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Disturbed Placental Imprinting in Preeclampsia Leads to Altered Expression of DLX5, a Human-Specific Early Trophoblast Marker

Running Title: *Zadora et al.; Perturbed Imprinting Regulation in Preeclampsia*

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Abstract

***Background*—**Preeclampsia (PE) is a complex and common human-specific pregnancy syndrome associated with placental pathology. The human-specificity provides both intellectual and methodological challenges, lacking a robust model system. Given the role of imprinted genes in human placentation and the vulnerability of imprinted genes to loss of imprinting changes, there has been extensive speculation, but no robust evidence, that imprinted genes are involved in PE. Our study aims at investigating whether disturbed imprinting contributes to PE.
***Methods*—**We first aimed at confirming that PE is a disease of the placenta by generating and analysing genome-wide molecular data on well-characterized patient material. We performed high-throughput transcriptome analyses of multiple placenta samples from normal and PE patients. Next, we identified differentially expressed genes (DEGs) in PE placenta, and intersected them with the list of human imprinted genes. We employed bioinformatics/statistical analyses to confirm association between imprinting and PE, and to predict biological processes affected in PE. Validation included epigenetic and cellular assays. Regarding human-specificity, we established an *in vitro* invasion-differentiation trophoblast model. Our comparative phylogenetic analysis involved single-cell transcriptome data of human, macaque and mouse preimplantation embryogenesis.
***Results*—**We found disturbed placental imprinting in PE and revealed potential candidates, including *GATA3* and *DLX5,* with poorly explored imprinted status and no prior association with PE. Due to loss of imprinting *DLX5* was upregulated in 69% of PE placentas. Levels of DLX5 correlated with classical PE marker. DLX5 is expressed in human, but not in murine trophoblast. The $DLX5^{high}$ phenotype resulted in reduced proliferation, increased metabolism and ER stress-response activation in trophoblasts *in vitro*. The transcriptional profile of such cells mimics the transcriptome of PE placentas. Pan-mammalian comparative analysis identified *DLX5* as a part of the human-specific regulatory network of trophoblast differentiation.
***Conclusions*—**Our analysis provides evidence of a true association between disturbed imprinting, gene expression and PE. Due to disturbed imprinting, the upregulated *DLX5* affects trophoblast proliferation. Our *in vitro* model might fill a vital niche in PE research. Human-specific regulatory circuitry of *DLX5* might help to explain certain aspects of PE.

Key Words: preeclampsia/pregnancy; genome-wide analysis; epigenetics; ER stress; Trophoblast, Genomic imprinting

Clinical Perspective

What is new?

- Unbiased analysis of genome-wide molecular and clinical data identifies DLX5 as an imprinted target gene with novel placental function in PE.
- We observe that DLX5 is paternally imprinted in the human placenta, and its expression is dysregulated in PE.
- We provide evidence for a mechanistic coupling between PE and disturbed placental imprinting (loss of imprinting) as a causal role in PE.
- Our data indicate that DLX5 has a role in trophoblast proliferation and differentiation (syncytialization).
- DLX5-induced overexpression in trophoblasts can faithfully model PE in a cell culture system, signifying the contribution of a single transcription factor and providing a potential cellular model both for further research and for analysis of drugable targets.
- DLX5 is expressed in the human, but not in mouse trophoblasts, underlying the human specificity of PE. Our study highlights the diverged cellular function of DLX5 during mammalian embryonic evolution.

What are the clinical implications?

- Our analysis supports the view that PE is not a single, but a heterogeneous disease, with disturbed imprinting commonplace.
- Unsupervised clustering analysis identified three distinct transcriptomic classes of PE, not clustering with the intrauterine growth restriction (IUGR). Our clusters are in conjunction with previously suggested classification of PE (early vs late onset), but subdivides early onset PE further.
- Our study identifies an early PE cluster that can be clearly characterized by elevated DLX5 levels, disturbed epigenetics and similar clinical manifestation.
- We find that levels of *DLX5* correlate with a placenta-derived PlGF circulating biomarker. Whether DLX5 will have a utility as a biomarker is unclear as its loss of imprinting is not observed in all instances (69% of all PE).
- Our cellular model has potential for further clinically-related research including analysis of drugable targets.

Preeclampsia (PE) is a complex, heterogeneous syndrome characterized by high blood pressure (≥140/90 mmHg) after the 20th week of pregnancy in association with proteinuria (≥300 mg/l per 24-hour)[1]. PE is the first sex-specific cardiovascular risk factor[2], affecting 2-8% of human pregnancies, and remains a leading cause of maternal and perinatal mortality[3].

Despite the considerable research efforts, the etiology of PE is not fully understood. PE is assumed to be associated with reduced fetal trophoblast invasion and impaired remodeling of maternal spiral arteries leading to poor uteroplacental perfusion. The improper placentation process triggers oxidative and endoplasmic reticulum (ER) stress, and results in defective protein synthesis in the placenta. As a consequence, dysregulated expression of inflammatory, angio- and antiangiogenic factors is observed[4] in PE. Currently, the only treatment is delivery, pinpointing to the potentially central role of the placenta (or maternal-foetal interaction more generally) in the disease[5].

The placenta has unique epigenetic features, including low levels of genomic DNA methylation and a specific expression pattern of imprinted genes, which are the prime candidates to be associated with the evolution of intrauterine development[6]. As epigenetic disruption of imprinted gene was associated with certain diseases, the potential involvement of disturbed regulation of imprinted genes in PE has been also intensively discussed[7-11].

Curiously, PE is not confirmed in other mammals, and considered as being human specific (even though a possible eclampsia event was observed in a gorilla)[12]. The human-specificity of PE generates real challenges. First, despite intensive research, the genetic background of the human-specificity and the etiology of PE remain poorly understood. Second, although rodent models have been suggested, no animal models have proven suitable.

As placentation is a diverse process even among primates[13], genes associated preferentially with placental expression (e.g. imprinted genes) are potential candidates for understanding human-specificity. This suggestion is largely centered on the understanding a) that dosage of imprinted genes can have phenotypic impact, b) that the placenta is a "hotspot" for the activity of imprinted genes, and hence c) that loss of imprinting will be likely to affect matters at the maternal-foetal interface.

Despite the abundant speculation that disruption of imprinting and PE might be mechanistically coupled, the evidence is at best limited. Indeed, a recent review of the genetics of PE[14] reports no robust evidence for a mechanistic coupling with imprinting. What evidence there has been is circumstantial, often negative or not replicable[15-19]. A further barrier to effective research - until recently[20] - has been the lack of a definitive list of genes imprinted in the human placenta.

To get a better understanding of this highly complex disease, we aim at confirming our hypotheses a) that PE is not a single, but a heterogeneous disease of the placenta; b) that it is associated with improper trophoblast function; c) that epigenetic turbulence can result in abnormal expression of imprinted genes, which in turn d) compromise proper maternal foetal interaction and contribute to PE; e) specifically in human. Our experimental strategy is based on a cross-disciplinary approach that employs the current catalog of human imprinted placental genes, combines the generation and analyses of genome-wide molecular data with well-characterized patient material, followed by experimental validation. We employ molecular, cellular technologies and bioinformatic analyses to predict biological processes affected in PE, followed by wet-bench validation. We also aimed at establishing an *in vitro* model to fill a vital niche in PE research, explicitly because there is no robust animal model system. Finally, we data-

mine existing single cell transcriptome data to possibly shed light on the human specific nature of PE.

Methods

Detailed methods are provided in the Data Supplement.

Patients

Microarray data of human placenta and decidua samples were described earlier[21]. The study also consists of placental and decidual tissues from 56 preeclamptic women (PE) and 28 women with normotensive and uncomplicated pregnancies described earlier[22]. The PE group was subdivided into PE + IUGR (n = 14) and PE without IUGR (n = 42). Furthermore, the PE group was divided into early-onset PE (delivery <34 gestational week) and late-onset PE (delivery ≥34 gestational week). The Regional Committee of Medical Research Ethics in South-Eastern Norway approved the study, and all the subjects gave informed written consent.

The healthy (n=5) and PE (n=5) primary trophoblast cells were isolated from human placenta samples obtained from HELIOS Klinikum in Berlin. Human placenta sampling was approved by the Regional Committee of the Medical Faculty of Charité Berlin.

Statistics

Data is presented as mean ± SEM (for normally distributed data) or median with inter-quartile range (for non-normally distributed data). Normality was assessed by Kolmogorov-Smirnov tests. Techniques for each analysis are specified in the figure legends.

$p<0.05$ was considered statistically significant.

Results

Cluster analysis identifies three distinct transcriptome patterns of preeclamptic placenta

To determine which genes have aberrant expression in PE, we sought to first better understand whether PE is one disease or many and then to look for genes misregulated across all PE subtypes. We compared placenta and decidua samples from 24 PE patients and 22 healthy women. Nineteen (19/24) PE placental samples generated three distinct molecular groups of PE (PE_P1-3) (**Fig. 1A**), while control samples (16/22) generated two groups (C1, C2). Five (5/24) PE and four (4/22) healthy placental samples grouped with the opposite cluster. While placental PE_P1 and PE_P3 contain samples from early-onset PE, PE_P2 included samples mainly from late-onset PE. We did not reveal any significant grouping for the IUGR phenotype. An alternative clustering method based on Euclidean distances, identified the same three PE clusters (**Fig. S1A**). In contrast to placental samples, the PE and control decidua samples appeared to scatter randomly (**Fig. S1B**), supporting that clinically established PE is a placental, and not a decidual disorder. The "negative" result for the decidua in addition provides a negative control for false/artefactual clustering.

