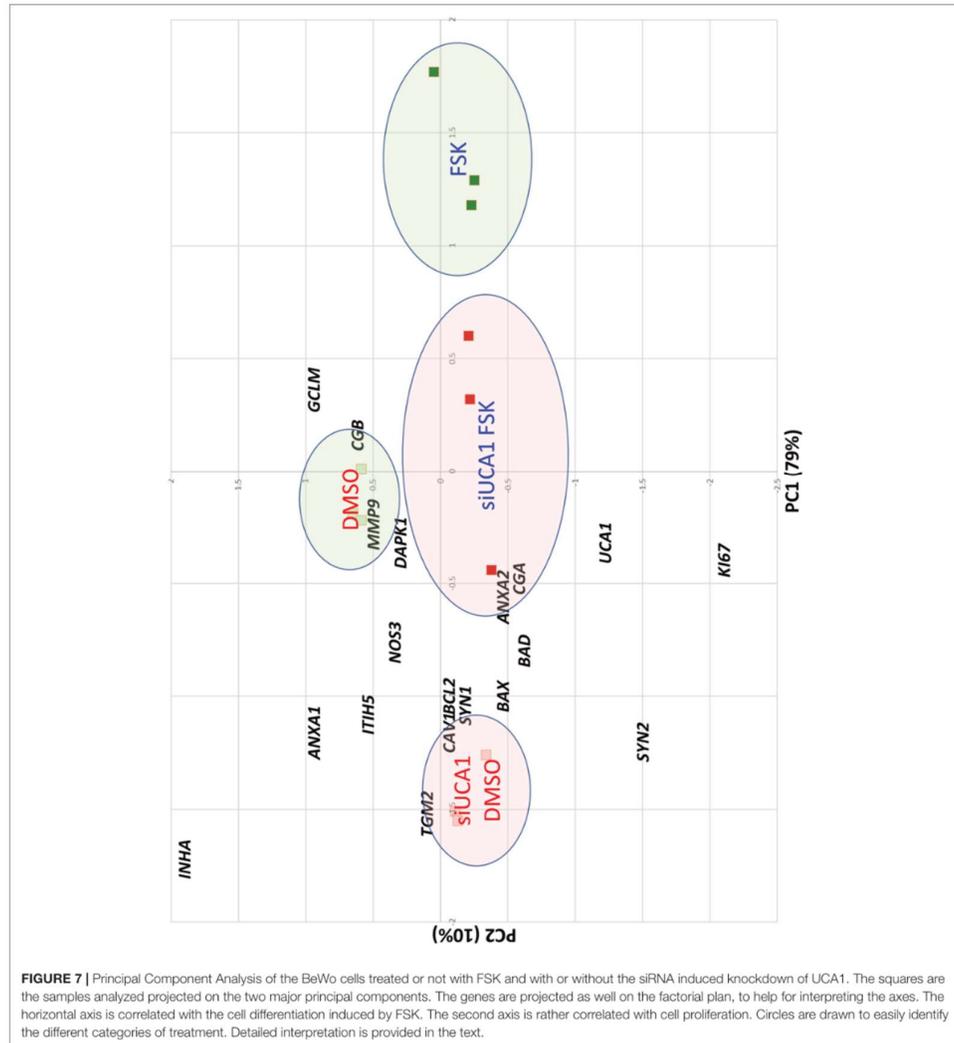
A statistical test by two-ways ANOVA revealed significant modifications for most genes except *GCLM* and *DAPK1* (Table 3 and Figure 6). The knockdown of UCA1 by itself affected all the genes, but *MMP9*. The FSK effect was significant in all the genes except UCA1, *CGB*, *KI67* (in addition to *GCLM* and *DAPK1*). Finally, there was a significant interaction effect between the FSK treatment and the UCA1 KD in the case of UCA1, *CGB*, *SYNCYTIN2*, *TGM2*, *ANXA2*, *ITIH5*, and *BAD*. These interaction effects indicate a differential effect of the UCA1 KD according to the FSK treatment leading to cell fusion.

Interference BETWEEN UCA1 and the Trophoblast Differentiation Factor STOX1 in BeWo Cells

Previously, we have identified STOX1 as a major actor of the syncytialisation process in BeWo cells. More specifically, we showed that the short isoform of STOX1, STOX1B, antagonizes cell fusion when overexpressed and that disturbing the STOX1A/B balance mimics specific gene perturbations seen in PE CTBs in three well-characterized cell lines BeWoA, BeWoB and BeWoC, the first two overexpressing STOX1A and STOX1B, 20–30- and 6-fold, respectively (Ducat et al., 2020). In the present paper, analyzing exon-level microarrays, we show now for the first time that STOX1 isoforms are differentially spliced when the BeWo cells fuse under forskolin (Supplementary Figure 3), strengthening the idea that the two isoforms are associated with different pivotal stages of trophoblast differentiation.

To explore the putative role of UCA1 in the syncytialization in normal and pathological conditions, we silenced this lncRNA using a specific small interfering RNA (si-RNA) in BeWo cells lines permanently transfected either with an empty plasmid (encoding G418 resistance), or with the same plasmid encoding for the expression of either STOX1A or STOX1B (These cells are





called BeWoC, BeWoA or BeWoB, respectively). The cells were transfected with the si-UCA1 or a scrambled siRNA, si-SCR, used as control. We analyzed by qPCR a panel of 8 genes (including UCA1). The choice of these 8 genes was motivated by the fact that strong correlations exist between the 19 genes analyzed in the first experiment. The chosen genes are representative of the different pathways analyzed (proliferation-KI67, oxidative

stress-INHA, trophoblast differentiation-SYN1, apoptosis-BCL2, BAX, invasion-MMP9, endocrine differentiation-CGA). The results are presented as a histogram (Figure 8A), with statistical values presented in Supplementary Table 3 (ANOVA), and as a PCA analysis, where the two first axes account for 40 and 19.5% of the variance (Figure 8B). BeWoC cells (represented by squares) harbor the same profile with the 8 genes from the

TABLE 3 | Statistical analyses of individual gene effects following UCA1 knock-down \pm FSK treatment (significant values are in red fonts).

Overall tests of univariate models			Tests of univariate effects		
Y variable	F	Prob.	Source	F	Prob.
UCA1	22.504	0.00029659	Si treatment	56.948	6.6307×10^{-05}
			Fusion	4.288	0.07213567
			Si treatment*Fusion	6.275	0.03665572
CGB	8.208	0.00797657	Si treatment	10.373	0.01222583
			Fusion	0.283	0.60936093
			Si treatment*Fusion	13.967	0.00572781
CGA	127.930	4.243×10^{-07}	Si treatment	43.849	0.00016558
			Fusion	336.346	8.0411×10^{-08}
			Si treatment*Fusion	3.596	0.09452054
GCLM	2.521	0.13152052	Si treatment	4.143	0.07620217
			Fusion	0.975	0.3524659
			Si treatment*Fusion	2.445	0.15650114
NOS3	14.589	0.00131394	Si treatment	15.149	0.0045949
			Fusion	28.613	0.0006966
			Si treatment*Fusion	0.004	0.95003544
CAV1	5.155	0.02833346	Si treatment	7.939	0.02257829
			Fusion	7.147	0.02821257
			Si treatment*Fusion	0.379	0.55505375
KI67	13.163	0.00184323	Si treatment	34.327	0.00037894
			Fusion	0.891	0.37283458
			Si treatment*Fusion	4.272	0.07257943
SYN2	23.107	0.00027009	Si treatment	39.848	0.00022968
			Fusion	18.244	0.00271954
			Si treatment*Fusion	11.229	0.0106638
TGM2	51.718	1.3944×10^{-05}	Si treatment	63.473	4.4987×10^{-05}
			Fusion	84.869	1.5606×10^{-05}
			Si treatment*Fusion	6.811	0.0311362
ANXA2	39.218	3.939×10^{-05}	Si treatment	23.397	0.00129298
			Fusion	43.573	0.00016922
			Si treatment*Fusion	50.685	0.00010006
ITIH5	56.208	1.0168×10^{-05}	Si treatment	37.157	0.00029085
			Fusion	125.667	3.5958×10^{-06}
			Si treatment*Fusion	5.801	0.04260521
SYN1	10.209	0.00413556	Si treatment	15.939	0.00399228
			Fusion	14.682	0.00500579
			Si treatment*Fusion	0.005	0.94569912
ANXA1	96.898	1.2527×10^{-06}	Si treatment	45.870	0.00014171
			Fusion	243.711	2.8262×10^{-07}
			Si treatment*Fusion	1.114	0.32213654
MMP9	5.171	0.02810927	Si treatment	1.134	0.31791426
			Fusion	13.560	0.00619729
			Si treatment*Fusion	0.819	0.39182403
INHHA	137.727	3.1788×10^{-07}	Si treatment	40.502	0.00021729
			Fusion	372.037	5.4151×10^{-08}
			Si treatment*Fusion	0.641	0.44650823
BAX	21.540	0.00034606	Si treatment	36.796	0.00030055
			Fusion	25.245	0.00102124
			Si treatment*Fusion	2.580	0.14686479
BCL2	8.774	0.00654947	Si treatment	13.427	0.00636233
			Fusion	12.786	0.00723255
			Si treatment*Fusion	0.110	0.74867557

(Continued)

TABLE 3 | Continued

Y variable	Overall tests of univariate models		Tests of univariate effects		
	F	Prob.	Source	F	Prob.
DAPK1	2.339	0.14975061	Si treatment	2.106	0.18480454
			Fusion	2.027	0.1923598
			Si treatment*Fusion	2.884	0.12788132
BAD	32.681	7.7268 × 10 ⁻⁰⁵	Si treatment	55.723	7.1629 × 10 ⁻⁰⁵
			Fusion	36.270	0.0003154
			Si treatment*Fusion	6.050	0.03934242

previous analysis: in the condition where the KD of UCA1 is combined with FSK treatment, the expression profile is close to the one of BeWoC cells without FSK and not treated with the siUCA1. Overall, the FSK effects were all oriented in the same direction of the first axis (clear to dark colors), which can thus be interpreted as a mark of FSK-induced trophoblast differentiation. A strong difference between BeWoA, BeWoB and the control BeWoC comes from the variation along the second axis, showing that in this case, STOX1-overexpressing cells in the presence of forskolin are positioned with increasing abscissae, suggesting that this axis correlate with STOX1-driven differentiation in the presence of forskolin. When UCA1 is KD, the effect of STOX1 are less pronounced on the second axis (red squares, lozenges-BeWoA and circles-BeWoB). In the absence of FSK the STOX effects are less obvious and the dots are all in the middle of the graph.

Interestingly, the gene expression profiles are very peculiar in BeWoB cells under FSK treatment when analyzing UCA1 effects. On the first axis, UCA1 KD is not able to modify strongly the x-axis in these cells (from ~2.3 to 1.5, $\Delta = 0.8$), while in control BeWoC, the modification on the x-axis ranges from ~1.9 to -0.5, $\Delta = 2.4$, and in BeWoA from ~2.2 to 0.5, $\Delta = 1.7$. The effect on gene differentiation was thus less reduced in BeWoB cells, comforting the idea that BeWoB overexpression and UCA1 KD act similarly but not synergistically against trophoblast fusion. This may be associated to the fact that STOX1B overexpression is associated with deficits of syncytialization (Ducat et al., 2020).

DISCUSSION

In the placenta, the STB acts as a barrier between the mother and fetus, and functions in gas exchange, nutrient, waste transport and hormone production. The STB is mitotically inactive and is formed by the constant cell-cell fusion of the underlying mononuclear CTBs. STB fragments are continuously shed into the maternal circulation. Thus, maintenance of the STB requires a finely regulated turnover. Excessive or restricted CTB-STB fusion may lead to PE, IUGR, and implantation failure (Gaster et al., 2009).

Although derived from human choriocarcinoma, the BeWo cells, display structural and physiological features of human primary trophoblast (Burres and Cass, 1986; Ramos et al., 2008; Orendi et al., 2010) and have been largely used as a model to study the process of trophoblasts fusion induced by forskolin

treatment (Chen et al., 2008; Zhou et al., 2013; Wang et al., 2014). Several transcriptomic studies have helped to identify important genes involved in trophoblast fusion (Kudo et al., 2004; Depoix et al., 2011; Shankar et al., 2015; Zheng et al., 2016). However, the majority of these studies have focused on the role of protein-coding genes. The role that ncRNAs could have in the trophoblast fusion remains to be explored.

Here, we have conducted a microarray transcriptomic analysis of BeWo cells under forskolin treatment (BeWo-FSK) and focused our analysis on the differentially expressed ncRNAs relative to controls (BeWo-CO). We identified a number of ncRNAs (antisense-RNAs, lncRNAs, miRNAs) which might be involved in the process of syncytialization *in vivo*. We have generated a network displaying the putative regulatory interactions among the differentially expressed ncRNAs and differentially expressed genes (DEGs) in the BeWo-FSK relative to the BeWo-CO. The analysis of this network shows that the majority of ncRNAs targets are involved in cell proliferation and metabolism. Topological analysis of the network identified miR193b as a principal hub of the network. Hallmarks enrichment analysis shows that most targets of miR193b are down-regulated genes involved in cell cycle progression such as CCND1, CCNA2, or BUB1B. Previous studies have shown that miR193b acts as tumor suppressor by repressing cell proliferation (Mazzu et al., 2017; Zhang et al., 2017; Bhayadia et al., 2018). In the context of placental development, it has been reported that miR193b-3p overexpression significantly decreases the migration and invasion of the trophoblast cells (HTR-8/SVneo) by targeting the 3'UTR of TGF-beta2 (Zhou et al., 2016). The miR193b has been found consistently up-regulated in PE and IUGR placentas in our study and others (Ishibashi et al., 2012; Xu et al., 2014; Zhou et al., 2016; Awamleh et al., 2019). This miRNA is thought to contribute to these pathologies because of its inhibitory effect on the migration and invasion of trophoblasts. Here we show that miR193b is also involved in the process of syncytialization. A prior, and key step in syncytialisation, is the acquisition of fusion competence, which requires the CTB to exit the cell cycle (Lu et al., 2017). Therefore, by targeting genes involved in the control of cell cycle progression, miR193b could play a pivotal role in this crucial step of the process of syncytialisation. Overexpression of miR193b in PE could negatively impact placental development by accelerating CTB-STB fusion, thus leading to a premature depletion of the pool of CTBs necessary to ensure the constant renewal of the STB. Alternatively, increased miR193b expression in PE could reflect a mechanism seeking to

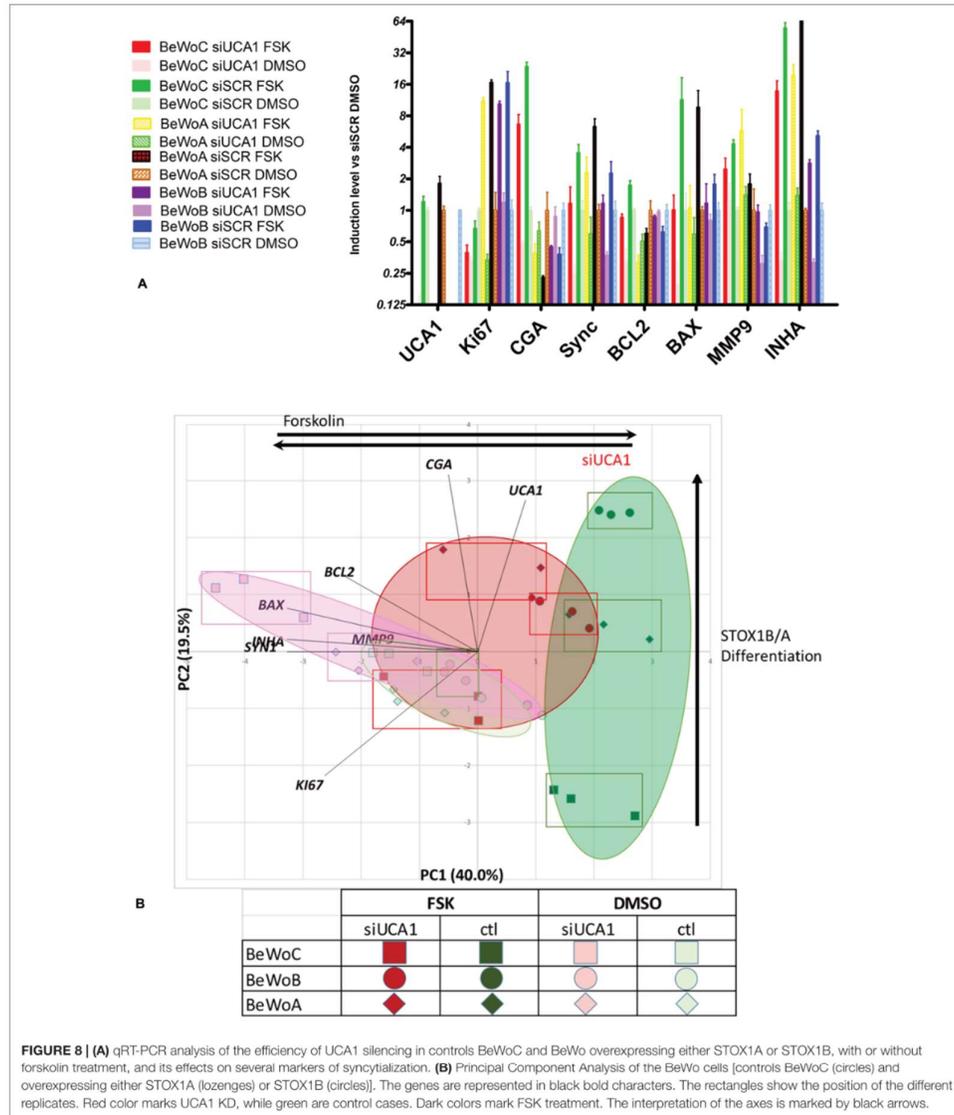


FIGURE 8 | (A) qRT-PCR analysis of the efficiency of UCA1 silencing in controls BeWoC and BeWo overexpressing either STOX1A or STOX1B, with or without forskolin treatment, and its effects on several markers of syncytialization. **(B)** Principal Component Analysis of the BeWo cells [controls BeWoC (circles) and overexpressing either STOX1A (lozenges) or STOX1B (circles)]. The genes are represented in black bold characters. The rectangles show the position of the different replicates. Red color marks UCA1 KD, while green are control cases. Dark colors mark FSK treatment. The interpretation of the axes is marked by black arrows.

compensate an excessive apoptotic shedding of the syncytium by facilitating the entering of CTB into the fusion process.

Since a dysfunctional syncytium could be at the origin of placental diseases, we systematically searched for lncRNAs

involved in the CTBs fusion that were deregulated in placentas from PE or IUGR. An exhaustive literature search, as well and the reanalysis of datasets available in the GEO Database resulted in the identification of three lncRNAs consistently up-regulated

in PE and IUGR and involved in syncytialization: miR193b, miR365a and UCA1.

UCA1 (Urothelial Cancer Associated 1), is a lncRNA initially identified in a bladder cancer cell line (Wang et al., 2006). The involvement of lncRNAs in placental diseases has been previously described for the HELLP syndrome, a serious complication of preeclampsia (van Dijk et al., 2012). In the case of UCA1 high expression has previously been reported in different types of cancer. UCA1 promotes cell proliferation, tumor progression, migration and drug resistance. UCA1 mediates the transcriptional regulation at an epigenetic level by interaction with chromatin modifiers (EZH2, CTCF, YAP...), by direct regulation via chromatin looping and/or by sponging miRNAs (Neve et al., 2018). The oncogenic functions of UCA1 have been extensively studied, but its role in development and differentiation remains unknown. A recent study, using the HTR-8/SVneo and JAR trophoblast cells suggests that UCA1 could inhibit trophoblast cell invasion and proliferation by down-regulating JAK2 (Liu et al., 2020). Knockdown of UCA1 in these cells suppressed the apoptotic rate and accelerated cell proliferation. Increased expression of UCA1 in PE had been reported previously and confirmed by our study (Liu et al., 2020). In addition, increased expression of UCA1, specifically in the STB of preeclamptic placentas, has been reported (Gormley et al., 2017). Thus, these data suggest that similar to miR193b, UCA1 might contribute in driving CTBs toward syncytialisation by inhibiting the genes involved in cell proliferation. The increased expression of UCA1 in the preeclamptic syncytium could reflect an increase in the turnover of the syncytium, to compensate for an increased apoptotic rate (Sharp et al., 2010; Fogarty et al., 2013). However, suppression of UCA1 in the HTR-8/SVneo and JAR cells decreased their apoptotic rates, indicating that UCA1 could also be involved in the induction of apoptosis (Liu et al., 2020). To explore more deeply the role that UCA1 might play in the process of syncytialization, we silenced its expression in the BeWo cells (BeWo-CO and BeWo-FSK) using a specific small interfering RNA (siRNA). Our results show that silencing UCA1 results in the downregulation of proliferation, attenuation of the expression of several markers of syncytialization, and most significantly downregulation of the expression of the antiapoptotic marker BCL2. As mentioned above in the HTR-8/SVneo and JAR cells, the Knockdown of UCA1, suppressed the apoptotic rate and accelerated cell proliferation (Liu et al., 2020). Thus, suppression of UCA1 in BeWo cells seems to have a different impact concerning the proliferation and apoptosis rates. These differences could be attributed to the fact that these cell lines are akin to different types of CTBs or either to particularities linked to the tumoral transformation process undergone by these cells. Nevertheless, our results on the BeWo cells are consistent with other studies indicating that in many cell types, UCA1 stimulates proliferation and inhibits apoptosis (Jun et al., 2018; Liu et al., 2018; Chen et al., 2019; Li et al., 2019; Wang et al., 2019). One limit of our study is that we did not carry out cell visualization experiments of the fusion; we can nevertheless assume that the alterations of syncytialisation marks that we observe here will likely be associated to phenotypic effects, that will have to be further assessed in future works.

Having in hand a model of altered fusion by STOX1A (BeWoA) or STOX1B (BeWoB) overexpression we attempted to evaluate the effect of UCA1 KD in these specific models. The overexpression of STOX1B (inhibiting fusion) led to more limited gene alterations than the other cells when forskolin was added in the Knock down of UCA1, suggesting that the alterations of gene expression induced by STOX1B overexpression render the UCA1 down-regulation alterations less visible.

Interestingly, hypoxia which plays a central role in the development of PE is known to induce UCA1 expression through HIF1A in both cancer cell lines and primary cultures of STB (Yuen et al., 2013; Xue et al., 2014; Zhu et al., 2019; Wang et al., 2020). In addition, it has been shown that UCA1 inhibits ischemia/reperfusion-induced apoptosis in cardiomyocytes (Chen et al., 2019; Wang et al., 2020). Given that ischemia/reperfusion is a known hallmark of severe PE and knowing that UCA1 has been detected as overexpressed specifically in the STB of severe preeclamptic patients (Gormley et al., 2017), it is tempting to speculate that UCA1 overexpression could reflect a protective mechanism aimed to attenuate apoptosis. However, in other cell types UCA1 has proved to exert pleiotropic effects, thus it might be involved in many other aspects of the trophoblast physiology. Although the BeWo cells are a good model to study the cell fusion process they are transformed carcinoma-like cells missing many trophoblast functions. Therefore, a more in-depth analysis of the functions of UCA1 in the placenta requires more physiological models such as primary trophoblast cultures, or placental organocultures. Another direction for future work is the assessment of the specific effects of the short isoforms of UCA1 that appear to be specifically modified when fusion occur. To note, we have recently demonstrated that alternative splicing is a general feature of placental disease, affecting hundreds of genes (Ruano et al., 2021). This could be achieved by lentiviral transformation of the cell lines, or even primary cultures with this short isoform. Finally, to understand better how UCA1 is actually functioning is an interesting challenge for further studies.

METHODS

Cell Culture

BeWo cell lines were cultivated in F12 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in 6-cm diameter plates, up to 60% confluence and with 50 µg/ml of geneticin G-418. The generation of the BeWoA (overexpressing constitutively STOX1A), BeWoB (overexpressing constitutively STOX1B) and BeWoC cell lines is described in detail in Ducat et al. (2020). The concentration of forskolin chosen for this study was based on preliminary studies (Ducat et al., 2020). At the end of the treatment, total RNA was extracted as previously described (Ducat et al., 2016). The siRNA against UCA1 was Ambion silencer select provided by ThermoFisher scientific. After plating in 12-well plates (1 ml) at 70% confluency, the cells were transfected the next morning using RNAiMAX transfection

reagent (Invitrogen). Each well was transfected with 200 μ l of OptiMax™ with 1 μ l of RNAiMax and 0.5 μ l of siRNA or si scrambled at 5 pmol/ μ l, following the manufacturer's protocol. To induce syncytialization the cells were treated 1 day later with 20 μ M forskolin or vehicle (DMSO) for 72 h.

Microarray Assay

One hundred ng of RNA per sample were analyzed using the ClariomD (Affymetrix) microarray assay. Library preparation, hybridization and data acquisition were performed by GENOMIC platform according to manufacturer's instructions. Gene and exon level expressions were processed and extracted from the ClariomD microarray using the Transcriptomic Analysis Console (TAC) provided by Affymetrix.

Quantitative Reverse Transcribed-PCR

Five hundred nanograms of total RNA were reverse transcribed with MMLV using the Invitrogen kit and random primers. qPCR was carried out under standard conditions in a LightCycler480 (Roche) in 96 well plates as previously described (Ducat et al., 2020), with a Sybrgreen kit from BioLine (Meridian Bioscience). In the case of UCA1, the analysis was carried out using a TaqMan probe and the Roche LightCycler® TaqMan® Master. The PPIA gene (cyclophilin) was used as reporter in all experiment, since we have shown previously an excellent stability of this gene in trophoblast cells. All the cell qPCR experiments were carried out three to four independent times, and every time in triplicates. Primers for the different genes are listed as **Supplementary Table 4**.

Functional Annotation of the Differentially Expressed Genes (DEGs)

For the functional annotation of the DEGs we performed Over-representation analysis (ORA) using the WebGestalt¹ bioinformatics resource (Liao et al., 2019). Databases interrogated include: Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Hallmarks. The significance of the detected enrichments was calculated using the Benjamini and Hochberg multiple test adjustment.

Gene Set Enrichment Analysis (GSEA)

GSEA was conducted using GSEA software from the Broad Institute². The BeWo fusion gene set was generated using the top up-regulated and down-regulated genes after 72 h forskolin treatment reported by Shankar et al. (2015). We used as input the gene expression matrix generated by the Transcriptomic Analysis Console (Affymetrix) including all samples and replicates. The permutation value was set as 1,000. *P*-values were corrected for multiple testing and the cutoff for significant enrichment corresponds to an FDR < 0.25.

Prediction of ncRNAs Targets

To identify targets for the differentially expressed ncRNAs in the BeWo-FSK relative to BeWo-Co cells we used *ad hoc* databases.

¹<http://bioinfo.vanderbilt.edu/webgestalt>

²<https://www.gsea-msigdb.org/gsea/index.jsp>

These include miRBase³, starBase v2.0⁴ and the DianaTools LncBase v.2⁵.

ncRNAs Regulatory Network

The ncRNAs and corresponding differentially expressed targets were used to generate a regulatory network. The network was constructed, visualized and analyzed using the Cytoscape 3.2.1 software⁶ and its complementary applications (Shannon et al., 2003). The centrality parameters of the network were analyzed using the Cytoscape application NetworkAnalyzer (Shannon et al., 2003). Two topological parameters Betweenness Centrality (BC) and node degree were used to identify hub genes. The network is available as a **Supplementary XML File**.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ebi.ac.uk/metagenomics/>, E-MTAB-9416 and <https://www.ncbi.nlm.nih.gov/genbank/>, GSE148088.

ETHICS STATEMENT

This study was approved by the Ethics Committee and CCPPRB (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale) of Paris Cochin. All patients gave their written consent for the use of their placenta and blood samples. For Angers, the collection and use for research purpose (including genetic analyses) of placentas from pregnancies complicated with IUGR or healthy pregnancy have been approved by the Ethics Committee of Angers. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DV, FM, and CM conceived the work and drafted it. DV performed qRT-PCR experiments. SJ performed the microarray work. GG drafted the manuscript and contributed human samples. CR and CA performed RNA and qPCR experiments and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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³<http://www.mirbase.org/>

⁴<http://starbase.sysu.edu.cn>

⁵<http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=lnCBase/index>

⁶<https://cytoscape.org/>

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.633937/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 4. Genome-wide screen to identify candidate genes involved in trophoblast fusion

“Or watch the things you gave your life to, broken,
And stoop and build ’em up with worn-out tools:
[...] you’ll be a Man, my son!”
If,
Rudyard Kipling.

Introduction

During early placentation, trophoblast cells soon differentiate into villous CTB that will remain in the tissue as pool of trophoblast reserve, proliferating and differentiating to generate the other trophoblast subtypes: STB, EVT and GC. EVT acquire a migratory phenotype during CTB differentiation through endothelial-to-mesenchymal transition and are responsible for deep invasion of the maternal uterine wall to ensure remodelling of the maternal spiral arteries. On the other hand, GCs are responsible for the secretion deep into the maternal tissues of enzymes that favour the degradation of the ECM, to aid the deep implantation of the placenta (Aplin, 1991; Red-Horse et al., 2004). We are interested in the STB.

The STB is a multinucleated syncytium generated through the process of cellular fusion of the mononucleated CTB. It consists of a unique structure that covers the surface of all placental villous trees and is in direct contact with the maternal blood, ensuring exchanges of nutrients between the maternal and foetal circulations, as well as acting as a barrier and secreting key hormones that regulate the progression of gestation (Gude et al., 2004; Guttmacher et al., 2014). The STB is a very metabolically active layer, continually expanding to accommodate for the growing nutritional needs of the developing foetus. Overtime, aged nuclei in the syncytium are eliminated thorough the formation of protrusions called syncytial knots that are released in the maternal blood stream for clearance. This phenomenon is balanced by continuous fusion of new CTB into the syncytium, ensuring the continuous renewal of the STB layer (Loukeris et al., 2010).

As we mentioned in **Chapter 3**, alterations of syncytialisation have been found in placentas from pregnancy pathologies such as PE and IUGR, these pathological modifications imply an increase in CTB proliferation over differentiation, resulting in insufficient renewal of the aging STB layer. The reduced STB function is accompanied by placentation defects associated with hypoxia and reperfusion-injury which further damage the STB, resulting in release of pro-inflammatory cytokines and anti-angiogenic factors in the maternal blood stream that foster the development of ED and systemic side effects (Huppertz and Kingdom, 2004; Langbein et al., 2008; Sheridan et al., 2012). More recently, the detrimental effects of the cytokine type-I interferon, increased in pregnancy pathologies IUGR, pre-term birth and foetal death, have been linked with inhibition of syncytialisation (Buchrieser et al., 2019). Similarly, the protein levels of NLRP7, involved in innate immune response, were found upregulated in IUGR placentas and were conducive to inhibition of trophoblast differentiation pathways, towards both STB and EVT cell fates (Abi Nahed et al., 2019). We also showed how ncRNAs involved in trophoblast differentiation, lncRNA UCA1 and miRs miR193b and miR365a, are upregulated in PE and IUGR placentas (Apicella & Ruano et al., 2021). Therefore, the understanding of this process will help shed light on mechanisms at the core of placental pathologies. Moreover, some molecular mechanisms involved in syncytialisation have been found in common with other cell types where fusion occurs such as cancer, osteoclast and myoblast and therefore novel data could be of interest for these fields as well (Degrelle et al., 2017; S e et al., 2011; Uygur et al., 2019).

