BAINBRIDGE, Shannon A.

Summary of Progress - *"Role of FGL2 in the Pathophysiology of Preeclampsia"*

The overarching hypothesis of my 2015 Preeclampsia Vision Grant is that preeclampsia (PE) is a spectral disorder which encompasses several subclasses of placenta pathology, driven by different underlying causes. Using molecular profiling I have previously been able to identify 3 distinct subclasses of PE, each of which appears to demonstrate distinct underlying disease processes, including: PE subclass 1) maternal maladaptation to pregnancy; PE subclass 2) placental malperfusion; and, PE subclass 3) heightened immune activation at the maternal-fetal interface. In the current project I sought to better understand this third subclass of PE patients - women whom I believe represent an immune-driven form of this disease. In the original grant proposal I identified a specific placental protein – FGL2- which demonstrated unique overexpression in this immune-drive subclass of PE, and hypothesized that placental FGL2 may play a contributing role to the disease progression in these PE cases through its pro-inflammatory and pro-coagulatory activity. Within the initial proposal the following research aims were identified as key steps towards confirming this hypothesis. Included below is a summary of the progress made to-date on each of the proposed research aims.

Aim 1: Characterize lesions within the human placenta that are specifically associated with the immune-driven subclass of PE, using a systematic placental histopathology approach

In order to successfully complete this aim, I have been working in close collaboration with Dr. David Grynspan, the perinatal pathologist at the Children's Hospital of Eastern Ontario. Together we have developed a synoptic placental pathology examination tool which allows for standardized collection of information pertaining to the presence/absence and severity of 34 different placental lesions relevant to placental pathology observed in cases of PE (we have recently submitted a manuscript to AJOG related to the development of this examination tool – see deliverables below). We have successfully completed the blinded placental pathology evaluation of 147 cases of PE (along with healthy controls) and were able to identify specific placental lesions that were unique to each subclass of PE that we previously identified through molecular profiling[1,2]. We specifically determined that women with the immune-driven subclass of PE demonstrated placental lesions consistent with placental villous maldevelopment, fetal vascular malperfusion and maternal-fetal interface disturbance (Table 1). Interestingly the pathology score for each of these placental lesions was positively correlated to the relative protein expression of FGL2 in matched placental tissue samples. These novel findings are currently being written up in a manuscript which we anticipate submitting for peer-review at the Journal of Clinical Investigation (JCI) in January 2017.

Table 1. Placenta histopathology defines molecular subclasses of preeclampsia

	PE Subclass 1 (N=60)	PE Subclass 2 (N=56)	PE Subclass 3 (N=31)	
Placental lesion	**Mean Pathology score (SD)**			**P-value**
A. ***Lesions consistent with Maternal Malperfusion***				
Distal villous hypoplasia[γ]	--	1.23 (0.73)	--	2.204e-11
Infarctions[γ]	--	1.02 (0.80)	--	1.093e-07
Villous maturation[γ]	--	0.87 (0.34)	--	1.149e-08
Syncytial knots[γ]	--	1.15 (0.61)	--	9.688e-09
B. ***Lesions consistent with Fetal Vascular Malperfusion***				
Avascular fibrotic villi[ŧ]	--	--	0.18 (0.40)	0.000443
C. ***Lesions consistent with Maternal-Fetal Interface Disturbance***				
Massive perivillous fibirin deposition (MPFD)[ŧ]	--	--	0.27 (0.47)	0.000243
Maternal floor infarction pattern[ŧ]	--	--	0.09 (0.30)	0.01804
D. ***Lesions consistent with Placental Villous Maldevelopment***				
Delayed villous maturation[γ]	--	--	0.45 (0.52)	0.000580

BAINBRIDGE, Shannon A.

Summary of Progress - *"Role of FGL2 in the Pathophysiology of Preeclampsia"*

Aim 2: Determine the effect of FGL2 overexpression in human trophoblast cells on immune-regulated trophoblast invasion and spiral artery remodelling, using an ex vivo human decidua-trophoblast co-culture model

To complete the current aim we needed to create an overexpression vector for human FGL2 and package it into lentivirus. We have been successful in generating these human FGL2 overexpression lentivirus particles (hFGL2), along with an empty vector control lentivirus particles (pWPI). As a first step, we infected two commonly used human placenta cell lines: an immortalized cell line of 1st trimester extravillous trophoblast cells (HTR-8/SVneo), which demonstrate an invasive phenotype, and a choriocarcinoma cell line (BeWo) that mimics *in vivo* formation of single layer of fused cells, a syncytium. Infection with the hFGL2 lentivirus resulted in a modest overexpression in HTR-8/SVneo cells but a robust overexpression in BeWo cells (Figure 1A). We then evaluated the effect of FGL2 overexpression in functional assays of these trophoblast cell lines.