A subset of early onset preeclampsia correlates with clinical symptoms

Is there an association between the PE gene clusters and clinical data? We clustered 36 Patient`s clinical and biomarker parameters on their relative values (**Table S1**)**.** Our analysis suggests that gene expression profile can be related to clinical disease phenotyping, but only to a certain extent (**Fig. 1B** and **Fig. S2A, B**). Besides a related transcriptome, PE_P1 also shared a similar clinical profile. By contrast, most of the patients from PE_P2 generated a cluster together with PE samples that had diverse transcriptome, and patients from PE_P3 had a diverse clinical manifestation.

We also performed correlation analyses of clinical and gene expression data across all Control and Patient samples to identify genes associated with any of the clinical phenotype. We found correlated gene expression with maternal PlGF (e.g. *PAPPA2*, *SPAG4*, *ENG* and *LEP)* and sFlt1 levels (e.g. *SPAG4*, *ENG*, *ANKRD37* and *ERRFI1)*. Among the imprinted genes, the expression of both DLX5 and GATA3 correlated with diastolic BP and anti-correlated with GA (**Fig. S2C**). DLX5 also correlated with sEndoglin (positive) and with baby weight (negative) markers.

All preeclamptic clusters have disturbed guidance signalling

We then analyzed differentially expressed genes (DEGs) in each PE cluster, compared to control samples. Each cluster contained over a thousand unique DEGs with 366 dysregulated genes common to all three clusters, (p-value < 0.05) (**Fig. 1C** and **Table S2**). To characterize the clusters, and find possible interactions, datasets containing 3525, 2634 and 4073 DEGs corresponding to PE clusters PE_P1, PE_P2, and PE_P3 respectively, were analyzed using Ingenuity Pathway Analysis (IPA) and GOrilla gene ontology tools. *Axonal guidance* was the top pathway in all three PE clusters, supporting the view that disturbed angiogenesis and cell-cell communication between endothelial cells and the trophoblasts via the mechanism of axonal guidance is a common feature of PE[23, 24]. Other than this universal property, the three clusters exhibited unique features (**Fig. S3** and **Table S2**).

Imprinted genes exhibit differential expression in preeclamptic placenta

Next, we focused on differentially expressed imprinted genes in PE. We used a merged list of genes of the Geneimprint database (184 validated and putative human imprinted genes) and the recent list of imprinted human placental genes (223, maternal:paternal allelic expression ratio, 80:20)[20] (**Table S3**). Our analysis identified 150 imprinted/putatively imprinted genes that were

dysregulated in at least one of the three PE clusters (p-value <0.05) (**Fig. 1D** and **Table S3**). Maternally and paternally expressed genes (MEGs and PEGs) were affected according to their relative commonality (23%, 59/257 vs 17%, 25/150; χ^2=1.9, p-value=0.16) (**Fig. 1F** and **Table S3**). This was observed also when M/P allelic expression ratios were considered at different stringencies (**Fig. 1G**). The mean expression of MEGs was however significantly downregulated compared to PEGs (**Fig. 1H**). Nevertheless, among the most significantly deregulated genes we identified both MEGs (e.g. *DLX5, APOBEC2*, *CD74*) and PEGs (e.g. *GATA3*, *CYP2J2*) (**Fig. 1E, F**). In our study, the two most significantly dysregulated imprinted genes were *GATA3* and *DLX5* (p-values <$2.51x10^{-6}$) and <$3.65x10^{-9}$, respectively). Further, we focus on *DLX5*, which is a MEG in human lymphoblasts and brain[25], but its imprinted status in the placenta is not yet explored.

DLX5 is upregulated in preeclamptic placenta

We used qRT-PCR to confirm the upregulation of *DLX5* in PE placental samples (**Fig. S4, B**). We then confirmed these findings in a second independent patient cohort of 56, compared to 28 controls. Although *DLX5* was significantly upregulated in both early-onset (p-value<0.0001) and late-onset (p-value<0.01) PE (**Fig. 2A**), and in all the three PE clusters, its expression level varied between placental samples. Nevertheless, altogether 69% of PE samples (n=56) could be associated with *DLX5* overexpression. We also confirmed increased DLX5 protein expression in PE placenta tissues by Western blotting (**Fig. S4C, D**). Correlation analysis of gestation age (GA) to *DLX5* expression in Control or PE placentas excluded GA-related changes in *DLX5* differential expression (**Fig. S4E**). In tissues and cells derived from a pregnancy-related tissue panel *DLX5* expression was detected in placenta and in trophoblasts, but was less pronounced in the decidua (**Fig. S4F, G**). Immunofluorescent staining detected co-expression of DLX5 with

cytokeratin-7 (CK7), a trophoblast-specific marker (**Fig. 2B**). Immunohistochemistry confirmed elevated DLX5 protein expression in placental tissues of early-onset PE (**Fig. 2C**).

To associate our findings with clinical PE biomarkers, we compared the placental expression of *DLX5* with the "anti-angiogenic" sFlt1 and sFlt1/PlGF ratio and with "pro-angiogenic" PlGF concentration in maternal serum[26]. While there was no significant correlation between *DLX5* expression and sFlt1 levels, we observed a negative correlation (r=-0.35; p-value=0.017) with PlGF concentrations, comparing PE and healthy woman (**Fig. 2D**). This observation suggests that placental *DLX5* expression is associated with decreased levels of a maternal circulating placental biomarker in PE.

Loss of imprinting results in elevated gene expression of *DLX5*

To investigate if alterations in the predicted imprinting status of *DLX5* could be responsible for its overexpression in the PE placentas, we performed a LOI assay[27]. We measured expression of the silenced allele in placental samples carrying the heterozygous *DLX5* SNP (rs73708843). Primers are provided in Table S4. Of 97 placental tissues, 42 were genotyped as heterozygous (43.3%), including 16 control (16.5%) and 26 PE (26.8%) placentas. The mean expression of the putatively inactive *DLX5* allele was 58% in PE placenta samples when setting the expression of the non-imprinted allele for each individual sample 100%. Control samples also exhibited LOI but with significantly less frequent (19%) activation of the imprinted *DLX5* allele (**Fig. 3A, B**). Sequencing of cDNA through the SNP (rs73708843) on three placenta samples confirms the single allelic expression from *DLX5* (**Fig. 3C**). Importantly, we found a correlation (r=0.314; p-value=0.046) between LOI and *DLX5* expression, suggesting that the overexpression phenotype of *DLX5* was associated with its LOI (**Fig. 3D**). We also inspected CpG methylation levels at the *DLX5* locus in PE placental compared to healthy control samples (20 vs 20)[28], and identified

significant CpG hypomethylation in PE samples (**Fig. 3E** and **Table S5**). Furthermore, the methylation level of several CpGs inversely correlated with *DLX5* expression in these samples (8 vs 8) (**Fig. 3F**). Collectively, we interpreted our data as the altered methylation at the *DLX5* locus in PE results in LOI, and affects gene expression.

Upregulated *DLX5* affects genes associated with cell growth, proliferation, survival and movement

To decipher the physiological effect of elevated DLX5 expression on trophoblasts, we stably overexpressed the human DLX5 protein in trophoblast cells *in vitro*. For the overexpression studies, we used the *Sleeping Beauty* transposon-derived expression system[29] in SGHPL-4 cells, derived from first trimester extravillous trophoblasts (**Fig. S5A-C**). Immunohistochemistry revealed a predominant nuclear localization of DLX5 in the DLX5-overexpressing SGHPL-4 cells ($DLX5^{High}$) (**Fig. S5D**). To observe the global effects of elevated DLX5 expression, we performed microarray transcriptome profiling of $DLX5^{High}$ and control (Control) cells. A total of 3650 DEGs (p-value<0.05; 771 genes at FDR<0.05) were identified upon DLX5 overexpression (**Fig. S6A** and **Table S6**). IPA revealed significant gene enrichment involved in cardiovascular system development and function. The most significant terms describing molecular and cellular functions include cellular growth and proliferation, cell death and survival or cellular movement and development (**Fig. S6B**). The top pathways include *interferon-*, *death receptor signalling* and *superpathway of cholesterol biosynthesis*. Importantly, several affected pathways are common between PE placental samples and the *in vitro* model. These include deregulated *axon guidance-, IL-8-, neuregulin receptor signalling*, *TR/RXR, RAR* and *PCP pathway, antigen presentation pathway*, *unfolded protein response* and *NRF2-mediated oxidative stress responses* (**Table S6**).