Syncytialisation has been widely explored thanks to the use of primary trophoblast cells, which, once isolated from the placenta, spontaneously fuse in culture within the first 24 hours. Additionally, the choriocarcinoma cell line BeWo has proved fundamental in clarifying the mechanisms behind syncytialisation. The BeWo mononucleated cells undergo syncytialisation upon treatment with the active compound FSK forming small syncytia in culture; the morphological changes are associated with deep transcriptomic, proteomic, and secretory changes that resemble the expression profiles of the placental syncytium. Moreover, contrarily to primary cells, this cell line can be maintained in culture long-term, allowing for easier genetical engineering (Orendi et al., 2010).

Figure 4.1 gives an overview of the main molecular classes involved in syncytialisation and on the downstream effects of FSK in the BeWo cell model.

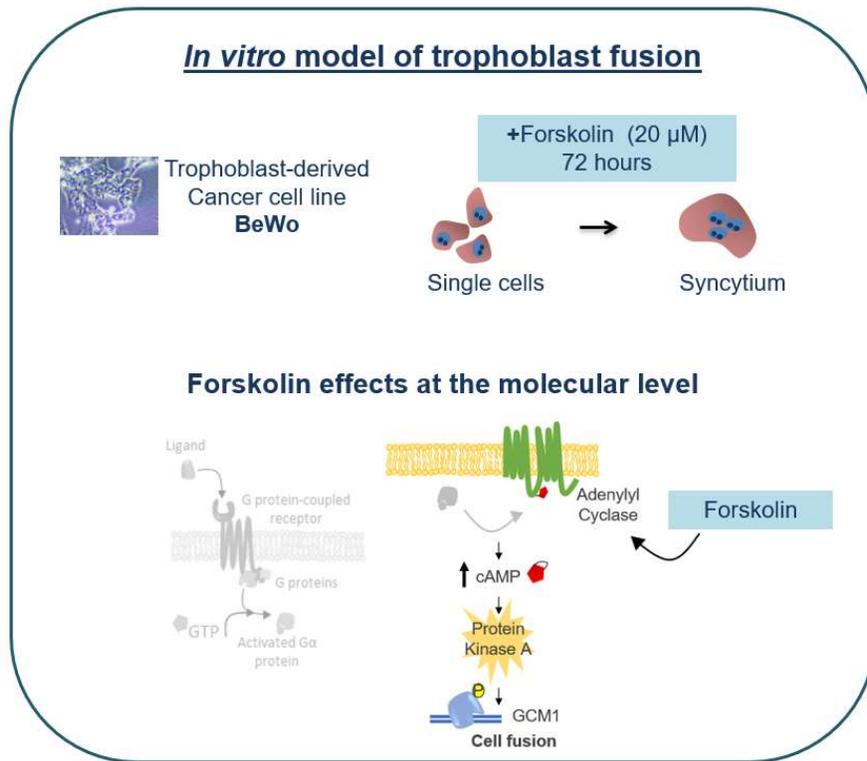
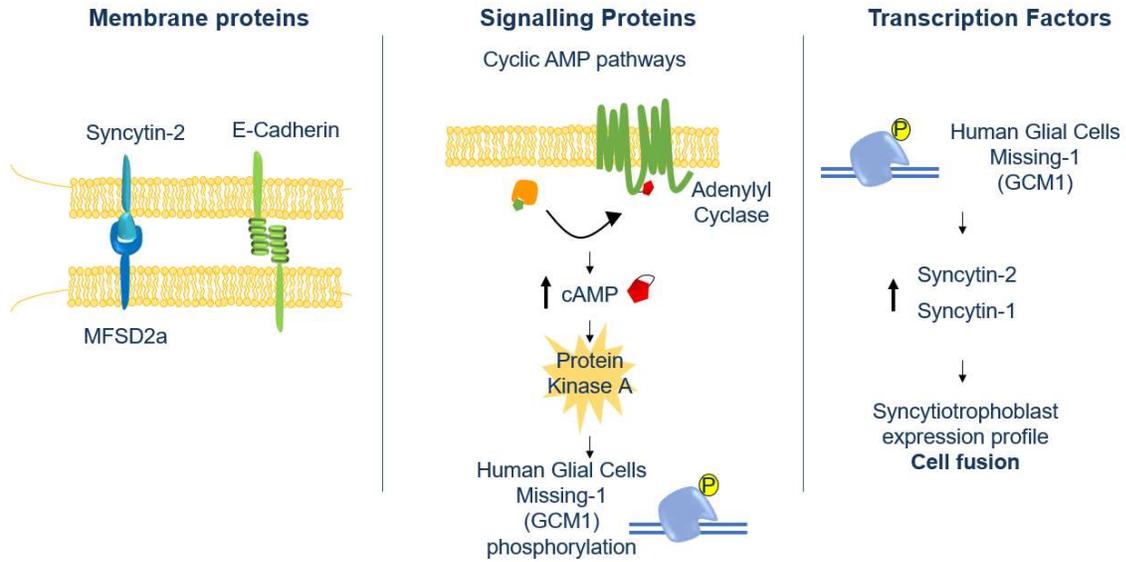


Figure 4.1. Syncytialisation at the molecular level. An overview of the molecular classes known to play a key role in trophoblast syncytialisation (TOP) and of the effects of Forskolin in the BeWo cell model (BOTTOM).

FSK is a potent activator of adenylate cyclase activity, dramatically increasing the intracellular levels of cyclic AMP (cAMP). cAMP signalling is in fact a key regulator of trophoblast fusion and cAMP levels are enhanced by hCG hormone secreted by the STB itself in a positive feedback (Rao et al., 2004; Weedon-Fekjær and Taskén, 2012). Downstream, cAMP triggers the activation of protein kinase A (PKA) and protein kinase C (PKC), which are spatially regulated thanks to the interaction with multiple membrane organisers such as the 11 identified A-kinase-anchoring proteins (Gerbaud et al., 2015; Omata et al., 2013). Through the activation of ERK/MAPK cascades, the transcription factor Glial Cell-missing 1 (GCM1) is activated through a series of post-translational modifications (phosphorylation, desumoylation and acetylation), resulting in transactivation of fusogenic genes such as Syncytin-1 and 2 as well as Syncytin-2 receptor major facilitator superfamily domain containing 2 (MFSD2) (Baczyk et al., 2009; Chang et al., 2011; Chen et al., 2010; Delidaki et al., 2011; Lin et al., 2011). Moreover, cAMP signalling triggers the upregulation of estrogen-related receptor γ (ERR γ) in differentiating trophoblast cells. ERR γ is critical for the biogenesis of mitochondria and activation of metabolism, as well as being directly involved in promoting the endocrine phenotype of the STB (Poidatz et al., 2015).

Syncytin-1 and 2 are membrane proteins of retroviral origins encoded by the human endogenous retrovirus W-1 (ERVW-1) and Endogenous retrovirus group FRD member 1 (HERV-FRD-1) genes respectively, which are pivotal for STB formation. Interestingly, homologues of retroviral origins have been found in eutherian mammals, as well as in reptiles and other orders where a placenta is present (Mi et al., 2000; Roberts et al., 2016). Other key membrane proteins include adhesion molecules in Gap- and Adherens junctions such as E-cadherin, that allows cell-cell interaction and formation of trophoblast aggregates prior to syncytialisation. Its shedding mediated by ADAM12 is then necessary to release the membranes and allow for fusion to occur (Aghababaei et al., 2015; Coutifaris et al., 1991). Syncytialisation is a very complex process orchestrated by many transcription factors, other than the aforementioned GCM1, GATA3 and Twist1 are used as nuclear markers to distinguish between CTB and STB respectively (Buchrieser et al., 2019; Degrelle and Fournier, 2018). While GATA3 is critical to maintain CTB identity, inhibiting GCM1 as well as EVT-differentiation pathways, Twist1 expression increases during syncytialisation in parallel to GCM1 upregulation (Chiu and Chen, 2016; Lu et al., 2016).

To the best of our knowledge, no untargeted approach has been carried out to investigate syncytialisation. The scope of this study was therefore to design a genome-wide screen making use of CRISPR/Cas9 technology to identify novel candidate genes involved in trophoblast differentiation, using the BeWo cell line as a model of villous trophoblast. The trophoblast fusion that results in the formation and renewal of the syncytium is a complex process of cell differentiation, which entails a reprogramming of the gene expression profile of the CTB to become STB. *In vitro* quantifications of epigenetic changes during syncytialisation have shown how DNA methylation as well as histone modifications are necessary steps for STB differentiation to occur successfully (Jaju Bhattad et al., 2020; Milano-Foster et al., 2019; Shankar et al., 2015). The untargeted approach we propose here has the aim and the potential of identifying key master regulators of trophoblast function (on the transcriptome as well as the epigenome) as well as key proteins required for the process of fusion itself. The main output of such genome-wide screen for trophoblast syncytialisation is a list of candidate-genes that once inactivated result in an impaired ability to differentiate into STB-like cells. If in this list were to feature genes previously associated with syncytialisation such as GCM-1 and syncytin-1/2, these would correspond to a positive control and help to confirm the validity of the screen design in identifying key players in this process.

Genome-wide screens involve the selective targeting of each gene in the genome by introducing a loss-of-function mutation, to then observe the effects of this mutation on the phenotype of interest (i.e. differentiation of BeWo cells into STB-like cells). The CRISPR/Cas9 system allows the targeting of a wide range of genes in the genome; it is based on the use of the bacterial endonuclease Cas9 which interacts with an engineered small RNA molecule (~20 nucleotides), the single-guide RNA (sgRNA), complementary to the gene of interest. Once co-expressed in the cell, the sgRNA and Cas9 form an active complex that, guided by the complementarity between the sgRNA and the gene of interest, binds the double-stranded DNA (dsDNA). The endonuclease activity of the Cas9 is dependent on the presence of a short sequence (2-6 nucleotides) called protospacer adjacent motif (PAM) at the target site; 3-4 nucleotides upstream of the PAM the Cas9 introduces a dsDNA-break (DSB) (Jinek et al., 2012; Murugan et al., 2017). The cell repairs this DSB either by the Non-Homologous End Joining (NHEJ) pathway, which results in Insertions and Deletions (INDELs) usually causing a knock-out of the gene, or more rarely by homologous recombination (HR) (Mao et al., 2008; Shrivastav et al., 2008). Just by changing the sequence of the sgRNA is possible to target a different region of the genome, withstanding the presence of a PAM sequence in the

complementary strand. To perform genome-wide screens, libraries of sgRNAs have been developed to target different ranges of genes across the genome, from the whole genome, protein-coding genes only, specific gene sets such as kinases. In this study, we use the Brunello library targeting only protein-coding genes for a total of 19,114 human genes (Doench et al., 2016; Sanson et al., 2018). An overview of the protocol is given in **Figure 4.2**.

Trophoblast fusion – CRISPR/Cas whole-genome screen

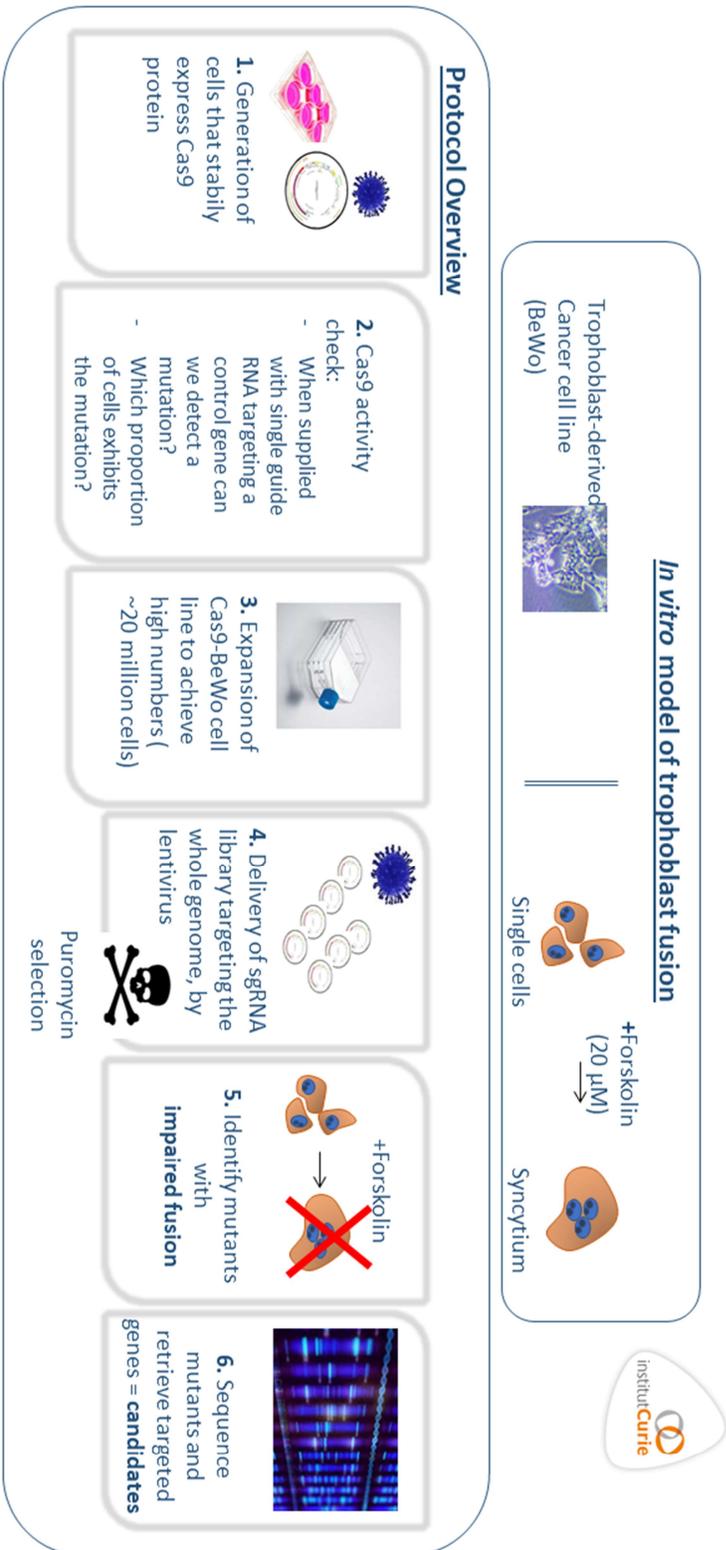


Figure 4.2. Overview of the process of whole-genome screening with CRISPR/Cas9

While we managed to define the experimental parameters for some of the steps in **Figure 4.2**, we could not bring the project to completion. Given that the nature of the results is very technical, rather than of biological relevance, in this chapter we will present the **Results** section first, in a discursive manner, to clarify the rationale behind the different experimental strategies, followed by the technical details relative to the protocols and the reagents used in the **Methods** section, to conclude with the **Discussion** section.

Results

Step 1 and 2. Generation of cells that stably express Cas9 and Cas9 activity test.

The first step of the whole genome screen is to generate the model cell line that constitutively expresses the Cas9 endonuclease, while the sgRNA library targeting the whole genome will be supplemented in a separate experiment and is encoded by lentiviral vectors. The use of such a two-vector system has shown to increase efficiency of sgRNA expression, while reducing health and safety risks, since the library is targeting the human genome and accidental contamination with a single virus carrying both the enzyme and the guide, could result in the introduction of mutations (Lino et al., 2018; Sanjana et al., 2014).

The BeWo cell line has extensively been used as a model of villous CTB, with about 14,000 references in Pubmed (King et al., 2000). Upon FSK administration, this cell line undergoes differentiation into STB, resulting in deep changes at the transcriptional and proteomics level, modification of the secretory profile and finally with the formation of small syncytia. For the purpose of a whole-genome screen, we used BeWo wild type cells (**BeWo-WT**) as well as a modified cell line BeWo IFI6-Blasticidin (**BeWo-IFI6**). These cells have been stably modified by transfection with a vector carrying a blasticidin resistance cassette under the control of the IFI6 gene promoter, since this gene had been found strongly downregulated in FSK-treated BeWo, transcriptomic dataset published in (Ducat et al., 2020). FSK treatment of BeWo-IFI6 cells resulted in a reduction of the blasticidin cassette expression levels. This cell line was developed in order to facilitate the isolation of cells that had impaired differentiation abilities, since FSK-treated cells in this scenario are expected to lose the blasticidin resistance, and if grown in medium containing blasticidin, should die over-time, leaving only the mutants with impaired fusion to survive.

A wide variety of vectors encoding for Cas9 from *S. pyogenes* (SpCas9) are commercially available, or accessible from plasmid depositories such as Addgene; among these, there are standard vectors that can be used in transfection, as well as lentiviral vectors for transduction experiments. In order to identify the subpopulations of transfected/transduced cells that positively express Cas9, depending on the vector of choice, antibiotic selection (i.e. blasticidin or hygromycin B in our approach) can be used, as well as through isolation of cell populations

expressing a fluorescent tag, co-expressed with Cas9. These tags are usually fluorescent proteins such as GFP or mCherry, encoded from the same promoter as the Cas9 and automatically cleaved by the cell during translation, due to the presence of an intercalating sequence 2A self-cleaving peptide. The assumption is that a clone that expresses the detectable fluorescent tag, will at the same time express the Cas9 protein. The identification and separation of these positive clones can be done by flow cytometry.

Once the Cas9-expressing cell line (Cas9+BeWo) has been developed, the ability of the endonuclease to interact with the specific sgRNA and catalyse the insertion of a DSB at the desired locus, resulting in mutation, needs to be tested through a *Cas9-activity test*. In this experiment, the Cas9-expressing cell line is transduced with a lentiviral vector that encodes a sgRNA targeting a control gene (sgRNA-CTRL), and encoding for an antibiotic selection cassette (i.e. puromycin). After this step, Cas9+BeWo are treated with puromycin, to ensure that the resulting clones co-express the Cas9 and the sgRNA-CTRL. Therefore, if the Cas9 enzymatic activity is functional, a DSB should occur at the targeted locus and INDELs should be detectable. By quantifying the proportion of cells of the Cas9+BeWo line that carry a mutation at the locus, it is possible to estimate the proportion of cells expressing a functional Cas9. To detect the mutation, primers targeting the flanking region of the cut site are used to amplify the region in a standard Polymerase Chain Reaction (PCR) experiment, the amplicons are then sequenced by Sanger sequencing. The ICE v2 software (SYNTHEGO) is used to estimate the different detected mutations, and the proportion of cells carrying these mutations, by comparing the sequence in Cas9+BeWo treated with sgRNA-CTRL guides and untreated cells (Hsiau et al., 2019). In the current study, we performed the *Cas9-activity test* with two sgRNAs targeting an exonic and an intronic region of phosphatidylinositol glycan anchor biosynthesis class A (PIGA) gene, located on chromosome X, respectively sgRNA-273 and sgRNA-274.

To develop Cas9-expressing cell lines three different approaches have been tested and are briefly described below.

Antibiotic kill curves.

The vectors used throughout the screen have different antibiotic resistance cassettes: blasticidin, puromycin and hygromycinB. For blasticidin, previous testing in the lab had shown 2 μ g/ml to be the optimal concentration, over a 2-week selection time. For the other two antibiotics, the minimal concentrations to achieve negative selection and exposure time were established by antibiotic kill curves. BeWo-WT cells were plated in duplicates, and different concentrations of either puromycin (0, 1, 1.5, 2.5, 3, 4, 5, 6, 7, 8, 9 μ g/ml) or hygromycin B (0, 2.5, 5, 10, 20, 30, 50, 75, 100 μ g/ml) were tested. In **Figure 4.3**, cell confluency is plotted after each day of exposure to the antibiotic, compared to the untreated control. Wells where no more live cells were detectable were considered to have been negatively selected. To avoid toxicity, the lowest concentrations of antibiotics that achieved negative selection within the 7-day period was selected. For puromycin selection, the antibiotic is used at a final concentration of 4 μ g/ml for a total of 6 days. While for hygromycin B, 75 μ g/ml for 6 days.

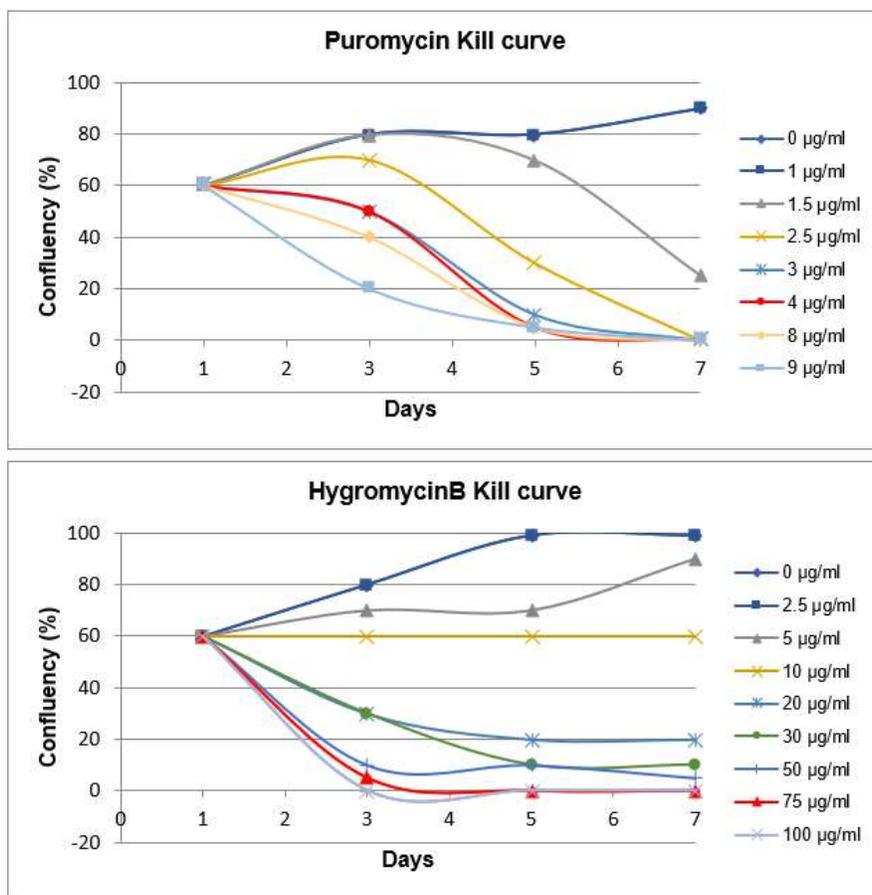


Figure 4.3. Puromycin and Hygromycin B kill curves. The plot shows the cell confluency of BeWo-WT cells treated with different concentrations of antibiotics over an 7-day period. In the Puromycin kill

curve plot (TOP), the distributions for 5, 6 and 7 $\mu\text{g/ml}$ are not shown, since they completely overlapped with 4 $\mu\text{g/ml}$.

Cas9-P2A-mCherry lentiviral vector

In a first attempt to generate the Cas9-expressing cell line, BeWo-IFI6 cells were transduced with a lentiviral vector encoding for Cas9 gene from *S. pyogenes* (SpCas9), linked to a mCherry molecule by the self-cleaving peptide 2A; mCherry positive (mCherry+) cells are detectable by flow cytometry by measuring fluorescence emission in the red spectrum (550-650 nm) (Shu et al., 2006). It is proposed that the proportion of mCherry+ cells correlates to the percentage of Cas9-expressing cells.

The transduction of the lentiviral vector was performed in triplicates, however only two of the wells could proliferate and will be referred to as Clone1 and Clone2. BeWo-IFI6 Clone1 and Clone2 cells present a mixture of mCherry+ and mCherry negative (mCherry-) cells; after flow cytometry quantification of the cells more than 80% were found to be mCherry+ (Representative results for Clone1 in **Figure 4.4a**). mCherry+ cells were further divided into two populations: mCherry+LOW where the recorded fluorescence was around 10^2 , and mCherry+HIGH. For each of the clones, the two populations of cells were isolated by Fluorescence-activated Cell Sorting (FACS) on Day1, and the purity of the mCherry fractions established by flow cytometry quantification. For both clones, the purity of the isolated mCherry+LOW and HIGH fractions was below <90% (**Figure 4.4b**); we could in fact still detect mCherry- cells, varying from 3.5 to 14.5%. These isolated, unpure mixtures were then replated in normal culture conditions, with the aim of expanding the mCherry+ population. At Day9 and Day14 the proportion of the different mCherry fractions was tested by flow cytometry, results are shown in **Figure 4.4b** for Clone1 and Clone2 isolates. In all samples, an increase in the mCherry- population is observed over the 2-week period, reaching up to 45.5% of the total cells, with a corresponding decrease of mCherry+HIGH and LOW populations. These results suggest that mCherry+ cells (supposedly corresponding to Cas9+ cells) were counter-selected in culture; an alternative explanation is that the expression of the viral vector becomes extinguished over-time. In the literature, Cas9 transgenic expression in mammalian cells has been associated with activation of the p53 apoptotic cascade, which could explain why the mCherry+ cells showed a selective disadvantage in normal cell culture conditions (Enache et al., 2020).

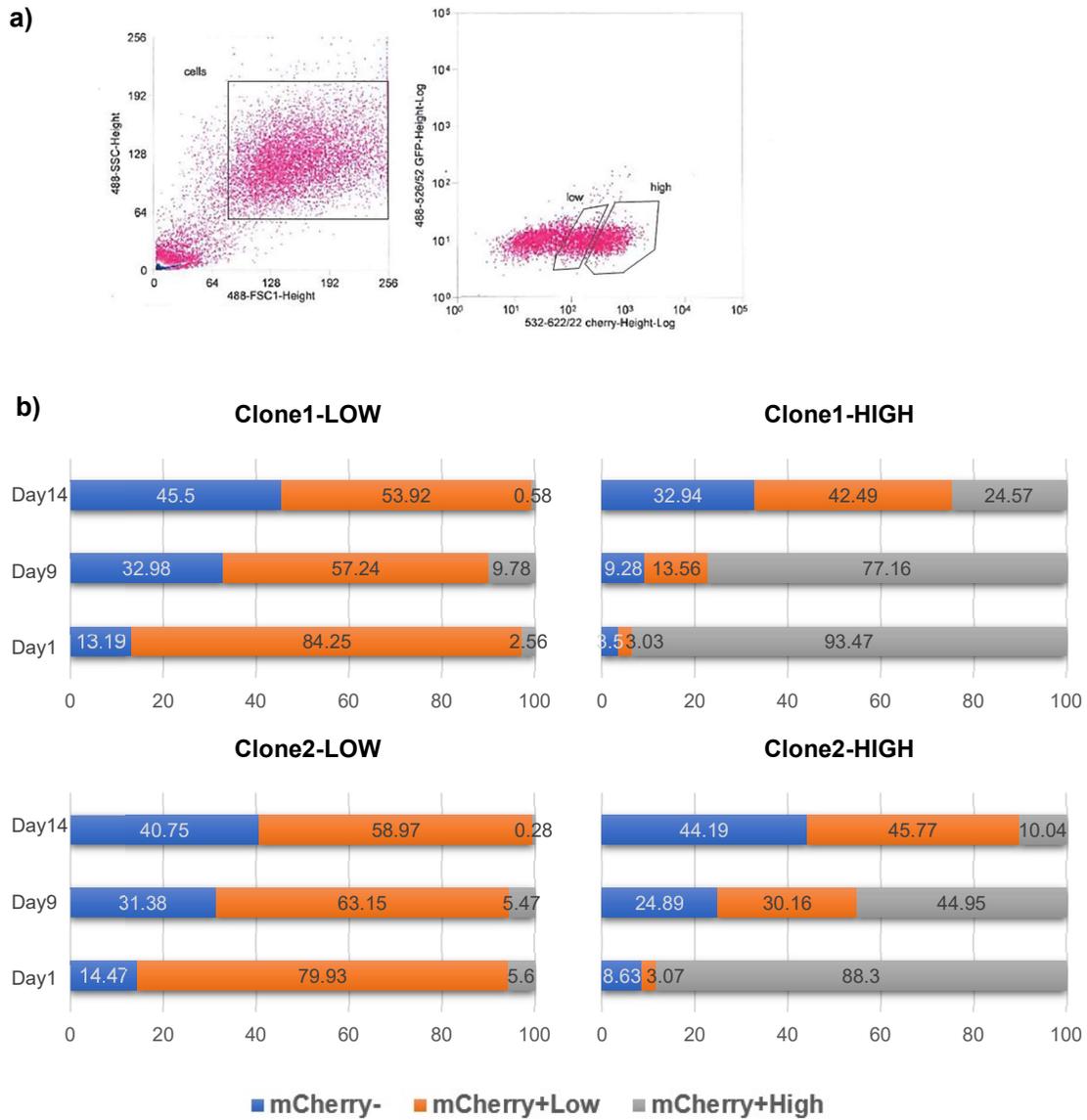


Figure 4.4. Proportion of mCherry positive cells in BeWo-IFI6-Cas9-mCherry cell lines. a) Representative results from FACS analysis of mCherry signal in clone1 and clone2 populations. On the left, SSCvsFSC plot showing the gated live BeWo cells; on the right GFPvsmCherry signal plot, the two gates divide mCherry+ cells in mCherry+LOW population (relative fluorescence $\sim 10^2$) and mCherry+HIGH. b) Proportion of mCherry-, mCherry+LOW and mCherryHIGH cells in Clone1 and Clone2 cell lines, over a two-week period. SSC = side scatter, FSC = forward scatter.

Cas9-P2A-BlasticidinR lentiviral vector.

Since the Cas9-expressing cell line could not have been generated with the Cas9-mCherry construct, as alternative approach, we tried using a lentiviral vector encoding SpCas9 linked to a blasticidin resistance cassette. Since the BeWo-IFI6 line used throughout the screen already carries a blasticidin resistance cassette, a BeWo-WT cell line was used.

After transduction of BeWo-WT cells with this vector, a 2-week antibiotic selection with blasticidin (2 µg/ml) was carried out and surviving clones pulled together, referred to as **BeWo-Cas9-Blasti** cell line. The expression of Cas9 was confirmed by immunofluorescence (representative image in **Figure 4.5**, Cas9 stained in red, nuclei in blue). In addition, Cas9 functionality was assessed by *Cas9 activity test* which showed very limited mutation rates for both tested sgRNAs, 0% for sgRNA-273 and 3-7% for sgRNA-274 treated samples. Therefore, the Cas9 expressed by BeWo-Cas9-Blasti cells was deemed not functional, and the cell line discontinued.

