PROJECT TITLE: Towards understanding placental FN processing in preeclampsia and its utility as an early predictor.

Funding obtained from the Preeclampsia Foundation Canada Vision Grant has contributed to the completion of both objectives, whereby we identified the novel oxygen sensor, JMJD6, as a key regulator of FN processing in the human placenta, that is aberrant in preeclampsia (PE). In addition, we have made extensive progress in establishing the diagnostic potential of FN cargo of placental exosomes as a predictive biomarker for PE. Data stemming from objective 1 has been published in the journal, Frontiers in Cellular and Developmental Biology (PMID: 34055782). Data from objective 2 has been compiled into a manuscript that is currently in submission to the Journal of Extracellular Vesicles. The proposed research has also culminated in an oral presentation at the annual meeting of the Society for Reproductive Investigation (SRI) in Paris, France (March 12-16, 2019) and has been selected for a poster presentation at the SRI to be held in Denver, Colorado, USA (March 15-19, 2022). The following is a summary of major findings from the two objectives.

Objective I - Investigate the involvement of JMJD6 in regulating trophoblast FN deposition in physiological and pathological (preeclamptic) conditions.

Results: Examination of FN transcript levels by qPCR analysis revealed that *FN1* mRNA levels were unchanged in early-onset preeclampsia (E-PE) *vs.* pre-term control (PTC) placentae (**Figure**

1A). In contrast, Western blotting (WB) and densitometric analysis showed markedly increased FN protein levels in E-PE and L-PE (late-onset preeclampsia) relative to both term control (TC) and PTC placentae (Figure 1B), suggesting an overall increase in this marker in PE pathology. Given that FN primarily localizes to developing blood vessels as well as the mesenchyme in placentae, we employed a primary cell model of placental mesenchymal stem cells (pMSCs) isolated from first trimester, term, and ΡE pregnancies. pre-term Flow confirmed cytometry analyses the predominant expression of mesenchymal stromal cell surface markers CD29, CD73, CD90 and CD105 and lack of hematopoietic markers, CD34 and CD45, in representative 7-week and PE pMSCs (Figure 1C). immunofluorescence (IF) Notably, data showed striking alterations in FN appearance in PE pMSCs when compared





to first trimester (8-week) and term controls (**Figure 1D**). Particularly, while FN existed in a fibrillar/elongated cell-associated network and localized to the peri-cellular region in first trimester and term pMSCs, it was clustered and disorganized in pMSCs from PE pregnancies (**Figure 1D**). This was accompanied by significantly elevated FN protein levels in PE-derived pMSCs relative to term pMSCs (**Figure 1E**).

Placental hypoxia is a key culprit in the pathogenesis of PE, and previous work from our laboratory revealed that the novel oxygen sensor, JMJD6 (Jumonji C domain containing protein 6), is enzymatically inactive in this pathology. Following siRNA knockdown of JMJD6 (siJMJD6) in Term



pMSCs following overexpression (OE) of either wildtype (WT) or mutant (Mut JmjC)

JMJD6 in Term pMSCs. One-way ANOVA, **p<0.01

pMSCs kept at 8% O₂, WB revealed significantly elevated FN levels (Figure 2A). To determine whether JMJD6 loss influenced FN spatial distribution in control Term pMSCs, we performed IF for FN upon siJMJD6 and found pericellular accumulation and disorganized fibril deposition FN (Figure 2B), similar to FN appearance in PE pMSCs. The Jumonji C domain of JMJD6 is central to its enzyme function; hence, to uncover the contribution of this domain to JMJD6 function in regulating FN, we generated JMJD6 plasmid constructs by mutating the iron-binding site in the JmjC domain, and transfected them into pMSCs. WT JMJD6 overexpression significantly reduced FN protein levels (Figure 2C). In contrast, overexpression of Mut JmiC

did not significantly affect the levels of both monomeric and dimeric/multimeric forms of FN (**Figure 2C**), confirming the importance of the JmjC domain in mediating the effects of JMJD6 on FN.

Since JMJD6 functions as a lysyl hydroxylase, we investigated the contribution of this role to FN processing in pMSCs from normotensive control and PE pregnancies. Notably, *in silico* analysis of the FN protein revealed the presence of several domains important for FN-FN binding and dimerization. Using a hydroxylation prediction software (RF Hydroxysite), we determined that among these sites, two domains contained lysine residues that had a significant likelihood of being targeted for hydroxylation. Hence, we generated short synthetic peptides spanning the two sites on FN (FN1726-1733 and FN1877-1884) (**Figure 3A**) and performed an *'in vitro* hydroxylation' reaction with recombinant JMJD6 enzyme at varying concentrations of Fe²⁺ and O₂. MALDI-TOF mass spectrometry was performed on the reaction products to analyze potential shifts in mass

stemming from post-translational modifications. Notably, we detected a 16 dalton shift in mass of one of the FN peptides (FN₁₈₇₇₋₁₈₈₄) in the presence of JMJD6 enzyme, corresponding to the addition of a single hydroxyl group (**Figure 3B**). When the enzymatic reaction was performed in the absence of Fe^{2+} , the shift in mass, as well as dissolution of the primary peptide mass peak was less pronounced relative to when the reaction was carried out in the presence of Fe^{2+} .