Elevated expression of *DLX5* models certain aspects of preeclampsia

To test how well our DLX5^{high} *in vitro* model mimics global transcriptional changes in PE, we compared transcriptome profiles of Control and DLX5^{high} cells with the three placental PE transcriptome clusters (PE_P1-3). Hierarchical clustering of relative gene expression levels of Control, DLX5^{high} lines (6 vs 6), PE_P1-3 and healthy placenta samples revealed that the transcriptome of DLX5^{high} cells clusters with the three PE groups (PE_P1-3), whereas Control cells cluster with control placenta samples (**Fig. 4A**). Additionally, we asked if it was possible to correlate transcriptomes according to their DLX5 expression levels (**Fig. S7A**). Placenta samples were ordered according to their DLX5 expression levels. Importantly, the transcriptomes of DLX5^{high} PE clustered with cultured DLX5^{high} samples, while the low_DLX5 PE were clustered with Control SGHPL-4 cells, suggesting that the overexpression of DLX5 in trophoblasts could model certain aspects of PE (**Fig. S7B**).

Identifying genes with correlated expression dynamic to DLX5

As the target genes of the transcription factor DLX5 in placenta are not known, we thought to identify genes whose expression is correlated with DLX5. Thus, we subjected a merged dataset of our two microarrays (total 58 samples) to weighted gene correlation network analysis (WGCNA). We aimed at identifying gene modules of correlated gene expression. This approach allowed us to detect several gene modules containing a total of 3,000 genes. Using pairwise ranked correlation analysis of 79 genes, associated with DLX5 across 58 samples (**Fig. 4B**), we identified putative targets of DLX5. The list contains several genes, previously associated with PE[30-35], including genes involved in cell growth, proliferation and differentiation (e.g. *GREM2*, *KIT*, *ERRFI1*), angiogenesis (e.g. *VEGFC*, *PAPPA2, GPR126*), cytokine and growth hormone signalling (*GBA, CXCR7*), immune response (*ISG15*, *HERC5*, *IFIT1*), pregnancy specific

proteins (*PSG2-4*), as well as the paternally imprinted, tissue factor pathway inhibitor-2 (*TFPI2*), involved in regulation of cell invasion and proliferation.

Upregulation of *DLX5* in trophoblasts is associated with disturbed epigenetics

To address the limitations of our initial approach using placental microarray data, we performed transcriptome analysis employing RNAseq on freshly isolated, purified human trophoblasts from control and PE placentas (5 vs 5). In the PE_T1 trophoblast sample, DLX5 was highly upregulated, and we observed 1466 genes commonly dysregulated in both PE_T1 and DLX5^{high} with 641 genes exhibiting the same pattern of expression, including *KIT*, *SOCS2*, *KLF5*, *BEX2*, *ERRFI1*, as well as DNA methyltransferases (*DNMT1* and *DNMT3B*) (**Fig. 4C**). In addition to common features, PE_T1 sample exhibited further DEGs involved in the regulation of DNA methylation and histone modification, such as TET gene family (*TET1-3*), *SETDB1*, *SIRT1* and HDACs, indicating that a subset of PE might be associated with severe epigenetic disturbances. As a likely consequence, PE_T1 sample is characterized by the deregulation of several imprinted genes (including *DLX5*), but also potentially mutagenic transposable elements, such as LINE-1 and SVA[36, 37] (**Fig. 4D, E**).

Upregulation of *DLX5* reduces trophoblast proliferation

The significant increase of DLX5 expression in PE placenta samples prompted us to explore the possible mechanism of DLX5 in the pathogenesis of PE. To characterize the DLX5^{high} phenotype we have performed several cellular assays, inspired by the Pathway analyses. To determine whether the DLX5^{high} phenotype affects trophoblast proliferation, we used time-lapse microscopy, and observed reduced cell proliferation of DLX5^{high} cells by 45%, compared to control after 48h incubation (**Fig. 5A**). Significantly, reduced trophoblast proliferation of DLX5^{high} was confirmed by a high-throughput sampler cell count and microtiter plate test (MTT)

colorimetric assay (**Fig. 5B, C**). DLX5 overexpression had only a slight, not significant effect on cell apoptosis as indicated by scoring apoptotic cells within 48h of incubation (**Fig. S8A, B**).

Elevated *DLX5* expression affects the metabolic profile of the trophoblast

Cell proliferation, growth and metabolism are tightly linked processes. To determine whether reduced proliferation was associated with altered metabolism, we monitored metabolic parameters in DLX5^{high} cells. We determined extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), indicators of mitochondrial respiration and glycolytic activity, respectively (**Fig. 5D-F**). Compared to control, metabolic profiling detected elevated level of ECAR and maximal OCR values, suggesting an accelerated metabolism of DLX5^{high} cells (**Fig. 5E, F**). Furthermore, DLX5^{high} cells displayed increased spare respiratory capacity (SCR) values, when compared to Control cells (**Fig. 5E**). In principle, the increased energetic demand could reflect a response to increased stress or cell survival challenges.

***DLX5* expression responds to endoplasmic reticulum stress**

Abnormal placentation in PE results in a series of biological stresses. To investigate the potential role of DLX5 in stress response, we monitored reactive oxidative species (ROS) production and the effect of induced endoplasmic reticulum (ER) stress. While, we did not detect elevated ROS production in DLX5^{high} cells (**Fig. 5G**), DLX5 expression was sensitive to induced ER stress in BeWo choriocarcinoma cells, expressing DLX5 at a readily detectable level. In a hypoxia-reoxygenation challenge assay, DLX5 expression increased significantly upon ER stress in a severity-dependent manner (**Fig. 5H** and **Fig. S9A**). Furthermore, in our DLX5^{high} transcriptome, we observed upregulation of several genes involved in the unfolded protein response pathway (UPR), associated with ER stress response (**Fig. S9B**). Importantly, eight of these genes, *INSIG1*, *SREBF1*, *HSP90B1*, *ATF6*, *MBTPS1*, *PPP1R15A*, *XBP1*, *HSPA2* were also dysregulated in our

PE placenta samples (**Fig. S9C**). While enhanced DLX5 level appears to trigger the cellular stress response, DLX5 expression increased with syncytium formation as evidenced by Forskolin treatment of BeWo cells, suggesting a potential role of DLX5 during the syncytialization process (**Fig. S9D**).

***Dlx5* expression shifts from post-implantation to pre-implantation stage of embryogenesis during evolution**

DLX5 is primarily known as a transcription factor regulating morphogenesis and tissue homeostasis[38-40], and it is mostly characterized during post-implantation embryogenesis. As we observed *DLX5* is expressed in trophoblast, we sought to monitor its expression pattern in pre-implantation embryos. We performed comparative single-cell RNAseq data analysis on mouse, macaque and human embryos collected at early embryonic developmental stages[41-43]. In human, the expression of *DLX5* appears at the transition from E4 to E5 stage (**Fig. 6A**), rendering *DLX5* one of the earliest expressed genes in the human trophoectoderm (**Fig. 6B**). Curiously, *Dlx5* expression is shifted toward late trophoectoderm in macaque (**Fig. 6C**) and is not detectable in murine pre-implantation embryos (**Fig. 6A**). To check if *Dlx5* is expressed at later stages of placenta development in mice, we performed placental immunostaining (E14.5 and E15.5) in Dlx5-LacZ$^{+/-}$ animals. Although we observed a weak positive LacZ staining in the Dlx5-LacZ$^{+/-}$ animals on the external muscular layer of the placenta, the signal was not significantly different from the control (**Fig. S10**), suggesting that *Dlx5* is not involved in murine placentation. Surprisingly, only 33 common trophectoderm marker genes could be identified between mouse and human. In human, among the early TE markers, *DLX5* exhibits the highest activation of expression, followed by *RGS13*, *NDRG2*, *ODAM*, *SLC38A1* as well as *ID3*, *HAND1*, *DLX3*, *GCM1* (**Fig. 6D**). Among the genes expressed differentially in human vs mouse preimplantation

embryos, *GREM2*, *GPR126*, *USP53* and *EFNB3* are putative targets of DLX5 (**Fig. 5B**). Intriguingly, *GREM2*, *GPR126*, *USP53* are also upregulated in PE (**Fig. 1B**), suggesting that the dysregulation of maternally expressed DLX5 and its putative targets might explain certain features of the human-specific nature of PE**.** The genes expressed in the same clusters might share transcriptional networks (**Fig. 6E, F** and **Table S7**). Interestingly, the maternally, but not the paternally expressed genes form characteristic clusters during human embryogenesis.