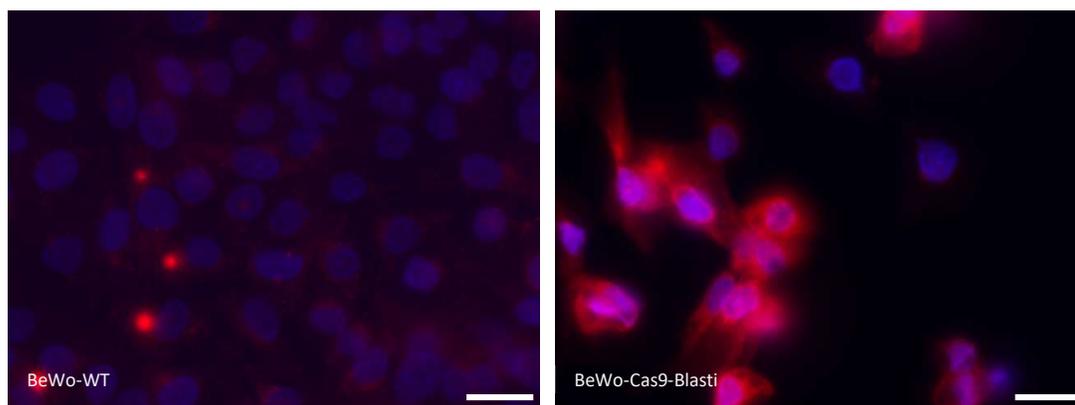


Figure 4.5. Cas9 expression is confirmed in BeWo-Cas9-Blasti cell line by immunofluorescence. BeWo-WT cells have been used as negative control (left) and BeWo-Cas9-Blasti (right). Blue signal corresponds to DAPI staining of nuclei, while red signal corresponds to Alexa495 signal corresponding to Cas9. A faint background signal (red) can be seen in BeWo-WT sample, while in BeWo-Cas9-Blasti cell line strong Cas9 signal localised in the cytoplasm and nucleus of most of the cells. Scale bar = 50 µm.

Cas9-P2A-HygromycinBR transfection vector.

Given the interest in using the BeWo-IFI6 cell line, we tested a new circular vector encoding for Cas9 linked with self-cleaving peptide 2A to an Hygromycin B resistance cassette. This vector is a circular plasmid that can be delivered by standard transfecting agents such as products based on cationic lipids (e.g. Lipofectamine™, ThermoFisher). BeWo-IFI6 were transfected and clones carrying the vectors were isolated, after a 1-week selection with Hygromycin B four different clones could be expanded from this newly created **BeWo-IFI6-Cas9Hyg** cell line. Clone #3 was not able to expand in cell culture, showing highly impaired proliferation.

The results of the *Cas9 activity test* performed on the other three cell lines, Clone#1, Clone#2 and Clone#4 are presented in **Table 4.1**.

Table 4.1. Cas9 activity test results for BeWo-IFI6-Cas9Hyg clones

<i>INDEL%</i>	<i>Clone1</i>	<i>Clone2</i>	<i>Clone4</i>
<i>sgRNA-273</i>	1	2	1
<i>sgRNA-274</i>	63	68	13

We were able to establish that the 273-sgRNA was not functional, also according to other collaborators. However, mutations at the PIGA intronic locus targeted by sgRNA-274 could be detected in all tested BeWo-IFI6-Cas9Hyg clones, with mutation rates of 63%, 68% and 13% respectively for Clone#1, Clone#2 and Clone#4. Given its highest mutation rate, Clone#2 was selected for further validations and a second *Cas9 activity test* showed stable Cas9-activity over-time, reporting an efficiency of 65%. To be more conservative, this value was chosen to approximate the Cas9-mediated mutation rate in this cell line, which was used in the rest of the experiments and will be referred to as **BeWoIFI6-Cas9Hyg-CL2**.

Figure 4.6 summarises the results from the three different approaches to develop the Cas9-expressing cell line.

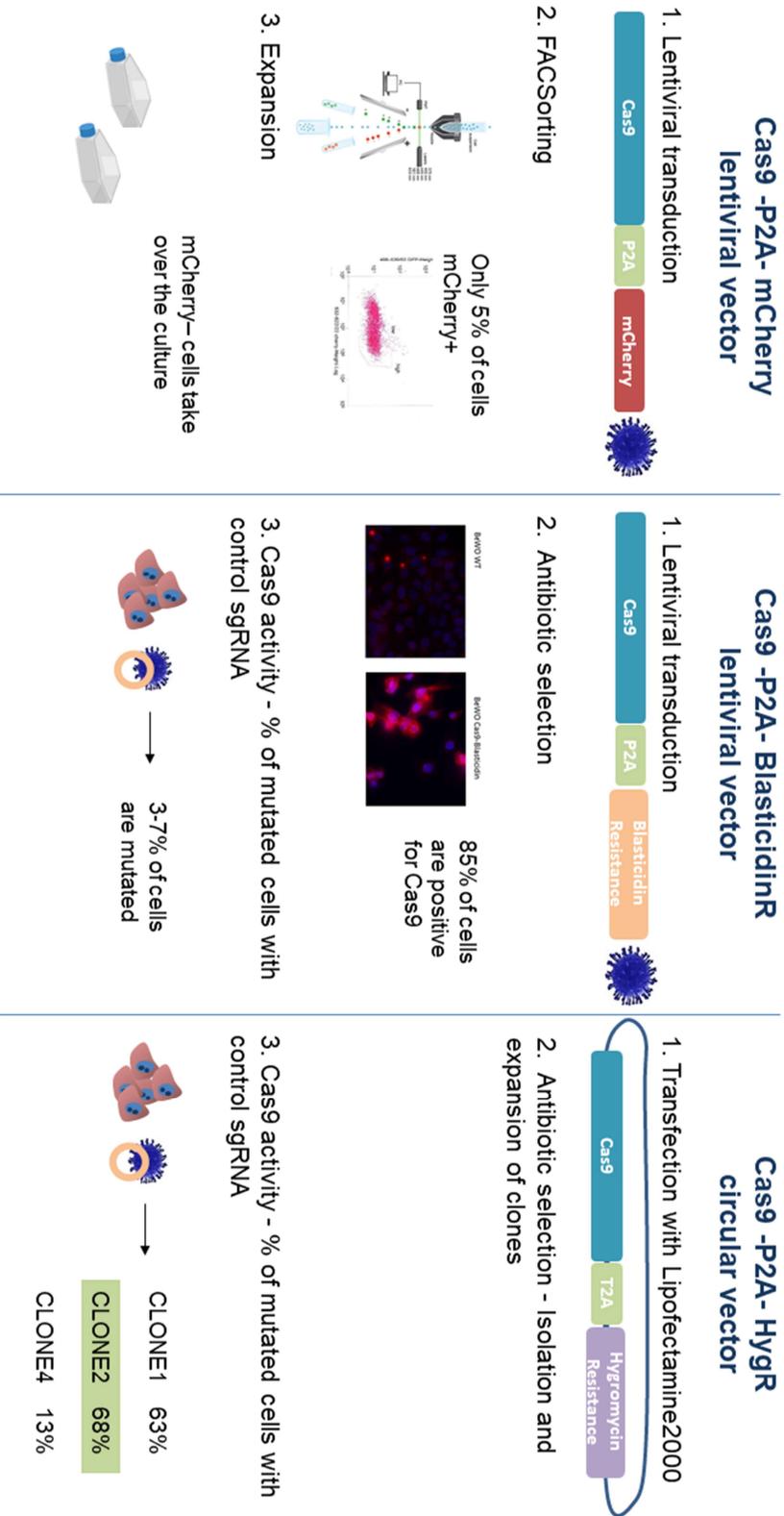


Figure 4.6. Cas9-expressing cell line generation: Summary of experimental strategies.

Step3. Defining Genome-Wide Screen parameters. How many cells do we need?

When performing a Genome-Wide Screen, we need to set defined values for experimental parameters such as guide representativity and desired multiplicity of infection (MOI). These parameters, together with Cas9 activity rates will directly impact the number of cells needed to perform the screen.

The representativity, or coverage, refers to the number of cells that express each sgRNA in the library. According to previous studies, we have aimed at a representativity of 500x, where each of the sgRNAs in the Brunello library should be found at least 500 times in our cell population (Joung et al., 2017; Peng et al., 2015; Shalem et al., 2015). Since the number of guides in the Brunello library is of approximately 80,000 (77,441), the number of transduced cells we need to achieve a representativity of 500x is:

$$\text{Transduced cells (500x)} = 80000 \cdot 500 = 40 \cdot 10^6 \text{ cells}$$

This is the minimum number of cells we should ensure to retain throughout the screen in order to maintain a representativity of 500x. Another critical parameter is the MOI, which corresponds to the transduction efficiency of the viral library, expressed in values varying from 0 to 1. In other words, the MOI is the percentage of transduced cells obtained with a given volume of viral library. This measure has been used in genome-wide screens to estimate the proportion of cells in the transduced population that will carry a unique sgRNA or multiple sgRNAs, by applying the Poisson Law of probability (Abedon and Bartom, 2013):

$$P(n) = \frac{m^n \cdot e^{-m}}{n!}$$

Where **P(n)** is the probability that **n** particles of virus are present per cell, **m** is the effective number of transduced cells (i.e. MOI), and **e** is Euler's number (2.718...). Varying the MOI we can calculate the probability that cells in the transduced population carry a certain number of sgRNAs. If MOI = 1, corresponding to 100% of transduced cells, we can estimate the following:

- Cells containing 1 sgRNA: $P(1) = 36.79\%$
- Cells containing 2 sgRNA: $P(2) = 18.39\%$
- Cells containing 3 sgRNA: $P(3) = 6.13\%$

When the totality of cells in an experiment are transduced, only a small proportion is expected to carry a single molecule of sgRNA (36.79%), while the rest will carry more than one sgRNAs. These different sgRNAs present in the cells will mediate Cas9-induced DSB in multiple locations of the genome, depending on the specific target of each sgRNA. Therefore, since the mutations occurred in multiple genes, the individual contribution to the phenotype will be harder to determine. To reduce the proportion of cells that are transduced with multiple sgRNAs, traditionally, a MOI ~0.3 is used (Joung et al., 2017; Peng et al., 2015; Shalem et al., 2015). We selected a MOI = 0.5 which corresponds to 30.32% of cells containing a unique sgRNA, and only 0.07% of cells containing two sgRNAs, since this set-up has previously been used with the Brunello lentiviral library (DeWeirdt et al., 2020; Sanson et al., 2018).

The number of cells to be used in the screen is then adjusted for the MOI, since the previously calculated number of transduced cells for a representativity of 500x (Transduced cells(500x)) corresponds only to the 50% of the total population (MOI = 0.5):

$$\text{Total number of cells} = \frac{\text{Transduced cells}(500x)}{\text{MOI}} = 80 \cdot 10^6 \text{ cells}$$

This “Total number of cells” needs to be further corrected according to the observed Cas9 activity rate in the Cas9-expressing cell line. In the BeWoIFI6-Cas9Hyg-CL2 cell line selected for the screen the Cas9 activity rate is 65%, therefore we would need to plate an additional 35% of cells in which we do not expect Cas9-mediated mutations to occur. The “Final Total number of cells” will therefore be:

$$\text{Final Total number of cells} = \frac{\text{Total number of cells}}{\text{Cas9 activity rate}} =$$

$$\text{Final Total number of cells} = \frac{\frac{\text{Transduced cells}(500x)}{\text{MOI}}}{\text{Cas9 activity rate}} = 123 \cdot 10^6 \text{ cells}$$

To better be able to estimate the growth of BeWoIFI6-Cas9Hyg-CL2 cells, we also defined their doubling time, which is ~45 hours; considerably slower than BeWo-WT cells, which have a duplication time of about 36 hours, this could be due to the detrimental effects of Cas9-expression on cellular fitness.

Step4. Optimisation of viral library transduction for the selected MOI.

In our study, we set out to use the Brunello lentiviral library, specifically optimised for Cas9-mediated gene knock-out screens, to minimise off-target effects as well as for targeting the protein-coding region of genes, with multiple non-overlapping sgRNAs (Doench et al., 2016). This library targets 19,114 human genes, encoding an average of 4 sgRNAs per gene, for a total of 76,441 guides. Additionally, 1000 non-targeting sgRNAs are included as internal control (Doench et al., 2016; Sanson et al., 2018).

The aim of this step is to establish the volume of viral library to use in transduction of BeWoIFI6-Cas9Hyg-CL2 cells, during the genome-wide screen, to achieve a MOI of 0.5. This can be done through library titration experiments, assessing the proportion of successfully transduced cells for a given volume of viral library. The results presented here are still preliminary and further optimisations are needed to achieve MOI closer to the set value of 0.5.

Since the lentiviral backbone encodes for a puromycin resistance cassette, to establish which volume of viral library yields which proportion of transduced cells, we performed a viral library titration and counted the number of puromycin-resistant cells. A small-scale titration was performed: for each titration point we transduced two wells of a 6well plate, one well was treated with puromycin (4 µg/ml) for 6 days (Puro+) and the other without puromycin (Puro-). At the end of the 6 days, only successful transduced cells in the Puro+ wells survive, and transduction efficiency, corresponding to the percentage of transduced cells, was calculated as follow:

$$\text{Transduction efficiency} = \frac{\text{Cells in Puro +}}{\text{Cells in Puro -}} \cdot 100$$

The different volumes of Brunello library stock tested were (µl): 0, 0.03, 0.1, 0.3, 1, 3 and the results are shown in **Figure 4.7**. Increasing transduction efficiencies were observed for increasing volumes of viral library, with the exception of the highest volume of library (3 µl) which showed a 50% reduction in transduction efficiency (**Figure 4.7a**). This is explained by the fact that at these levels, the lentiviral library causes a drastic reduction in cell viability, milder detrimental effects are observed also for 1 µl (**Figure 4.7b**). By looking at the trend of transduction efficiency in **Figure 4.7a**, a window of linear correlation between transduction

viral volume and transduction efficiency can be observed between 0.1 and 0.3 titration points. Interestingly, the volume of 0.1 μl resulted in a MOI ~ 0.3 .

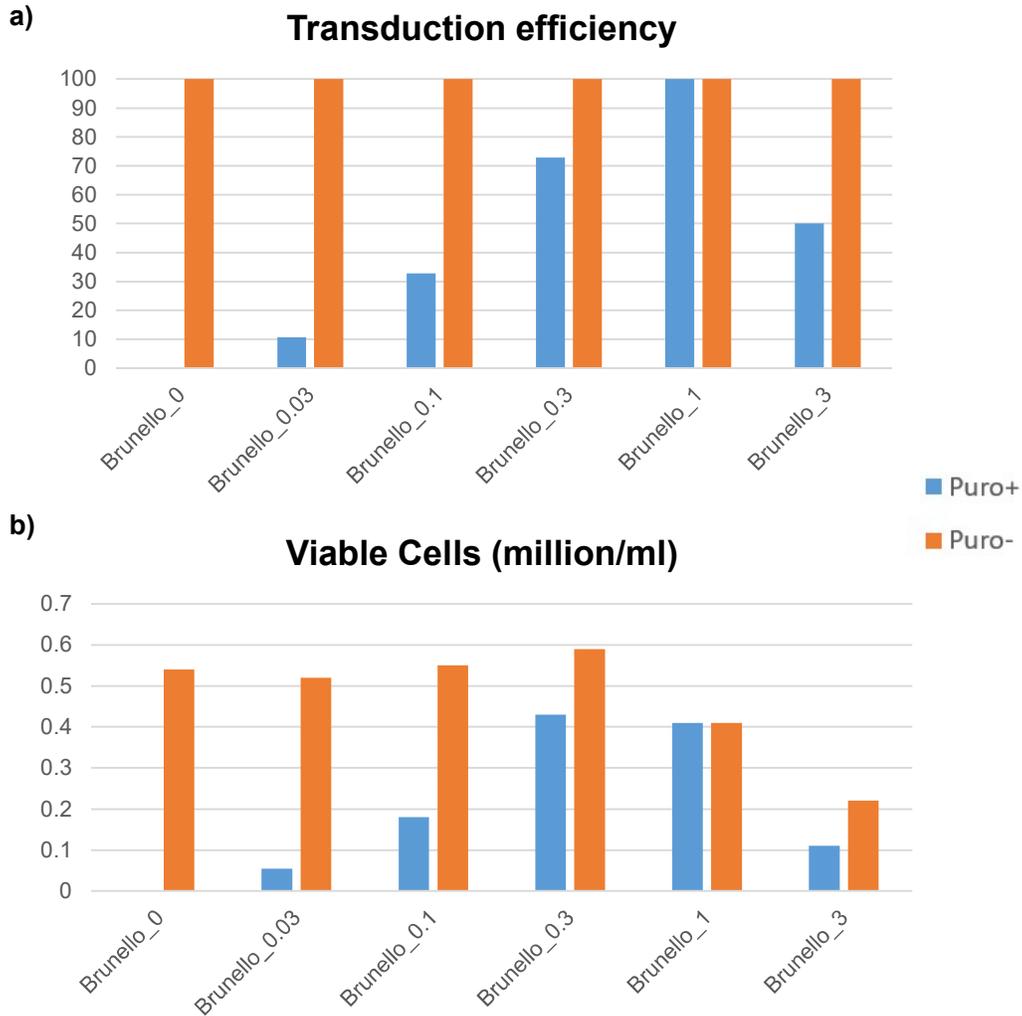


Figure 4.7. Small-scale Brunello lentiviral library titration results. Different volumes of Brunello library were tested (μl), 0, 0.03, 0.1, 0.3, 0.1, 3, and the transduced cells were either grown in medium containing puromycin (4 $\mu\text{g/ml}$) (Puro+), or without (Puro-) **a) Transduction efficiency.** The percentage of cells in each Puro+ titration point was calculated in relation to the corresponding number of cells in the untreated sample (Puro-). **b) Viable cells expressed as million/ml.** Viability was assessed by trypan blue staining with automated cell counter Vi-CELL™ XR, after trypan blue staining.

We used this linear range, to define the linear relation between viral volume and transduction efficiency, to extrapolate the volume of virus needed to achieve a transduction efficiency of 50% (= MOI 0.5). We further corrected this volume to account for a scaling-up in plate dimensions, in order to perform a second library titration in T150cm² flasks, identifying 3.85

μl of viral library as the volume needed to achieve a MOI of 0.5. However, the transduction efficiency observed in this large-scale setting was of 62.4%, rather than the estimated 50%. Small variations are to be expected between culture containers of different sizes, such as 6well plates and T150, and additional titrations in T150 cm^2 flasks, around the value of 3.85 μl , are needed to find the optimal volume for a MOI of 0.5.

Step5. Identification of mutants with impaired ability to undergo CTB differentiation into STB.

Using negative selection of fusing cells: IFI6-Blasticidin approach.

As previously mentioned, we initially set out to perform a positive screen, in which Cas9-mediated mutants in genes involved in trophoblast fusion should survive over the cells with unaffected CTB differentiation genes. The premises of this approach are summarised in **Figure 4.8**. In this set-up, the BeWo-IFI6-Cas9Hyg-CL2 cells used in the screen, upon forskolin administration, by differentiating into STB-like cells are expected to have reduced fitness.

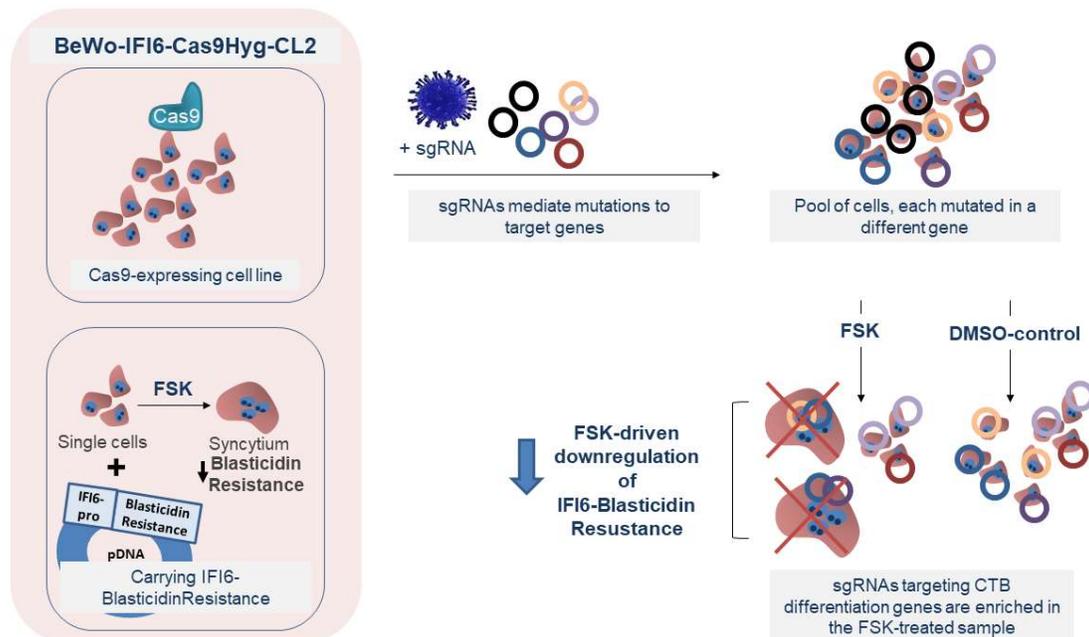


Figure 4.8. Positive screen experimental approach based on the FSK-driven downregulation of IFI6-BlasticidinResistance in differentiating BeWo-IFI6-Cas9Hyg-CL2. IFI6-pro = promoter of the IFI6 gene, FSK = forskolin, CTB = cytotrophoblast.

This cell line is in fact a derivative of the BeWo-IFI6 cell line, previously generated in the team, in which a blasticidin resistance cassette is under the control of the IFI6 promoter, chosen for its being downregulated upon FSK-treatment. In the context of the genome-wide screen, the expectation is therefore that Cas9-mediated mutations in genes involved in trophoblast differentiation should interfere with the ability of BeWo-IFI6 cells to respond to FSK-treatment and as a result the fitness of these clones in blasticidin medium should remain unaltered. This would result in the death of FSK-treated, differentiating BeWo cells only, allowing specific collection of FSK-treated unresponsive clones. The majority of these clones should present Cas9-sgRNA-mediated mutations targeting genes involved in CTB differentiation into STB.

To test this experimental design, we assessed the mortality rates by monitoring cell confluency at the light microscope, of BeWo-IFI6-Cas9Hyg-CL2 cells grown in complete medium with blasticidin (8 µg/ml), treated either with FSK or vehicle control containing dimethyl sulfoxide alone (DMSO) (since FSK is prepared in DMSO), over a period of 20 days. However, even though slight differences can be seen up to Day 5, since DMSO-treated cells show increased confluency over-time with higher rates compared to FSK-treated cells, no detectable selection is seen in the latter. At Day 16 and 19, even FSK-treated samples showed considerable increase in cell confluency, indicating the presence of a high proportion of proliferatively active cells in the population (**Figure 4.9**).

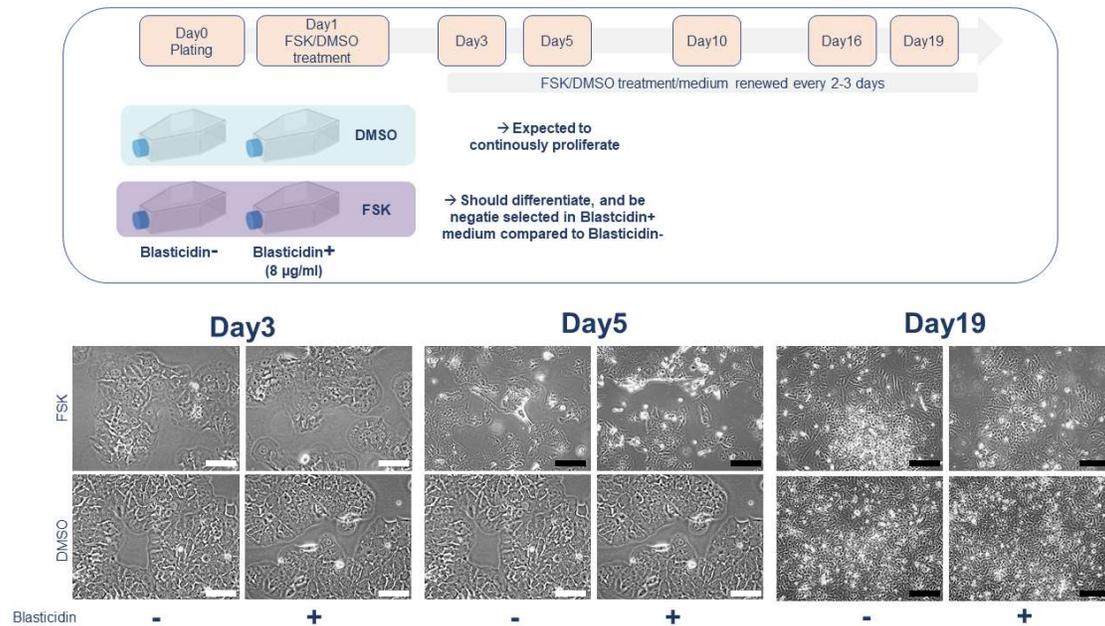


Figure 4.9. Bewo-IFI6-Cas9Hyg-CL2 cells are not differentially selected after FSK treatment, in blastcidin+ or blastcidin- media. FSK = forskolin. White/Black scale bar = 100 µm / 150 µm.

To assess whether higher blastcidin concentrations were needed to negatively select FSK-treated cells, we repeated the same experiment over a 1-week period for FSK-treated and DMSO control cells grown in increasing concentrations of blastcidin: 0, 8, 20, 40, 80 and 100 µg/ml. In this case, 20 and 40 µg/ml of blastcidin caused a decrease in proliferation rates in both FSK and DMSO-treated cells, similarly to 80 and 100 µg/ml where these were even more pronounced and accompanied by signs of toxicity such as cytoplasmic aggregates. Therefore, blastcidin concentrations > 20 µg/ml are too elevated and cause toxicity even in the DMSO-control. However, none of the used concentrations cause differential effects between FSK-treated and DMSO-treated cells. This prompted us to devise a new experimental strategy to differentially distinguish between FSK-treated differentiating and unresponsive cells.

Harnessing nuclear markers of trophoblast differentiation state: CTB vs STB.

For the purpose of the screen, we wanted to harness specific markers of CTB-like and STB-like states to isolate these distinct populations in FSK-treated samples. While in DMSO-treated samples, BeWo cells should retain a CTB-like state characterised by proliferation and cell growth, in the FSK-treated samples, the activation of the cAMP cascade activates syncytialisation and the cells can shift towards a STB-like state. However, cells in which key fusion genes have been knock-out by the Cas9-sgRNA complex should be unable to initiate the differentiation process and would retain a CTB-like phenotype. We chose the nuclear transcription factors Ki67, GATA3 and GCM1 to distinguish between CTB and STB respectively. In primary trophoblasts as well as in BeWo, the CTB population is characterised by high levels of the proliferative marker Ki67 and of trophoblast lineage determinant GATA3 (Apicella & Ruano et al., 2021; Buchrieser et al., 2019; Chiu and Chen, 2016; Cierna et al., 2016). Therefore, untreated BeWo as well as cells unable to differentiate, that retain a CTB-like phenotype, are expected to express Ki67 (Ki67+) and GATA3 (GATA3+). On the other hand, during CTB differentiation into STB, GATA3 and Ki67 downregulation is accompanied by increase in the nuclear expression levels of fusion transcription factor GCM1 (Chiu and Chen, 2016); hence, differentiating BeWo cells are proposed to be GCM1+.

To assess the expression levels of these markers in the two populations (DMSOvsFSK), flow cytometry was used, yet the presence of small syncytia from FSK-treatment poses a limitation in the use of standard flow cytometry techniques which are based on capillarity and processing of cells, one by one. To tackle this issue, we opted for a protocol based on the isolation of the nuclei from the cells, based on the 10XGenomics protocol for nuclei isolation from single cell suspension, designed for single nuclei RNA-sequencing experiments (10XGenomics, 2021).

Since we needed to first check the validity of this approach, we used BeWo-WT cells, in case that the expression of IFI6-Blasticidin and Cas9-Hygromycin constructs could alter the physiology of the cells and complicate the interpretation of the results. We first treated BeWo-WT cells with DMSO/FSK for 72 hours, then proceeded with the isolation of the nuclei. Once the nuclei were isolated, they were immune-stained with antibodies against Ki67, GATA3 and GCM1, and the proportion of CTB-like and STB-like fractions in DMSO/FSK-treated samples quantified by flow cytometry, according to the relative expression of these nuclear markers. As

a control for the purity of the samples, to distinguish between debris and intact nuclei, the expression of the nuclear pore complex Nup107, expressed on the nuclear membrane, was quantified and the relative signal of Ki67, GATA3 and GCM1 was acquired only in Nup107+ events.

The results presented here are to be considered as very preliminary, since they originate from duplicated experiments only. The nuclei isolation protocol has not been fully optimised yet, even though single nuclei can be obtained in DMSO-treated samples, large clumps are always detected in FSK-treated samples. The clumping is usually associated with damage to the nuclear membrane which leads to DNA leakage responsible for the formation of aggregates (Neely and Bao, 2019). This is reflected in the extensive number of debris visible in the flow cytometry plots relative to FSK-treated samples (debris are characterised by negative signal for antibody Mab414, corresponding to low levels of nuclear marker Nup107) (**Figure 4.10a**). However, differences in the proportions of debris have not been tested for statistical significance between FSK and DMSO-treated samples. The putative increase in fragility of FSK-treated nuclei could be due to changes in the nuclear membrane as a result of the FSK-induced syncytialisation, hindering our ability to collect the STB-like nuclei. For all tested markers, no major differences can be seen between DMSO/FSK-treated samples; however, in FSK-treated cells a small peak of GATA3- cells, suggestive of STB-like state, is detectable and corresponds to ~10% of the tested nuclei (**Figure 4.10b, green circle**).