Contrarily to our original hypothesis, FGL2 overexpression did not affect the invasive or migratory capabilities of HTR-8/SVneo cells (Figure 1 B-C). These results suggest that the FGL2 overexpression observed in the immune-driven subclass of PE may not alter trophoblast invasion and spiral artery remodelling. While opposed to our original hypothesis, these data are directly in line with the results we obtained through our detailed histopathology investigation of the placentas of women with this subtype of PE, as detailed in Aim 1, indicating that in the immune-driven subtype of PE, there may not be a significant role for placental underperfusion in the underlying pathophysiology. We are currently attempting to replicate these results using 1st trimester human placental tissue explants co-cultured with decidual tissue. To date, we have successfully mastered the co-culture methods and have achieved similar levels of FGL2 overexpression in the primary placenta tissue explants. Co-culture

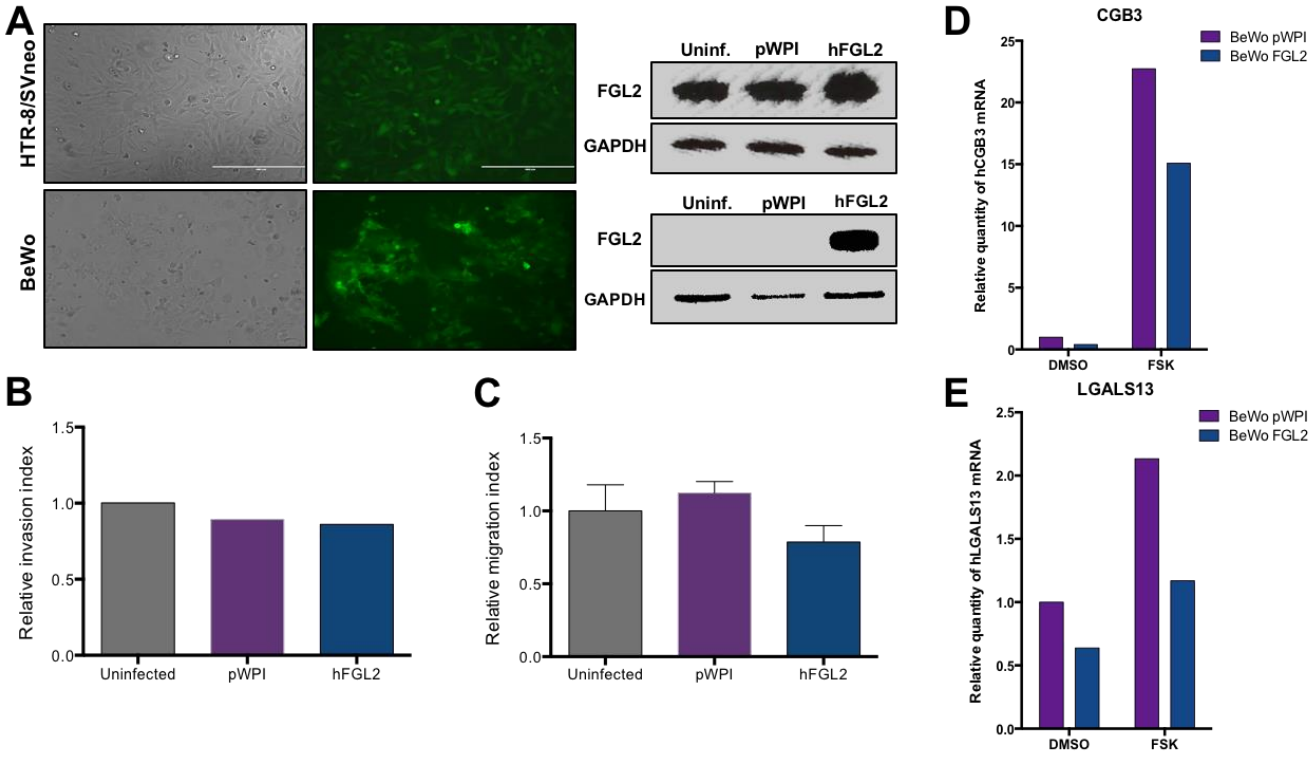

Figure 1: Effect of FGL2 overexpression on function of two human trophoblast cell lines. A) GFP fluorescent pictures and Western blots demonstrating successful infection of HTR-8/SVneo and BeWo cells. Cells were infected with lentivirus containing an empty vector (pWPI) as a negative control or with an overexpression vector (hFGL2). B) Invasive capacity of infected HTR-8/SVneo cells was measured using a Boyden chamber matrigel invasion assay. No change was observed (n=2). C) Migratory capacity of infected HTR-8/SVneo cells was measured using a Boyden chamber migration assay. No change was observed (n=3). D-E) Syncytium-forming capacity was measured in infected BeWo cells using a forskolin fusion assay followed by qPCR quantification of placental-specific genes: CGB3 (D) and LGALS13 (E). FGL2-overexpressing BeWo cells express lower levels (n=1).

experiments to assess alterations in trophoblast invasion and spiral artery remodelling are currently ongoing and we anticipate having results for this model in the next 2 months.