Discussion

There certainly has been much speculation that disturbed regulation of imprinted genes might be involved in the development of PE, however prior evidence could not establish significant association between them[14-19]. Here we provide robust evidence for a mechanistic coupling between PE and disturbed placental imprinting. Our experimental strategy first aimed at identifying differentially expressed genes (DEGs) in PE placentas by analysing genome-wide molecular data on well-characterized patient material. The list of DEGs was than intersected with the current catalog of human imprinted placental genes. Employing the novel set of genes could clarify certain important issues regarding the long-term debated list of imprinted genes in the human placenta. Our strategy revealed several potential candidates, supporting the hypothesis that disturbed imprinting and PE could be indeed associated. Our candidate list included imprinted genes that were previously associated with PE, however their expression deregulation could not be convincingly connected to LOI[15]. *CYP2J2* and *CD74* belonged to a category of genes whose deregulation was already implicated in PE, but their epigenetic disturbance was not considered as a contributing factor[21, 22]. Our strategy also identified genes that were not yet implicated in PE, and their imprinted status is poorly explored in placenta (APOBEC2, *GATA3*,

DLX5). Curiously, APOBEC2, an enzyme involved in controlling DNA-based parasites, such as viruses and transposable elements, appeared on the list of MEGs deregulated in all three clusters of PE. The most significantly affected imprinted genes in PE were *GATA3* and *DLX5*. *GATA3* could be an excellent candidate for further research, as it was previously reported to inhibit trophoblast invasion[44], thought to be a key process in PE. Here, we focus on *DLX5*, a transcription factor of the Distal-less homeobox protein family. *DLX5* is involved in developmental processes of the limb, brain and bone in both mice and human[38, 45]. However, its placental function is not characterized.

We show that *DLX5* is expressed in human villous and extravillous trophoblast, and is controlled by imprinting. *DLX5* is upregulated in ~70% of PE patients. The upregulation of *DLX5* in PE is associated with its "leaky" expression from the imprinted allele (LOI). In contrast to previous studies[27, 46], we find a correlation between expression of an imprinted gene and its LOI in PE. Our data mining[28, 47] also reveals differential CpG methylation of the *DLX5* locus in PE placentas. Although our analysis does not rule out other mechanisms of DLX5 expression regulation, such as transcriptional or microRNA regulation, we provide evidence of an association between disturbed imprinting gene expression and PE.

The spatial and temporal regulation of cell proliferation and differentiation is crucial for successful pregnancy. The first half of gestation is characterized by a series of trophoblast proliferation and differentiation processes, building mature villi and extravillous structures[48, 49]. Upregulation of DLX5 resulted in reduced (~45%) proliferation of the trophoblast. Curiously, the decreased trophoblast cell proliferation was accompanied by increased oxygen consumption. Why might poorly proliferating trophoblast cells require an accelerated metabolism? We hypothesized that DLX5 overexpression could sensitize trophoblasts to stress. As a result, the

cells require increased metabolic activity to overcome this state-of-affairs. Indeed, the transcriptome analysis of DLX5^{high} trophoblast revealed several affected pathways acting as stress inducers, such as *unfolded protein response pathways,* increased *interferon-*, and *death receptor signalling.* Thus, the upregulation of *DLX5* could be a factor contributing to an accelerated placenta "ageing" process and elevated ER stress, resulting in stressed syncytiotrophoblast and consequently increased shedding of inflammatory factors into the maternal circulation[50, 51]. While enhanced DLX5 level triggers the cellular stress response, DLX5 expression increases with syncytium formation, suggesting a role of DLX5 in regulating the syncytialization. Our single cell transcriptome data mining of human preimplantation embryos establishes DLX5 as a key marker of trophectoderm differentiation. We propose that DLX5 is involved in regulating a delicate balance between proliferation and differentiation processes of the trophoblast. A disturbance of this key process has been previously associated with PE[52, 53].

In contrast to trophoblasts, *DLX5* overexpression is associated with enhanced cell proliferation in various cancer cells[54-56]. Thus, *DLX5* might affect proliferation either negatively or positively during early development or in cancer, respectively. The response to overexpressed DLX5 possibly depends on cell type specific target genes. Either way, DLX5 appears to be a key gene in determining the developmental decisions of trophoblast cells.

We modeled the effect of DLX5 upregulation in an *in vitro* system, overexpressing DLX5 in SGHPL-4 trophoblast cells (DLX5^{high}). Since artificial, exogenous overexpression of a gene in cells could alter normal cellular function due to accumulation of unprocessed proteins, we asked how faithfully DLX5^{high} cells mimic PE. Importantly, the transcriptome of DLX5^{high} cells resembled that of the PE transcriptomes, and several dysregulated pathways were commonly seen both *in vitro* (DLX5^{high}) and *in vivo* (PE samples). Thus, the DLX5^{high} phenotype can model

several features of PE *in vitro*, in a cell culture system, signifying the impact of the deregulated DLX5 in the pathogenic phenotype of PE.

Given the barriers put towards analysis of PE by its human specificity, our *in vitro* model system has a considerable potential for downstream analyses. Nevertheless, our study does not suggest that there could be a single explanation to PE. We identified three distinct transcriptomic clusters of PE placenta (PE_P1-3). That the clusters could be related to previously established categories of PE, such as early-/late-onsets of PE[57], supports the view that PE is a heterogeneous placental disease, and does indeed come in several discrete forms. While PE_P2 matches late-onset PE placentas, PE_P1 and PE_P3 can be considered as subdivisions of early-onset PE. The uncovered heterogenic nature of PE would call for validating a panel of subclass-specific biomarkers for future diagnostic procedures. The DLX5 overexpression phenotype is detectable in all the three clusters, but is most pronounced in PE_P1/2. Levels of *DLX5* correlated with placenta-derived PlGF circulating biomarker. Whether DLX5 will have a utility as a biomarker is unclear as its loss of imprinting was not observed in all instances (69% of PE). Importantly, while the PE_P2/3 clusters have no clear clinical pattern, PE_P1 patients exhibit characteristic clinical phenotypes. Nevertheless, more samples need to be analyzed to securely relate the characteristics of the PE_P1 cluster to clinical disease phenotyping.

Our RNAseq data analysis revealed that a subset of PE is connected to disturbed epigenetic gene regulation. The global epigenetic turmoil is likely associated with the observed differential expression of genes regulating DNA methylation, resulting in the deregulation of transposable retroelements (REs) and imprinted genes. Curiously, the mechanisms of regulating imprinting and repressing REs by DNA methylation share several common features[58]. In fact, genomic imprinting is speculated to be a by-product of the genome's defense mechanisms

against retroviruses and REs[59, 60]. Curiously, a domesticated RE-derived gene (Syncytin-1), implicated to have a key role in placental development[61-63] has been associated with PE[64, 65]. Here, in a subset of PE, we observed the reactivation of the human-specific L1_HS and SVA-F elements, capable of transposition in the human genome[37, 66].

Besides trophoblast, dysregulation of DLX5 expression in other tissues has been reported to contribute to diseases. Downregulation of DLX5 in endometrial glands could also complicate early stages of pregnancy that could later manifest in IUGR or PE[67]. LOI of the maternally expressed DLX5 in lymphoblastoid cells contributes to Rett syndrome (RTT), a disorder associated with GABAergic dysfunction[68, 69]. Dysregulation of DLX5 impairs the differentiation of GABAergic neurons[70]. Curiously, GABA can increase hCG secretion in human placenta[71], indicating possible placenta–brain endocrine interactions regulated by imprinting. By contrast to Rett syndrome, we do not detect differential expression of DLX6 in PE, suggesting that the dysregulation of DLX5 in PE is not associated with expressional changes of DLX6 (not regulated by imprinting). We propose that DLX5 and DLX6 are regulated differently in brain and placenta.

DLX5 is expressed in the human, but not in mouse trophoblast. Comparative single cell transcriptome analysis revealed a differential expression of DLX5 between human, macaque and mouse preimplantation embryogenesis, highlighting the diverged cellular function of DLX5 during mammalian embryonic evolution. While the *DLX5* gene is highly conserved across different mammalian species (>95% exon sequence similarity in human, macaque and mouse), its upstream, 2-10kb (potential regulatory) region is much faster evolving (>80% and <10% sequence similarity between human vs macaque and between human vs mouse, respectively). Indeed, despite of the conserved coding structure, DLX5 expression appears to be gradually

shifted toward earlier developmental stages during mammalian evolution. While DLX5 is not expressed in preimplantation embryos in mice, its expression peaks at the stage of TE and ICM/epiblast separation, marks TE committed cells, and is regulated by imprinting in human. In addition to DLX5, we could identify further differentially expressed genes between human and mice TE. Among these genes, *GREM2*, *GPR126*, *USP53* and *EFNB3* are also putative targets of *DLX5,* suggesting that the function of a *DLX5* regulated circuitry has been redefined during mammalian evolution. Importantly, *GREM2*, *GPR126*, *USP53* are also upregulated in PE, and thus might be also associated with the human-specific nature of PE.

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Disclosures

None.

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Figures Legends

Figure 1. Gene expression analysis of preeclamptic and healthy placenta samples.