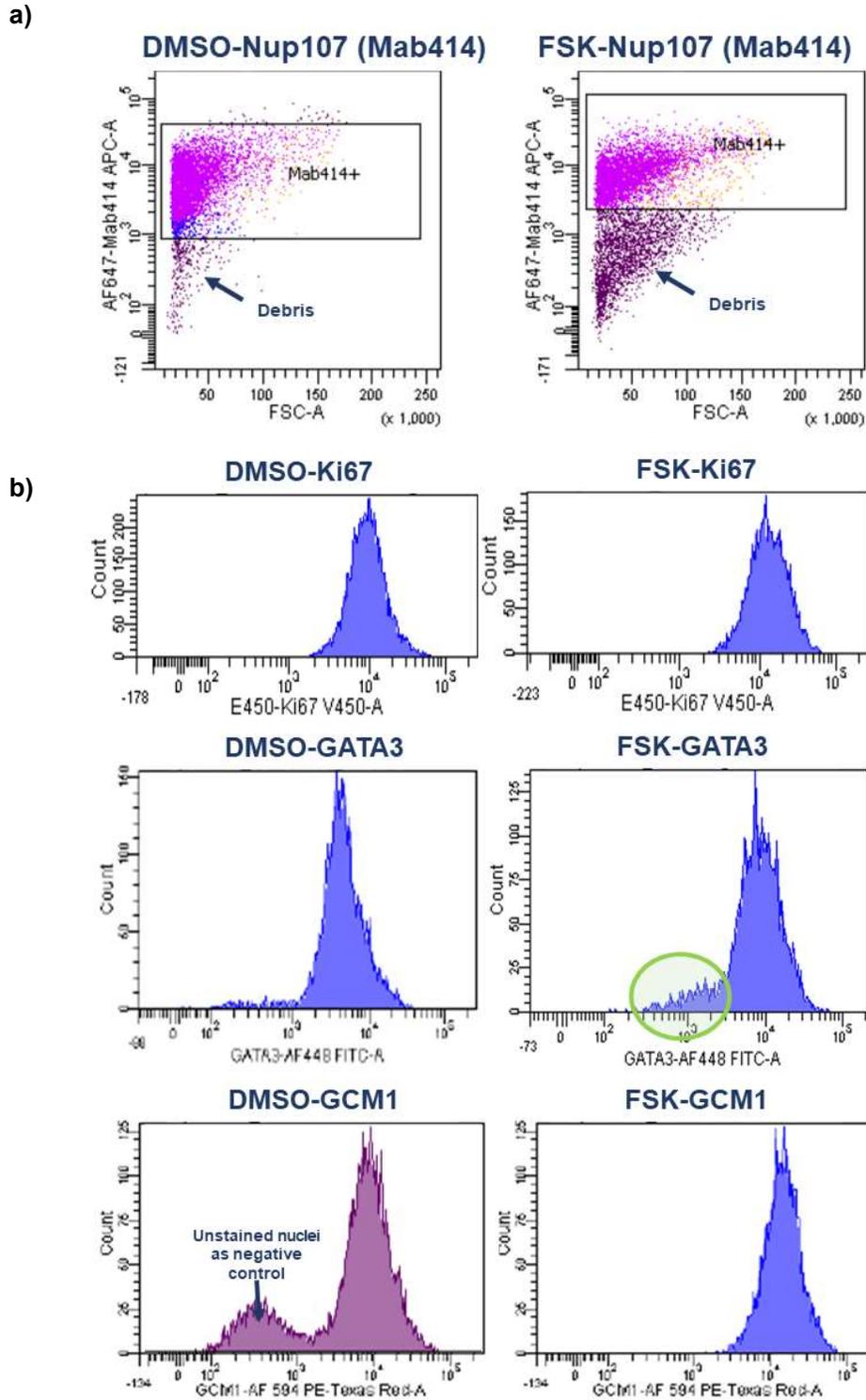


Figure 4.10. Flow cytometry assessment of nuclear markers: Ki67 and GATA3 as markers of CTB-like state, GCM1 as marker of STB-like state. a) Flow cytometry plot showing the signal relative to Mab414 antibody against nuclear membrane marker Nup107 in DMSO and FSK treated BeWo-WT cells. Mab414+ events correspond to intact nuclei and are identified by the gate, while the

rest of the events correspond to cellular debris. Only the Mab414+ nuclei have been analysed for the expression of Ki67, GATA3 and GCM1. **b) Flow cytometry plots assessing the proportion of cells expressing Ki67, GATA3 and GCM1** in DMSO and FSK treated BeWo-WT cells. In the DMSO-GCM1 graph in pink, the peak of cells negative for GCM1 corresponds to a population of unstained nuclei that have been used to spike the sample prior of analysis by flow cytometry, to check whether the GCM1 signal was strong enough to be distinguished from the negative population. FSK = forskolin.

Methods

Collaboration.

This work has been carried out as a collaboration with the CRISPRit platform, Institut Curie, as part of Dr. Margueron's Team. In particular, our team has closely collaborated with Dr. Raphael Margueron, Auréline Boré, Dr Michel Wassef and Camille Fouassier, for expertise, experimental design, protocols and specialised facilities.

Cell lines, culture conditions, forskolin treatment.

The human choriocarcinoma cell line BeWo was used, available from ATCC (CCL-98). Cells have been grown according to manufacturer's instruction, in Ham's F-12 Nutrient Mix, GlutaMAX™ (Gibco™, REF: 31765, LOT: 2040426), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco™, REF: 10500-064, LOT: 2036257K) and Penicillin-Streptomycin (Gibco™, REF: 15070-063, LOT: 2009159) used at final concentration of 100 U/mL. Cells are grown at 37 °C, with 5% CO₂. For sub-culture, adherent cells have been grown at densities between 30-90%, trypsin-EDTA (Gibco™) has been used for gentle detachment, by incubating at 37°C for 1-2 minutes and resuspending detached cells in complete medium. For induction of cytotrophoblast differentiation in BeWo cell lines, 20 μM of FSK in DMSO was administered in complete medium for 72 hours, changing medium every second day. Control cells were treated with the vehicle only, equivalent volume of DMSO in complete medium.

Cas9 vectors, viral transduction, and transfection conditions.

To generate Cas9-expressing cell lines the following vectors have been used:

- lentiviral vector LentiCRISPRv2GFP was a gift from David Feldser (Addgene plasmid # 82416; <http://n2t.net/addgene:82416> ; RRID:Addgene_82416). This vector has been modified by CRISPRit platform at the Institut Curie, replacing GFP sequence with mCherry (Walter et al., 2017).

- lentiviral vector encoding for pSpCas9 and Blasticidin resistance cassette lentiCas9-Blast was a gift from Feng Zhang (Addgene plasmid # 52962; <http://n2t.net/addgene:52962>; RRID:Addgene_52962) (Sanjana et al., 2014).
- circular vector encoding for pSpCas9 and Hygromycin B resistance cassette pSpCas9(BB)-T2A-HygR was a gift from Jorge Ferrer (Addgene plasmid # 118153; <http://n2t.net/addgene:118153>; RRID:Addgene_118153) (Miguel-Escalada et al., 2019).

For viral transduction 3×10^5 cells have been plated in each well of a 6well plate the day before transduction. For transduction, 50 μ l of virus have been resuspended in 2 ml of complete medium with polybrene (Sigma-Aldrich, TR-1003-G, LOT: 3136750) at a final concentration of 10 μ g/ml, and cells incubated with the viral mix at 37°C for 24 hours, then medium has been replaced with fresh complete medium, containing antibiotics according to the resistance cassette in the vector (Blasticidin 2 μ g/ml (InvivoGen, REF: ant-bl-1), Hygromycin B 75 μ g/ml (Gibco™, REF: 10687010, LOT: H044-51US)).

For plasmid transfection, pSpCas9(BB)-T2A-HygR has been resuspended at a final concentration of 1 μ g/ml in Opti-MEM™ (Gibco™), supplemented with 10% FBS, after having been pre-incubated with Lipofectamine™ 2000 (Invitrogen™) according to manufacturer's instructions. Cells have been incubated for 24 hours with Opti-MEM™ (Gibco™), supplemented with 10% FBS, and DNA-Lipofectamine mixes at 37°C. Then, the medium was replaced with complete medium supplemented with 75 μ g/ml Hygromycin B (Gibco™).

Cas9 activity test.

To test the activity of the Cas9 in the Cas9-expressing cell lines produced we performed a *Cas9-activity test*, measuring the rate of mutation in cells transduced with control sgRNAs targeting the control gene PIGA located on chromosome X, coordinates (GRCh38 build) chrX:15,319,456-15,335,523.

We used two guides gRNA-274 and gRNA-273 cloned in the same lentiviral backbone of the library, encoding for a puromycin resistance cassette.

gRNA-274 (TGGTAAACCATGATATGCTG) targets one of the intronic regions and induces the double-stranded break (DSB) mediated by the Cas9 on position 15,332,254, while gRNA 273 (TGCTCAGGTACATATTTGTT) targets an exonic region, inducing the DSB at in 15,331,580.

For the *Cas9 activity test*, 3×10^5 Cas9-expressing cells have been plated in each well of 6well plate, in duplicates for each of the conditions: un-transduced, viral vector gRNA-274, viral vector gRNA-273. The day after plating, 5 μ l of viral vector have been resuspended in 2ml of complete medium with polybrene (Sigma-Aldrich, TR-1003-G, LOT: 3136750) at final concentration of 10 μ g/ml for transduction of gRNA-274 and gRNA-27 wells, the other two wells have been left untreated, with the addition of complete medium with polybrene alone. Cells in 6well plates have been incubated at 37°C for 24 hours. Then, the transduction medium was replaced with fresh complete medium (Puro-) for half of the wells, and fresh medium containing puromycin (4 μ g/ml) (Puro+) for the other half. Medium has been replaced accordingly every two days for 6 days. On the morning of the 7th day, when no surviving un-transduced cells were detected in Puro+ well, cells from all wells were collected by scraping followed by centrifugation at 0.2 RCF for 5 minutes. Cell pellets were treated for genomic DNA extraction according to manufacturer's instruction, with NucleoSpin Tissue kit (Macherey-Nagel, Ref:740952.50).

To test if the targeted regions were modified by Cas9, they are first amplified by PCR using primers flanking the targeted locus (**Table 4.2**), with GoTaq® Flexi DNA Polymerase kit (Promega), annealing temperature 64 °C. PCR products are run on 2% agarose gel and the bands are sliced and purified before Sanger sequencing with forward primer.

Table 4.2. PCR primers used to amplify targeted loci for Cas9 activity test.

<i>Primers</i>	<i>Sequences</i>
<i>PIGA_274_Forward</i>	GCCATGCTGAGACGGTTTAG
<i>PIGA_274_Reverse</i>	AGTTTTGGTGAGGCTGCAAT
<i>PIGA_273_Forward</i>	TGTTTGTAAGCACCGAGCTG
<i>PIGA_273_Reverse</i>	ACCGTGCCTCAGCTACTC

Sequencing results are analysed with the free tool from SYNTHOGO ICE v2 which calculates for each sample the percentage of INDELS observed in comparison to the untreated cell line,

which we use here as a measure of the proportion of cells in the sample that presented a Cas9-mediated mutation (Hsiau et al., 2019).

Immunostaining for Cas9 cellular expression.

To test the proportion of BeWo-Cas9-Blasti cells that express Cas9, a Cas9 immunostaining experiment was performed, using BeWo-WT as negative control. In a 24well plate, sterile glass coverslips were placed at the bottom of each well, and 8×10^4 cells for each cell line plated in duplicates and grown respectively in complete medium + Blasticidin (2 $\mu\text{g}/\text{ml}$) for BeWo-Cas9-Blasti, and complete growth medium for BeWo-WT. After 24 hours incubation at 37 °C, cells were washed in cold PBS four times. For fixation and permeabilization, cells were incubated at -20°C in 500 μl cold methanol (previously stored at -20°C) for 8 minutes, followed by four washes in cold PBS. Blocking was performed by 1 hour incubation with cold blocking buffer (PBS, 1%BSA) at room temperature, followed by overnight staining with primary antibody Rabbit αCas9 used at 1:100 dilution (custom antibody developed by the ANTICORPS THÉRAPEUTIQUES (TAB-IP) ET ANTICORPS RECOMBINANTS platform at Institut Curie, REF: A-P-R#56) in blocking buffer, in cold room (4°C). The following morning, the primary antibody was removed with 4 washes in cold PBS, followed by secondary antibody incubation in blocking buffer for 45 minutes at room temperature, in the dark: donkey anti-Rabbit IgG (H+L)-AlexaFluor594 (Invitrogen #A-21207) used at 1:500. After 4 washes in cold PBS cells were stored in PBS at 4°C in the dark. For imaging, the coverslips coated with fixed and stained cells were removed from the 24well plate and mounted onto glass slides using 10-20 μl of DAPI Fluoromount-G® (SouthernBiotech), for 5 minutes at room temperature. Before imaging at the fluorescent microscope, mounted slides were incubated at 4°C for additional minimum 30 minutes to improve fluorescence signal. Fluorescent images were acquired with epifluorescence microscope.

Viral library, library titration.

The Brunello lentiviral library (Addgene #73178) was used. The library consists of lentiviral vectors encoding for a total of 77,441 sgRNAs targeting 19,114 human genes, with an average of 4 guides per gene; the library also includes 1,000 control guides that do not target any gene

(Doench et al., 2016; Sanson et al., 2018). The viral backbone encodes for a puromycin resistance cassette.

The small-scale library titration was carried out in 6well plates, in each well 2.2×10^5 BeWo-IFI6-Cas9Hyg-Clone2 cells were plated and incubated for 24 hours at 37°C. Transduction has been carried out as described above, testing the following volumes of lentiviral library: 0, 3µl, 1µl, 0.3 µl, 0.1 µl, 0.03 µl. After 24 hours at 37°C, the medium was changed to either Puro+ or Puro-, every 2 days for 6 days. On the 6th day, the cells were collected by trypsin-ETDA treatment for 1minute at 37°C and resuspended in complete medium (300 µl trypsin + 700µl medium). The number of viable cells in each well was counted with automated cell counter Vi-CELL™ XR, after trypan blue staining, and transduction efficiency calculated as ration between the number of cells in Puro+ condition divided by the number of cells in Puro-, expressed as percentage. Moreover, by plotting the transduction efficiency versus the titration points, the linear range of library volumes correlating with transduction efficiency was identified.

The large-scale library titration was carried out in T150cm² cell culture flasks, in each of three flasks 5.3×10^6 BeWo-IFI6-Cas9Hyg-Clone2, in complete medium (Ham's F-12 Nutrient Mix, GlutaMAX™ (Gibco™, REF: 31765, LOT: 2040426), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco™, REF: 10500-064, LOT: 2036257K) and Penicillin-Streptomycin (Gibco™, REF: 15070-063, LOT: 2009159) used at final concentration of 100 U/mL, blasticidin (2 µg/ml) and hygromycin B (75 µg/ml)) and incubated overnight at 37°C. The following day, viral library transduction was performed. The volume of viral library estimated from the linear distribution obtained from the small-scale titration to correspond to MOI=0.5 was used: 3.85 µl for 20 ml of medium in T150 cm²flask, resuspended in growth medium, with polybrene at final concentration of 10 µg/ml. Of the three flasks, two were transduced with 3.85 µl of viral library and one with polybrene alone. Flasks were incubated at 37°C for 24hours, then the medium was replaced with complete medium either with or without puromycin (4 µg/ml), replaced every 2days. The flasks correspond to the three conditions:

- Virus-PURO+: to check that un-transduced cells have died
- Virus+PURO+: number of transduced cells
- Virus+PURO-: total number of cells

The proportion between Virus+PURO⁺ and Virus+PURO⁻ was calculated to estimate library transduction efficiency and effective MOI after 6 days of selection; cells were detached with trypsin-EDTA and the number of viable cells established with automated cell counter Vi-CELL™ XR, after trypan blue staining.

Antibiotic selection and kill curves.

Optimal blasticidin concentrations and exposure time had been previously determined in the laboratory, using blasticidin (InvivoGen, REF: ant-bl-1) at a final concentration of 2µg/ml for two weeks. For puromycin (Gibco™, REF: A11138-02, LOT: 2068873) and Hygromycin B (Gibco™, REF: 10687010, LOT: H044-51US) kill curves, at Day0, 1.4×10^5 BeWo-WT cells/well were plated in 12well plates and incubated at 37°C for 24 hours. At Day1, control medium was added to each well, supplemented with different concentration of antibiotics, in duplicate wells. Tested concentrations for puromycin in µg/ml were 0, 1, 1.5, 2.5, 3, 4, 5, 6, 7, 8 and 9, while for hygromycin B were 0, 2.5, 5, 10, 20, 30, 50, 75, 100 µg/ml. Cell confluency was assessed by light microscopy in each well, at days 1, 3, 5 and 7; kill curves were determined by plotting cell confluency at each time point, for each of the tested concentrations.

Genome-Wide Screen parameters: representativity, MOI, cell numbers, duplication time.

For the current genome-wide screen, based on existing literature, sgRNA representativity was set at 500x, aiming for a MOI of 0.5. (DeWeirdt et al., 2020; Joung et al., 2017; Peng et al., 2015; Sanson et al., 2018; Shalem et al., 2015). These two parameters were used to estimate the numbers of cells needed for the screen, considering the size of the library as described in the Results section. Briefly, given that the Brunello library includes approximately 80,000 (77,441) guides, for a representativity of 500x, 40×10^6 cells are needed. Setting the MOI to 0.5, this number was corrected for the MOI by division, estimating the number of cells to 80×10^6 cells. Finally, the Final total number of cells was calculated by correcting further by the observed Cas9 activity rate in BeWo-IFI6-Cas9Hyg-CL2 cells of 0.65. Dividing the Total number of cells by 0.65, the Final total number of cells needed for the screen is estimated to be 123×10^6 cells. Duplication time for the BeWo-IFI6-Cas9Hyg-CL2 was calculated by

monitoring cell growth in T75cm², over a 1-week period, counting cell number with Malassez counting chamber every 2-days:

$$\text{Doubling Time} = \frac{\text{number of days} * \log(2)}{\log(\text{Final Concentration}) - \log(\text{Initial Concentration})}$$

Testing effects of FSK-treatment on Blasticidin resistance and survival in BeWo-IFI6-Cas9Hyg-CL2 cells.

On Day0, 2.45 x 10⁶ BeWo-IFI6-Cas9Hyg-CL2 cells were plated in each of four T75cm² flasks, two flasks were grown in complete medium without blasticidin (Blasticidin-), while the other two in complete medium with blasticidin at 8 µg/ml (Blasticidin+); cells were incubated for 24 hours at 37°C. On Day1, for each of the two growth conditions, one flask was treated with 20 µM forskolin, in DMSO, while the other flask with equivalent volumes of forskolin only. Cells were grown in these conditions for up to 20 days, changing medium and treatment every 2-3 days and assessing confluency and survival by light microscopy.

To test different ranges of blasticidin concentrations, a similar experiment was repeated in 6well plates, 2.1 x 10⁵ cells per well; for each tested concentration 2 wells were plated, one to be treated by FSK and the other by DMSO alone as described above, and confluency and survival were assessed by light microscopy on Day4, 6 and 7. The blasticidin concentrations tested were: 0, 8, 20, 40, 80, 100 µg/ml.

Flow cytometry of isolated nuclei.

To assess first the validity of the use of nuclear markers of CTB-like state (Ki67, GATA3) and STB-like state (GCM1), we tested the isolation of nuclei and quantification of nuclear markers protocol in BeWo-WT cells. On Day0, 2.45 x 10⁶ BeWo-WT cells were plated in T75cm² flasks in growth medium, on Day1 half of the flasks were treated with FSK (10µM) diluted in DMSO and added to fresh growth medium, while the other half was treated with equivalent volumes of DMSO alone. The treatment was carried out for 72 hours, refreshing the medium with FSK/DMSO every 48hours. On Day4 cells were harvested by Trypsin-EDTA treatment as previously described and treated for isolation of nuclei, following the 10XGenomics

protocol for nuclei isolation from single cell suspension, designed for single nuclei RNA-sequencing experiments (10XGenomics, 2021). Briefly, cells were centrifuged at 300 rcf for 5 min, removing the supernatant and resuspended in cold Lysis Buffer (10mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, 0.1% Nonidet P40 Substitute in Nuclease-Free Water, at 4°C), then incubated on ice for 5-6 minutes for lysis to occur. Lysates were centrifuged for 5min at 700 rcf, at 4°C to precipitate the nuclei. After removing the supernatant, the nuclei were washed in Wash Buffer (1x PBS with 1% BSA, at 4°C) and thoroughly resuspended by gentle pipetting. This step was repeated once by centrifugation, removal of supernatant and additional resuspension in wash buffer. The nuclei in solution were counted at the Malassez counting chamber, by staining with trypan blue at 1:10 dilution) and diluted to a final concentration of 10⁶ nuclei/ml.

The nuclei suspensions were then treated for immunostaining. For each condition DMSO-treated cells/FSK-treated cells, four different samples were prepared by immunostaining with the following combinations of primary antibodies: Mab414 – Ki67, Mab414 – GATA3, Mab414 – GCM1, unstained. For each sample 500 µl of nuclei solution, corresponding to 5 x 10⁵ nuclei, were used. Primary antibodies were diluted in 100 µl of Wash Buffer, and samples incubated on ice for 30 minutes, followed by washing in cold Wash Buffer, centrifugation for 10 min at 700 rcf, at 4°C. Secondary antibodies were added, diluted in 100 µl/sample, and incubated for 10 minutes on ice in the dark. Then, the conjugated antibodies were added directly into each of the samples, incubated on ice for a further 15 minutes. For the final wash, cells were resuspended in 1 ml of Wash Buffer, centrifuged 10 min at 700 rcf, at 4°C; after removal of the supernatant, the stained nuclei were resuspended in 300 µl of Wash Buffer and transferred to flow cytometry tubes, with filtered cap.

The complete list of antibodies, with relative working concentrations is found in **Table 4.3**.

Table 4.3. Antibodies used for nuclear staining of CTB and STB markers.

<i>Antibody</i>	<i>Species</i>	<i>Specifics</i>	<i>Reference</i>	<i>Stock concentration (mg/ml)</i>	<i>Working volume/5*10⁵ nuclei (µg)</i>
<i>αGATA3</i>	rabbit	monoclonal (EPR16651)	abcam #ab199428	0.6	0.12
<i>αRabbit IgG (H+L)-AlexaFluor488</i>	donkey	polyclonal	Invitrogen #A-32790	2	0.5
<i>αKi67 conjugated-eFluor450</i>	rat	monoclonal (SolA15)	eBioisience #48-5698-82	0.2	0.125
<i>αGCM1</i>	mouse	monoclonal (R-06)	Santa Cruz #sc-101173	0.1	1
<i>αMouse IgG (H+L)-AlexaFluor594</i>	goat	polyclonal	Invitrogen #A-11032	2	2
<i>αNup107 conjugated-APC</i>	mouse	monoclonal (Mab414)	Covance #MMS-120P	1	2.5

Discussion

Trophoblast syncytialisation and STB renewal are key processes for placental development, and increasing evidence links STB defects with pregnancy pathologies such as IUGR and PE (Bastida-Ruiz et al., 2019; Colson et al., 2020; Wang et al., 2019). For these reasons, the focus of this project was to expand our current knowledge of this phenomenon and identify novel candidate genes involved in syncytialisation. Here we have for the first time attempted to tackle the problem of trophoblast fusion from a global perspective, by mean of a genome-wide screen based on the CRISPR/Cas9 system. Even though we have not yet been able to perform the screen and identify novel putative markers of syncytialisation, we have succeeded in overcoming some of the technical hurdles posed by this complex experimental design.

First, we have successfully generated a BeWo cell line that not only stably expresses the Cas9 gene, but also shows functional endonuclease activity, having detected sgRNA-mediated INDELS at the control gene PIGA in 65% of the cell population. Cas9 has been previously used in the BeWo cell lines to successfully generate targeted knock-out of specific genes-of-interest to investigate their role on syncytialisation, like HtrA4, HSPE1 and SIK1 (Kovács et al., 2019; Mansilla et al., 2021; Msheik et al., 2019). In this study however, we struggled to generate a functional Cas9-expressing cell line and three different vectors, two lentiviral vectors and one plasmid DNA, had to be tested. These differences can be explained by the fact that, during a gene-of-interest knock-out experiment, the goal is to obtain clones in which the target gene has been successfully modified and detrimental mutations can be detected at the locus. In this scenario, the experimental procedure consists in delivering the Cas9 gene and the sgRNA targeting the gene-of-interest to a pool of cells; after the CRISPR/Cas9 system has been delivered, the cells are treated so to isolate different clones, usually starting from individual single cells, which will then be tested by sequencing of the targeted region to identify the clones that carry loss-of-function mutations. In this case, the rate of Cas9 activity in the whole transfected population is of no interest, since only the outcome is tested, i.e. the presence of a mutation in any given number of cells. In a genome-wide screen however, the Cas9 expressed by the cell line needs to be functional in a large proportion of the population since each cell will receive a different sgRNA targeting one of the genes in the genome, and the assumption is that all cells will be able to undergo Cas9-mediated gene editing; however, the validity of such an assumption is strictly dependent on the Cas9 activity rate.

Essentially, the key difference between the two approaches is the fact that in the targeted approach we are only looking for a successful outcome, without investigating the qualities of the Cas9 protein which gave rise to the detected mutation, while in a genome-wide screen we are aiming for consistency and reproducibility of the Cas9 functional properties across the population of cells. In the latter case therefore, many parameters will interject in our ability to produce a satisfactory cell line expressing functional Cas9. Among these factors, the genetic variability of the cell line might influence the PAM sequence of a specific sgRNA, affecting the efficiency of the Cas9-sgRNA complex to catalyse the DSB at the locus. Other critical factors include the cellular localisation of Cas9 which should preferentially be targeted to the nucleus and in some cellular systems additional nuclear localisation signal sequences have been found to be beneficial, as well as the relative levels of expression of the Cas9 itself and how these levels influence the overall cellular fitness (Enache et al., 2020; Lino et al., 2018).

Another important issue that we have encountered is on the matter of the screen design: how can we select the trophoblasts that carry a mutation that impairs their ability to differentiate into STB? We have first tried to generate a cell line in which the blasticidin resistance gene is under control of the promoter of the IFI6 gene, found to be deeply downregulated in BeWo cells after forskolin treatment in the dataset we generated in (Ducat et al., 2020). However, even with high concentrations of blasticidin ($> 40 \mu\text{g/ml}$), FSK-treated BeWo-IFI6-Cas9Hyg-CL2 cells do not die differently than untreated cells. This could be due to insufficient downregulation of blasticidin gene (previously reported around 4 to 8x), given that wild type BeWo cells die within 2-weeks exposure from $2\mu\text{g/ml}$ blasticidin.

Having failed to identify this issue early on during the optimisation of the screen, even though we would not have been able to harness the IFI6promoter-blasticidin construct, for further protocol optimisations (e.g. library titration experiments) we kept using the BeWo-IFI6-Cas9Hyg-CL2 cell line since it successfully expressed the functional Cas9. We then went on to design a quite innovative strategy based on the isolation of nuclei from FSK/DMSO- treated cells, and quantification of nuclear markers of CTB-like and STB-like state by flow cytometry; respectively, proliferation marker Ki67 and trophoblast lineage transcription factor GATA3, and fusion transcription factor GCM1 (Buchrieser et al., 2019; Chiu and Chen, 2016; Cierna et al., 2016). To first test the validity of this approach, we used BeWo-WT cells. While the results presented here are to be considered as preliminary, since the flow cytometry experiments have

been carried out only in duplicates, they seem to show a very high proportion of FSK-treated cells (72 hours) that do not show the expected changes at the protein level, since there were no differences in ki67 and GCM1 signals, and only 10% of the FSK-treated population showed a decrease in GATA3 levels.

Even though in BeWo cells drastic changes in response to FSK-treatment have been observed at the transcriptomic, DNA methylation and histone modification levels, in our and other teams (Ducat et al., 2020; Shankar et al., 2015; Zheng et al., 2017); our flow cytometry results seem to suggest that these changes might be occurring only in a small proportion of cells in the population. This is in contrast with the reports of syncytialisation rates calculated based on immunofluorescence experiments that assessed the proportion of STB-like BeWo expressing specific nuclear markers such as GATA3 for CTB-like status, Twist1 and GCM1 for STB-like phenotype, or by the number of nuclei in a single membrane structure. These studies presented observed fusion rates between 60-90%, in BeWo cell cultures treated with a range of FSK concentrations (20-100 μ M), for an exposure time varying from 48 to 72 hours (Al-Nasiry et al., 2006; Degrelle and Fournier, 2018; Omata et al., 2013; Shankar et al., 2015; Shao et al., 2021). However, in the work of Kudo *et al.* (2003), only 10% of FSK-treated cells is reported as having taken part in fusion. In this study, the team generated two BeWo cell lines expressing histone-2B fused to either GFP or DsRed2 fluorescent tags, allowing the differential detection of the two lines by flow cytometry. The cell lines were grown in co-culture and treated with FSK (100 μ M) for 48 hours, followed by flow cytometry assessment of cellular co-expression of the fluorescent tags, implying that fused cells would have colocalization of green and red signals. In this study however, very little technical details are given on the sample preparation for flow cytometry and is not clear how the issue of the small syncytia has been tackled (Kudo et al., 2003). Nonetheless, specialised flow cytometry techniques have been developed especially to deal with large particles in the context of cardiomyocytes and myocytes biology (Conboy et al., 2010; Kannan et al., 2019).

Interestingly, in the present study we observed GATA3 reduction, associated with a STB-like phenotype, in only 10% of the FSK-treated cells, suggesting similar fusion rates as observed by Kudo *et al.* (2003). The discrepancy between the syncytialisation rates measured by fluorescence imaging and flow cytometry could be explained by the large differences in the number of events that are assessed by the two methods. In fact, while in fluorescence imaging only a few hundreds of cells are usually analysed at best, and the selection can be user-

dependent, in flow cytometry we are in the range of thousands, which could explain a rather over-estimation of fusion rates in experiments that rely solely on microscopy assessments. Comparative measures from toxicology and diagnostic studies in solid tumours however showed overall consistency between these two methods (Jung et al., 2010; Szánthó et al., 2018).

If the proportion of BeWo FSK-treated cells that indeed shifts from a CTB to a STB-phenotype is < 20%, this would pose a major limitation in using this cell model for genome-wide screening, given the rarity of the phenotype of interest – differentiation of the CTB into STB – and the resulting high signal-to-noise ratio. In this scenario, only 20% of the wild type BeWo cells undergoes syncytialisation detectable by monitoring the nuclear markers we propose such as GATA3 downregulation. Therefore, 80% of the cells do not present any detectable changes. By performing the screen in these conditions, mutant clones in which Cas9-sgRNA has mediated a knock-out of syncytialisation genes that have an impaired ability to undergo syncytialisation will have the same phenotype of this unresponsive population of cells (80% of the total sample), rendering the detection of candidate gene enrichment statistically challenging if not impossible. Nevertheless, before drawing final conclusions, it is worth to repeat the flow cytometry experiments we have presented here, testing increasing concentrations of FSK as well as longer exposure times (e.g. 5 days). Alternatively, the use of iPSc could provide an additional avenue for genome-wide screening of trophoblast fusion genes, given the opportunity to specifically differentiate them into trophoblast subtypes and the increasing resources relative to the use of CRISPR/Cas9 in iPSc (Chen et al., 2013; Ihry et al., 2019; Kojima et al., 2017; Li et al., 2019, 2018; Navarro-Guerrero et al., 2021).

Final Discussion and Future Perspectives

The scope of this PhD thesis was to increase the current understanding of placenta physiology at the molecular level, investigating a wide variety of mechanisms. As any other organ, placental function is regulated at different levels: the genetic sequence in the foetal genome that resides within the placental cells will contribute to the overall phenotype, transcriptomic and epigenetic mechanisms of regulation will determine which genes are expressed and at which level, defining the regulatory networks that orchestrate placental functions at any given moment during the nine months of gestation. Finally, being a complex organ, the individual contribution of each cell type will also have a dramatic impact on the overall output of each placental function.

Even though this project is built upon four main axes of research related in the four chapters, it encompasses two main themes: the contribution of genetics and transcriptomics (OMICS) to the placenta in health and disease, and the need to better characterise the syncytiotrophoblast given its pivotal role in placental function.

OMICS in human placenta

« élément sous droit, diffusion non autorisée »

The issue of the syncytiotrophoblast

In the placenta, the STB is responsible for the exchange of nutrients between maternal and foetal circulation, the filtering of metabolic waste products from the foetal blood stream and for the secretion of key hormones that promote foetal growth, expansion of the STB itself, vascularisation and angiogenesis of the placenta (Regnault et al., 2002).