Contrary to the lack of findings in the HTR-8/SVneo invasive cell line, we did observe functional differences in the villous BeWo cell line following FGL2 overexpression. In this cell line, FGL2 overexpression impaired the process of syncytial fusion – a critical process in terminal trophoblast differentiation (Figure 1D-E). Defects in syncytium formation are known to play an important role in the pathophysiology of placental dysfunction in preeclampsia and could be an interesting mechanism to pursue further. We are currently investigating methods to replicate this defect using 1st trimester human placental tissue explants.

Aim 3: Develop a mouse model of placenta-specific FGL2 overexpression to evaluate its causal role in the development of placental dysfunction and maternal signs of PE in late gestation

To complete this aim, we first generated an overexpression vector of mouse FGL2 (mFGL2), packaged into a lentivirus. As a very high concentration of viral particles is needed for this technique to work *in vivo*, several batches of lentivirus were made and their concentration measured, in order to choose the optimal one. This lentivirus was tested *in vitro* in a mouse cell line, and caused a robust overexpression of FGL2 (Figure 2A-B). We have made significant progress in mastering the techniques needed to complete this experiment: intra-peritoneal injections of mice for superovulation, collection, handling, treatment and *in vitro* culture of mouse embryos, optimization of culture media and lentivirus concentrations, etc. We have now been able to successfully generate trophoblast specific overexpression of a GFP lentivirus vector (Fig 2D) and have now moved on to test out our FGL2 overexpression vectors. We anticipate successful trophoblast-specific FGL2 overexpression in embryos within the next two months.