(A) Hierarchical clustering analysis of microarray data identified three PE groups in placenta: PE_P1 (blue), PE_P2 (yellow), and PE_P3 (green) and two control groups: C1 and C2 (gray, dashed). (Control placenta n = 22, PE placenta n = 24). The onset of PE (by gestational age at delivery) for each sample is indicated. The PE+IUGR samples are indicated by a star (*). **(B)** Clustering analysis of clinical data - Heatmap representing pairwise correlation between Patients based on the clinical data (Suppl. Mat, Table S1). Hierarchical cluster dendrogram was calculated using ranked correlation and complete linkage method on relative values from the clinical variables (n = 36). Height of dendrograms represents the Euclidian distance. **(C)** Venn diagram of differentially expressed genes (DEGs) in three PE clusters (DEG on p-value < 0.05). Top up- (red) and downregulated (blue) genes for each cluster are shown. **(D)** Imprinted genes in PE. Heatmap representing differential expression of imprinted genes in the three PE clusters vs controls (DEG p-value < 0.05). Log_2-fold change of differential expression of maternally **(E)** and paternally **(F)** expressed genes (MEGs and PEGs), in all PE cases (DEG p-value < 0.05). Colored are genes with log_2-fold change ≥ 0.5 (red) and ≤ -0.5 (blue). **(G)** Distribution of differentially expressed MEGs and PEGs in PE according to different criteria for maternal to paternal allelic expression ratio (M[%] – P[%]): 60-40, 70-30, as well as 90-10. **(H)** Wilcoxon test on the mean log_2-fold change expression of differentially expressed MEGs and PEGs in PE.

Figure 2. DLX5 is upregulated in PE placenta.

(A) Quantitative PCR confirmed increased DLX5 mRNA level in placentas of a second PE cohort (values are as mean ± SEM; Control: 1.053 ± 0.0745, n = 28; EO-PE+IUGR: 1.447 ± 0.066, n = 9; LO-PE+IUGR: 1.71 ± 0.238, n = 5; EO-PE: 1.532 ± 0.08, n = 20; LO-PE: 1.304 ± 0.09, n = 22) (*p < 0.05; **p < 0.005; ***p < 0.0005; ANOVA, Bonferroni's multiple comparisons test). **(B)** Double immunofluorescence staining of term human placenta tissue indicated nuclear expression and co-localization of DLX5 to the cytokeratin 7 (CK7), a trophoblast-specific marker. The DLX5 staining was positive in the nucleus of both villous and extravillous trophoblasts. **(C)** Immunohistochemistry staining on placental villous tissue from healthy pregnancy (Early Control, GA 31 and 34 weeks) and PE (GA 31 and 34 weeks) for human DLX5 confirmed increased DLX5 protein expression in early-onset PE placenta. **(D)** Correlation of *DLX5* expression and placental biomarker. Placental *DLX5* expression significantly negatively correlated to the serum PlGF in the PE group but not in controls. (Control n = 27, PE n = 48, Spearman rank correlation).

Figure 3. Loss of imprinting (LOI) of DLX5 in PE placenta.

(A) Analysis of mean LOI levels for DLX5 in healthy and PE placenta samples. Values are presented as a mean ± SEM of LOI (Control: 0.1943 ± 0.04765, n = 16; Early-onset PE: 0.5661 ± 0.08516, n = 12, Late-onset PE: 0.6349 ± 0.08437, n = 14) (**p < 0.001, ***p < 0.0001; one-way ANOVA, Bonferroni's multiple comparisons test). **(B)** Distribution (number) of DLX5 heterozygocities exceeding particular LOI. **(C)** Allelic expression analysis of the imprinted DLX5 in placenta samples exhibiting the allele-specific expression, but no LOI. cDNA of the heterozygous placenta samples for the SNP (rs73708843) were sequenced. **(D)** LOI correlated

with DLX5 expression in placenta (p = 0.046, Spearman rank correlation). **(E)** CpG methylation of DLX5 locus. Log_2-fold change of CpG methylation level in PE (Control, n = 20; Early-onset PE n = 20). Hypomethylated CpG sites are shown (DMR at adj.p-value ≤ 0.05). **(F)** Pairwise Spearman rank correlation of CpG methylation and DLX5 expression in placenta (Control, n = 8; Early-onset PE n = 8; FDR p-value: *p < 0.05, **p < 0.01).

Figure 4. Intersection of PE transcriptomes with the DLX5^{high} transcriptome.

(A) Hierarchical clustering (Spearman rank correlation, average linkage) and bootstrapping (1000 replicates) of the transcriptomes of SGHPL-4 cells with PE Cluster PE_P1, PE Cluster PE_P2 and PE Cluster PE_P3 transcriptomes (Placenta PE) and control placenta samples (Placenta control). The equal number of control placenta samples to the number of samples in each PE cluster was chosen randomly. **(B)** Weighted gene co-expression network analysis (WGCNA) across 58 samples gave several modules containing a total of ~ 3000 genes. Identification of DLX5 target genes in placenta and trophoblast cells. Clustered pairwise correlation matrix of identified 79 genes across 58 samples (Spearman rank correlation, threshold 0.6 and -0.55, p-value < 0.05, Euclidian distance). **(C)** A comparison between the log_2-fold change of the differentially expressed genes in trophoblast sample PE_T1 and genes differentially expressed upon DLX5 overexpression in SGHPL-4 trophoblast cell line. 641 genes heaving the same differential expression pattern were common in both datasets. **(D)** and **(E)** Relative expression of transposable LINE1 and SVA elements in control trophoblast samples and PE sample PE_T1. In PE_T1 the young members of the TE families L1PA3, L1PA2, L1HS, SVA-E and SVA-F are upregulated (*p < 0.05, ***p < 0.005, Kolmogorov-Smirnov (KS) test, Benjamini and Hochberg's (BH) false discovery rate (FDR)).

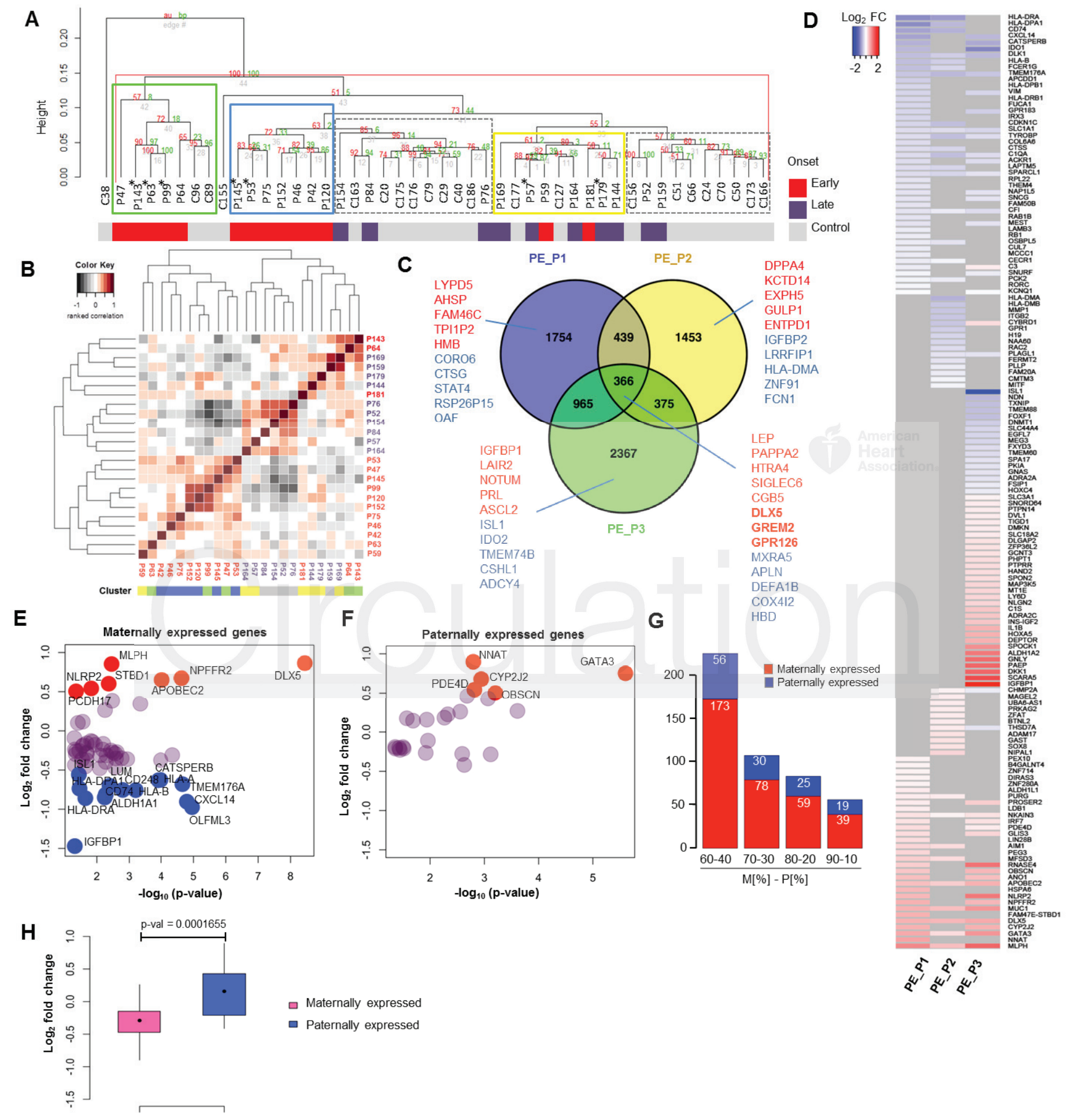
Figure 5. DLX5 decreases SGHPL-4 cell proliferation.

