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Gestational differences in murine placenta: Glycolytic metabolism and pregnancy parameters

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ABSTRACT

The placenta is a complex and essential organ composed largely of fetal-derived cells, including several different trophoblast subtypes that work in unison to support nutrient transport to the fetus during pregnancy. Abnormal placental development can lead to pregnancy-associated disorders that often involve metabolic dysfunction. The scope of dysregulated metabolism during placental development may not be fully representative of the *in vivo* state in defined culture systems, such as cell lines or isolated primary cells. Thus, assessing metabolic function in intact placental tissue would provide a better assessment of placental metabolism. In this study, we describe a methodology for assaying glycolytic function in structurally-intact mouse placental tissue, *ex vivo*, without culturing or tissue dissociation, that more closely resembles the *in vivo* state. Additionally, we present data highlighting sex-dependent differences of two mouse strains (C57BL/6 and ICR) in the pre-hypertrophic (E14.5) and hypertrophic (E18.5) placenta. These data establish a foundation for investigation of metabolism throughout gestation and provides a comprehensive assessment of glycolytic function during placental development.

1. Introduction

The placenta is necessary for fetal development and provides the interface for nutrient, gas, and waste exchange between the mother and fetus [1,2]. It is composed of distinct trophoblast cell types (lineages) that develop during pregnancy [3]. Early embryonic development occurs under low oxygen conditions that support anaerobic glycolysis [4,5]. Under these low oxygen conditions, placental cells are thought to promote glycolysis and suppress mitochondrial metabolism [5–7]. As oxygen availability increases, a shift away from anaerobic glycolysis occurs. The placenta is in a pre-hypertropic state throughout midgestation (E0.5-E16.5) but undergoes a dramatic shift with an overall expansion in volume and increase in nonproliferative growth in the hypertrophic phase as it nears the end of gestation (E16.5-birth) [8–10]. Therefore, effective regulation of placental metabolism is necessary to maintain a healthy pregnancy [11].

Pregnancies with male offspring have been associated with having a greater risk of pregnancy-associated disorders than pregnancies with female offspring, particularly in conditions of nutrient restriction [5,8,9,12–17]. It is believed that this difference between male and female outcomes may be due to the ability of the female placenta to better adapt to environmental influences [6,17,18]. Additionally, in cases where both males and females experience preeclampsia, male placentas exhibit a stronger phenotype, exhibiting higher expression of pro-apoptotic, hypoxic, and pro-inflammatory molecules compared to placentas from their female counterparts [19]. While these studies have identified that placentas from female offspring have a better ability to adapt metabolically, they shed little light on functional differences in male and female placentas under normal conditions.

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The energy provided by glycolytic metabolism is important for proper development; however, direct measures of glycolytic function in placental tissue are limited. Current methods to assay glycolysis include: quantifying glucose uptake and lactate production, enzymatic activity at rate-limiting steps, and determination of metabolite levels using mass spectrometry or nuclear magnetic resonance spectrometry [20]. While these methods are effective at measuring glycolytic function, they often require radioactive isotopes, are not conducive to high-throughput analysis, and/or can require substantial amounts of tissue. However, the development and implementation of the extracellular flux analysis has resulted in an automated system capable of assessing metabolic function with minimal sample requirements.

Extracellular flux analysis of isolated primary placental cells and cell lines has recently been used to investigate several aspects of cellular metabolism [21–23]. In monolayer cell culture, extracellular flux analysis is a relatively uncomplicated assay that uses glucose, oligomycin, and 2-deoxy-D-glucose (2-DG) to determine glycolysis, glycolytic capacity, glycolytic reserve, and non-glycolytic proton production rate (PPR). While cultured cells have been used to increase our knowledge of cellular metabolism, assaying glycolysis in intact tissue would provide a more accurate representation of the *in vivo* state. Additionally, extracellular flux analysis of *ex vivo* tissue rather than cultured cells, will allow for analysis of different time points in gestation and reduce confounding environmental factors related to primary cell isolation and cell culture. A few reports have adapted extracellular flux analysis to intact tissue; including single skeletal muscle fibers, white adipose tissue, and brain sections; however, these tissues are relatively homogeneous in composition in contrast to the multilineage, highly-vascularized placenta [24–27]. In this study, we hypothesized that glycolysis would decrease as gestation progresses, while pregnancy parameters increase with age, regardless of fetal sex or mouse strain.

In this study, we have developed a method to analyze glycolytic parameters in *ex vivo* tissue of the mouse placenta that retains in utero placental architecture and is representative of *in vivo* trophoblast lineages. In addition, we have shown that there are few changes in glycolytic parameters based on gestational age or sex; however, there are dramatic differences in pregnancy parameters.

