THE ROLE OF PROGESTERONE IN REGULATING GLUCOSE METABOLISM IN THE UTERINE EPITHELIUM

BY

MALIA DANIELLE BERG

DISSERTATION

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Doctoral Committee:

Assistant Professor Matthew Dean, Chair and Director of Research
Professor David Miller
Professor Romana Nowak
Professor Matthew Wheeler

Abstract

Early embryonic loss in cattle occurs in 40-60% of pregnancies. The uterus and developing embryo need glucose. The embryonic demand for glucose increases by the blastocyst stage. How glucose is regulated to match the changing needs of the embryo is unclear. Glycogen is composed of thousands of glucose molecules and is present in the uterus of many species. Our lab has shown that the glycogen content of the bovine uterine epithelium was low on day 11 of the cycle when progesterone is high. Therefore, our objectives were to 1) make an immortalized bovine uterine epithelial (BUTE) bovine uterine fibroblast (BFIB) cell lines to investigate the hormonal control of glycogen metabolism in the uterine epithelium, 2) to elucidate the role of progesterone on glycogen breakdown in BUTE cells, 3) determine if acid α-glucosidase (GAA) is present in the endometrium and 4) determine how progesterone regulates expression of glucose metabolizing enzymes. Fresh endometrial biopsies were collected from a Holstein dairy cow, digested into a single cell suspension, and plated to allow the formation of individual colonies. Cells were immortalized with large T-antigen, and colonies with cobblestone or spindle-like morphologies were isolated, expanded, and confirmed to be epithelial (BUTE) or fibroblasts (BFIB).

To determine if progesterone was glycogenolytic, BUTE cells were treated with IGF-1 (50 μg/ml to stimulate glycogenesis) and treated with progesterone for 48 hours. Progesterone decreased glycogen levels in BUTE cells by 99%. RU486, which antagonizes the nuclear progesterone receptor (nPR), did not block progesterone's effect. BUTE cells expressed all five membrane progesterone receptors (mPRs; α, β, γ, δ, and ε), and a mPR agonist (Org OD 02-0) reduced glycogen levels in BUTE cells by 94%. In vivo, immunohistochemistry showed that the bovine uterine epithelium expressed high levels of mPRα. These results suggest that

progesterone acts through mPRs to stimulate glycogenolysis in BUTE cells. Neither progesterone nor Org OD 02-0 changed intracellular cAMP concentrations. In agreement, the adenylyl cyclase activator forskolin increased cAMP concentration but did not decrease glycogen levels. We found that progesterone increased phospho-AMPK levels by 18% at 24 hours compared to the control. Supporting these results in vivo, phospho-AMPK levels in the uterine epithelium were high in the bovine uterine epithelium during the luteal phase when glycogen levels were low. However, the AMPK activator (A-769662) did not reduce glycogen in BUTE cells. Dorsomorphin, an AMPK inhibitor, did not block the effect of progesterone on glycogen breakdown. Meanwhile, BUTE cells treated with D942, which increases intracellular AMP concentrations, showed a 92% decrease in glycogen levels compared to the control group. AMP allosterically activates glycogen phosphorylase, which would directly decrease glycogen levels. In human Ishikawa cells that express mPRs but not nPRs, progesterone and Org OD 02-0 also decreased glycogen levels. Glycogen can also be broken down in lysosomes by acid α-glucosidase (GAA). GAA was expressed in the luminal epithelium and BUTE cells. However, progesterone did not alter GAA protein levels or activity.

To identify other pathways regulated by progesterone, BUTE cells were treated with progesterone for 24 hours, and RNAseq was performed. BUTE cells treated with progesterone had 1,623 genes upregulated and 1,449 genes downregulated. GO analysis found several over-represented pathways linked to metabolism. Interestingly, 3 of 7 genes in the pentose phosphate pathway were altered. To determine if the pentose phosphate pathway was upregulated, BUTE cells will be treated with progesterone to measure NADPH. Progesterone increased the level of NADPH in BUTE cells.

In conclusion, our study has significant implications for understanding the role of progesterone in glucose and glycogen metabolism. We generated cell lines to model the bovine endometrium and showed that progesterone acts through the mPR in BUTE cells to stimulate glycogenolysis by glycogen phosphorylase. However, GAA is present in the cow endometrium but is not regulated by progesterone. The breakdown of glycogen could be crucial in providing glucose to endometrial tissue for glucose metabolism through the pentose phosphate pathway to increase NADPH within the cell.