(A) DLX5^{high} cells are less proliferative compared to WT cells as indicated by scoring dividing cells over 48h of incubation. After 48h of incubation cell proliferation of DLX5^{high} cells (39.58 ± 5.34) is reduced by 45% as compared to WT (72.92 ± 4.49) (***p = 0.001, ****p < 0.0001; 2-way ANOVA, Bonferroni's multiple comparisons test). TNFα at conc. 30ng/ml slightly decreased cell proliferation in both DLX5^{high} and WT cell (not significant). **(B)** DLX5^{high} cells exhibited decreased cell proliferation (n = 6, median 524.5, IQR: 343.8-843) as compared to WT (n = 6, median 1320, IQR: 679.8-2955) confirmed by cell count HTS assay (***p < 0.001; Mann Whitney test). **(C)** MTT viability assay confirmed decreased cell proliferation in DLX5^{high} cells (DLX5^{high} median 0.1883, IQR: 0.0454-0.242 vs. WT median 0.3319, IQR: 0.2226-0.3565) (****p < 0.0001; Mann Whitney test). **(D)** Effect of DLX5 on mitochondrial respiration in SGHPL-4 cells. OCR was measured under basal conditions followed by the sequential addition of oligomycin (0.75µM), FCCP (1µM), and antimycin A (1µM) + Rotenone (0.1µM) in WT (n = 6) and DLX5^{high} cells (n = 6). Data is normalized to the cell number. **(E)** Individual parameters for basal respiration (WT vs DLX5^{high}: 43.901 ± 1.705 vs 66.082 ± 3.213), maximal respiration (WT vs DLX5$^{high\ H}$: 109.226 ± 3.913 vs 162.244 ± 6.431), ATP production (WT vs DLX5^{high}: 34.266 ± 1.105 vs 46.415 ± 2.2), proton leak (WT vs DLX5^{high}: 9.635 ± 1.307 vs 20.85 ± 1.687), non-mitochondrial OCR (WT vs DLX5^{high}: 18.72 ± 2.989 vs 34.247 ± 5.773) and reserve capacity (WT vs DLX5^{high}: 65.325 ± 3.381 vs 90.866 ± 5.455) were extracted from the assay (*p < 0.05, ***p < 0.001, ****p < 0.0001; 2-Way ANOVA, Bonferroni's multiple comparisons test). **(F)** Mean basal ECAR level in WT and DLX5^{high} cells (DLX5^{high} median 26.27, IQR: 24.27-36.35 vs. WT median 22.38, IQR: 17.05-26.99) (**p = 0.01; Mann Whitney test). **(G)** ROS production in WT and DLX5^{high} cells measured by FACS. Data is presented as a mean

fluorescent intensity (MFI) of the fluorescent signal from the dichlorodihydrofluorescein (DCF) oxidized by ROS. DCFH-DA diffuses into the cell, becomes deacetylated by cellular esterases to non-fluorescent DCFH, which is next oxidized to fluorescent DCF by ROS. **(H)** DLX5 expression level upon induction of ER stress in BeWo cells. 20N – normoxia, 5/20 HR and 1/20 HR - cyclic condition of 6 hour of incubation in 5%/20% O_2 and 1%/20% O_2. Quantification of DLX5 level upon induction of ER stress indicates significant upregulation of its expression (*p < 0.01; Kruskal-Wallis test, Dunn's multiple comparisons test).

Figure 6. DLX5 expression in human, macaque and mouse early embryonic development. **(A)** Violin plots display the expression levels of *DLX5* during different stages of human, macaque and mouse early embryonic development. In humans, *DLX5* starts to be expressed at E4/E5 stage of development. In macaque, *DLX5* is expressed at E6 stage. *Dlx5* is not expressed in mouse pre-implantation embryo. **(B)** Single cell transcriptome analysis of human pre-implantation embryo reveals TE-specific *DLX5* expression. t-SNE analysis on human 353 single cells from E4-E5 stages where inner cell mass (ICM) and trophectoderm (TE) split from Morulae. We defined clusters of cell populations to identify genes expressing exclusively in those clusters. We defined the cell type for each cluster according to the known markers. **(C)** Single cell transcriptome analysis of macaque pre-implantation embryo demonstrates enriched *DLX5* expression in the late TE (*****p = 2.602E-12, ****p = 1.387E-06; Wilcoxon test). **(D)** Comparative analysis between human and mouse TE markers. For human TE markers, shown are genes with log_2-fold change > 3. ICM – Inner cell mass, PE – Primitive endoderm, TE – Trophectoderm. Single cell transcriptome analysis of human embryogenesis for maternally **(E)** and paternally **(F)** expressed genes. Clustering analysis of imprinted genes expressed during

early (oocyte, zygote, 2-cell and 4-cell stage), mid (8-stage, Morulae) embryogenesis and late blastocyst (179/257 maternally expressed genes, 86/150 paternally expressed genes).


A
Height
Onset
Early
Late
Control
B
Color Key
ranked correlation
Cluster
C
PE_P1
PE_P2
PE_P3
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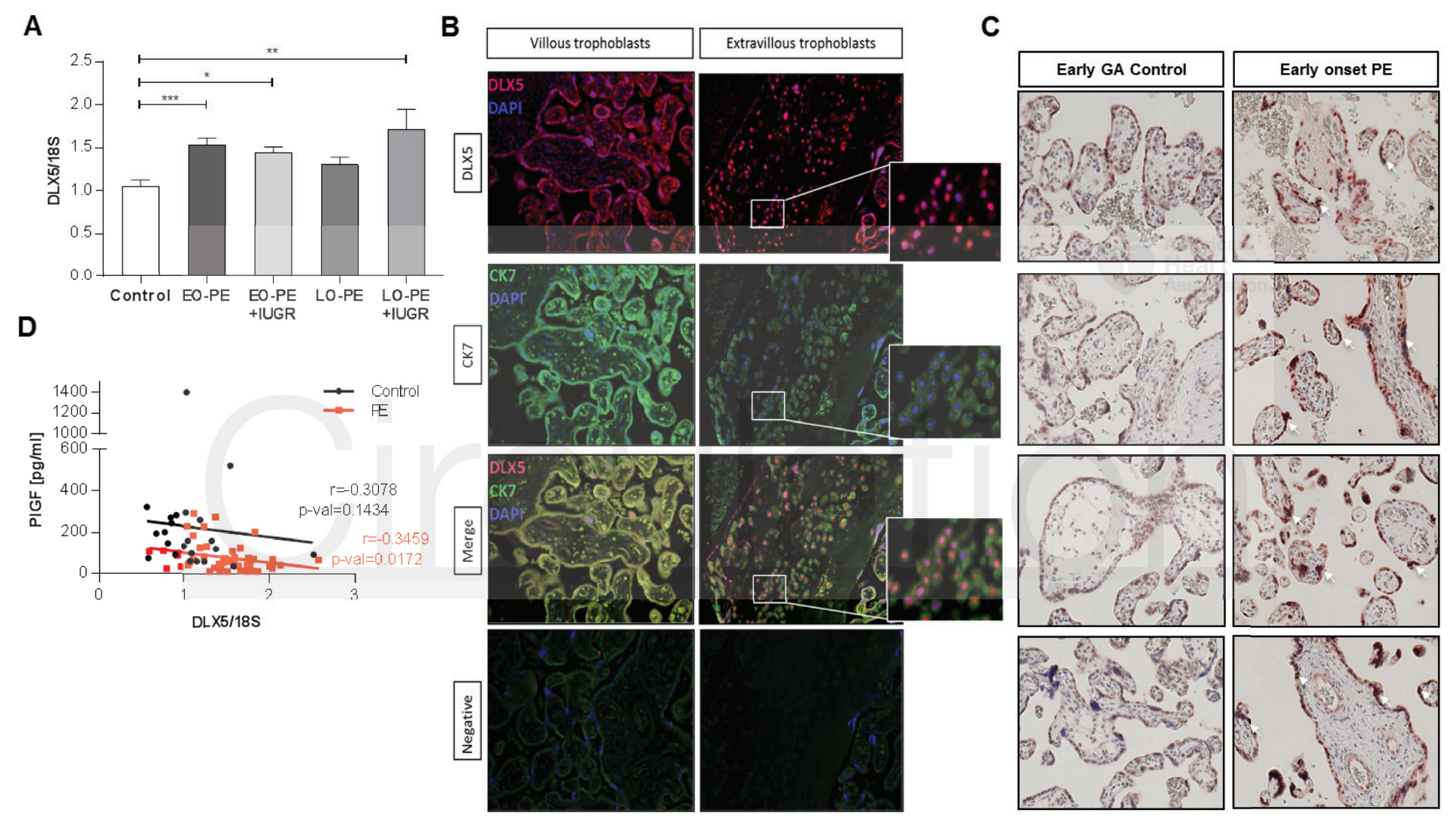
1453
366
965
375
2367
LYPD5
AHSP
FAM46C
TPI1P2
HMB
CORO6
CTSG
STAT4
RSP26P15
OAF
DPPA4
KCTD14
EXPH5
GULP1
ENTPD1
IGFBP2
LRRFIP1
HLA-DMA
ZNF91
FCN1
IGFBP1
LAIR2
NOTUM
PRL
ASCL2
ISL1
IDO2
TMEM74B
CSHL1
ADCY4
LEP
PAPPA2
HTRA4
SIGLEC6
CGB5
DLX5
GREM2
GPR126
MXRA5
APLN
DEFA1B
COX4I2
HBD
D
Log2 FC
-2
2
E
Maternally expressed genes
Log2 fold change
-log10 (p-value)
F
Paternally expressed genes
G
Maternally expressed
Paternally expressed
M[%] - P[%]
60-40
70-30
80-20
90-10
H
p-val = 0.0001655
Log2 fold change
Maternally expressed
Paternally expressed