The STB develops early on during placentation through the fusion of mononucleated CTBs, which constitute the pool of trophoblast cells in the tissue and through proliferation ensure self-renewal, while undergoing continuous differentiation to supply the STB with new nuclei. This continuous flux of CTB nuclei into the multinucleated STB allows for expansion of the STB necessary to meet the increasing metabolic needs of the developing foetus, as well as the replacement of aging nuclei (Gude et al., 2004; Gutmacher et al., 2014). Alteration of this balance, often seen in the form of increased CTB proliferation and reduced syncytialisation, results in dysfunctional STB characterised by oxidative stress and release of stress molecules. Morphological and functional alterations in the STB are a feature of pregnancy pathologies such as PE and IUGR (Mariana A Costa, 2016; Huppertz and Kingdom, 2004; Langbein et al., 2008; Roland et al., 2016; Sheridan et al., 2012).

Being such an important as well as peculiar aspect of placental development and function, the process of syncytialisation receives constant research efforts aimed at identifying novel candidate genes. The majority of studies focus on membrane proteins, kinases and phosphatases involved in signalling cascades, and transcription factors (M.A. Costa, 2016; Gerbaud and Pidoux, 2015). Instead, the focus to epigenetic mechanisms of regulation involved in syncytialisation is virtually non-existent, except for a few studies that showed the role of various miRs. The angiogenesis miRNA-17 family, including miR-17, 20a and b, has been found upregulated in preeclamptic placentas, associated with a 40% decrease in syncytialisation of the BeWo cell line, emblematic *in vitro* model for CTB differentiation into STB (Wang and Chen, 2012). miR-92a-1-5p and miR-27b-5p have been found to be strongly downregulated upon syncytialisation of BeWo cells, appearing to be important to retain the CTB identity. High levels of these miRs were found associated with downregulation of STB-specific genes such as hCG, as well as inhibition of syncytialisation rates. This was achieved through the dampening of cAMP-PKA and WNT2B cascades, respectively (Dubey et al., 2018;

Verma et al., 2021). Finally, miR-210 has been defined as master regulator of hypoxia-response genes, and together with HIF-1 α found to be upregulated in preeclamptic placentas, characterised by oxidative stress (Muralimanoharan et al., 2012). Recently it was shown how upregulation of miR-210 and HIF-1 α in CTB inhibits syncytialisation; this process can be reversed by upregulating cytoplasmic polyadenylation element-binding 2 (CPEB2) (Wang et al., 2019)

In 2019, we have reviewed epigenetic mechanisms of regulation in the healthy placenta and in preeclampsia, showing how they play a pivotal role in establishing gene expression patterns. Interestingly, many lncRNA are often found to be deregulated in preeclamptic placentas. Nonetheless, these lncRNA are usually investigated in cell models of EVT, characterised by high proliferative and migratory potential, but not in STB-like cells (Apicella & Ruano et al., 2019). We contributed significantly to this field by clarifying the role of non-coding RNAs confirmed deregulated in PE and IUGR, lncRNA UCA1 and miR-193b which play a significant role in trophoblast syncytialisation. In this study, we analysed transcriptional changes in the BeWo cell line upon FSK administration, associated with trophoblast differentiation towards STB, focusing on ncRNAs (Apicella & Ruano et al., 2021).

In FSK-treated cells compared to control, 800 and 1281 total genes were respectively upregulated and downregulated. Among them, 196 were regulatory ncRNAs, belonging to the classes of lncRNAs, miRs, circularRNAs, small nuclear RNAs and piRNAs. We then investigated their putative targets and relative expression in IUGR and PE placentas in our human placental transcriptomic dataset as well as previously published datasets. miR-193b was identified as key regulatory hub in differentiating BeWo, with 135 predicted targets, of which 108 downregulated in FSK-treated cells. miR-193b is also found upregulated in disease placentas, which supports the hypothesis of deregulation of trophoblast syncytialisation in these pregnancy pathologies. However, the overall effects of this deregulation on syncytialisation rates are not yet clear and further *in vitro* studies are needed.

Our major contribution in this work is relative to the role of UCA1 on syncytialisation. This lncRNA had been previously found upregulated in preeclamptic placentas and its deregulation in EVT model showed its role as negative modulator of proliferation and migratory capabilities of these cells (Liu et al., 2020). Here, we confirmed its upregulation in both PE and IUGR placentas and characterised its function in STB differentiation. Knock-down of UCA1 in FSK-

treated cells deeply altered the transcriptomic profile of these cells, reducing the changes associated with syncytialisation. This suggests that UCA1 is necessary for a successful syncytialisation (Apicella & Ruano et al., 2021). In summary, in physiological conditions, UCA1 upregulation has a negative impact on trophoblast proliferation and migration, challenging CTB differentiation into EVT, while it promotes CTB syncytialisation and it is necessary for transcriptional changes that occur during this process (Apicella & Ruano et al., 2021; Liu et al., 2020). Therefore, the abnormal upregulation of UCA1 in PE and IUGR placentas would suggest a tipping of the balance towards STB formation, rather than CTB self-renewal.

In the literature, contrasting evidence has been found regarding the relative placental composition in CTB and STB in pregnancy pathologies PE and IUGR, and the differences have been reconnected to the severity of the disease phenotype (Roland et al., 2016; Sheridan et al., 2012). More often however, an increased CTB proliferation is observed, resulting in STB morphological abnormalities in PE and IUGR placentas, due to reduced STB renewal and expansion (M.A. Costa, 2016; Huppertz and Kingdom, 2004). This is accompanied by evidence that *in vitro* syncytialisation of primary trophoblasts isolated from PE and IUGR placentas is impaired (Langbein et al., 2008; Pijnenborg et al., 1996; Roland et al., 2016). However, increased CTB proliferation at the expense of the STB, as well as excessive STB formation driven by fusion genes upregulation would equally result in defective STB renewal, leading to an increase in STB senescence and accumulation of syncytial knots. In PE and IUGR placentas, abnormal accumulation of STB apoptotic and senescence markers has been extensively reported and supports the hypothesis of early placental aging being a hallmark of pregnancy pathologies, even though the details on how this outcome is reached are yet to be clarified (Manna et al., 2019; Roland et al., 2016).

In our work, we observe an increase of UCA1 in diseased placentas and we have shown a role of this gene in promoting successful STB differentiation; nonetheless, there is no evidence at this stage that the increased UCA1 levels correspond to an increase in STB formation. To the contrary, this finding could support the notion that, in pregnancy pathologies, syncytialisation is impaired to such a degree that, despite the upregulation of fusion genes such as UCA1, co-existing faulty mechanisms impede CTB differentiation into STB. In fact, pivotal fusion proteins Syncytin-1 and 2 as well as fusion transcription factor GCM1 have been found downregulated in PE placentas, in previous studies as well as in our own dataset (Chen et al.,

2006, 2004; Ruebner et al., 2013; Vargas et al., 2011; Zhuang et al., 2014). Experimental models that investigate the effects of alterations in multiple fusion genes simultaneously could aid in unravelling the overall effects on syncytialisation and self-renewal rates, as well as clarifying the temporal sequence of events.

On our quest to increase our understanding of syncytialisation, we set out to design a genome-wide screen to identify candidate genes with an untargeted approach. We used the CRISPR/Cas9 technology together with an optimised sgRNA library targeting more than 19,000 protein-coding genes in the human genome (Sanson et al., 2018). We have tested a wide variety of experimental approaches in order to optimise the genome-wide screen for trophoblast syncytialisation in the BeWo cell model. However, it is still necessary to find optimal experimental conditions to best distinguish differentiating cells in the FSK-treated BeWo population, and non-competent cells in the same sample. It appears that, depending on the experimental method of detection of differentiating STB, in the BeWo cell model the fusion and differentiation rates alike vary across different studies (Al-Nasiry et al., 2006; Degrelle et al., 2017; Kudo et al., 2003; Omata et al., 2013; Shankar et al., 2015; Shao et al., 2021).

While at the transcriptomic level we could detect deep changes between FSK-treated and control cells, evidenced by our studies in (Apicella & Ruano et al., 2021; Ducat et al., 2020), we failed to see such wide-spread changes at the protein level in the FSK-treated BeWo population, being restricted to only ~10% of cells. So how can we reconcile these findings and justify the use of the BeWo cell model? Changes in the transcript expression levels might be so profound that are detectable even when only a small proportion of the cells experiences them, justifying the use of this cell line when addressing the global impact of genetic perturbations, such as the siRNA-mediated knock-down of UCA1. Instead, if, like in a genome-wide screen, the aim is to assess the impact of mutations in virtually each cell of the population, other *in vitro* approaches might need to be explored, such as iPSc-derived trophoblasts (Chen et al., 2013; Kojima et al., 2017; Levy-Sakin et al., 2019; Li et al., 2018).

Conclusion

In these three years of PhD, under the expert and inspiring supervision of Dr. Daniel Vaiman and Céline Mehats, I have had the chance to explore the different domains of molecular regulation, by looking at the genetics, the transcriptome, the epigenetic mechanisms such as the ones mediated by ncRNAs. I did this in the field of reproductive biology, to which I was a novice, learning of the crucial role of placenta for the health of the foetus, *in utero* and later in its adult life. Even though the placenta is the first organ that the embryo meets and that will in turn meet its nutritional, hormonal, and immune needs for the whole duration of gestation, this organ still remains widely understudied. The elusive mechanisms that link placental health with developmental issues and long-term effects are only beginning to be understood.

It has been of great satisfaction to be able to contribute to the progress of knowledge in this field. Even though we cannot discern the whole picture yet, with this work we have laid a few pieces in the mosaic of placental function and regulation. Hoping that these findings will promote further validations and characterisations, and one day foster better understanding of pregnancy pathologies.

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Annexes

Annex 1

Genome Browser panels relative to Chapter 1 results

eSNPs on chromosome 6 and 11 mapped to regions associated with Histone modification marks relative to enhancer-like activity.

To preliminary investigate the potential mechanisms behind the correlation of cis-eQTLs observed on chromosome 6 and 11 with neighbouring genes, the location of eSNPs in relation to the genes in questions (TOB2P1 and ZSCAN9, LOC646029 and AQP11, CARD17 and CASP1P2) and to annotated histone modification marks was assessed by navigating the UCSC Genome Browser (Kent et al., 2002). As visualisation parameters for the map, the gene expression levels of the displayed genes are relative to the GTEx database, for 54 tissues, the Histone modification mark levels H3K26Ac associated with enhancer activity are from ENCODE database, relative to different cell models: lymphoblastoid cell line GM12878, human embryonic stem cells (H1-hESC), Human Skeletal Muscle Myoblasts (HSMM), Human umbilical vein endothelial cells (HUVEC), erythroleukemia (K562), neonatal Human Epidermal Keratinocyte (NHEK) and neonatal human lung fibroblast (NHLF). Candidate Cis-Regulatory Elements (cCREs) resulting from combination of ENCODE data from the different cell lines are displayed along the tracks as coloured boxes: red= promoter-like signature, orange = proximal enhancer-like, yellow = distal enhancer-like, pink = DNase-H3K4me3 relative to promoter-like features beyond 200bp from Transcription Start Site (TSS) and blue for CTCF binding site (The ENCODE Project Consortium, 2012). Finally, the common SNPs in the regions are annotated from the dbSNP dataset, release 153. The SNPs of interest have been highlighted in yellow, and their relative position along the tracks is defined with a vertical cyan line. The SNPs are colour-coded based on their functional annotations: in red protein-altering variants and splice site variants, synonymous codon variants in green and non-coding transcript or Untranslated Region (UT) variants in blue.

The 24 common eSNPs correlating with TOB2P1 and ZSCAN9 expression levels span a region of over 189kb and for visualisation purposes they have been displayed in three windows for

UCSC Genome Browser (**Figure A1.1, Figure A1.2a**). While many variants are upstream of both genes and do not colocalise with annotated regulatory regions, rs1225618 colocalise with the beginning of ZNF192P1 gene, at the core of a region with annotated high levels of H3K26Ac, with highest peaks corresponding to HUVEC signal. Additionally, this variant colocalises with promoter-like signature region (**Figure A1.1a**). Between TOB2P1 and ZSCAN9 sequences, enhancer H3K26Ac signature is high in K562 and NHEK, marked by multiple putative cCREs as identified from all cell types (ENCODE), rs1726891 colocalises within this region. Interestingly, labelled in blue, rs7206 is annotated as being 3'-UT variant for ZSCAN9 itself. The rest of the variants in this region localises within the promoters of neighbouring genes ZKSCAN4 and NKAPL, rs12000 is annotated as a missense variant (**Figure A1.1b**).

Both genes are expressed at relatively low levels, with average level of fluorescence (LOG₂) 4.55 and 4.59 respectively. In terms of residual variance, NKAPL presented a RV of 0.47 after modelling the expression levels by the 23 covariates; while for ZKSCAN4 this is considerably higher, with a value of 0.74. No ZSCAN4 significant eQTLs were found in the cis-eQTL RV0.7 dataset, but this could be due to the location of the probe that is used to assess global levels of expression of the gene. Further downstream we find the last 9 eSNPs (**Figure A1.2a**), of these, 3 fall within the region encoding for the transposase PiggyBac Transposable Element Derived 1 (PGBD1). This gene is highly expressed in the brain, however it is also found expressed at lower levels in many other human tissues, including the placenta at medium levels (Fagerberg et al., 2014). Rs1150724 overlaps with H3K26Ac marks, close to the promoter region of the gene, in between cCREs associated with putative enhancer function and promoter-like DNase-H3K4me₃ signature. Rs2142730 and rs1997660 are located progressively closer to the end of the gene and rs1997660, labelled in red, is annotated as being a missense variant. In our dataset, PGBD1 mean fluorescence levels (LOG₂) is 6.03, with a residual variance of 0.45, hence it has not been tested for potential correlation with the genotype dataset.

Of the 10 common eSNPs associated with gene expression levels of LOC646029 and AQP11, we can observe the relative location along the genome of 7 variants in a window of approximate 50kb (**Figure A1.2b**). The remaining three variants are located upstream (rs7943742) and downstream (rs10899397, rs12286317) of the window and did not overlap with annotated regulatory features. The first three variants rs7129556, rs10793257 and rs4945185, are located within the AQP11 gene: rs7129556 is close to the beginning of the gene, upstream of annotated

histone mark H3K26Ac peaks, rs10793257 overlaps with cCREs characterised as distal enhancer-like signature. All variants fall within the CLNS1A gene sequence, found on the antisense strand.

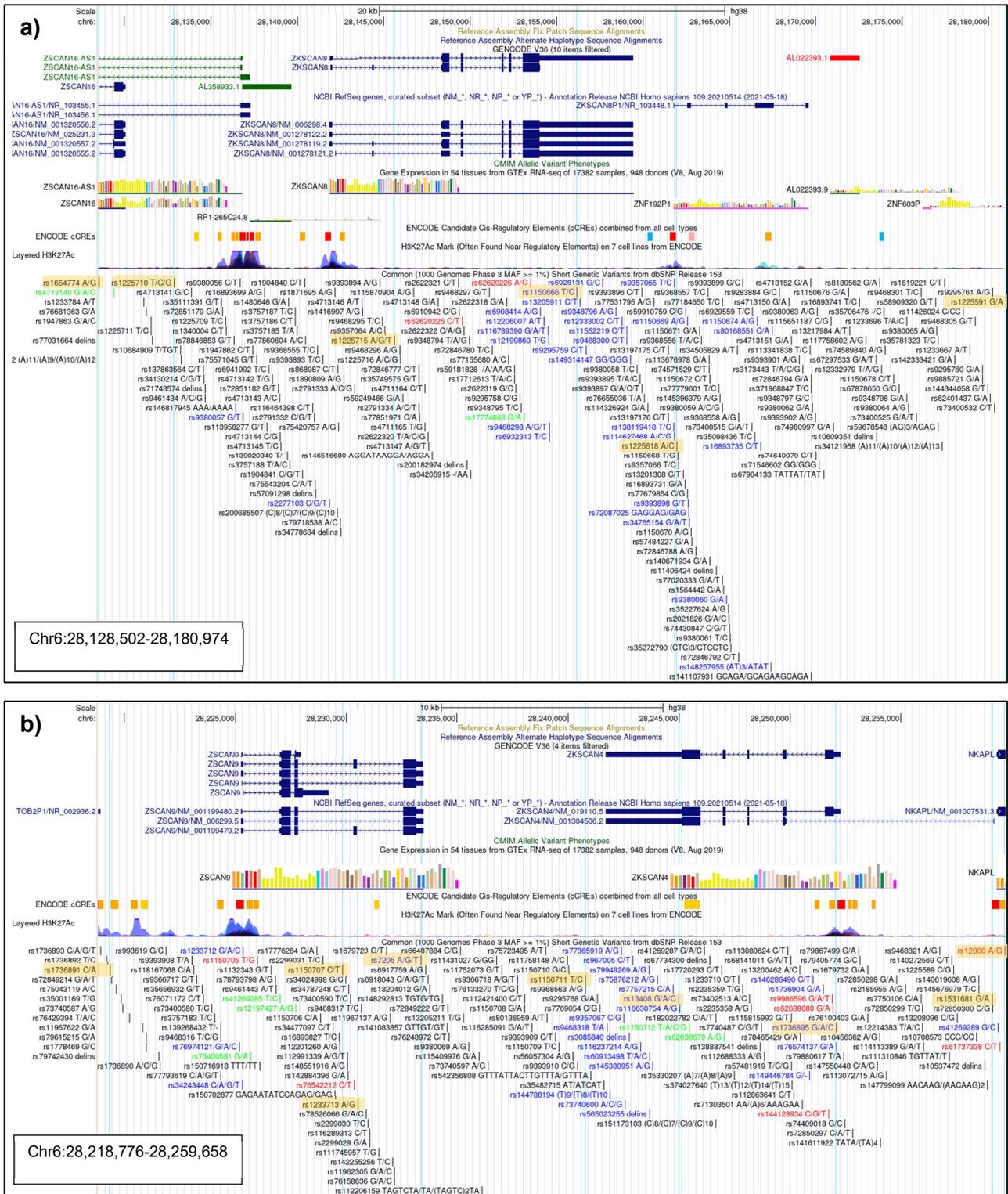


Figure 1. UCSC Genome Browser Panels for eSNPs associated with ZSCAN9 and TOB2P1.

a) Chromosomal coordinates Chr6:28,128,502-28,180,974. b) Chromosomal coordinates Chr6:28,218,776-28,259,658.

LOC646029 is not displayed in the depicted window, it is upstream of AQP11 andl coord would be found scrolling left-wise.

Only 3 eSNPs are in common between CASP1P2 and CARD17, considering that only 3 significant cis-eQTLs were found associated with CARD17. These are located in a window of 55kb that spans both genes. While enhancer-like H3K26Ac histone marks are found between the two genes, markedly from HUVEC, no variants colocalise in the region. Rs562441 and rs7934144 are at the very edge at the displayed window, in regions relatively desert of regulatory signals, however the latter is downstream of an enhancer-like cCRE. Being CARD17 on the antisense strand, both variant and enhancer signature are located upstream of the promoter. The last common eSNP rs1623342, labelled in blue is annotated, as 3'-UT variant for CARD17 itself; this same variant colocalises with strong histone H3K26Ac peaks in NHEK.

Annex 2

The Role of Epigenetics in Placental Development and the Etiology of Preeclampsia

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Review

The Role of Epigenetics in Placental Development and the Etiology of Preeclampsia

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Abstract: In this review, we comprehensively present the function of epigenetic regulations in normal placental development as well as in a prominent disease of placental origin, preeclampsia (PE). We describe current progress concerning the impact of DNA methylation, non-coding RNA (with a special emphasis on long non-coding RNA (lncRNA) and microRNA (miRNA)) and more marginally histone post-translational modifications, in the processes leading to normal and abnormal placental function. We also explore the potential use of epigenetic marks circulating in the maternal blood flow as putative biomarkers able to prognosticate the onset of PE, as well as classifying it according to its severity. The correlation between epigenetic marks and impacts on gene expression is systematically evaluated for the different epigenetic marks analyzed.

Keywords: preeclampsia; epigenetics; DNA methylation; non coding RNAs; miRNAs; histone post translational modifications; HOX genes; H19; miR-210

1. Introduction

PE affects ~2–5% of the pregnancies. This disease, characterized in the classical definition by hypertension and proteinuria, surging from the mid-gestation at the earliest, is often seen as a two-stage disease, where a placental dysfunction occurs, first without observable symptoms and is followed later by a symptomatic phase from the 20th week of gestation at the earliest. The placenta is central to the disease development [1]. During pregnancy, the cytotrophoblasts (CTs) invade and remodel the structure of the spiral arteries of the myometrium [2]. These changes cause a significant increase in blood flow to the placenta. In a classical vision of the disease etiology, it is said that deep invasion is deficient in preeclampsia [3]. It is generally acknowledged that in preeclamptic pregnancies, placentation is disrupted because the CTs fail to properly invade the myometrium and transform the spiral arteries [4]. This decreases the blood flow and alters the oxygenation of the placenta (causing hypoxia and hyperoxia events), triggering oxidative stress, necrosis and inflammation [5]. In a very stimulating paper, B. Huppertz challenges this classical understanding of PE etiology, by dissociating the defect of deep trophoblast invasion from preeclampsia but rather associating this defect with the Fetal Growth Restriction (FGR) phenotype [6]. In this vision, preeclampsia would rather be caused by a combination of *villous* trophoblast defects (which are not involved in invasion, contrary to *extravillous* trophoblast) and maternal susceptibility. He based his reasoning on the fact that invasion defects are actually not histologically visible in many cases of preeclampsia. This may be connected to mouse models of preeclampsia where no obvious fetal growth restriction occurs, consistently with the fact

that invasion is not important in rodent [7]. More accepted than this vision, the same paper strengthens the idea that hyperoxia rather than hypoxia is a major actor of the disease [6,8].

The preeclamptic placenta releases vasoactive molecules, pro-inflammatory cytokines, microparticles and syncytial fragments into the maternal circulation which ultimately cause a systemic endothelial dysfunction [9]. Epigenetics plays an important role in the regulation of the development and physiology of the placenta [10]. Besides, substantial epigenetic alterations, in the preeclamptic placenta and other affected tissues have been described and are likely playing a substantial role in the evolution of the disease [11–14].

2. Epigenetics and Normal Placental Development

2.1. Description of the Placenta and Placental Cells

The placenta is a temporary organ connecting the developing fetus to the uterine wall through the umbilical cord, to allow for nutrient absorption, thermal regulation, waste disposal and gas exchange via the mother's blood supply. In addition, the placenta produces hormones that support pregnancy and it acts as a barrier to fight against internal infection [15].

The human placenta at term has a discoid shape, an average diameter of 15–20 cm, a thickness of 2.5 cm in the center and a weight of about 500 g. Its surfaces are the chorionic plate on the fetus side and to which the umbilical cord is attached and the basal plate facing the maternal endometrium. Between the endometrium and the basal plate there is a cavity filled with maternal blood, the intervillous space, into which branched chorionic villi project. The chorionic villi are the structural and functional unit of the placenta. Their core is made of fibroblasts, mesenchymal cells, endothelial cells, immune cells such as Hofbauer cells (supposed to be macrophage-like) and fetal-placental vessels. The villi are covered by two layers of trophoblasts. The inner layer is composed of villous cytotrophoblasts (vCTs), which are highly proliferative and can differentiate into either outer layer villous syncytiotrophoblasts (SCT), which are in direct contact with the maternal blood or extravillous trophoblasts (EVTs), as shown in Figure 1.

2.2. Human Placental Development

The development of the human placenta has been described in detail elsewhere [16–18]. Briefly, the blastocyst implants into the uterine endometrium (decidua) via the trophoblast cells adjacent to the inner cell mass (ICM). From the trophoblast, the syncytium (SCT) emerges and spreads. Subsequently, CTs proliferate rapidly to form large finger-like projections (villi) that penetrate the entire depth of the SCT. Ultimately, the villi become filled with mesenchyme originated from the extraembryonic mesoderm. This mesenchyme will form fetal blood vessels which connect to the fetal circulation via the umbilical cord. The intervillous space subsequently becomes filled with maternal blood. The vCTs situated at the tips of the anchoring villi proliferate and stratify, forming highly compact cell columns breached only by channels carrying maternal blood toward and away from the placenta (Figure 1). The trophoblast cells within this structure are referred to as EVT, according to their external location relative to the chorionic villi. EVT situated close to the decidua, stop proliferating and develop invasive properties. These invasive EVT migrate deeply into the decidua, where they transform the uterine vasculature in order to supply the placenta maternal blood, a critical step in establishing uteroplacental circulation. As pregnancy progresses, the number of vCTs decreases and few is observable at term underneath the SCT.

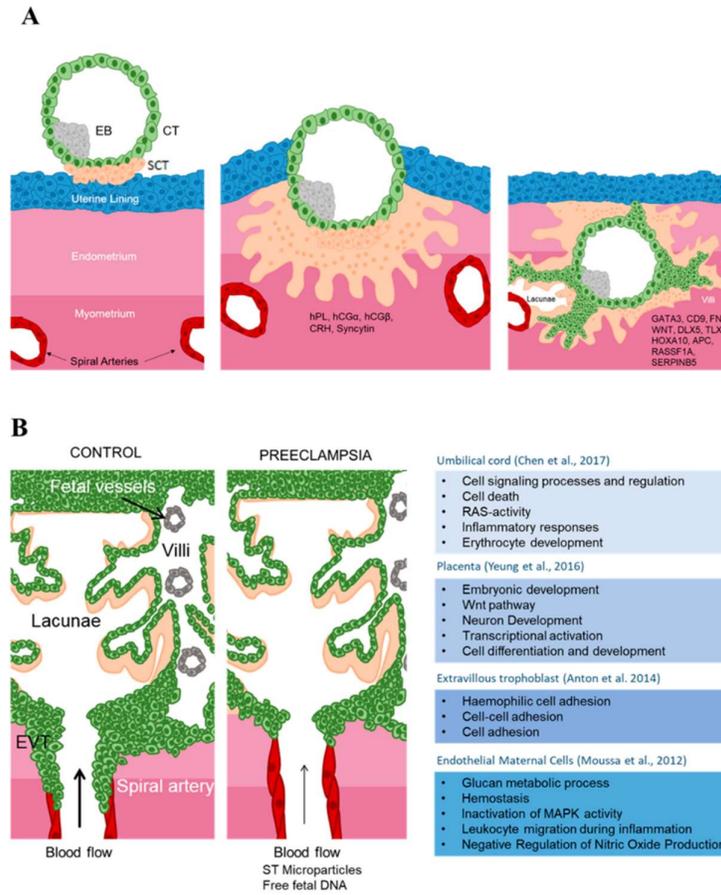


Figure 1. (A) Blastocyst implantation and Placenta Development: After recognizing the uterine lining, the blastocyst is formed by the embryoblast (EB) and the cytotrophoblast (CT). The cytotrophoblast starts to differentiate into Syncytiotrophoblast (SCT). SCT invades the endometrium towards the maternal spiral arteries located in the myometrium. deregulation of numerous genes is observed [19]. Lacunae develop in the syncytiotrophoblast, which will eventually constitute the intervillous space. Genes upregulated during villi formation are presented on the right figure [20]. Other cytotrophoblasts will invade the maternal spiral arteries by differentiating into Extravillous trophoblast. (B) Gene Ontology of genes differentially methylated in PE compared to control samples: (Left) in normal pregnancies, extravillous trophoblast (EVT) invades the maternal spiral arteries allowing for an increased blood stream towards the extravillous space. Nutrients cross the placenta, are directed towards the embryonic vessels and collected in the umbilical cord. In PE, decreased invasion of the EVTs induces poor spiral artery remodeling, leading to poor blood flow towards the placenta. Increased amount of microparticles from the syncytiotrophoblast and increased amount of free fetal DNA is observed in the maternal blood. (Right) Gene ontology of differentially methylated genes found in PE samples in different tissues affected during pregnancy: Umbilical cord, placenta, EVT, Endothelial Maternal cells (see text for detail).

2.3. Epigenetics Mechanisms in Placental Development

Epigenetic mechanisms are involved in the regulation of gene expression both during development and in differentiated tissues [21,22]. These mechanisms include DNA methylation, histone modifications and biogenesis and action of noncoding RNAs (ncRNAs). They regulate gene expression by modulating the accessibility to DNA of transcription factors and other regulatory proteins. In addition, ncRNAs also regulate gene expression at a post-transcriptional level. Epigenetic mechanisms are essential for cellular differentiation and therefore development, as summarized in Table 1

Table 1. Epigenetic mechanisms in placental development.

