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Technical note

A new pipeline for clinico-pathological and molecular placental research utilizing FFPE tissues

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ABSTRACT  
The placenta is at the core of many pregnancy pathologies, but we have limited knowledge about placental function because of two key research barriers: 1) lack of guidelines for sample collection and pathologic diagnosis; and 2) limited tools are available for molecular analysis of stored placental samples. We aimed to create a searchable, population-based placental database of pathologic diagnoses, and to validate molecular methods for gene expression studies of matching formalin fixed paraffin embedded (FFPE) placental blocks. Our database has over 1000 pregnancies coded for clinical diagnosis with corresponding FFPE blocks that are available for gene expression studies. RNA harvested from FFPE tissues is of sufficient quality for downstream applications. We successfully used this pipeline to identify FFPE placenta from term and preterm pregnancies, and compared their gene expression. The establishment of this platform, which links clinicopathological data and molecular gene expression, will increase our understanding of obstetrical diseases.

1. Introduction  
Archival formalin fixed paraffin embedded (FFPE) tissues are a stable and inexpensive source of genetic material. Archived pathology samples have been successfully used for advanced gene expression research in many organs, but not in the human placenta. The George Washington University (GWU) has a collection of placental FFPE blocks from every pregnancy between 2012 and 2017, but their utility for molecular studies has not been systematically validated. We describe a new pipeline for clinicopathological and molecular assessment of existing, archived FFPE placental samples which 1) employs a new database of clinicopathological electronic medical records (EMRs) and 2) utilizes FFPE placenta for downstream gene expression analyses. We exploit this pipeline to query the gene expression differences between term and preterm placenta using RNA-sequencing.

2. Materials and methods  
Per hospital policy between 2012 and 2017, the placenta from each delivery was sent for pathological examination. GWU’s placental archive of ~15,000 samples contains the following sections from these deliveries: one block with 2 umbilical cord sections and amniotic membrane roll, 2 blocks with full sections (maternal and fetal aspects) of the placental disc (central), and additional sections if there were gross abnormalities. GWU keeps FFPE blocks for 10 years.

Clinical data from pregnancies (maternal and fetal EMR) were extracted from the GWU’s Cerner EMR and entered into an IRB approved REDCap database. Placental pathology was re-scored by an expert pathologist and entered into the database following the “Amsterdam Consensus Guidelines” [1].

To acquire matched frozen and FFPE tissues, women were prospectively screened for uncomplicated pregnancy and delivery. Upon delivery, full thickness, central tissue cores of the placental disc were snap-frozen and stored at ~80 °C; the remaining placenta was processed as standard FFPE blocks for pathological assessment. Placenta were kept at room temperature and underwent two stages of formalin fixation – first in the delivery room, within 30–60 min of placenta delivery, and then again in the gross room. Length of fixation was variable, ranging from 1 to 3 days, depending on whether delivery occurred on a weekday or weekend. Only placenta without gross lesions or significant pathologic abnormalities were included in the study and matched with their frozen specimens.

After validation of our methods for FFPE tissues, 10 term and 10 preterm cases, defined as delivery before 34 weeks, were identified.
using our database.

We used standard kits and reagents for RNA isolation, cDNA synthesis, qRT-PCR and immunostaining, and followed manufacturers’ protocols. A detailed list of reagents, equipment, statistics, and primers can be found in Supplementary data.

RNA-sequencing was done by LC Sciences (Houston, TX). Details can be found in Supplementary data.

3. Results and discussion

Placental dysfunction is at the core of many pregnancy complications, yet our understanding of obstetrical diseases and their impact on child health is limited by a lack of tools to study the placenta. Since FFPE placenta are a potential goldmine of genetic material, that can be linked to EMRs, we sought to create a pipeline whereby clinicopathological questions could be investigated.

Our database of >1000 cases, is founded on a unique, population-based dataset that includes placenta and EMRs from both complicated and normal term pregnancies. Cases are coded for patient demographic data; gross (macroscopic) and microscopic placental pathology findings; gestational age; antenatal conditions and onset; pre- peri- and post-pregnancy status; and fetal outcomes (Table 1). Since GWU is located in the urban metropolis of Washington, D.C., this database is representative of the diverse populations of the community. Matching of maternal and fetal EMRs with systematic placental pathology reports into a searchable database now allows for interrogation of obstetric clinical questions, by enabling easy sample identification of the corresponding FFPE placental blocks.

It was previously unclear whether FFPE human placental blocks were useful for downstream genetic analysis and comparable to fresh frozen tissues since placental core sections are particularly thick and fibrous, limiting their solubility and requiring prolonged formalin fixation that can potentially degrade RNA [2–4]. Approximately 6 µg of RNA was obtained per 20 µm slice of FFPE placenta tissue. 260/280 absorbance ratios were ~2 for all samples, indicative of pure RNA. Quality of FFPE RNA (RNA integrity number, RIN, 2.38 ± 0.04, n = 20) was significantly lower (t (1,38) = 74.12, p < 0.0001) than matched frozen RNA frozen tissues (9.68 ±0.09, n = 20) and published values from fresh or RNA-later stored placenta [5,6]. Comparing RNA from FFPE placenta to RNA-later stored tissues is an important line of future work since RNA-later is superior to snap-freezing [5,6], and both methods of RNA stabilization likely provide higher RNA quality than FFPE extraction can. However, clinical samples continue to be stored in FFPE. Although lower RINs can result in higher qRT-PCR cycle threshold (Ct) values [5,6], the expression level of only 8% of placental genes are dependent on RIN [7] and there are methods to correct for differences in sample degradation [8]. Notably, RIN values are similar in FFPE tissues stored over time [9]; thus, samples identified using our pipeline undergo similar degrees of degradation and are suitable for comparison to one another. Moreover, RINs of ~2.4 are similar to that of long-term stored, snap-frozen placenta where Cis were detectable [5,6], and are par with RNA harvested from other FFPE tissues types, where RINS above 1.4 were successfully used in gene expression assays [10,11]. To our knowledge, no other method for FFPE tissues consistently returns higher RINs [12].