A
DLX5/18S
2.5
2.0
1.5
1.0
0.5
0.0
**
*
***
Control
EO-PE
EO-PE +IUGR
LO-PE
LO-PE +IUGR
B
Villous trophoblasts
Extravillous trophoblasts
DLX5
DAPI
CK7
DAPI
DLX5
CK7
DAPI
Merge
Negative
C
Early GA Control
Early onset PE
D
PIGF [pg/ml]
1400
1200
1000
600
400
200
0
Control
PE
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p-val=0.0172
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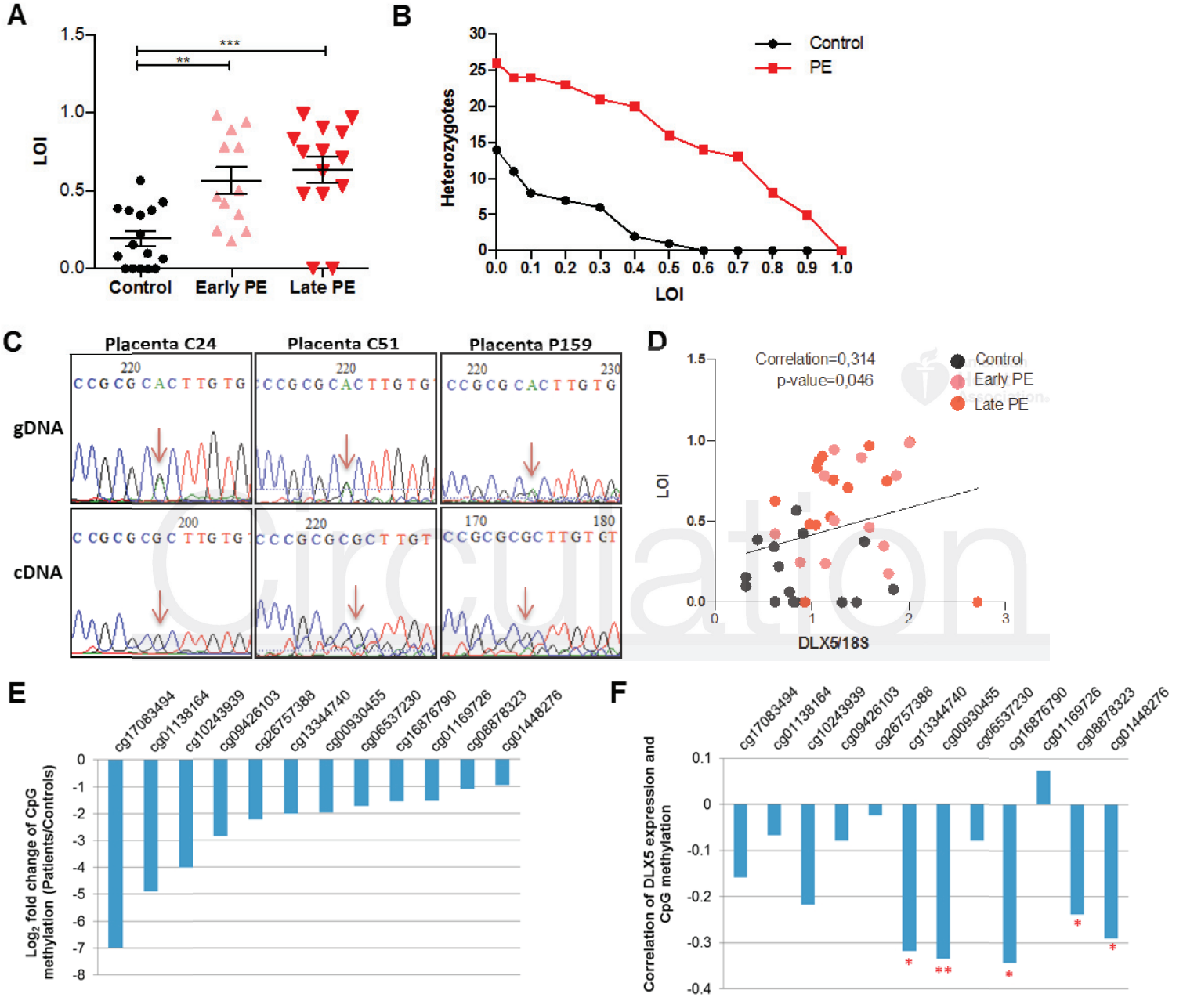
1
2
3
DLX5/18S