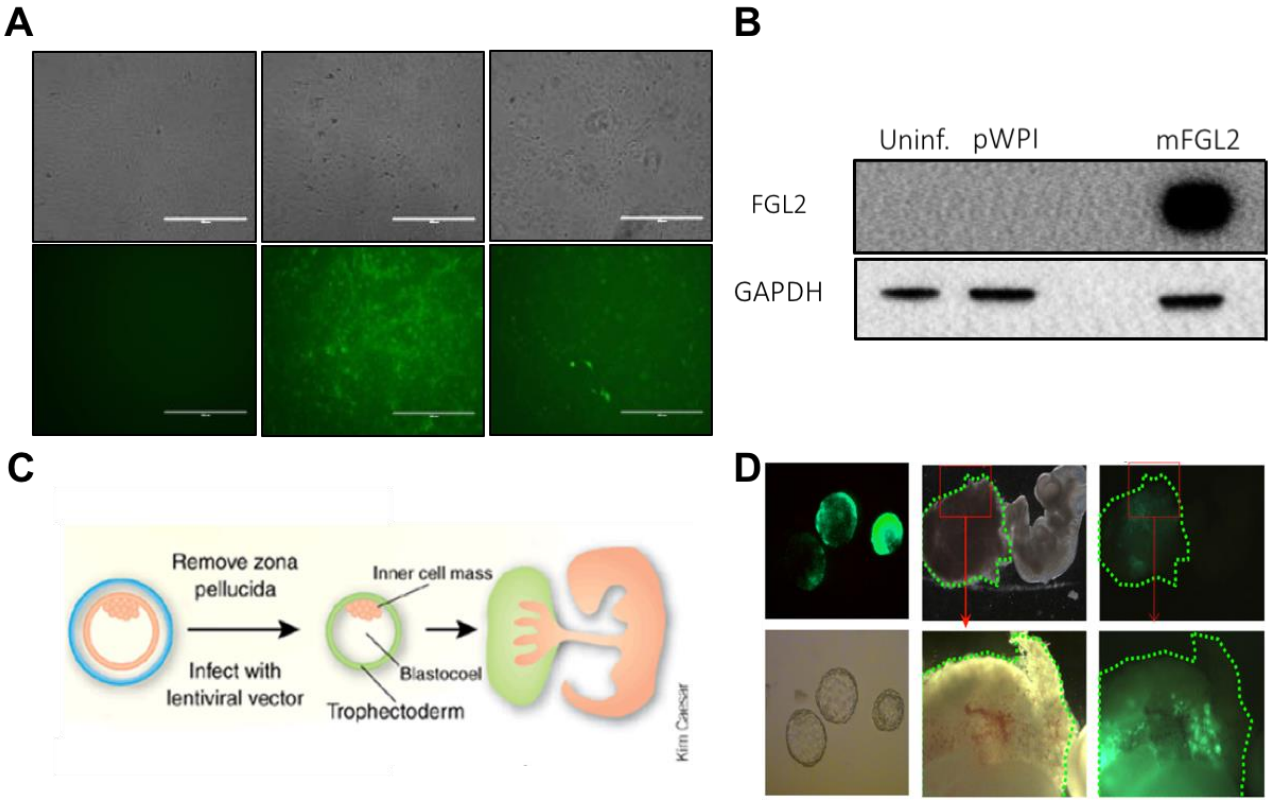

Figure 2: Testing of mFGL2 overexpression vector for *in vivo* use (A-B) and schematics of successful technique (C-D). A) GFP fluorescent pictures demonstrating successful infection of a mouse cell line. Cells were infected with lentivirus containing an empty vector (pWPI) as a negative control or with an overexpression vector (mFGL2). B) Western blot demonstrating successful overexpression of mFGL2 in a mouse cell line. C) Schematic representation of placenta-specific mFGL2 overexpression by lentiviral vector infection. D) Example of successful placenta-specific infection from previous attempts.

BAINBRIDGE, Shannon A.
Summary of Progress - *"Role of FGL2 in the Pathophysiology of Preeclampsia"*

Deliverables achieved since obtaining the Preeclampsia Foundation Vision Award:

The results summarized above have been disseminated in several ways, including:

- Aspects of this work have been presented in part at three invited platform presentations:
  - "Understanding the Molecular Underpinnings of Distinct Subclasses of Preeclampsia". International Federation of Placenta Association Annual Meeting, Portland, OR, USA, 2016.
  - "Identification of Distinct Subclasses of Disease In Preeclampsia". Frontiers in Reproduction Symposium, Woods Hole, MA, 2016.
  - "Using Metabolomic Profiling to Identify Distinct Subclasses of Placental Dysfunction in Preeclampsia", Placenta Association of Americas Satellite Meeting at the Society for Reproductive Investigation 2016 Annual Meeting, Montreal, QC.
- Aspects of this work have been presented in part by my trainees at 2 international scientific conferences and published in refereed conference abstracts:
  - Benton S, Fellus I, Kamalathasan R, El Demellawy D, Barrowman N, Grynspan D, Bainbridge SA. A Standardized Pathology Examination Tool to Improve Placental Pathology Reporting. Society for Reproductive Investigation Annual Meeting, Montreal, Quebec, March 2016. (Reproductive Sciences. 2016;23:319A-319A.)
  - Charette P, Cook D, **Bainbridge S**, Vanderhyden B. FGL2 is involved in the regulation of inflammatory response and leukocyte activation within the preeclamptic placenta. Society for the Study of Reproduction Annual Meeting, San Diego, July 2016.
- Aspects of this work have been presented in part at 4 local scientific conferences and work-in-progress seminars:
  - "FGL2 is involved in the regulation of inflammatory response and leukocyte activation within the preeclamptic placenta" Ottawa Hospital Research Day, November 2016.
  - "FGL2 is involved in the regulation of inflammatory response and leukocyte activation within the preeclamptic placenta" Cellular and Molecular Medicine Research Day, University of Ottawa, November 2016.
  - "FGL2 is involved in the regulation of inflammatory response and leukocyte activation within the preeclamptic Placenta". Southern Ontario Reproductive Biology Annual Meeting, Queen's University, May 2016.
  - "Investigating the role of FGL2 in placental development and preeclampsia" Center for Cancer therapeutics Work-In-Progress seminar, Ottawa Hospital Research Institute, April 2016.
- One manuscript has been submitted and is currently under review pertaining to the development of the histopathology tool outlined in Aim 1
  - Benton S, Grynspan D., **Bainbridge SA**. Placenta Pathology 2.0: Harnessing the power of clinical placenta pathology to better understand pregnancy complications. Submitted and under review, AJOG, November 2016.
- The results of Aim 1 are currently being prepared into a manuscript for submission to the Journal of Clinical Investigation – anticipated submission of Jan 2017.

Further, the project funded through this award has allowed for the training of one 4th year honors thesis student and 1 PhD student.

References:

1. Leavey K, **Bainbridge SA***, & Cox BJ*. Large scale aggregate microarray analysis reveals three distinct molecular subclasses of human preeclampsia. PLoS One. 2015 Feb 13;10(2):e0116508. (*Shared senior authorship)
2. Leavey K, Benton SJ, Grynspan D, Kingdom JC, **Bainbridge SA*** & Cox BJ*. Unsupervised Placental Gene Expression Profiling Identifies Clinically Relevant Subclasses of Human Preeclampsia. Hypertension. 2016 Jul;68(1):137-47. (*Shared senior authorship)