2. Methods

2.1. Institutional approval

Animals were mated overnight. Females were removed the following morning, checked for a copulation plug, and designated embryonic day 0.5 (E0.5). At the appropriate gestational days, animals were euthanized by CO2 followed by cervical dislocation under a Wright State University IACUC approved protocol. *Ex vivo* placental tissues were collected from ICR and C57BL/6 mice (Charles River) at E14.5 or E18.5.

2.2. Hematoxylin and eosin staining (H&E)

For H&E analysis, placentas were isolated and fixed in 4% paraformaldehyde overnight and dehydrated through a series of ethanol washes. *Ex vivo* placental tissues were subsequently isolated using a 2 mm punch (Integra Miltex, #33-31) and paraffin embedded. 10 μm sections were cut, deparaffinized, rehydrated, and H&E stained using standard procedures [28].

2.3. Quantitative PCR analysis (qPCR)

Ex vivo placental tissue or whole placentas from E14.5 ICR mice were isolated and placed into RNA*later* (Thermo Fisher, AM7020). Tissues were homogenized in Trizol (Thermo Fisher, 15-596-018), and total RNA was isolated using Direct-zol RNA miniprep columns (Zymo Research, R2050). Expression of mRNA was assessed by qPCR using SYBR green, as previously described [29,30]. Briefly, total RNA (1 μg) was reverse transcribed using the iScript RT kit for SYBR green and qPCR reactions were then prepared using the iTaq Universal SYBR green supermix (BioRad), according to the manufacturers instructions. Validated primers for *Epcam*, *Gcm1*, *Syncytin-a* (*SynA*), *Prl3b1*, and *Prl2c2* were obtained from BioRad (BioRad Prime PCR Assays, Table 1). These genes were chosen to represent all trophoblast subtypes present in the different layers of the placenta at E14.5 (decidua, junctional zone, labyrinth), including labyrinth trophoblast progenitors (*Epcam*), syncytiotrophoblast (*Gcm1*, *Syncytin-a*), spongiotrophoblast and trophoblast giant cells (*Prl3b1, Prl2c2*). Quantitative PCR reactions were conducted in triplicate on equal amounts of each cDNA sample, for each gene, and data was compiled and analyzed using CFX Manager software applying the ΔΔCq method and normalized relative to *Ppia, Ywhaz*, and *H2afz* [31]. Gene expression was presented as relative expression normalized to multiple reference genes to minimize error in normalization. These reference genes were chosen based on stable expression in both the whole placenta and *ex vivo* placental tissue samples.

2.4. Analysis of glycolytic function

Glucose (G8270), oligomycin (75351), and 2-DG (D8375) were purchased from Sigma. XF24 Sensor Cartridges (101174-100), XF24 Islet Capture Plates (101122-100), XF24 Microplates (100777-004), XF Base Minimal Media (102353-100), and Glycolysis Stress Test Kit (103020-100) were obtained from Agilent. Lineage-intact, *ex vivo* placental tissue was isolated using a 2 mm punch (Integra Miltex, #33-31). *Ex vivo* tissue (2 × 2 mm) was weighed, placed into assay media (525 μl, XF Base Minimal Media plus 2.0 mM L-glutamine; untreated) or assay media containing the glycolytic inhibitor, 2-DG (100 mM; treated), where the tissue remained for approximately 30 min while all samples were collected. Tissue was subsequently placed into microwells of an XF24 Islet Capture Plate and secured using mesh screens. The glycolytic assays on *ex vivo* tissue used sequential injections of 62.5 mM glucose, 10.0 μM oligomycin A, and 100 mM 2-DG. Three measurements were taken at each metabolic condition using a mix/wait/measure protocol of 2/2/2 min. The mouse labyrinthine placental progenitor cell line, SM10, under differentiated conditions, was also assayed for glycolytic rates for comparative analysis. SM10 cells (1×10^4) were plated on XF24 Microplates and allowed to incubate for 24 h at 37C/5% CO_2, and then treated with TGF-β2 (5 ng/ml) for 72 h to induce terminal differentiation [23,32–34]. SM10 cells were assayed under the similar conditions to *ex vivo* placental tissue, but with differing drug concentrations: glucose (10 mM), oligomycin A (1.0 μM), and

Table 1
qPCR Primers, ID and Accession Number.

Gene	BioRad ID	Entrez Accession Number
Epcam	qMmuCED0049724	17075
Gcm1	qMmuCED0045702	14531
Syncytin-a	qMmuCED0003216	214292
Prl3b1	qMmuCID0011919	18776
Prl2c2	qMmuCID0041440	18811
Ppia	qMmuCED0041303	268373
Ywhaz	qMmuCED0027504	22631
H2afz	qMmuCED0040907	51788