A
LOI
Control
Early PE
Late PE
B
Heterozygotes
LOI
Control
PE
C
Placenta C24
Placenta C51
Placenta P159
gDNA
cDNA
D
Correlation=0,314
p-value=0,046
Control
Early PE
Late PE
LOI
DLX5/18S
E
Log2 fold change of CpG methylation (Patients/Controls)
F
Correlation of DLX5 expression and CpG methylation
cg17083494
cg01138164
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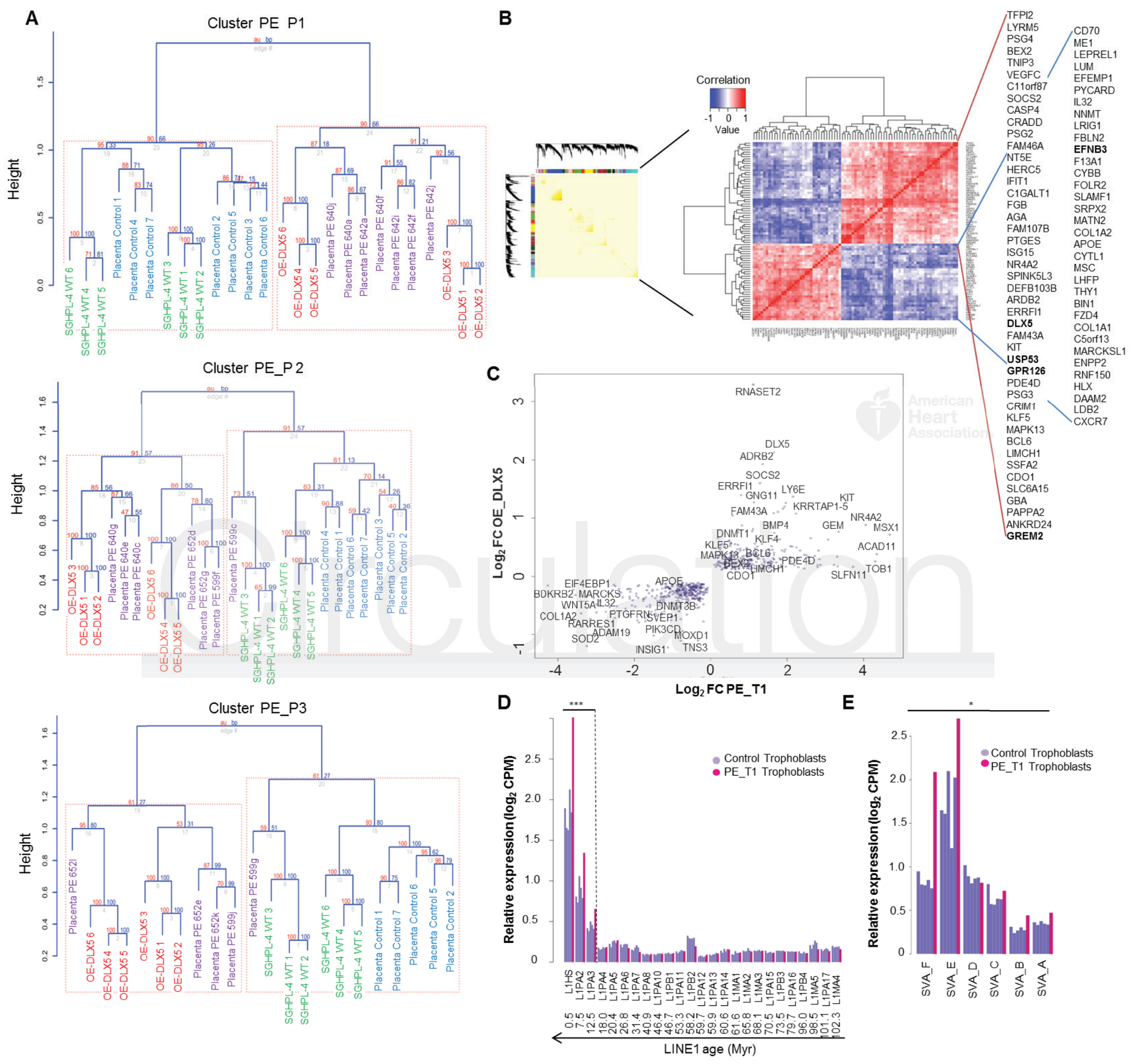
A
Cluster PE P1
Height
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SGHPL-4 WT 5
Placenta Control 1
Placenta Control 4
Placenta Control 7
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SGHPL-4 WT 1
SGHPL-4 WT 2
Placenta Control 2
Placenta Control 5
Placenta Control 3
Placenta Control 6
OE-DLX5 6
OE-DLX5 4
OE-DLX5 5
Placenta PE 640j
Placenta PE 640a
Placenta PE 642a
Placenta PE 640f
Placenta PE 642i
Placenta PE 642f
Placenta PE 642j
OE-DLX5 3
OE-DLX5 1
OE-DLX5 2
B
Correlation
-1 0 1
Value
TFPI2
LYRM5
PSG4
BEX2
TNIP3
VEGFC
C11orf87
SOCS2
CASP4
CRADD
PSG2
FAM46A
NT5E
HERC5
IFIT1
C1GALT1
FGB
AGA
FAM107B
PTGES
ISG15
NR4A2
SPINK5L3
DEFB103B
ARDB2
ERRFI1
DLX5
FAM43A
KIT
USP53
GPR126
PDE4D
PSG3
CRIM1
KLF5
MAPK13
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GBA
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CD70
ME1
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APOE
CYTL1
MSC
LHFP
THY1
BIN1
FZD4
COL1A1
C5orf13
MARCKSL1
ENPP2
RNF150
HLX
DAAM2
LDB2
CXCR7
Cluster PE_P 2
Height
OE-DLX5 3
OE-DLX5 1
OE-DLX5 2
Placenta PE 640g
Placenta PE 640e
Placenta PE 640c
OE-DLX5 6
OE-DLX5 4
OE-DLX5 5
Placenta PE 652d
Placenta PE 652g
Placenta PE 599f
Placenta PE 599c
SGHPL-4 WT 3
SGHPL-4 WT 1
SGHPL-4 WT 2
SGHPL-4 WT 6
SGHPL-4 WT 4
SGHPL-4 WT 5
Placenta Control 4
Placenta Control 1
Placenta Control 6
Placenta Control 7
Placenta Control 3
Placenta Control 5
Placenta Control 2
C
RNASET2
DLX5
ADRB2
SOCS2
ERRFI1
GNG11
LY6E
KIT
KRRTAP1-5
FAM43A
NR4A2
BMP4
GEM
MSX1
DNMT1
KLF4
KLF5
ACAD11
MAPK13
BCL6
PDE4D
BEX2
LIMCH1
CDO1
SLFN11
TOB1
EIF4EBP1
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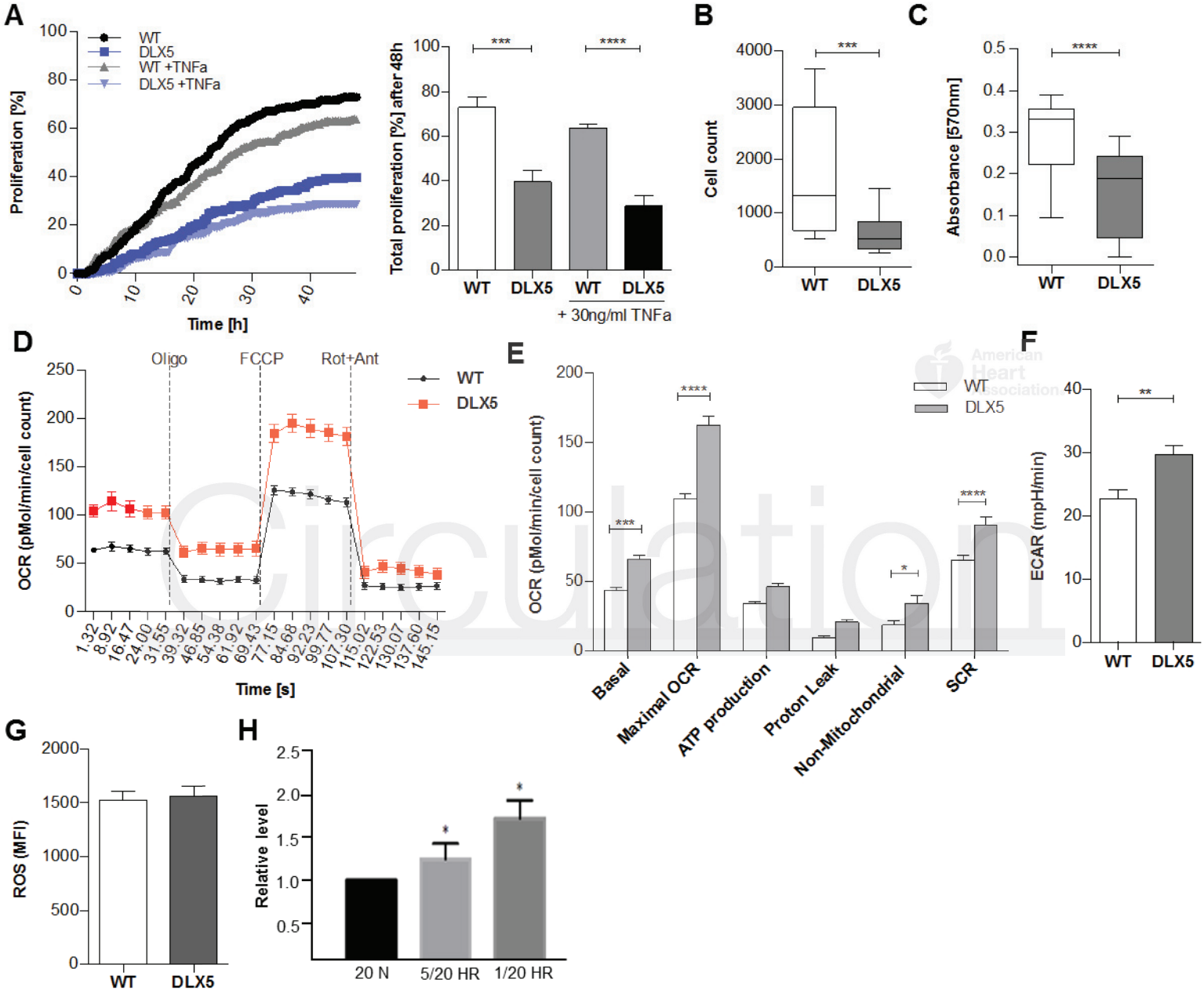
ADAM19
MOXD1
SOD2
INSIG1
TNS3
Log2 FC OE_DLX5
Log2 FC PE_T1
American Heart Association
Cluster PE_P3
Height
Placenta PE 652l
OE-DLX5 6
OE-DLX5 4
OE-DLX5 5
OE-DLX5 3
OE-DLX5 1
OE-DLX5 2
Placenta PE 652e
Placenta PE 652k
Placenta PE 599j
Placenta PE 599g
SGHPL-4 WT 3
SGHPL-4 WT 1
SGHPL-4 WT 2
SGHPL-4 WT 6
SGHPL-4 WT 4
SGHPL-4 WT 5
Placenta Control 1
Placenta Control 7
Placenta Control 6
Placenta Control 5
Placenta Control 2
D
***
Relative expression (log2 CPM)
Control Trophoblasts
PE_T1 Trophoblasts
L1HS
L1PA2
L1PA3
L1PA4
L1PA5
L1PA6
L1PA7
L1PA8
L1PA10
L1PB1
L1PA11
L1PB2
L1PA12
L1PA13
L1PA14
L1MA1
L1MA2
L1MA3
L1PA15
L1PB3
L1PA16
L1PB4
L1MA5
L1PA17
L1MA4
0.5
7.5
12.5
18.0
20.4
26.8
31.4
40.9
46.4
46.7
53.3
58.2
59.7
59.9
60.6
61.6
65.8
68.1
70.5
73.5
79.7
96.0
98.5
101.1
102.3
LINE1 age (Myr)
E
*
Relative expression (log2 CPM)
Control Trophoblasts
PE_T1 Trophoblasts
SVA_F
SVA_E
SVA_D
SVA_C
SVA_B
SVA_A


A
WT
DLX5
WT +TNFa
DLX5 +TNFa
Proliferation [%]
Time [h]
Total proliferation [%] after 48h
WT
DLX5
WT
DLX5
+ 30ng/ml TNFa
B
Cell count
WT
DLX5
C
Absorbance [570nm]
WT
DLX5
D
Oligo
FCCP
Rot+Ant
WT
DLX5
OCR (pMol/min/cell count)
Time [s]
E
WT
DLX5
OCR (pMol/min/cell count)
Basal
Maximal OCR
ATP production
Proton Leak
Non-Mitochondrial
SCR
F
ECAR (mpH/min)
WT
DLX5
G
ROS (MFI)
WT
DLX5
H
Relative level
20 N
5/20 HR
1/20 HR