Confirming the quality of RNA, known placental housekeeping genes Ywhaz, CycT1, and Top1 [13,14] were detected in every FFPE sample, demonstrating that RNA degradation did not preclude qRT-PCR (Fig. 1A). Variability in Ct increased in proportion to gene length, possibly due to reduced transcript stability [7] and increased mRNA fragmentation. However, comparable Cis between fresh frozen and FFPE RNA suggest that expression in FFPE placenta tissues is representative of placental expression, and is consistent with findings from other tissues where snap frozen and FFPE processed samples’ gene expression correlate strongly (r = −0.9 [11,15,16]). Identification of these housekeeping genes in FFPE placenta allow for normalizing inter-sample

### Table 1

<table>
<thead>
<tr>
<th>Criteria Information</th>
<th>% in population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Race</td>
<td></td>
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<tr>
<td>Black</td>
<td>40%</td>
</tr>
<tr>
<td>White</td>
<td>47%</td>
</tr>
<tr>
<td>Other</td>
<td>13%</td>
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<tr>
<td>Ethnicity</td>
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<tr>
<td>Hispanic</td>
<td>9.2%</td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td>90.8%</td>
</tr>
<tr>
<td>Body Habitus (body mass index, BMI)</td>
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</tr>
<tr>
<td>Normal, BMI less than 25</td>
<td>37%</td>
</tr>
<tr>
<td>Overweight, BMI within 25 and 30</td>
<td>25%</td>
</tr>
<tr>
<td>Obese, BMI greater than 30</td>
<td>28%</td>
</tr>
<tr>
<td>Gestational Age</td>
<td></td>
</tr>
<tr>
<td>Term, 37 weeks or more</td>
<td>88%</td>
</tr>
<tr>
<td>Preterm, less than 37 weeks</td>
<td>12%</td>
</tr>
<tr>
<td>Pathology condition</td>
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</tr>
<tr>
<td>Chorioamnionitis</td>
<td>30.5%</td>
</tr>
<tr>
<td>Fetal inflammatory response</td>
<td>19.0%</td>
</tr>
<tr>
<td>Maternal stromal vascular lesions</td>
<td>18.0%</td>
</tr>
<tr>
<td>Meniscitum-Related changes</td>
<td>18.0%</td>
</tr>
<tr>
<td>Intervillosus thrombi</td>
<td>12.0%</td>
</tr>
<tr>
<td>Gestational hypertensive disorders</td>
<td>10.0%</td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>3.4%</td>
</tr>
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</table>

**Summary of term and preterm cohorts**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Preterm</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age</td>
<td>~39.85 ± 0.3</td>
<td>~26.5 ± 1.3</td>
</tr>
<tr>
<td>Sex of fetus (M/F)</td>
<td>3/7</td>
<td>6/4</td>
</tr>
<tr>
<td>Delivery type (VD-CS)</td>
<td>5/5</td>
<td>7/3</td>
</tr>
<tr>
<td>Obese</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Chronic HTN, PEC</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Other delivery indications</td>
<td>Breech (2)</td>
<td></td>
</tr>
</tbody>
</table>

**Cervical insufficiency or short cervix (5)**

**h/o LEEP (1)**

**Intrauterine fetal demise (1)**

**Non-reassuring fetal heartrate (1)**

**Non-reassuring fetal heartrate (1)**

**Placental abruption (2)**

**PPROM (3)**

**Precipitous delivery (1)**

**VD: vaginal delivery.**

**CS: caesarian section.**

**Chronic HTN, PEC: Chronic hypertension and preeclampsia with severe features.**

**PPROM: preterm premature rupture of membranes.**

**h/o LEEP: history of loop electrosurgical excision procedure.**

Data analyzed by Chi-squared test and student t-test for categorical and parametric data, respectively.

loading variability [17,18] and make qRT-PCR analysis feasible.

We identified term and preterm FFPE placenta using our database (Table 1) and successfully performed gene expression analysis by RNA-sequencing, which was then validated by qRT-PCR (Fig. 1B–E). There were 1394 upregulated and 2235 downregulated genes in preterm placenta compared to term (Fig. 1F). Hierarchical clustering reveals distinct expression signatures between term and preterm placenta, that cluster closely within their groups (Fig. 1G). Notably, our findings agree with microarray data from second trimester versus term, fresh, frozen, human placenta. For genes that are significantly dysregulated in both datasets, there is an ~87% concurrence in the fold change direction [19]. High gene overlap, despite methodological differences, indicates the validity of placental FFPE for RNA-sequencing.

A greater proportion of down-regulated differentially expressed
genes (DEGs) may reflect premature loss of late-gestation placental factors, exhibited by disruptions to cell cycling and steroid biosynthesis (Fig. 1H), which are gestationally regulated processes [20, 21]. Placenta steroid disruption is one pathway significantly disrupted in preterm birth that deserves further investigation [22]. Alcoholism and systemic lupus erythematosus (SLE) were the most represented pathways and are known risk factors for preterm delivery [23, 24]. Likely the DEGs in alcoholism and SLE overlap with genes related to inflammation and maternal immune activation, which are comorbidities for some of the clinical conditions indicated in our preterm cohort.

Our novel pipeline presents a non-bias method to examine placental functions associated with preterm birth and other obstetric complications. The creation of this database combined with validation of FFPE placenta samples as suitable genetic material provides a tool for identifying biomarkers and pathways for further investigation.

**Acknowledgements**

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/...
References