Prolyl and lysyl hydroxylation are critical post-translational modifications that are essential for the



subsequent glycosylation of collagen, another key extracellular matrix protein¹. As a readout to study potential FN glycosylation, examined FN we association to the sugar-binding lectin, Concanavalin A (Con A) by coimmunoprecipitation analysis following treatment with minoxidil, а pharmacological inhibitor of protein lysyl hydroxylation. We found that minoxidil treatment of Term pMSCs resulted in increased FN-Con A binding, indicative of enhanced glycosylation (Figure 3C left panel). Mimicking conditions of lysyl hydroxylase inhibition, total FN glycosylation was also augmented following JMJD6 knockdown in term pMSCs (Figure 3D), suggesting that JMJD6-mediated hydroxylation of FN negatively impacts on its glycosylation. Collectively, these data indicate that JMJD6 is required not only for FN lysyl hydroxylation. also but for its glycosylation, a key step required for FN secretion and matrix formation.

Objective II – Establish the diagnostic potential of FN cargo of placental exosomes as a predictive biomarker for preeclampsia

Results – To address this aim, total exosomes were isolated from maternal plasma by differential centrifugation and filtration as previously described². Subsequently, placenta-derived exosomes were isolated with a biotinylated anti-human PLAP antibody using streptavidin-agarose beads². Exosomal size and concentration were validated by NanoSight Nanoparticle Tracking Analysis



Figure 4 – Isolation and characterization of placental exosomes across gestation. (A) Nanosight nanoparticle tracking analysis of placenta-derived PLAP-positive exosomes from control and L-PE pregnancies. (B) WB for PLAP, CD63 and TSG101 in control and L-PE placental exosomes. (C) WB and densitometry for FN in control and L-PE placental exosomes across gestation (G1 to G4). (D) WB for FN in control non-placental exosomes across gestation. One-way ANOVA, ***p<0.001

(NTA), which revealed a peak particle size at ~ 100-150 nm, typical of exosomes (Figure 4A). As well, our placental exosome quality was validated by positive WB for CD63, TSG101 and PLAP (Figure **4B**). In addition, derived from exosomes the placenta are positive for the placenta-specific enzyme placental-type alkaline phosphatase (PLAP), thereby purification enabling the and characterization of placentaderived exosomes in maternal circulation^{3,4}. FN content in total and placental exosomes was longitudinally examined in the maternal sera of normotensive control and preeclamptic women during four gestational windows: namely 10-14, 16-22, and 26-32 weeks of gestation and at delivery (termed G1-G4, respectively). Notably, in control placental exosomes, FN was significantly

elevated at G4 relative to G1, G2 and G3, while this effect was not observed in L-PE placental exosomes (**Figure 4C**). Interestingly, in contrast to the trend observed in placental exosomes, FN is largely unchanged across G1 to G4 in circulating non-placental exosomes (comprising the fraction of exosomes derived from maternal plasma but excluding placenta-derived PLAP-positive exosomes) (**Figure 4D**), thereby highlighting a true placental contribution to its overexpression in PE.

When FN levels in placental exosomes were directly compared to those in controls, they were significantly augmented in L-PE as early as G1 and continuing throughout gestation (**Figure 5A**). Once again, the increase in FN levels in L-PE was specific to placenta-derived exosomes, since FN in total circulating exosomes and those derived from the non-placental immunoprecipitated fraction, was largely unchanged in L-PE (**Figure 5B**). Examination of FN in E-PE pathology revealed that similar to L-PE, FN levels were significantly increased in E-PE placental exosomes compared to age-matched normotensive controls (**Figure 5C**), suggesting that the rise in FN may not be specific to the time of PE onset. Interestingly, WB FN in placental exosomes from gestational hypertension (GH; isolated elevation of blood pressure after 20 weeks of gestation)⁵

showed a high degree of variability at each gestational age (perhaps reflective of the heterogeneity of the disease), with only G1 and G3 demonstrating a significant increase (**Figure**



5D). This further underscores the finding that augmented FN in placental exosomes in preeclampsia is unique to this multi-factorial syndrome. At the same time, the significant increase in FN in GH at G1 suggests that general endothelial dysfunction (most likely causative for maternal hypertension) is also a major contributor to excessive FN release via placental exosomes. Lastly, to evaluate the diagnostic ability of placental exosomal FN in accurately predicting PE pathology. we constructed Receiver operating characteristic (ROC) curves for FN in E-PE, L-PE and GH. We found that the diagnostic accuracy of FN in predicting L-PE at G1 and G2 was the highest (area under the curve (AUC) 0.9777 at G1 and 0.9378 at G2; Figure 6A) while it was the lowest for GH prediction regardless of gestational window (AUC in range of 0.5750 -0.7800). Interestingly, similar ROC values

were found for E-PE and L-PE at G3 (AUC 0.8056 and 0.8533 respectively; Figure 6B).

Conclusions and Impact: Preeclampsia is a devastating disorder of pregnancy that is notoriously challenging to predict. Currently, there are no tools to placental health/function assess in crucial gestational periods that can aid clinical diagnosis and predict adverse pregnancy outcomes and maternal longterm sequelae. Funding obtained from the Preeclampsia Foundation Canada Grant Vision has facilitated the identification of placental exosomal FN as a factor that is uniquely and robustly elevated in both early- and late-onset preeclampsia, as early as the first trimester of gestation. The increase was



detected significantly prior to the onset of maternal symptoms; therefore, targeting this important

period of development for assessing predictive biomarkers, perhaps in combination with other early screening parameters, is necessary for the development of prophylactic therapy for this complex disorder. Overall, research stemming from our initial proposal adds a crucial piece to the puzzle of what goes wrong in preeclampsia by uncovering the molecular pathways responsible for dysregulated FN, which is critically required for proper placental development. Importantly, this work will also facilitate early diagnosis, and timely intervention for preeclampsia, ultimately improving maternal health outcomes.

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