Epigenetic Mechanism	Target	Cell Type	Biological Relevance	Reference
H3K9/27me3	MMP-2, MMP-9	Human placenta	Related to trophoblasts motility and invasion	[23]
H3K4 acetylation + H3K9 methylation	Maspin	Human placenta	Negatively correlated with human trophoblasts motility and invasion	[24,25]
Acetylated H3	Pregnancy-Specific Glycoproteins	JEG-3	Inhibition of HDACs in JEG-3 cells up-regulated PSG protein and mRNA expression levels	[26]
HDAC3	GCMa	Cell Line	HDAC3 associates with the proximal GCMa-binding site (pGBS) in the syncytin promoter and inhibits its expression	[27]
Acetylation of H2A and H2B		Murine TSCs	Decreases the EMT and invasiveness of murine TSCs while maintaining their stemness phenotype	[28]
H3K4Me2; H4K20me3	Genome Wide	SCTs	H3K4Me2 co-localizes with active RNAP II in the majority of STB nuclei	[29]
H3K27me3	Genome Wide	vCT	H3K27me3 highly represented in vCT	[30]
lncRNA TUG1	RND3	HTR-8/SVneo, JEG-3	TUG1 epigenetically silences RND3 transcription by interacting with EZH2 involved in cellular proliferation, migration and invasion in trophoblasts	[31]
lncRNA RPAIN	C1q	HTR8/SVneo	Inhibition of proliferation and invasion. Inhibits C1q expression	[32]
lncRNA MALAT1		JEG-3	Regulates proliferation, migration, invasion and apoptosis	[33]
lncRNA MEG3		HTR8/SVneo and JEG-3	Regulates migration and apoptosis	[34]
lncRNA MIR503HG		JEG-3	Regulates migration and invasion	[35]
lncRNA LINC00629		JEG-3	Regulates migration and invasion	[35]
lncRNA SPRY4-IT1	HuR	HTR8/SVneo	Regulates migration and apoptosis/interferes with the β -catenin Wnt signaling	[36,37]
lncRNA H19	Binds small RNAs and proteins	vCT, JAR	Regulates proliferation and apoptosis	[38]
miR-141-3p and miR-200a-3p	Transthyretin (TTR)	syncytialized BeWo	Inhibits TTR expression by directly binding to the 3'UTR of TTR. Regulate thyroxin uptake by the SCT	[39]
miR-34	Plasminogen activator inhibitor-1 (PAI-1), SERPINA3	JAR	Regulates invasion	[40,41]
miR-155	Cyclin D1	HTR-8/SVneo	attenuates trophoblast proliferation	[42]
miR-17_92, miR-106a_363, miR-106b_25, miR-675	GCM1		attenuate differentiation of trophoblasts	[43]
C19MC miR cluster	NOMOL, Igf1R	JEG3 cells HTR8/SVneo	restricts trophoblast proliferation impaired migration	[44] [45]
methylation of gene body	DAXX	Human placenta	Loss of methylation during both vCT syncytialization to SCT and EVTs differentiation to invasive EVTs	[46]
methylation of gene promoter	APC	Human placenta and choriocarcinoma cells	trophoblast invasiveness	[47]
hypomethylated promoter	MASPIN	Human placenta	inhibits EVTs migration and invasion	[24,25,48]
Hypermethylated promoter	RASSF1A	Human placenta; JAR; JEG3	Possible role in cytotrophoblast development through its effects on ID2	[49]
Genome wide methylation	PMDs (Partially Methylated Domains)	human placenta: Chorionic Villi	genes involved in immune response, Epithelial-mesenchymal transition and inflammation	[50–52]
Genome wide methylation	Genome Wide	human SCTs compared to vCTs	hypomethylated SCTs compared to vCTS	[53]
Genome wide methylation	Genome Wide	BeWo and BeWo + Forskolin	DNA methylation status of numerous genes regulated at the expression level were altered by forskolin-induced fusion	[54]
Methylation	HOX genes: TLX1, HOXA10, DLX5	Human placenta	Increased methylation across gestation correlates with decreased expression. Involved in SCTs differentiation	[46]
Genome wide methylation	Genome Wide	Side-population trophoblasts, vCTs and EVTs	Each cell population has a distinctive methylome	[55,56]
Methylation	Cdx2; Eomes; Plct1; Tfap2c	Mice trophoblast stem cells (TSCs)	methylation regulates the expression of genes involved in the establishment of the TSCs	[57–59]
Methylation	Genome Wide	Blastocyst	hypomethylation of the trophoctoderm compared to the inner cell mass	[60]

2.3.1. DNA Methylation

The best studied epigenetic mechanism in the placenta is DNA methylation, the covalent addition of a methyl group to a cytosine, usually in the context of cytosine-phospho-guanine (CpG) dinucleotides. Several reviews have been dedicated to the role of this mechanism in placental development [10,12,61]. Also, several high-throughput analyses have been performed to analyze the methylation epigenetics of the developing placenta (Table 2, Supplementary Table S1 for the details).

Table 2. Summary of DNA methylation studies in developing placenta using genome-wide approaches.

Sample	Method	GEO ID	Findings	Reference
First-trimester and term placenta and maternal blood	Illumina HM450		2944 hypermethylated CpG sites in the first and 5218 in third trimester placenta.	[62]
First-trimester placenta and maternal blood	MeDIP-Seq and Illumina HM450		3759 CpG sites in 2188 regions were differentially methylated	[63]
Placenta (first, second and third trimester)	Illumina HM450 and MethylC-Seq & RNA-Seq	GSE39777	Identification of partially methylated domains (PMDs) and differences between placenta and other tissues	[51]
Placenta (first, second and third trimester)	Illumina HM27		Increase in overall genome methylation observed from first to third trimester.	[64]
Term placenta	MeDIP + custom microarray		Tissue-specific differentially methylated regions in the placenta	[65]
Various human trophoblast populations	Illumina HiSeq 2000	GSE109682	Human trophoblasts are different from somatic cells in terms of global CpG methylation	[56]
Methylation profiles of E18.5 term placenta of WT and Hltf ^{-/-} mouse	Illumina HiSeq 2000 (Mus musculus)	GSE114145	Hltf-gene deletion alters the epigenetic landscape of the placenta.	[66]
Fetal placental tissue of both sexes in GR ^{+/+} vs. GR ^{+/-} mice	Illumina HiSeq 2000	GSE123188	GR mutation in mice changes the epigenome of placental tissue in a sex-specific manner	[67]
Human placentas	Illumina HM450	GSE108567	Adjusting for batch effects in DNA methylation	[68]
Epigenetic mechanism of mouse embryo development	Illumina HiSeq 2500 (Mus musculus)	GSE104243	H3K27me3 and DNA methylation in extraembryonic and embryonic lineages	[69]
Samples from different normal human tissues	Illumina HM450	GSE103413	Identifying candidate imprinted genes	Database, unpublished
Bisulphite and oxidative bisulphite converted placental DNA	Illumina HM450	GSE93429	Hydroxymethylcytosine and methylcytosine profiles in the human placenta	[70]
Methylation in first and third trimester placental samples	Illumina Genome Analyzer Iix	GSE98752	Complex Association between DNA Methylation and Gene Expression	[71]
DNA Methylation in Human Fetal Tissues and Human IPSC	Illumina HM450	GSE76641	DNA methylation and transcriptional trajectories in human development.	[72]
DNA methylation of fetal membranes, trophoblasts and villi 2nd trimester	Illumina HM450	GSE98938	Genome-scale fluctuations in the cytotrophoblast epigenome	Database, unpublished
Developing mouse placenta	Illumina HiSeq 2000	GSE84350	DNA Methylation Divergence and Tissue Specialization in the Developing Mouse Placenta	[73]
Villous cytotrophoblasts samples	Illumina HM450	GSE93208	DNA methylation profiling of first trimester villous cytotrophoblasts	[52]
Placental tissue collected at term.	Illumina HM450	GSE71719	DNA methylation and hydroxymethylation assessment.	[74]
DNA from chorionic villus from the 1st trimester and maternal blood cell samples	Illumina HiSeq 2000 (Homo sapiens)	CSE58826	DNA Methylation Predictors of Gene Expression in the 1st Trimester Chorionic Villus	Database, unpublished
Methylation patterns of human placenta, blood neutrophils and somatic tissue	Illumina HiSeq 2000 (Homo sapiens)	GSE59988	The human placenta exhibits a dichotomized DNA methylation pattern compared to somatic tissues	[75]
mRNA and DNA methylation profiling of Dnmt3a/3b-null trophoblasts	Illumina HiSeq 2000 (Mus musculus)	CSE66049	Maternal DNA methylation in early trophoblast development	[76]
Imprinted differentially methylated regions in hu-man villous trophoblast and blood samples	Illumina MiSeq (Homo sapiens)	CSE76273	Polymorphic imprinted methylation in the human placenta	[77]
Placental villous explant culture in different growth conditions	Illumina HM450	GSE60885	Genome-wide DNA methylation identifies trophoblast invasion-related genes.	[78]
Trophoblast methylation in NLRP7 knockdown	Illumina HM450	CSE45727	NLRP7 alters CpG methylation	[79]
Bisulphite converted DNA	HumanMethylation27 Illumina BeadChip	CSE36829	Epigenome analysis of placenta samples from newborns	Database, unpublished
First trimester, second trimester and full-term placentas	HumanMethylation27 Illumina BeadChip	GSE31781	Widespread changes in promoter methylation profile in human placentas.	[80]
Chorionic villus and maternal blood cell samples	HumanMethylation27 Illumina BeadChip	GSE23311	DNA Methylation Analysis in Human Chorionic Villus and Maternal Blood Cells	[81]

Differentiation of Stem Cells

Contrary to mice, a Trophoblast Stem Cell (TSC) population has not yet been clearly identified in humans, thus limiting our capacity to study the role of DNA methylation in the early stages of trophoblast differentiation. A recent study has addressed this question using a side-population trophoblasts, a candidate human TSC [55], isolated from first trimester placenta. The comparison of

the methylomes of this side-population trophoblasts and the methylomes of vCTs and EVT_s all isolated from the same first trimester placenta, showed that each population had a distinctive methylome [56]. In comparison to mature vCT_s, side-population trophoblasts, showed differential methylation of genes and miRNAs involved in cell cycle regulation, differentiation and regulation of pluripotency. In addition, the comparison of the methylomes and transcriptomes of vCT_s and EVT_s revealed the methylation of genes involved in epithelial-mesenchymal transition (EMT) and metastatic cancer pathways, which could be involved in the acquisition of the invasive capacities of the EVT_s. However, this study, as many others, failed to establish a systematic correlation between hypermethylation of the genes and downregulated expression. Therefore, the authors conclude that although CpG methylation is involved in the trophoblasts differentiation, it cannot be the only regulatory process.

Regulation of Homeotic Genes

Several studies have identified and established the importance of the transcription factors of the homeobox gene family (HOX) in the development of human placenta [82–86]. Most HOX genes have been found stably hypo-methylated throughout gestation, suggesting that DNA methylation is not the primary mechanism involved in regulating HOX genes expression in the placenta. However, these genes show variable methylation patterns across gestation, with a general trend towards an increase in methylation over gestation. Three genes (*TLX1*, *HOXA10* and *DLX5*) present slightly increased methylation while their mRNA expression decreases throughout pregnancy, supporting a role for DNA methylation in their regulation [46]. Down-regulation of these genes using siRNAs specific for *DLX5*, *HOXA10* and *TLX1* in primary trophoblasts leads to loss of proliferation and to an increase in mRNA expression of differentiation markers, such as *ERVW-1*. This suggests that loss of these proteins is required for proper SCT development [46].

Placental Development and Cancer Pathways

The early steps of placentation are reminiscent of the invasive properties of malignant tumors. Studies on DNA methylation in cancer cells and placental cells have highlighted similarities in their epigenomes, particularly, a widespread hypomethylation throughout the genome and focal hypermethylation at CpG islands. Hypomethylation within the placenta is not uniform but occurs in large domains (>100 kb) called partially methylated domains (PMDs) which are regions of reduced DNA methylation that cover approximately 40% of the placental genome [51]. PMDs are unique to a few different tissue types that include the placenta, cultured and cancer cells [50,51,87]. Placental genes within PMDs tend to be tissue-specific and show higher promoter DNA methylation and reduced expression as compared with somatic tissues [51]. A genome-wide comparison of DNA methylation changes in placental tissues during pregnancy and in 13 types of tumor tissues during neoplastic transformation revealed that megabase-scale patterns of hypomethylation distinguish first from third trimester chorionic villi in the placenta [52]. These patterns mirror those that distinguish many tumors from the corresponding normal tissues. The genomic regions affected by this hypomethylation encompass genes involved in pathways related to EMT, immune response and inflammation, all of them associated to cancer phenotypes. Moreover, the authors observed that hypomethylated blocks distinguish vCT_s before 8–10 weeks of gestation and after 12–14 weeks of gestation. The analogy between early placentation and malignant tumors at the epigenetic level is further stressed by studies analyzing the methylation status of the promoters of several tumor suppressor genes (*RASSF1A*, *SERPINB5* also known as APC and *Maspin*, respectively) in the developing placenta and human choriocarcinoma cell lines (JAR and JEG3) [25,49]. These studies show that promoter DNA-methylation regulates the expression of these tumor suppressor genes which in turn affects the migration and invasive capacities of the trophoblastic cells (As summarized in Table 1).

2.3.2. Non-coding RNAs and Epigenetic Regulation of Placenta Development

Definition

A non-coding RNA (ncRNA) is defined as an RNA molecule that is not translated into a protein. Classes of non-coding RNAs include transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), small RNAs such as microRNAs (miRNAs), siRNAs, piRNAs, snoRNAs, snRNAs, exRNAs, scaRNAs and the long ncRNAs [88]. The role of these molecules in placental development, physiology and pathology has been recently reviewed in detail [89]. Here we will discuss solely the role of miRNAs and long ncRNAs in the epigenetic control of placental development.

MiRNA and Normal Human Placental Development

The miRNAs are single stranded RNA molecules of 19–24 nucleotides, which act primarily by degrading mRNA transcripts or inhibiting translation of miRNA in to proteins [90]. To date, more than 2000 human miRNAs have been discovered, which appear to regulate 50% of human RNAs [91]. A large number of miRNAs detected in the placenta are expressed from a gene cluster located on chromosome 19 (C19MC) [92,93]. This cluster includes 46 intronic miRNA genes that express 58 miRNA species. These miRNAs are primate-specific, and they are expressed almost exclusively in the placenta (and are thus termed trophomiRs). In the human placenta, the expression of C19MC miRNAs is detected as early as 5 weeks of pregnancy and the expression gradually increases as pregnancy progresses [94]. An imprinted, paternally expressed, CpG-rich domain has a regulatory role in C19MC expression [95]. This DMR, is hypermethylated in cell lines that do not express C19MCs [96]. The C19MC region contains genomic transposable elements called “Alu repeats”, which have been implicated in recombination and gene duplication events. Because of their sequence complementarity it has been proposed that several C19MC miRNAs could be responsible of the targeting and degradation of transcribed Alu elements. Also, the C19MC miRNAs are expressed in embryonic and in stem cells but their expression drops considerably when these cells differentiate, which may indicate a role in the maintenance of an undifferentiated state [97–101]. Several members of the C19MC cluster are expressed at much higher levels in vCT compared with EVT and overexpression of the C19MC cluster results in reduced migration of the extravillous trophoblast line HTR8/SVneo [45]. The chromosome 14 miRNA cluster (C14MC) is another miRNA cluster that is expressed in the placenta [102]. This cluster includes the miRNAs: miR-127, miR-345, miR-370, miR-431 and miR-665. These miRNAs have been involved in the regulation of the immune suppressive, anti-inflammatory response and also in the regulation of the ischemia/hypoxia response [103]. The expression of the C14MC members generally declines during pregnancy [104].

The miR-675 is expressed from the first exon of the H19 long non-coding RNA. Up-regulation of miR-675, which is controlled by the stress-response RNA-binding protein HuR, restricts murine placental growth. Deficiency of H19, promotes placental growth and miR-675 overexpression decreases cell proliferation, likely through targeting Igf1R [105]. Consistent with these findings, the expression of miR-675 rises toward the end of murine pregnancy, when placental growth decelerates. In addition, miR-675 restricts proliferation in JEG3 cells, likely through binding to the nodal modulator 1 (NOMO1) protein [44].

Several other miRNAs are likely involved in placental development by inhibiting genes associated to regulation of trophoblast fate, invasion and proliferation (Let-7a, miR-377, miR-145, members of the miR-17_92 cluster, members of the miR-106a_363 and miR-106b_25 clusters, miR-155, miR-34, miR-141-3p and miR-200a-3p) [106,107]. As additional examples of regulation, miR-431 inhibits invasion of trophoblast cells by targeting the ZEB1 gene [108], miR-106a~303 inhibits trophoblast differentiation by targeting hCYP19A1 and hGCM1 [43], miR-34 targets SERPINA3, a key gene in a variety of biological processes and highly deregulated in placental diseases [41].

These miRNAs regulate diverse processes such as trophoblast physiology, proliferation and invasion (some mentioned in Table 1 and reviewed in Reference [107]).

lncRNA and Normal Human Placental Development

Long non-coding RNAs (lncRNAs) are RNAs greater than 200 nucleotides in length that do not encode a protein product. They are expressed with cellular and temporal specificity and have been involved in many cellular events, including the regulation of gene expression, post-transcriptional modifications and epigenetic modifications, imprinting and X-chromosome inactivation [109]. They act as scaffolds (binding other RNAs or proteins), signals and antisense decoys and engage in transcriptional interference. Usually a single lncRNA has multiple functions. The function of lncRNAs in placental development is poorly understood, mostly inferred from studies on placental pathologies. Nevertheless, lncRNAs have been involved in a number of critical trophoblast functions, from proliferation, invasion and migration, to cell cycle progression [110]. H19 was one of the first lncRNAs to be discovered [111]. H19 is located within a large imprinted domain on chromosome 11, at ~100 kb downstream of IGF2. H19 and IGF2 are reciprocally imprinted that is, for H19 only the maternal allele is expressed, while for IGF2, only the paternal allele is expressed [112]. H19 expression could be regulated by PLAGL1, a zinc finger transcription factor, in the human placenta [113]. Two major functions have been described for H19, specifically as a modulator for binding small RNAs and proteins [114] and as a source of the miRNA mir-675 (see above). H19 has variable levels of biallelic expression in the placenta (reports suggest between 9% and 25% expression occurs from the imprinted allele) until 10 weeks of gestation by which time H19 expression is mostly restricted to the maternal allele [115]. H19 expression is restricted to intermediate and vCT and is not found within SCTs in the human placenta. H19 down-regulation in trophoblast cells leads to inhibition of proliferation and apoptosis [116]. Many other lncRNAs have been involved in placental development, including lincRNA SPRY4-IT1, MIR503HG, LINC00629, MEG3, MALAT1, RPAIN and TUG1 [31–37]. The study of the expression of these lncRNAs during placental development and the manipulation of their expression *in vitro* in choriocarcinoma cell made it possible to infer their possible function in the context of placental development (Table 1).

2.3.3. Histone Modifications in the Developing Placenta

Histone modification is the process of modification of histone proteins by enzymes, including post-translational modifications, such as methylation, acetylation, phosphorylation and ubiquitination. Histone modifications participate in gene expression regulation by modulating the degree of chromatin compaction [117].

Our knowledge concerning the role of histones modification in human placentation is scarce and refers mostly to studies in mice. Methylation frequently occurs on histones H3 and H4 on specific lysine (K) and arginine (A) residues. Histone lysine methylation can lead to activation or to inhibition, depending on the position in which it is located. For instance, H3K9, H3K27 and H4K20 are considered as important ‘inactivation’ markers, that is, repressive marks, because of the relationship between these methylations and heterochromatin formation. However, the methylation of H3K4 and H3K36 are considered to be ‘activation’ marks [118,119].

The heterochromatin methylation marker H3K27me3 was found to be highly active in vCT. That was explained by rapid and transient repression of genes at the time of SCT formation. SCTs nuclei were also found enriched for H4K20me3 [30]. However, this report contrasted with another study reporting that the CTs were enriched with H3K4me3 and that the SCTs were transcriptionally activated by the chromatin marker H3K4me2, which co-localized with active RNAP II in the majority of SCT nuclei [29]. In mouse and other mammals, H3 arginine methylation predisposes blastomeres to contribute to the pluripotent cells of the ICM, which appears to require higher global levels of H3 arginine methylation than the TE/trophoblast lineage [120]. Nevertheless, these lower modification levels in the trophoblast lineage are indispensable for normal placental development.

Acetylation, which in most cases occurs in the N-terminal conserved lysine residues, is also an important way to modify the histone proteins, for example, acetylations of lysine residues 9 and 14 of histone H3 and of lysines 5, 8, 12 and 16 of histone H4 by Histone Acetylases (HATs). Acetylation is generally associated with the activation or opening of the chromatin. On the contrary,

de-acetylation of the lysine residues by histone deacetylases (HDACs) leads to chromatin condensation and inactivation of gene transcription. Oxygen (O₂) concentrations strongly influence placental development partially through modifications of the histone methylation codes. Initially, the gestation environment is hypoxic and O₂ concentration increases during development. Hypoxia-inducible factor-1 (HIF-1), consisting of HIF-1 α and ARNT subunits, activates many genes involved in the cellular response to O₂ deprivation [121]. HIF-1 is also known to recruit and regulate HDACs [122,123]. Moreover, HIF-1 has been found to bind specific sites on the promoter of the H3K9 demethylases thereby inducing their expression. In particular, it induces JMJD1A and JMJD2A that remove dimethyl marks on H3K9me₂, JMJD2B [124,125] which removes trimethyl marks (H3K9me₃) and more weakly JMJD2C which converts H3K9me₃ to me₂ [126]. Studies in rodents have shown that HIFs have important roles in the regulation of TSCs differentiation by integrating physiological, transcriptional and epigenetic inputs. Thus, the crosstalk between HIF and the HDACs is required for normal trophoblast differentiation [123,127].

Another example of histone modification during placentation, is the acetylation of histones H2A and H2B by the CREB-binding protein (CBP). CBP acts as an acetyltransferase that decreases the EMT and invasiveness of murine TSCs while maintaining the properties of stem cells [28].

Trophoblastic fusion depends on the regulation of GCMA activity by HATs and HDACs. Human GCMA transcription factor regulates expression of syncytin, which in turn mediates trophoblastic fusion. It has been demonstrated that CBP-mediated GCMA acetylation underlies the activated cAMP/PKA signaling pathway that stimulates trophoblastic fusion [27]. Human pregnancy-specific glycoproteins (PSG) are the major secreted placental proteins expressed by the SCTs and represent early markers of cytotrophoblast differentiation. Pharmacological inhibition of HDACs in JEG-3 cells up-regulated PSG protein and mRNA expression levels. This correlated with an increase in the amount of acetylated histone H3 associated with PSG promoter [26]. Combined acetylation at H3K9 and H3K4 methylation also activates Maspin, a tumor suppressor gene which is negatively correlated with human trophoblasts motility and invasion [24,25]. The invasive capacity exhibited by EVT is attributed in part to the extracellular matrix degradation mediated by matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9. Differential expression of these MMPs and their tissue inhibitors (TIMPs) has been associated to histone H3K9/27me₃ [23].

2.3.4. Imprinting and Placental Development

Placentation and the Materno-Fetal Conflict

Pregnancy in Eutherian mammals is an immunological challenge as reviewed recently [128]. To note, an ancestral inflammatory response in pregnancy and parturition also exist in marsupials (metatherians), as recently observed [129,130]. Other mechanisms are equally conserved in the formation of the placenta, in particular the fusion mechanisms of cytotrophoblasts into syncytiotrophoblasts that are mediated by retroviruses, in eutherians as well as in metatherians [131].

Once the placenta is formed, it will allow nutrients to transit from the mother circulation to the fetal circulation. In the context of the maternal-fetal conflict hypothesis, tightly regulating the placentation process and limiting placental growth is crucial for the mother survival. The genes controlling this regulation are expected to be found different between viviparous and non-viviparous species. For this, mammals appear as an excellent model as a group of ~4500 species divided into egg-laying animals (prototherians, Platypus and Echidnae, 5 species), animals with a short-lived placenta (metatherians, Marsupials ~250 species) and viviparous species with a long-lived placenta (eutherians, i.e., all the other mammals, where gestation length can be up to 22 months in the African elephant). One major difference found between the genome of placental species and non-placental species of mammals is the presence of imprinted genes only in the first group.

Definition of Imprinted Genes and Links with Viviparity

Imprinted genes are genes that are expressed from either the maternal or the paternal allele, mainly through differentially methylation mechanisms. Their existence leads to dramatic phenotypic differences in animal hybrids according to the sense of the cross. For instance, interbreeding of lions and tigers results in two morphologically different animals, if the male is the lion or the male is the tiger, leading to a liger or a tigon, respectively [132]. While the tigon has a size like that of its parents, the liger is the largest existing felid (up to >400 kg) and several hypotheses have been raised to explain this fact, mostly connected to the existence of imprinted genes. Experimentally, in the 80s, Solter and Surani carried out nuclear transfer experiments that demonstrated in mice the necessity of a paternal and maternal genome to foster healthy development [133]. Androgenetic embryos lead to the production of a hypertrophic placenta while gynogenetic embryos had a very small placenta and a stunted embryo. Similarly, in humans, development from two paternal genomes leads to hydatiform moles, where the placenta is composed of grapelike vesicles, whereas parthenogenic development leads to the apparition of teratomas [134].

As far as we know today, imprinting is closely associated to viviparity. The sequencing of the platypus genome in 2008 [135] revealed syntenic regions that are relatively well conserved with the eutherian and marsupials, albeit no evidence of imprinted gene can be found in Monotremes. This may be since acquisition of imprinting in a species seems to be associated to the progressive acquisition of CpG islands (besides other mechanisms, such as chromosome translocations or retrotransposons insertions), that appear absent from the platypus genome [136,137]. In marsupials (metatherians), where the placenta is short-lived, the number of imprinted genes is more limited than in eutherian mammals. Two imprinted regions are well conserved between metatherians and eutherians such as the PEG10 and the H19-IGF2 regions [135]. Similarly, an exhaustive analysis of the transcriptome of chicken failed to identify imprinted genes, while allele specific expression does exist [138,139]. The evidence collected therefore strongly links these genes with the placenta presence. Besides, imprinted genes may have a strictly paternal or strictly maternal expression. Series of inactivation experiments in mice indicated that paternal genes tend to increase placental growth while maternal genes tend to limit this growth [140].

Example of the H19-IGF2 Cluster; Cross Species Conservation of Imprinted Genes

A well-known example of this is the H19-IGF2 cluster localized distally at 11p15.5 in humans and 7qF5 in mice. In both species, the structure of the locus is conserved (about 100 kilobases separating the two genes, with differentially methylated regions inside IGF2 and nearby H19). An IMC (Imprinting Control Region), located 3 kb from the starting point of H19 has also been identified, with seven binding sites for the ZNF transcription factor CTCF. H19 is expressed exclusively from the maternal allele, while IGF2 is expressed from the paternal allele. In mice, a placental specific promoter of Igf2 was discovered. The selective inactivation of this promoter [141] leads to a strong decrease of placental development and placental growth. By contrast, the inactivation of H19, leads to placental and fetal overgrowth [142]. Amongst other imprinted genes that affect placental and fetal growth besides H19 and IGF2 are paternally expressed genes, generally identified in mice (Peg1, Peg3, Rasgrf1, Dlk1) and maternally expressed genes (Igf2r, Gnas, Cdkn1c, Grb10).

Interestingly, in mice, the decoy receptor of Igf2, Igf2r is imprinted and with a maternal profile of expression. In humans, surprisingly, the imprinting status of IGF2R seem to be erratic, polymorphically imprinted according to the human individual analyzed. This was first published in 1993 [143] that showed that 2 out of 14 fetuses had an exclusive expression from the maternal allele. Recently it was shown that IGF2R is duly imprinted in macaques [144], showing that even in primates, the imprinting status can vary between relatively close species. Overall, it appears that many placental imprinted mouse genes are biallelic in their expression in humans [145]. Reciprocally, in a study aiming at identifying novel imprinted genes in the human placentas, we compared variants of the placental DNA versus those of cDNAs from the same placentas using SNP microarrays [146,147]. In addition to

four known imprinted genes (IPW, GRB10, INPP5F and ZNF597), we could identify 8 novel imprinted genes in the human placentas (ZFAT, ZFAT-AS, GLIS3, NTM, MAGI2, ZC3H12C, LIN28b and DSCAM). Using a mouse cross allowing the following of the allelic origin, we found an astonishing variegation of the imprinting status: only Magi2 was imprinted in the mouse species.

Imprinted genes may have a general impact on the global methylation status of the placenta. For instance, recently a polymorphism located at the IGF2/H19 locus was shown associated to placental DNA methylation and birth weight in association with Assisted Reproductive Technologies usage [148].

Imprinted genes deregulation in the placenta is linked to placental diseases, as reviewed in References [149,150]. In a recent study, Christians and coworkers, analyzed a list of 120 imprinted genes in relation with global expression of 117 placental samples, including PE and Intra Uterine Growth Restriction (IUGR) cases [151]. The authors identified a significant correlation between birth weight and the expression level of imprinted genes but without significant differences between paternally versus maternally expressed genes. Imprinted genes were also more heavily deregulated in preeclampsia than other genes and in this case paternally expressed genes were down-regulated, while maternally expressed genes were up-regulated. The trend was similar for IUGR. Interestingly, the two human-specific microRNA clusters (C19MC and C14MC), both appear to be imprinted (paternally and maternally expressed) for C19MC and C14MC, respectively, clusters that have been duly studied by the team of Yoel Sadovsky [45,89,152]. Recently, we identified duplication in the 19q13.42 imprinted region encompassing the C19MC cluster [153], from a male 26 weeks fetus with severe IUGR, suggesting that a double dose of the miRNA could contribute to the disease. This suggests links between miRNA regulation, imprinting status and the putative consequences for fetal health and growth.

3. Epigenetic Alterations in Preeclampsia

3.1. DNA Methylation Alterations in Preeclampsia

Anomalies of DNA methylation in preeclampsia have been analyzed from different cellular sources. Besides the analysis of placental cells, investigators have analyzed circulating maternal blood cells or cell-free DNA, as well as maternal endothelial cells (much less accessible, though) and cord-blood white blood cells (of fetal origin). A list of genes of which methylation was found altered is presented as Table 3.

A summary of epigenetic mechanisms at work in PE is shown in Figure 2.

3.1.1. Methylation Alterations in the Preeclamptic Placenta

Common Alterations of Gene Expression in PE are Associated to Methylation Alterations

Numerous studies revealed altered expression of various genes in the pathological placentas (as synthesized previously [154]). These alterations of gene expression are partly explained by the existence of epigenetic deregulations. In PE, numerous methylation deregulations have been found in the pathological compared to control placentas, some studies (but not all) taking into account the gestational age, a recurrent issue when normal and pathological placentas are compared, for which there is often a more than 6 weeks difference [155–160]. The different techniques used to analyze methylation globally are presented in a previous review [161]. These epigenetic changes probably originate from the abnormal placental environment in PE (or IUGR), characterized by alternations of low oxygen tension and hyperoxia. As mentioned above, hypoxia *per se* induces the expression of the Hypoxia-Inducible factor (HIF1 α), which binds to Hypoxia Responsive Element activating the transcription of various genes related with angiogenesis and metastasis-associated genes [162]. Overall, abnormal oxygen signaling in the placental context leads to increased concentrations of Oxygen Reactive Species (ROS) [163]. Oxidative stress may drive an accelerated ageing of trophoblast cells, which could be key to understand the origin of placental disorders. Indeed, several studies

emphasized alterations of telomere length (a mark of ageing) in preeclamptic pregnancies, with a drastic augmentation of short telomeres in PE, especially in Early Onset PE (EOPE) [164–166]. This senescence may be induced by alterations of the management of oxidative stress [167–169]. The accelerated transformation of vCTs into SCTs will lead to a decrease life expectancy of the placenta and an alteration of its capacity to bring the gestation harmoniously to its normal term.

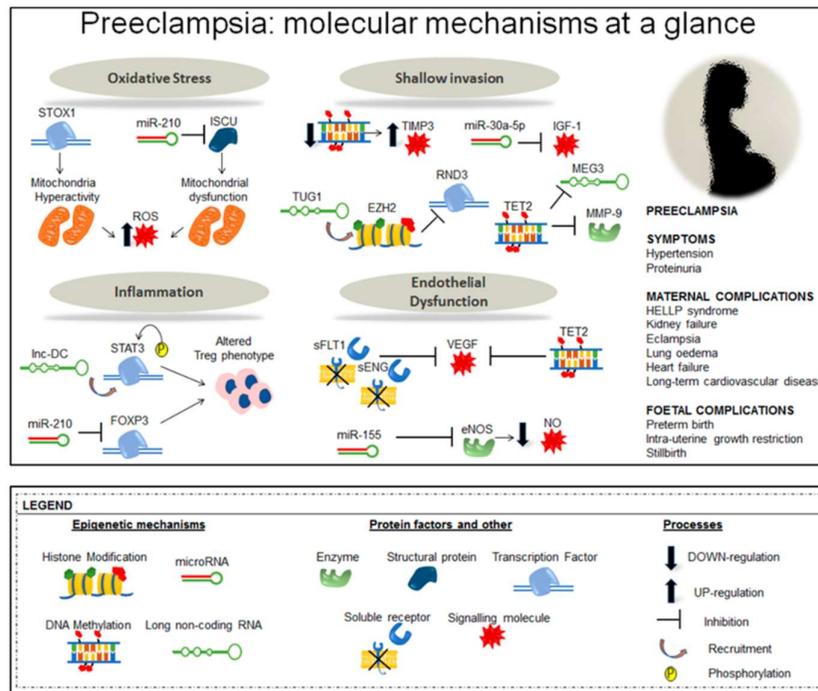


Figure 2. Overview of the molecular mechanisms at play in preeclampsia. Annotations: eNOS = Endothelial Nitric Oxide Synthase; EZH2 = Enhancer of Zeste Homolog 2; FOXp3 = Forkhead box P3; IGF-1 = Insuline-like Growth Factor 1; ISCU = Iron-sulfur cluster; Lnc-DC = Long non-coding RNA DC; miR-30a-5p = microRNA 30a-5p; miR-155 = micro-RNA 155; miR-210 = microRNA 210; MMP-9 = *Matrix Metalloproteinase-9*; NO = Nitric Oxide; RND3 = Rho Family GTPase 3; ROS = Reactive Oxygen Species; sFLT1 = Soluble fms-like tyrosine kinase *receptor-1*; STAT3 = Signal transducer and activator of transcription 3; STOX1 = Storkhead Box 1; TET2 = Tet methylcytosine dioxygenase 2; TIMP3 = TIMP Metallopeptidase Inhibitor 3; TUG1 = long non-coding RNA taurine-upregulated gene 1; VEGF = Vascular Endothelial Growth Factor.

It is well known that persisting environmental variations induce changes in the epigenetic marks, including DNA methylation. These marks can either be mere biomarkers or participate actively in regulating genes to overcome the changing environmental conditions (although gene expression changes are often disconnected from methylation alterations).

Overall, several of the genome-wide studies showed that the methylation profiles differ between early and late onset of preeclampsia (EOPE and LOPE), suggesting a different etiology between these two types of PE [170–173]. EOPE shows more pronounced genome-wide hypermethylation changes

than LOPE, probably since it is caused by earlier alterations allowing the epigenetic reprogramming to install earlier, in reason of the earlier cellular stress [171,174].

Using the Illumina Methylation 450 BeadChip Array, Yeung and coworkers, identified 303 differentially methylated regions in PE, 214 hyper and 89 hypomethylated, after adjusting for gestational age. The genes located nearby or encompassing hypermethylated regions were enriched in gene-ontology (GO) terms such as “ATP transport”, in KEGG pathways, such as “steroid hormone biosynthesis”, “cellular senescence” and Reactome pathways, such as “Vpr-mediated induction of apoptosis by mitochondrial outer membrane (SLC25A6 and SLC25A4)”. The annotation of clusters also revealed an alteration of clusters of homeobox genes, (especially HOXD genes), Wnt2 cell signaling; fertilization and implantation genes; reactive oxygen species signaling (NOX5) and cell adhesion (ALCAM) genes [158]. Amongst the most recent studies, Leavey and coworkers used a novel approach based upon bioinformatics to sort 48 human PE samples through their transcriptome profile before subjecting them to methylation analysis, using the Illumina Human methylation450K array. This made it possible to divide the preeclamptic cases into two groups associated to abnormal methylation marks nearby ‘immunological’ genes or more ‘canonical’ EOPE cluster, with for instance abnormally methylated CpG in FLNB, COL17A1, INHBA, SH3PXD2A, as well as in the gene body of FLT1 [160].

In 2015, the study of Zhu and coworkers [175] was the first to analyze simultaneously methylation and hydroxymethylation in the PE placentas. Hydroxymethylation results from the hydroxylation of methyl-Cytosine is a first step towards the active demethylation of DNA through the action of Ten+Eleven Translocation enzyme (TET) proteins, and could play an important role in gene expression regulation [176]. The authors showed that the methylation level is higher in gene promoters and gene bodies in PE versus control placenta. Surprisingly most of the clustering of the genes that were altered, either by methylation or by hydroxymethylation were associated with nervous system development, neurotransmitters, neurogenesis, which are presumably not relevant in a non-neural tissue as the placenta. Nevertheless, positive regulation of vasoconstriction was also enriched as a GO term, as well as regulation of nitrogen compounds, two pathways that have a clear biological sense in terms of placental diseases pathophysiology (association with vascularization and with the modulation of oxidative/nitrosative stresses).

Table 3. Differentially methylated genes in preeclampsia.

Cell Type	Gene	Methylation State in PE	Possible Target	Reference
Placenta and maternal plasma	<i>SERPINB5</i>	Hypomethylated	Trophoblast Invasion	[177]
First-trimester maternal white blood cell and placenta samples	<i>ABCA1</i>	Hypomethylated	Cholesterol transporter in macrophages	[178,179]
First-trimester maternal white blood cell, placenta samples, umbilical cord blood	<i>GNAS</i>	Hypomethylated	Diabetes, hypertension and metabolic diseases	[178,179]
First-trimester maternal white blood cell and placenta samples	<i>TAPBP</i>	Hypomethylated	Peptide loading in the Histocompatibility complex	[178]
First-trimester maternal white blood cell and placenta samples	<i>DYNLL1</i>	Hypomethylated	Phosphate metabolic processing	[178]
First-trimester maternal white blood cell and placenta samples	<i>ORPD1</i>	Hypomethylated	Opioid Receptor	[178]
Placenta	<i>TIMP3</i>	Hypomethylated	Metalloprotease Inhibitor	[180]
Placenta	<i>P2RX4</i>	Hypomethylated	Apoptosis and Inflammation	[170]
Placenta	<i>PAPPA2</i>	Hypomethylated	Insuline-like growth factor regulator	[170]
Placenta	<i>DLX5</i>	Hypomethylated	Trophoblast proliferation and differentiation	[181]
Placenta	<i>KRT15</i>	Hypomethylated	Cytoskeleton	[182]
Placenta	<i>SERPINA3</i>	Hypomethylated	Inhibition of inflammation, pathogen degradation and tissue remodeling	[183]
Placenta	<i>FNI</i>	Hypomethylated	Cell adhesion, trophoblast proliferation, differentiation and apoptosis	[182]
Placenta	<i>TEAD3</i>	Hypomethylated	Cell homeostasis, Inflammation, Coagulation, complement activation	[184]
Placenta	<i>JUNB</i>	Hypomethylated	TNF signaling pathway	[182]
Placenta	<i>PKM2</i>	Hypomethylated	Cellular metabolism	[182]
Placenta	<i>NDRG1</i>	Hypomethylated	Trophoblast invasion	[182]

Table 3. Cont.

Cell Type	Gene	Methylation State in PE	Possible Target	Reference
Placenta	<i>BHLHE40</i>	Hypomethylated	Inhibition of trophoblast differentiation	[171]
Placenta	<i>INHBA</i>	Hypomethylated	Inhibition of trophoblast differentiation	[171]
Placenta	<i>CYP11A1</i>	Hypomethylated	Trophoblast autophagy and steroidogenic pathway	[184]
Placenta	<i>HSD3B1</i>	Hypomethylated	Steroidogenic pathway	[184]
Placenta	<i>TEAD3</i>	Hypomethylated	Steroidogenic pathway	[184]
Placenta	<i>CYP19</i>	Hypomethylated	Steroidogenic pathway	[184]
Placenta	<i>CRH</i>	Hypomethylated	Cortisol bioavailability in the placenta	[184]
Placenta	<i>TFPI-2</i>	Hypomethylated	Block in endothelial dysfunction	[185]
Placenta	<i>VEGF</i>	Hypomethylated	Angiogenesis	[186]
Umbilical cord blood, placenta samples	<i>IGF2</i>	Hypomethylated	Embryonic development and fetal growth	[179,187]
Placenta and Peripheral Blood	<i>GNAI2</i>	Hypomethylated	Blood pressure	[188]
Placenta	<i>CAPC</i>	Hypomethylated	Macrophage function	[189]
Placenta	<i>GLI2</i>	Hypomethylated	Embryo development	[189]
Placenta	<i>KRT13</i>	Hypomethylated	Cytoskeleton	[189]
Placenta	<i>LEP</i>	Hypomethylated	Cell homeostasis and metabolism	[190]
Placenta	<i>LP1</i>	Hypomethylated	Lipid metabolism	[191]
Placenta	<i>CEBPα</i>	Hypomethylated	Transcription stimulation of LEP promoter	[191]
Placenta	<i>SH3PXD2A</i>	Hypomethylated	Trophoblast invasion and podosome formation	[191]
Placenta	<i>NCAM1</i>	Hypomethylated	Trophoblast-trophoblast interactions and adhesion	[174]
Cord blood samples	<i>HSD11B2</i>	Hypomethylated	Cortisol transmission from the mother to the fetus	[192]
Placenta	<i>WNT2</i>	Hypermethylated	Placental and cell signaling	[158,193]
Placenta	<i>SPESP1</i>	Hypermethylated	Fertilization	[158]
Placenta	<i>NOX5</i>	Hypermethylated	Reactive Oxygen Species signaling	[158]
Placenta	<i>ALCAM</i>	Hypermethylated	Cell Adhesion	[158]
Placenta	<i>IGF-1</i>	Hypermethylated	Placental, trophoblast function, fetal growth.	[194]
Placenta	<i>SOX7</i>	Hypermethylated	Embryonic development and cell fate	[155]
Placenta	<i>CDX1</i>	Hypermethylated	Trophoblast invasion restriction	[155]
Placenta	<i>CXCL1</i>	Hypermethylated	Chemokine inducer of angiogenesis	[155]
Placenta	<i>ADORA2B</i>	Hypermethylated	Placenta impairment and fetal growth restriction	[155]
Placenta	<i>FAM3B</i>	Hypermethylated	Cytokine activity	[182]
Placenta	<i>SYNE1</i>	Hypermethylated	Nuclear organization and structural integrity	[182]
Placenta	<i>AGAP1</i>	Hypermethylated	Cellular development, assembly and function	[182]
Placenta	<i>CRHBP</i>	Hypermethylated	Cortisol bioavailability in the placenta	[190]
Placenta and maternal blood	<i>STAT5A</i>	Hypermethylated	Transcription activation	[195]
Placenta and maternal plasma	<i>RASSF1A</i>	Hypermethylated	Tumor suppressor gene	[177]
Placenta	<i>PTPRN2</i>	Hypermethylated	Phosphate metabolic processing	[173]
Placenta	<i>GATA4</i>	Hypermethylated	Placenta Growth	[173]
Placenta	<i>YWHAQ</i>	Hypermethylated	Cellular response to reduce oxygen levels	[196]
Placenta	<i>TNF</i>	Hypermethylated	MMP-9 stimulation, Immune system activation, cell survival, migration and differentiation	[174]
Placenta	<i>COL5A1</i>	Hypermethylated	Extracellular matrix	[174]
Placenta	<i>CDH11</i>	Hypermethylated	Trophoblast anchoring to the decidua, syncytiotrophoblast differentiation	[174]
Placenta	<i>HLA-G</i>	Hypermethylated	Maternal Immune tolerance and immune rejection	[197]

The major modifications of methylation occurring in preeclampsia are presented as Figure 3.

Limits of the Genome-Wide, Multicellular Approach for Preeclampsia Methylation Profiling

As mentioned earlier, a recurrent criticism of genome-wide comparisons between normal and PE placenta is linked to the fact that in general placental samples of PE patients are collected at earlier terms than controls. However, the existence of methylation profiles for control placentas throughout gestation [51] now allows to make the part between the effect of the placental ageing and the effects of the pathology per se. Other limits of these approaches are the complexity of the cell material, the variation between the degree of severity of the disease or the various statistical tests that are used in the different studies. Also, several studies have brought attention to the lack of reproducibility in high-throughput genomic, transcriptomic and epigenomic studies. This has been recently discussed by Komwar and coworkers in a recent study where they analyze the sources of variation in preeclampsia high-throughput studies and propose a methodology to ensure reproducibility and thus facilitate the integration of data across studies [198].

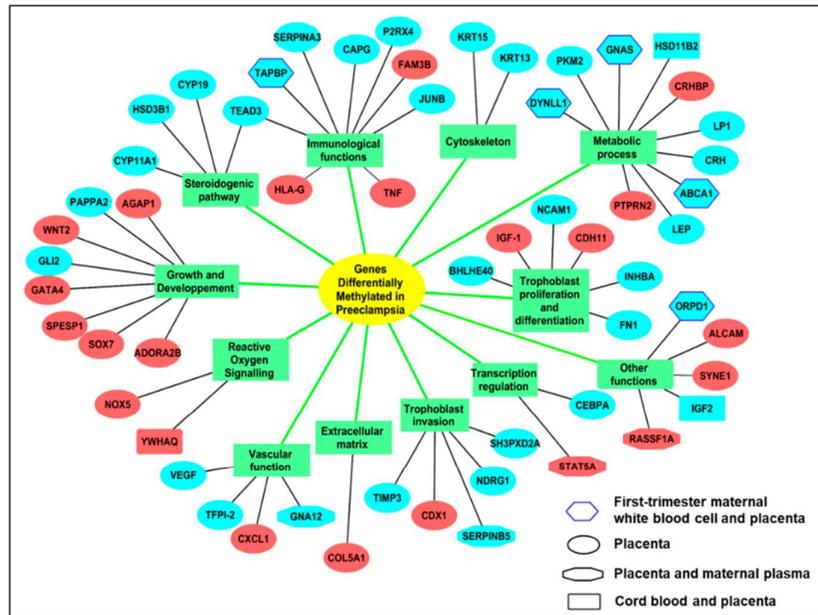


Figure 3. Overview of major methylation alterations in preeclampsia. The main pathways are shown in green boxes. The significant alterations in methylation may be associated either to increased or decreased gene expression (hypermethylated in red and hypomethylated in blue).

Single-Cell Analysis, the Next Frontier to Methylation Epigenomic Approaches

Gene-scale expression studies were recently carried out at the single cell scale [199] and have provided evidence of gene expression shifts during the CT, SCT and EVT differentiation steps, as well as, allowed the reconstructions of differentiation trajectories. This has also been analyzed by genome-scale DNA methylation analysis. Gamage and coworkers have analyzed by RRBS side population trophoblasts, CTs and EVTs from human first trimester placentas [56]. Forty-one genes involved in EMT and metastatic cancer pathways were found methylated between CT and EVTs, possibly contributing to the invasive phenotype of these cells. In the BeWo cell model where fusion can be induced by forskolin, RRBS analysis performed before and after fusion, showed altered methylation of genes involved in cell differentiation and commitment, together with a gain in transcriptionally active histone marks such as H3K4me3 [54]. Such approaches are for the moment difficult to transpose to placenta pathophysiology. Instead, several systems where methylation influences normal placental function have been studied. As an example, we present below the epigenetic regulation of genes involved in placental invasion and PE.

An Example of Specific Gene Alterations of Methylation: Regulation of Invasion

MMPs are well-characterized proteins involved in trophoblast invasion and angiogenesis during pregnancy. They constitute a family of 23 Zn²⁺ and Ca²⁺-dependent proteases that degrade the extracellular matrix. This family of proteins presents abnormal concentration and behavior in placental diseases such as PE [200], placenta accreta and placenta percreta [201,202]. This has been recently reviewed for preeclampsia [203]. A decreased level of MMP-2 and MMP-9 reduces the remodeling of

spiral arteries in early gestation. Besides, other MMPs, such as MMP-1 and MMP-14, may also have a role in this disease. Epigenetic mechanisms are at work for controlling MMP gene expression.

Li and coworkers observed that TET2 is involved in the demethylation of the *MMP-9* promoter, this being associated to the downregulation of the protein and contributing to trophoblast shallow invasion [204].

TIMP3, a MMP inhibitor, shows the highest methylation reduction (over 15%) in EOPE compared to control placentas with an inverse correlation between methylation level and gene expression suggesting an increased transcription of TIMP3 in PE placentas [180,189]. Low levels of TIMP3 lead to poor invasion of the trophoblast and placenta hypoperfusion. Moreover, TIMP3 may be able to inhibit angiogenesis by blocking vascular endothelial growth factor binding to its receptor contributing to impaired placenta blood vessels development. Also, genetic variations of the gene have been associated with cardiovascular disorders and hypertension.

3.1.2. Maternal Blood Epigenetic Marks in Preeclampsia

Alterations in the levels of many plasma and serum proteins have been associated with PE. In 2013, White and coworkers showed that PE was favoring hypermethylation in white blood maternal cells using the methylation-27k arrays from Illumina [205]. *GRIN2b*, *GABRA1*, *PCDHB7* and *BEX1* were found differentially methylated, with an enrichment of the neuropeptide signaling pathway. The re-analysis of methylation of genes known to be involved in PE revealed that in maternal circulating leukocytes, CpG sites from 4 genes associated with PE, *POMC*, *AGT*, *CALCA* and *DDAH1*, showed differential methylation in PE compared to control, with moderate methylation differences (<6%) [206]. These 4 genes are known to alter immunomodulation and inflammatory response, suggesting that at least alterations of the placental physiology in preeclampsia have epigenomic consequences on maternal circulating cells.

During pregnancy, 3 to 6% of cell-free DNA in the maternal blood plasma is derived from the placenta. Oxidative stress in PE leads to increased trophoblast apoptosis and the release of SCT microparticles and a five to ten-fold increase in circulating fetal DNA in the maternal bloodstream compared with control counterparts [207,208]. These free fetal molecules and their methylation status have been proposed as a non-invasive biomarker of fetal and placental pathologies before the onset of symptoms. This has been shown for *Maspin*, for which the unmethylated version has a median methylation more than 5.7 fold higher in PE than control pregnancies [209,210]. Another epigenetic marker of preeclampsia is the methylation of *RASSF1A* (Ras Association domain-containing protein 1) promoter [177,211,212].

3.1.3. Maternal Endothelial Cells

There is limited access to maternal vessels in pregnancy, nevertheless DNA methylation was assessed from this material in 2012 using the 27K methylation array of Illumina [213]. From 14,495 genes interrogated by the array, 65 genes were identified as hypomethylated in PE. Clustering leads to identify biological processes such as smooth muscle contraction, thrombosis, inflammation, redox homeostasis, sugar metabolism and amino acid metabolism. These alterations of the maternal endothelium suggest potential effects on cardiovascular life of the mother after preeclampsia. Focusing on collagen metabolism, the authors revealed an increased expression of *MMP1* and *MMP8* in vascular smooth muscle cells and infiltrating neutrophils of omental arteries of preeclamptic women, which was associated with reduced methylation in the promoters of both genes in pathological patients compared to control patients [213]. In the same study, several other MMPs, showed reduced hypomethylation in PE patients albeit with lower significance [214,215]. Moreover, pregnant women under dietary supplementation may restore the reduced methylation in the promoters of these genes and be protected against the development of PE. Interestingly, all these MMPs genes are located in chromosome 11, which may be indicative of a specific sensitivity of this chromosome to epigenetic changes caused by oxidative stress during the development of the pathology. The same team reported

the reduced methylation in the promoter region of *TBXAS1* gene in correlation with increased gene and protein expression of thromboxane synthase in vascular smooth muscle, endothelium and infiltrating neutrophils [215]. Increased levels of thromboxane synthase induce the overproduction of thromboxane A₂, a potent vasoconstrictor and platelet activator, contributing to hypertension and coagulation abnormalities classically related to PE.

3.1.4. Cord Blood Cells

In 2014, Nomura and coworkers analyzed the global methylation profile of cord blood cells using the LUMA technique [161] and failed to observe an actual difference but with a limited number of controls samples (5) [216]. Genome-Wide Methylation analysis using the 450K microarray tool on neonatal cord blood DNA showed a significant genome-scale hypomethylation in neonatal cord blood DNA associated with EOPE, with 51,486 hypomethylated and 12,563 hypermethylated CpGs [187]. In this study the most differential methylated genes were associated with inflammatory pathways, cholesterol and lipid metabolism, including *IL12B*, *FAS*, *PIK31* and *IGF1*. Deregulation of both metabolic pathways may increase the risk of cardiovascular diseases in the fetus [187]. The same microarray approach allowed to identify 5001 mostly hypermethylated regions in umbilical cord white blood cells and 869 mostly hypomethylated regions in the placenta [217]. In the cord blood cells, the gene networks enriched were involved in cardiovascular system development, cell cycle, cancer, cell morphology, infectious diseases, suggesting specific alterations that could have long-term consequences on the fetal health.

Some studies focused on mitochondrial DNA, showing hypomethylation in PE cord blood cells. The most affected loci are keys in mitochondria functionality: D-loop (control of mitochondrial DNA replication), Cytochrome C oxidase subunit 1 gene (respiratory chain) and *TF/RNR1* locus (necessary for protein synthesis) [218]. Increased copy of mitochondria is observed in the placenta and maternal blood during PE suggesting an adaptive response to stress [219,220]. This is also observed in mouse models of PE [221]. Hypomethylation in the D-loop may lead to increased mitochondrial replication explaining the pathological increase of mitochondrial DNA. Methylation assay in endothelial colony-forming cells present in cord blood from PE presents differential methylation level in genes related to RNA metabolic processes, cellular protein modification processes and in positive regulation transcription, as assessed with the EPIC Illumina array, interrogating over 850,000 CpG [222]. However, at later passages, an increased number of genes are abnormally methylated. This suggests that preeclampsia may drive an altered epigenetic program in endothelial cell precursors that will be the building bricks of the newborn vascular system and program later complications.

3.2. Non Coding RNAs

Non-coding RNAs have been found to be differentially expressed in preeclampsia by a number of sources. Some studies have focused on investigating differential expression patterns between PE placental samples of different severities versus control groups looking for miRNAs or lncRNA [223–226], without generally identifying consensual signatures. With the aim of identifying potential biomarkers that could be used diagnostically to predict preeclampsia onset, many groups have set out instead to identify molecules differentially expressed in the plasma of patients, which could potentially be detected by mean of a simple blood test [227]. lncRNA and miRNA are the two classes of non-coding RNAs that have dominated the scene of non-coding molecules in preeclampsia. Other classes of non-coding RNAs have been identified, such as circular RNAs, that appeared recently in the context of PE development and future research will help understand the role of these molecules in the regulation of gene expression and disease [228].

3.2.1. LncRNAs in Preeclampsia

Long non-coding RNAs are RNA molecules longer than 200 nucleotides which are involved in regulation of cell function through a wide range of mechanisms. lncRNAs are expressed in the nucleus

as single stranded RNA molecules, which can either function in their native form or undergo maturation through the addition of a 5' cap and polyA tail; however, they are never translated into a protein product [229]. They regulate cell function by a wide range of mechanisms: alteration of the stability of target mRNAs, direct recruitment of chromatin modification enzymes, segregation of transcription factors through specific binding sites contained within the lncRNA sequence, warehousing miRNA as 'miRNA sponges', a function shared with circular RNAs [230]. For a complete overview please see Reference [231].

Transcriptomic analyses of placenta and decidua total RNAs allowed identifying differentially expressed lncRNAs between PE and control patients, often with a difference between EOPE and LOPE [228,232,233]. Most of these lncRNAs had been previously identified in the field of cancer research, often associated with cell proliferation, migration and invasion [234]. As mentioned above, given the parallels between the features of cancer cells and the trophoblasts during placentation such as fast proliferation of the trophoblast, migration and invasion of the maternal tissues, immunotolerance [235–238], this did not come as a surprise and has prompted extensive in vitro research to elucidate the roles of these lncRNAs in trophoblast physiology.

In the present review, we will focus on a few lncRNAs that have been well characterized: MALAT-1, MEG3, RNA-ATB. Finally, we will give a brief overview on how in PE some lncRNAs regulate gene expression by altering chromatin methylation state of their target genes, through direct recruitment of histone methyltransferases, bringing as examples PVT1, TUG1 and DIAPH2-AS1. H19 was discussed above for its important role in placental development and miRNA encoding lncRNA.

MALAT-1

Metastasis associated lung adenocarcinoma transcript-1 (MALAT-1) was firstly identified in lung cancer; it is a lncRNA of over 8 kb [239]. MALAT-1 normally localizes in the nucleus where it forms nuclear aggregates called speckles involved in the regulation of splicing factors availability [240]. MALAT-1 is overexpressed in placental pathologies associated with uncontrolled trophoblast invasion [241], which prompted Chen and coworkers [33] to investigate its expression in PE. Comparing RNA levels in 18 PE placentas with matched controls, MALAT-1 was found significantly downregulated in PE placentas. Overexpression and downregulation of MALAT-1 in JEG-3 regulates cell proliferation and invasion, while inhibiting apoptosis [33]. These findings suggest that MALAT-1 deregulation could lead to poor invasion of the maternal endometrium, affecting the spiral arteries' remodeling and placenta development. Li and coworkers [242] have shown that the role of MALAT-1 is not restricted solely to the trophoblast but has a key role in regulating the angiogenesis and vascularization of the maternal decidua and fetal umbilical vasculature. MALAT-1 is expressed by mesenchymal stem cells (MSCs) in the maternal decidua and in the umbilical cord. These cells are pluripotent progenitors which are able of self-renewal and proliferation, differentiate to promote tissue regeneration, form de novo vasculature, angiogenesis and regulate immune system responses [243]. Li and coworkers (2017) observed a decreased MALAT-1 expression in MSCs from decidua and umbilical cord of preeclamptic pregnancies and set out to investigate its function in these cells. Similarly, MALAT-1 promotes proliferation and protects from apoptosis in isolated MSCs. Interestingly, coculture of MSCs with trophoblast cell line HTR-8/SVneo clearly showed how MALAT-1 overexpression could promote migration and invasion of the trophoblasts towards the MSCs layer. Coculture of the endothelial cell line HUVECs (Human Umbilical Vein Endothelial Cells) in supernatant obtained from MSCs which either expressed or had downregulated MALAT-1 showed how MALAT-1 promotes tube formation this process being dependent on Vascular Endothelial Growth Factor secretion. Finally, MALAT-1 over-expression increased the levels of the IDO protein, which activated macrophage maturation, proving its role in immune system regulation. These findings combined with the work of Chen and coworkers (2015) beautifully illustrates how MALAT-1 has a symmetric regulatory function in placentation: on the one hand, it promotes trophoblast proliferation, invasive and migratory potential and on the other hand, its expression in MSCs cells helps to attract and promote trophoblast

invasion, stimulates tube formation, promotes angiogenesis and vascularization. Increase in Reactive Oxygen Species caused MALAT-1 and VEGF downregulation in MSCc exposed to oxidative stress in a dose-dependent manner [242]. MALAT-1 downregulation in preeclampsia could therefore have a huge impact on placentation and further development of the placenta over the course of gestation. It is possible that a first triggering event maybe of immunological nature, causes an increase of oxidative stress during implantation which will then alter the expression level of many targets, including MALAT-1; based on the data, this consequent deregulation would have an impact on both trophoblast and MSCs physiology, culminating in preeclampsia.

MEG3

Maternally Expressed 3 (MEG3) is an imprinted lncRNA which is expressed in many different cell types and tissues and acts as a tumor suppressor and is downregulated in many types of cancer. Physiologically, MEG3 acts by stabilising p53 and activating apoptotic responses [244]. Zhang and coworkers [34] analysed MEG3 RNA levels in 30 placentas from preeclamptic women, compared to 30 control samples and found a statistically significant 80% downregulation. These results were consistent with those of Yu and coworkers [245] studying a cohort of 20 preeclamptic and 20 control placentas, finding that MEG3 RNA was only 28% of the RNA levels of the control group. To elucidate in more detail the function of MEG3 in placenta, Zhang and coworkers (2015) overexpressed MEG3 in two trophoblast cell lines (JEG3 and HTR-8/SVneo), showing enhanced antiapoptotic effects, while downregulation of MEG3 increased the apoptotic cells. Analysis of protein markers showed how MEG3 downregulation increased the levels of pro-apoptotic proteins such as Caspase-3 and Bax. These results contrast with what is observed in cancer, where MEG3 expression is rather associated with the activation of proapoptotic pathways, possibly suggesting a different mode of action of MEG3 in these cell types. Yu and coworkers [245] focused on the link between MEG3 expression and endothelial-mesenchymal transition (EMT). During implantation and placentation, the trophoblasts undergo EMT in order to be able to migrate and invade the maternal tissues. MEG3 downregulation correlated with increased E-cadherin levels and downregulation of mesenchymal markers such as N-cadherin, vimentin, slug (encoded by the gene SNAI2), in placental RNA and protein extracts, placental sections and in vitro tests (HTR-8/SVneo trophoblast cell line). Changes in MEG3 expression did not influence proliferation but MEG3 overexpression promoted migration and trophoblast invasion through matrigel matrixes [34,224]. Altogether, MEG3 protects from apoptosis, promotes migration and invasion by regulating endothelial-mesenchymal transition in trophoblast cells and therefore its downregulation possibly affects trophoblast invasion and placentation, playing a key role in preeclampsia. Consistently, the imprinting control region (IG-DMR) of the DLK1-MEG3 cluster was very recently found hypermethylated in human umbilical veins from preeclamptic pregnancies, with an altered expression of both imprinted genes, a lower secretion of nitrite, VEGF and a higher secretion of endothelin 1 (ET1) all factors able to mediate pathological mechanisms in the offspring from preeclampsias [246].

RNA-ATB

As with many other lncRNAs, lncRNA-activated by TGF β (RNA-ATB) was first discovered in cancer, upregulated in hepatocellular carcinoma, it promotes cell proliferation, migration and invasion [247]. It has been reported that in hepatocells RNA-ATB is expressed in response to TGF β and in fibroblasts; it can create positive feedback regulation by promoting TGF β paracrine release. Lnc RNA-ATB was found to be significantly downregulated in placental samples from women with preeclampsia. Moreover patients with EOPE showed an even stronger deregulation [248]. Given the proliferative, invasive and migratory features of trophoblasts and in particular extravillous trophoblast, Liu and coworkers (2017) investigated lncRNA-ATB function in trophoblast cell line HTR-8/SVneo, which is a standard in vitro model of extravillous trophoblast. While overexpression of lncRNA-ATB increased the proliferative, migratory and invasive potential of HTR-8/SVneo cells, the downregulation

caused a steep decrease in proliferation, migration and invasion, proving that this gene has an important role on the physiology of the extravillous trophoblast and that its deregulation could explain an aberrant implantation and endometrium invasion in preeclampsia, potentially being linked to incomplete spiral artery remodeling. Whether RNA-ATB regulates trophoblast function through the interaction with members of the miR200 family is yet to be determined. However, increased miR200 has been found to affect the development of endometrium receptivity, negatively impacting implantation [249]. In labor, miR200 is upregulated in the human uterus and has been associated with pre-term labor in murine studies [250]. It seems likely that an interaction between RNA-ATB and miR200 is required for correct placental development, gestation and delivery.

PVT1, TUG1 and DIAPH2-AS1: Regulating Gene Expression through Recruitment of Chromatin Remodeling Complexes

lncRNAs work through different mechanisms, depending on the specific lncRNA, the cell type, the downstream targets [231]. In the past few years a few lncRNAs have been identified in preeclampsia as potential modulators, among which PVT1, TUG1 and DIAPH2-AS1 adopt the same mechanism of action. lncRNA TUG1 is downregulated in preeclamptic placentas. Interference of TUG1 in trophoblast cell lines (JEG3 and HTR-8/SVneo) negatively affected cell proliferation and growth, migration and invasion, network formation, while it increased apoptosis [31]. Transcriptome analysis by RNA-sequencing of HTR-8/SVneo cells in which TUG1 was downregulated showed a prevalence of affected genes involved in cell growth, migration and apoptosis. Xu and coworkers (2017) identified RND3 as main downstream factor involved in the phenotypic effects of TUG1 downregulation. RND3 mRNA and protein levels were strongly upregulated in response to TUG1 interference in vitro and RND3 mRNA was upregulated in preeclamptic placenta. RND3 is also known as RhoE, a GTPase that acts as a tumor suppressor, negatively regulating proliferation, migration and invasion [251]. In vitro experiments beautifully elucidated the mechanism by which TUG1 modulates RND3 expression—TUG1 directly interacts with the histone modification factor Enhancer of Zeste Homolog 2 (EZH2) and recruits it to the RND3 promoter, where EZH2 drives the silencing of RND3 by tri-methylating H3K27, resulting in strong RND3 downregulation [31]. A year later, Xu and coworkers [252] identified another lncRNA PVT1, strongly downregulated in preeclamptic placenta, whose downregulation negatively affects proliferation and increases apoptosis of trophoblast cell lines. PVT1 was found to recruit EZH2 to the promoter of the transcription factor ANGPTL4, driving its repression by increase in repressive chromatin markers: this could partially explain the phenotypic effects of PVT1 deregulation. Feng and coworkers [253] uncovered a complicated regulatory network behind PAX3 deregulation in preeclampsia which is linked with decreased proliferation, invasion and migration of trophoblast cells [254]. PAX3 is a transcription factor downregulated in preeclamptic placentas and this correlates with DNA hypermethylation of the promoter region [171,254]. In this study, Feng and coworkers (2019) found that in preeclamptic placentas lncRNA DIAPH2-AS1 is upregulated along with the transcription factor HOXD8. In vitro experiments in HTR-8/SVneo cells clarified the regulatory network: under hypoxia the transcription factor HOXD8 is upregulated and induces expression of the lncRNA DIAPH2-AS1. DIAPH2-AS1 recruits lysine-specific demethylase 1 (LSD1) to the promoter of PAX3 where it alters the chromatin modification state, decreasing methylation of Histone H3. LSD1 can also modify DNA methyl-transferase 1 (DNMT1), stabilizing it. ChIP experiments showed enrichment of LSD1 and DNMT1 at the PAX3 promoter, which correlated with increased DNA methylation and mRNA repression. Interference of DIAPH2-AS1 was enough to reverse the phenotype and increase PAX3 levels [253]. These studies underscore that different epigenetic mechanisms regulate gene expression. It is possible that certain mechanisms are favored in different cell types and future studies will help identify the conserved regulatory networks that plays a role in the etiology of preeclampsia.

3.2.2. micro RNA and Preeclampsia

microRNAs in Preeclampsia

The first study on microRNAs (miRs) in preeclampsia was published in 2007. In this study, the expression levels of a subset of 157 miRNAs expressed in the placenta were tested by qRT-PCR in human placental samples from pregnancies without any complications, with PE, and with PE and small for gestational age (SGA) outcomes. 153 miRNA were detected in the placenta RNA samples and three of them were found to be upregulated in PE: miR-210, miR-155, miR-200b [255]. The first global transcriptomic analysis of microRNAs was performed with 20 PE placental samples and 20 controls, with microarray technology by Zhu and collaborators in 2009. Comparing gene expression profiles of the severe PE group with controls, 11 microRNAs were upregulated and 23 downregulated. Among them, many microRNAs are organized in chromosomal clusters: downregulated clusters are found in 13q31.3, 14q32.31, Xq26.2, Xq26.3, while upregulated clusters are found in 19q13.42 suggesting co-regulation profiles [256]. An integrative analysis was conducted comparing distinct datasets with the aim of identifying microRNAs–transcripts regulatory networks in preeclampsia, resulting in the construction of a map of putative microRNA–gene target interactions in developmental process, response to nutrient levels, cell differentiation, cell junction, membrane components [257].

Although many studies followed, most of them aimed at identifying differentially expressed miRs in placenta and in plasma samples from PE women. Fewer studies have focused in other cell types present in the placenta. For example, in fetal endothelial cells downregulation of miR-29a-3p and miR-29c-3p and upregulation of miR-146a is observed in PE patients [258]. Both miR-29a and miR-29c show proangiogenic functions by stimulating HUVECs proliferation and tube formation through VEGFA-induced and FGF2-induced cell migration pathways [259]. However, other studies suggest an antiangiogenic role of miR-29c through downregulation of the IGF-1 proteins at the post-transcriptional level [260,261]. On the other hand, miR-146a inhibits the de-novo formation of blood vessels in-vitro and reduces tube formation ability in HUVECs [260,261]. The study of the role that miRs may have in the different cell types present in the placenta is indispensable to understand the role of this molecules in the development of the disease. In the long term, it has also been shown that miRNA profiles in the neonate is altered following an hypertensive pregnancy; for instance the level of miR-146a at birth predict microvascular development three months later [262].

Many studies followed, aimed at identifying differentially expressed miRs in placenta and in plasma samples from PE women.

In this review, we will discuss the most well characterized microRNAs miR-210, miR-155 and give an overview of some of the research that has been carried out on circulating microRNAs, given their potential as clinically relevant biomarkers.

miR-210

miR-210 is a microRNA involved in the regulation of mitochondrial function and hypoxia response. It has been well characterized, in placentas as well as in different cancer and tissue types [263]. Most of the knowledge on the regulatory pathways that involve miR-210 comes from oncology research. miR-210 has been soon identified as one of the early hypoxia-response miRs, being directly regulated by the Hypoxia inducible factor 1 α (HIF-1 α) [264]. Under hypoxic conditions, miR-210 alters mitochondrial function promoting a metabolic switch to glycolysis. This is achieved by negative targeting of genes involved in the electron-transport chain, namely iron-sulfur cluster scaffold homolog (ISCU) and cytochrome C oxidase assembly protein (COX10). As a result, miR-210 also increases the levels of Reactive Oxygen Species (ROS) [265]. Under hypoxia, miR-210 and HIF-1 α establish a positive feedback regulation that maintains expression of both factors. This is achieved by miR-210 downregulation of the mRNA of Glycerol-3-Phosphate Dehydrogenase 1-Like, which would otherwise contribute to targeting HIF1 α to the proteasome for degradation. Conversely, stabilized HIF1 α directly activates miR-210 expression [266].

In endothelial cells, miR-210 is involved in regulating angiogenesis and vascularization which are fundamental processes in placenta development. Hypoxia causes miR-210 activation which protects endothelial cells from apoptosis and stimulates chemotaxis driven by VEGF, migration and tube formation [267]. In preeclampsia, miR-210 was first identified as upregulated in placenta samples by Pineles and collaborators (2007) using qPCR. In the first comprehensive study carried out with microarray technologies, miR-210 was consistently found upregulated in placenta of severe preeclamptic women, however in mild preeclampsia it was found to be downregulated, which might suggest different mechanisms at play or rather different metabolic states of the placenta, with a more pronounced ischemia in severe preeclamptic placentas [256]. Moreover, subsequent analyses identified significantly upregulated miR-210 in plasma samples from patients with preeclampsia [181]. In the context of PE miR-210 is involved in the mitochondrial dysfunction observed, which causes metabolic imbalance, excessive ROS production and cell damage. Similarly to what happens in cancer, miR-210 negatively regulates ISCU which is downregulated in preeclampsia samples, directly affecting mitochondrial architecture and functionality [268–270]. The deregulation of miR-210 was also found in the placentas of mice from a preeclamptic model [134].

miR-210 is also an important modulator of trophoblast physiology. In vitro studies using isolated primary trophoblasts and trophoblast cell line JAR, proved how hypoxia induces an increase in miR-210 levels. Artificial overexpression of miR-210 in JAR cells caused a significant downregulation of migration and invasion. In trophoblast cells, hypoxia and ROS can activate HIF1 α but more importantly NF κ -B p50—which is found upregulated in preeclamptic placenta tissues. NF κ -B p50 binds a consensus sequence in the miR-210 promoter, activating its expression. In trophoblasts, miR-210 interacts with a perfect match with the 3'-UTR of the transcription factor homeobox-A9 (HOXA9), causing both degradation of the mRNA and downregulation of translation. Another direct target is Ephrin-A3 (EFNA3), a ligand of the Ephrin binding receptors, in this case miR-210 binds the 3'UTR of the gene with an imperfect match, causing only translational downregulation. These two transcription factors activate expression profiles involved in migration, invasion and vascularisation [181]. Therefore, in trophoblast, miR-210 expression correlates with a negative regulation of migration and invasion, mediated by downregulation of EFNA3 and HOXA9, in response to hypoxia, ROS and activated NF κ -B signaling.

Further studies have identified additional downstream targets of miR-210 in preeclampsia, which are downregulated in preeclamptic samples and whose expression is altered upon miR-210 activation in cell models. A few examples are inflammation related molecules STAT6 and IL-4 [271], potassium channel modulatory factor 1 (KCMF1) [272], thrombospondin type I domain containing 7A (THSD7A) [273].

This mounting body of evidence highlights a key role of miR-210 in the development and maintenance of a preeclamptic phenotype. However it is still not clear which is the triggering event. It is possible that complications during implantation trigger an immune response which would create a pro-inflammatory environment, activating NF κ -B signaling, causing aberrant expression of miR-210 and all consequent downstream cascades. Recently, Chen and collaborator (2019) analysed the inflammatory profile of preeclamptic women, compared to patients which experienced healthy pregnancy [274]. The concentrations of proinflammatory cytokines (IL-6, IL-17) were higher in plasma samples from peripheral blood in the preeclampsia group. Moreover, Transforming Growth Factor β 1 (TGF β 1) levels were higher as well. TGF β 1 has the function of promoting the prevalence of a subset of regulatory T cells (Tregs) that maintain immunotolerance, allowing a successful implantation and avoiding an immune response against the foetal tissues. These Tregs are characterised by expression of the fork-head box p3 (Foxp3) transcription factor, which promotes an immunotolerant phenotype [275]. However, proinflammatory signals such as IL-6 cause the activation of T cells at the expense of Foxp3-positive Tregs, causing an activation of inflammatory responses. Zhao and coworkers showed that miR210 was upregulated in preeclamptic placentas and Foxp3 mRNA and protein levels were found downregulated, previous studies had shown evidence of direct regulation of Foxp3 by miR210,

suggesting a pivotal role of this microRNA in regulating the threshold of immunotolerance by altering the balance of Foxp3+ Tregs/activated T cells [276].

miR-155

miR-155 is upregulated in preeclamptic placentas [255]. This upregulation correlates inversely with the level of cysteine-rich protein 61 (CYR61) [277], which is a factor secreted by different cell types, including trophoblast, involved in promoting migration, invasion, angiogenesis and vascularisation [278,279]. miR-155 directly targets the 3'-UTR of CYR61 mRNA with a perfect match, causing transcriptional and translational repression. In vitro experiments (HTR-8/SVneo trophoblast cell line) showed how miR-155 inhibits CYR61-mediated expression of VEGF, inhibiting trophoblast migration [277]. Decreased trophoblast-mediated secretion of VEGF would negatively affect angiogenesis and vascularisation in the site of placenta development.

miR-155 regulates trophoblast proliferation and migration also by directly targeting the cell cycle gene Cyclin D1 [172]. Cyclin D1 is involved in cell cycle progression, migration and invasion of trophoblast lineages, downregulated in preeclamptic placentas at both mRNA and protein levels [280–282]. In vitro studies have shown how miR-155 through direct targeting of the 3'UTR of CyclinD mRNA downregulates mRNA and protein levels, negatively affecting migration, causing cell cycle arrest and decrease in proliferation in HTR-8/SVneo cells [42]. Exiting cell cycle is a step of terminal differentiation, which suggests how miR-155 overexpression, as found in preeclampsia, could lead to a premature differentiation of cytotrophoblasts, possibly inducing syncytialization. This phenomenon would cause depletion of the cytotrophoblast pool, accelerating placental aging.

In sum, miR-155 modulates proliferation, migration and invasion of trophoblasts and its expression can affect the phenotype of endothelial cells by negatively regulating VEGF release. miR-155 deregulation could have catastrophic consequences in placentation, deeply affecting trophoblast infiltration, vascularization and angiogenesis of the developing placenta.

Circulating miR-155

Maternal plasma from preeclamptic women presented significantly statistically higher levels of miR-155 [283]. In blood, microRNAs are quite stable and can travel through circulation, to be uptaken by different cell types, such as endothelial and immune cells, regulating gene expression [284]. Yang, Zhang and Ding (2017) showed how plasma levels of miR-155 positively correlate with proinflammatory cytokine interleukin-17 (IL-17) and with proteinuria and urine podocytes counts in women with preeclampsia. Similarly to miR-210, miR-155 promoter presents a binding site for NFκ-B and can be activated by this inflammation master regulator, which could suggest a similar pattern of regulation for miR-155 and pro-inflammatory factors, other than a direct interaction between these genes [285].

miR-155 in Endothelial Cells

Endothelial cells play a fundamental role in placentation given the copious vascularisation and angiogenesis that takes place in the maternal endometrium during placentation. In preeclampsia, pro-inflammatory factors and secreted molecules from the preeclamptic placenta produce an excessive activation of the maternal endothelium, resulting in endothelial dysfunction, culminating in inflammation, blood pressure changes, downstream systemic effects [286]. miR-155 has been found to be downregulated in human umbilical vein endothelial cells (HUVECs) from preeclamptic women, compared to HUVECs from healthy pregnant women [287]. This downregulation correlated with an increase in Angiotensin II Receptor 1 (AT1R) and increased phosphorylation of Extracellular Signal-regulated Kinases1/2 (ERKs), identifying AT1R as direct target of miR-155 [287]. Activation of the Angiotensin II- AT1R through ERK1/2 in endothelial cells causes cell cycle arrest and initiation of senescence pathways; miR-155 depletion-dependent increase in AT1R will render endothelial cells more sensitive to blood level of Angiotensin II, promoting endothelial damage [288].

miR-155 has been implicated in regulating Nitric Oxide (NO) production in endothelial cells. NO is a potent vasodilator and reduced levels of NO have been associated with preeclampsia etiology [289,290]. In vitro studies using HUVECs proved how endothelial Nitric Oxide synthase (eNOS) mRNA is a direct target of miR-155; proinflammatory stimuli upregulate miR-155 expression in these cells in vitro, downregulating eNOS and NO production [290]. As mentioned above, microRNAs can be found in plasma and miR-155 is upregulated in plasma of women with preeclampsia [69]. microRNAs can be free in circulation or travel inside vesicles and exosomes, which can be uptaken by target cells, activating signaling pathways, affecting expression profiles [291]. Shen and collaborators (2018) elegantly showed how exosomes from plasma samples of preeclamptic patients can affect eNOS mRNA and protein levels in HUVECs [292]. In particular, treatment of HUVECs in vitro with isolated exosomes from plasma of preeclamptic patients (compared to exosomes from control group) caused a statistically significant decrease in eNOS mRNA and protein levels, which correlated with decreased NO production. When analysing the composition of the exosomes, miR-155 was found to be upregulated in the preeclamptic group. Follow up in vitro tests proved how miR-155 located in the exosomes affects eNOS regulation in endothelial cells.

miR-155 in Vascular Smooth Muscle Cells

In arteries and arterioles, endothelial cells are interspaced by vascular smooth muscle cells (VSMCs) which thanks to their contractile properties allow vasoconstriction and vasodilation to occur, accommodating for changes in blood pressure. VSMCs generally present a contractile phenotype characterised by elongated spindle-like morphology, high concentration of contractile filaments. In response to external stimuli, they can switch to a synthetic phenotype characterised by loss of contractility markers, rhomboid morphology, increased proliferative and migratory potential; in this state VSMCs cells lose the ability to modulate vascular resistance [293]. Phenotypic regulation of VSMCs is driven by soluble guanylate cyclase (sGC) which increases intracellular levels of guanosine monophosphate (cGMP), key messenger molecule. cGMP is the substrate of cyclic GMP-dependent protein kinase (PKG) which activates downstream signaling pathways promoting VSMCs contractile phenotype. Nitric Oxide produced by endothelial cells positively modulates sGC activity, favouring vasodilation through enhancement of the VSMCs contractile phenotype [294,295].

In the presence of proinflammatory cytokine Transforming Necrosis Factor α (TNF α), miR-155 was found to be directly activated by NF κ -B in in vitro model of VSMCs. The upregulated miR-155 directly interacts with the 3'-UTR of the mRNA of PKG1 [296] and of the β 1 subunit of guanylate cyclase (sGC β 1), resulting in translational repression and mRNA degradation [297]. As a consequence of sGC β 1 downregulation, intracellular cGMP levels are strongly decreased and the downregulation of PKG1 inhibits downstream pathways [296,297]. Park and collaborators (2019) co-cultured HUVECs and VSMCs, observing higher cGMP accumulation in VSMCs, which is mediated by Nitric Oxide stimulation, produced by the endothelial cells [297]. This could be countered by ectopic miR-155 expression in VSMCs. miR-155 overexpressing in response to TNF α , mediating inhibition of the sGC/PKG pathway, causes downregulation of contractile protein markers. This results in a shift of VSMCs to a synthetic phenotype, assuming a rhomboid morphology, increasing proliferation and migration rates. Interestingly the pro-contractile effects of Nitric Oxide could be cancelled by miR-155 expression [296,297]. In placental vessels of preeclamptic placenta sGC β 1 mRNA levels are downregulated [297], given the evidence provided on miR-155 repression of the sGC/PKG pathway, we can imagine that PKG1 might be downregulated as well. In response to inflammation, both endothelial and smooth muscle cells are affected and in preeclampsia they overexpress miR-155 which alters their ability to produce and respond to vasodilation stimuli. Taken together, this evidence highlights the pivotal role of inflammation and miR-155 in the etiology of the preeclamptic phenotype.

Potential Biomarkers: microRNAs Circulating in Maternal Plasma

Since the identification of circulating small RNAs in plasma samples, the prospect of their potential use as diagnostic and predictive biomarkers has fueled extensive research [298]. In the context of preeclampsia, the finding that small microRNAs with placental origin can travel in the blood circulation and affect systemically different cell types opens new avenues for the understanding of the mechanisms of this complex disease [299,300].

In Table 4 are listed some of the microRNAs that have been found deregulated in plasma samples of preeclamptic patients. In several studies, groups of microRNAs differentially expressed have been analyzed for their potential as predictive biomarkers of the preeclamptic phenotype [301–305]. These studies show how blood levels elevation of PE-associated microRNAs can be predictive for the preeclamptic phenotype starting from the second trimester. Li and collaborators (2015) evaluated the predictive values of the upregulated micro-RNAs miR-152, miR-183 and miR-210 by plotting the corresponding receiver operating characteristic curves. In the second trimester samples, the Area Under the Curve (AUC) indicated strong predictive values and were respectively 0.93 for miR-210, 0.97 for miR-183 and 0.94 for miR-152. Interestingly, different studies investigated the predictive power of miR-210 and, even though all results highlighted its key role in preeclampsia and potential as diagnostic marker, the AUCs varied in a range between 0.7 and 0.94 [301–303,305]. This variation might be due to differences in patient cohorts, samples collections and handling; however, the fact that miR-210 still emerged as predictive biomarker is encouraging.

Winger and collaborators (2018) collected peripheral blood cells in preeclamptic and control patient group, analysing the expression levels of a subset of 30 microRNAs previously identified altered in preeclampsia. 48 samples were divided in a training and a validation group. Analysis of differentially expressed microRNAs in the training cohort identified a panel of 8 microRNAs with good prediction values (AUC > 0.75) and p value ≤ 0.05 : miR-1267, miR-148a, miR-196a, miR-33a, miR-575, miR-582, miR-210, miR-16. The panel was successfully validated and the use of the 8 microRNAs combined increased the prediction power of the tests [305].

From Table 4, it is possible to appreciate the heterogeneity of findings across different studies. These discrepancies in the repertoires of circulating miRNAs complicate the identification of useful biomarkers. This heterogeneity could partly be explained by the fact that preeclampsia is a complex systemic disease that develops over months of gestation; therefore, the panel of circulating molecules in blood samples might vary considerably depending of the time point at which samples are collected. Another possible explanation might reside in the wide range of different methodologies used for the extraction of circulating RNAs which introduce technical variability [306,307]. Moreover, there is mounting evidence on how the current techniques are able to detect only a small fraction of the total bulk of circulating RNAs (WO2009093254A2). Therefore, further research is still required to improve our technical knowledge so to design better, more consistent methodologies for the identification of circulating biomarkers, that might one day allow the design of diagnostic panels for effective early detection and prevention of preeclampsia.

3.2.3. Additional Considerations on the Analysis of lncRNA Functions

Possible Caveats of the Current Trophoblast In Vitro Models

Many of the lncRNAs found to be deregulated in preeclamptic placenta have previously been identified in cancers, where they have a role in regulating proliferation, migration, invasion and apoptosis. Most of these PE-associated lncRNAs have pro-survival and pro-migration properties, therefore downregulation is associated with activation of apoptosis, decreased migratory potential and proliferative rate.

Once they have been found to be downregulated in preeclamptic placenta, the main objective has been to investigate the molecular function of these lncRNAs in the context of placenta physiology and preeclampsia. In vitro studies have seen the use of classical cellular models of trophoblast, either

choriocarcinoma cell lines (JEG3 and BeWo) or artificially immortalized cell lines (HTR-8/SVneo). Through these in vitro studies it has been established that most of these lncRNAs regulate proliferation, invasion and migration of the trophoblast.

Table 4. Deregulated miRNA in preeclampsia.

microRNA	PE Placenta	PE Plasma	Function	Gene targets	AUC	References
miR-214	DOWN					[308]
miR-152		DOWN				[300]
miR-218	DOWN					[308]
miR-590	DOWN					[308]
miR-18a	DOWN	DOWN	Promoting trophoblast migration	SMAD2		[225,308]
miR-19a	DOWN					[308]
miR-19b1		DOWN	TGF β -signaling	SMAD factors		[225]
miR-379	DOWN					[308]
miR-411	DOWN					[308]
miR-195	DOWN					[308]
miR-223	DOWN					[308]
miR-363	DOWN					[308]
miR-542-3p	DOWN					[308]
miR-144		DOWN	Ischemia, hypoxia			[225]
miR-15b		DOWN	Angiotensin-renin system			[225]
miR-181a	UP	UP				[225,308]
miR-584	UP					[308]
miR-30a-3p	UP					[308]
miR-151	UP					[308]
miR-31	UP					[308]
miR-210	UP	UP		PTPN2	0.7 < AUC < 0.9	[225,255,300,302,303,305,308,309]
miR-17-3p	UP					[308]
miR-193b	UP					[308]
miR-638	UP					[308]
miR-525	UP					[308]
miR-515-3p	UP					[308]
miR-519e	UP					[308]
miR-517-5p	UP	UP			AUC = 0.7	[304]
miR-518b	UP	UP				[225,304,308]
miR-524	UP					[308]
miR-296	UP					[308]
miR-362	UP					[308]
miR-574-5p		UP			AUC > 0.7	[302]
miR-1233-3p		UP			AUC > 0.6	[302]
miR-155		UP			AUC > 0.7	[225,303]
miR-1267		UP			AUC > 0.8	[305]
miR-148a		UP	Immune response	HLA-G	AUC > 0.9	[305,310]
miR-196a		UP			AUC = 1	[305]
miR-33a		UP			AUC = 1	[305]
miR-575		UP			AUC > 0.9	[305]
miR-582		UP	Trophoblast invasion, migration	VEGF	1	[305,311]
miR-152	UP	UP	Immune response	HLA-G	AUC > 0.9	[256,301,312]
miR-183	UP	UP	Cell differentiation, apoptosis, invasion		AUC > 0.9	[255,301,313]
miR-215		UP				[225]
miR-650		UP				[225]
miR-21	UP	UP	Apoptosis			[225,314]
miR-29a		UP				[225]
miR-300		UP	Trophoblast differentiation	ETS-1		[315]

Annotations: AUC = Area Under the Curve; SMAD2 = Mothers Against Decapentaplegic Homolog 2; PTPN2 = Tyrosine-protein phosphatase non-receptor type 2; HLA-G = Histocompatibility antigen, alpha chain G; VEGF = Vascular endothelial growth factor; ETS-1 = E26 oncogene homolog 1; TGF β = Tumor growth factor β .

Have we completely unfolded the role of PE-associated lncRNA in the human placenta? Since lncRNAs have been previously identified in cancers, it is possible that the functions we have attributed them in the placenta are actually a result of the fact that we are analyzing them in cell lines that are cancer-like. Therefore, there is still the possibility that these lncRNAs have additional distinct functions in placenta that could be highlighted using more physiological placenta models. The recent development of placenta organoids from stem cells rises the hope for exciting new avenues, to explore these questions [316].

What about the Syncytiotrophoblast?

Migration, apoptosis, invasiveness and proliferation are functions shared between cancer cells and by cytotrophoblast (CTB) especially by the extravillous trophoblast (EVT) in the placenta, the in vitro investigations into PE associated lncRNAs have so far focused on EVT cell line models (e.g., JEG3, HTR-8/SVneo). However, it is important to highlight how transcriptomic data from placenta samples are a result of overall placenta gene expression levels. The extracted placental RNA comes from all the different cell types present in the tissue and the most abundant cell populations are represented by cytotrophoblasts and syncytiotrophoblasts (SCT). Even though it is true that CTB and EVT cells are fundamental for implantation and correct placental development, the syncytiotrophoblast is the functional core of the placenta itself, constituting the barrier for nutrient exchanges between fetal and maternal vasculatures and acting as secretory organ that hormonally regulates progression of gestation. Liu and coworkers (2017), in their work on RNA-ATB, showed a strong in situ hybridization staining of lncRNA-ATB in the syncytiotrophoblast layer of the placenta, reinforcing the idea that the syncytiotrophoblast might be equally affected by deregulation in the lncRNAs species [248]. Yu and coworkers (2018) work on MEG3 showed how MEG3 downregulation observed in preeclampsia correlates with an increase in adhesion molecule E-cadherin [224]. While it is true that this molecule is important for endothelial-mesenchymal transition, and its alteration would affect trophoblast invasion and EVT migration, E-cadherin downregulation after cytotrophoblast cell-cell interaction has been implicated in CTB syncytialization [317]. Suggesting that MEG3 might affect STB physiology as well.

Therefore, there are still potentially interesting questions to be raised: what are the effects of downregulated lncRNAs on the physiology of the CTB and SCT? Do we see an alteration of the proliferative state of the CTB, does this cause premature placental aging? Does this deregulation affect the differentiation potential of the CTB, affecting the balance between CTB renewal and SCT terminal differentiation? Do these lncRNAs have other functions, exclusive to placenta, other than the ones shared with cancer?

3.3. Histone Modifications

Few studies addressed the question of histone code modifications in PE. Chakraborty and coll. evidenced a HIF-KDM3A-MMP12 signaling cascade that promotes trophoblast invasion and trophoblast-directed uterine spiral artery remodeling in rat placenta and human placental cells. Hypoxia drives HIF activation and KDM3A expression, which in return will alter the histone methylation status of genes promoting development of the invasive trophoblast lineage and tissue remodeling, illustrated with trophoblast-derived MMP12 activation [318]. Hypoxia was also shown to affect the histone demethylase JMJD6 (Jumonji domain containing protein 6) and JMJD6 demethylase activity was shown to be drastically reduced in PE placenta as compared to Control Placenta [319]. Very recently, the expressions of HDACs were investigated in PE placentas and only HDAC9 was found downregulated both at the mRNA and protein levels in syncytiotrophoblast cells. Knock-down of HDAC9 in HTR-8/SVneo cells inhibits trophoblast cell migration and invasion. TIMP3, an inhibitory of MMP involved in invasion and tissue remodeling, is a direct target of HDAC9, identified by ChIP and is upregulated in the absence of HDAC9 [320].

3.4. Imprinting

Overall, preeclampsia cannot be considered an imprinting disease, despite the fact that a recent study showed that imprinted genes are more differentially expressed in PE than other genes, with paternally expressed genes (inducing placental growth) rather down-regulated and maternally expressed genes upregulated [151]. A systematic analysis of preeclampsia placental gene expression and imprinted genes was carried out in 2017 [321], which revealed altered expression of DLX5 in human PE placentas but with a rather mild deregulation (~2 fold). To be mentioned as well, the first gene identified by positional cloning in preeclampsia, STOX1, is imprinted in specific placental cell

subtypes [322,323]. The mutation originally found in STOX1 has rather a gain-of-function effect [323] and in fact, overexpression of STOX1 induces a preeclamptic expression profile and a preeclamptic phenotype in cells or in mice, respectively [7,324]. To note, however, we have no evidence that Stox1 is imprinted in mice, therefore it is suspected that the mere ectopic and untimely overexpression of this factor is the cause of the disease. The idea that an imprinted gene is implicated in preeclampsia has been cleverly substantiated by Jennifer Graves as early as 1998 [325] and she gave theoretical reasons why this should be the case. The future will tell us if more examples of imprinted preeclampsia-associated genes exist in the human genome.

4. Perspectives and Conclusions

The recent years have seen the emergence of an increasing number of studies focused on the role of epigenetics in the regulation of placental development and on its potential implication in placental pathologies. However, we still lack a precise picture on how these epigenetic modifications correlate with gene expression. In particular, we have a limited knowledge on how DNA-methylation or Histone modifications impact gene expression in normal and pathological placenta development. In addition, our knowledge on the mechanisms regulating the dynamics of the instauration of the different epigenetic marks across development is very scarce. Nevertheless, recent studies have started to reveal how epigenetics is involved in the regulation of important processes in placental development such as cell fate determination, *syncytialization* or EVT migration and invasion. The emergence of new technologies allowing the study of the epigenetic and transcriptomic profiles of the different cells types of the placenta will certainly greatly contribute to improve our understanding of epigenetics in placenta. Moreover, in the context of PE, to date, the studies analyzing epigenetic modifications have focused on the placenta, however the antiangiogenic and cytotoxic factors released by the PE placenta have the potential to induce epigenetics modifications in maternal target tissues (blood cells, endothelial cells). This could impact the future maternal and fetal health and deserves to be studied in detail. Overall, the comprehension of epigenetic regulation in preeclampsia both at the level of the placenta and other involved organs could provide new biomarkers and therapeutic targets to improve the management of this disease. For the moment, this has not been successfully applied as diagnostic or prognostic of preeclampsia. One explanation of this observation could be that the extraction of circulating RNAs from the plasma is still immature technologically, leading to discrepant results between various laboratories and absence of consensus in defining a panel of diagnostic miRNA. This may evolve in the future, leading to substantial exploitation of these markers in complex diseases, including preeclampsia.

Supplementary Materials: Supplementary materials can be found at <http://www.mdpi.com/1422-0067/20/11/2837/s1>. Table S1. High-throughput studies analyzing methylation profiles of different relevant tissues in the context of preeclampsia [42,43,47,54–73].

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