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List of Abbreviations

α: alpha

ABC: avidin-biotin complex

ADP: adenosine diphosphate

AI: artificial insemination

ATP: adenosine 5'-triphosphate

β: beta

BCA: bicinchoninic acid assay

BFIB: bovine uterine fibroblast

BSA: bovine serum albumin

BUTE: bovine uterine epithelial

cAMP: cyclic adenosine monophosphate

CL: corpus luteum

DMSO: dimethyl sulfoxide

DPC: days post-coitum

E2: estradiol

EGF: epidermal growth factor

ER: estrogen receptor

ERα: estrogen receptor alpha

ERβ: estrogen receptor beta

FBS: fetal bovine serum

FDR: False discovery rate

FSH: follicle-stimulating hormone

g: gram

G1P: glucose-1-phosphate

G3P: glyceraldehyde 3-phosphate

G6P: glucose-6-phosphate

G6PC: glucose-6-phosphatase

G6PD: glucose-6-phosphate dehydrogenase

GAA: acid α-glucosidase

GBE: glycogen branching enzyme

GDE: glycogen debranching enzyme

GE: glandular epithelium

GFP: anti-green fluorescent protein

GLUT: facilitative glucose transporter

GnRH: gonadotrophin-releasing hormone

GYG: glycogenin

GYS: glycogen synthase

hCG: human chorionic gonadotropin

HK: hexokinase

IGF-1: insulin-like growth factor 1

IHC: immunohistochemistry

ITS: insulin transferrin selenous acid

IVF: in vitro fertilization

KOH: potassium hydroxide

LE: luminal epithelium

LH: luteinizing hormone

µg: microgram

µl: microliter

µM: micromolar

mg: milligram

ml: milliliter

mM: millimolar

mRNA: messenger RNA

Na+: sodium ion

ng: nanogram

nM: nanomolar

PAS: periodic acid Schiff

PBS: phosphate buffered saline

PGF: uterine prostaglandin

PGF2α: prostaglandin F2 alpha

pGYS: phosphorylated glycogen synthase

PP1: protein phosphatase-1

PPP: pentose phosphate pathway

PR: progesterone receptor

PR-A: progesterone receptor alpha

PR-B: progesterone receptor beta

PVDF: polyvinylidene difluoride

PYG: glycogen phosphorylase

RIPA: radioimmunoprecipitation assay buffer

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SGLT: sodium-glucose linked transporters

TBS: tris buffer saline

TBS-T: tris buffer saline with tween

TCA: tricarboxylic acid

UDP: uridine diphosphate

UTP: uridine 5’-triphosphate

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Chapter 1 Introduction and Rationale

Early embryonic loss occurs in about 40-60% of pregnancies in dairy cattle and about 20-40% in human pregnancies detected by human chorionic gonadotropin (Miller *et al.*, 1980; Wilcox *et al.*, 1988; Zinaman *et al.*, 1996; Hansen, 2011). Several factors contribute to early embryonic loss in cattle and humans. These factors include embryonic abnormalities and an inadequate uterine environment. An essential nutrient for the developing embryo is glucose. The demand for glucose increases by the blastocyst stage, but it becomes toxic if too much glucose is present. However, how the uterus regulates glucose to match the changing needs of the embryo is unclear.

A potential way to regulate glucose concentration is glycogen. Glycogen is composed of thousands of glucose residues and is present in the uterus of many species. A study in rats compared glycogen concentrations in the uterus during the first seven days of pregnancy and found the concentration of glycogen was the highest on day 1, then decreased until day 4, and increased from day 5 to 7. Glucose levels in homogenized uteri were the highest on day 4 (Greenstreet and Fotherby, 1973). In mice, glycogen levels are decreased in the uterine epithelium on DPC 1.5 and 3.5 and increased in the implantation site at DPC 5.5 compared to proestrus (Chen *et al.*, 2022). Our lab has shown that the glycogen content of the bovine uterine luminal and glandular epithelium was lower on day 11 than on day 1 of the cycle (Sandoval *et al.*, 2021). Hence, progesterone-stimulated glycogenolysis may play a crucial role in providing glucose to endometrial tissue or the growing embryo. My research uses a bovine model to better understand the role of uterine glycogen during early pregnancy and it's regulation by progesterone.

Aim 1. Generate bovine endometrial cell lines to study hormonal control of glycogen metabolism. Cattle are important to both agricultural and biomedical research. My objective was to make an immortalized bovine uterine epithelial (BUTE) and bovine uterine fibroblast (BFIB) cell line to investigate the uterine environment in vitro further. Fresh endometrial biopsies were collected from a Holstein dairy cow, digested into a single cell suspension, and plated to allow the formation of individual colonies. Cells were immortalized with large T-antigen, and colonies with cobblestone morphology were isolated, expanded, and tested for keratin and vimentin expression by western blot and immunofluorescence. The clone with the highest expression of keratin and no detectable vimentin was used for all further experiments and was named BUTE cells. In contrast, the clone with the highest expression of vimentin and no detectable keratin was named BFIB cells. Western blots confirmed that BUTE and BFIB cells expressed the enzymes necessary for glycogen metabolism (glycogen synthase, glycogen phosphorylase, hexokinase, and glucose-6-phosphatase) and the nuclear receptors for estrogen α and progesterone. Both cell lines were free of mycoplasma. These cell lines will allow further investigation of glycogen metabolism, leading to a better understanding of how the uterus uses glycogen during pregnancy.

Aim 2. Determine how progesterone stimulates glycogen breakdown in bovine uterine epithelial cells. Early embryonic loss in cattle occurs in 40-60% of pregnancies, and the developing embryo needs glucose to survive. A better understanding of how progesterone regulates glycogen metabolism is necessary to determine glycogen's role within the uterus. To determine if progesterone stimulates glycogen breakdown, BUTE cells were treated with either progesterone, RU486 (nuclear progesterone receptor antagonist), or Org OD 02-0 (mPR agonist) for 48 hours to measure the amount of glycogen in each treatment. Progesterone, Org OD 02-0, and progesterone + RU486 decreased glycogen concentrations compared to the controls

(DMSO). RU486 did not block glycogenolysis, suggesting that progesterone is not acting through the nuclear progesterone receptor. However, Org OD 02-0, an mPR agonist, stimulated glycogenolysis. Further, progesterone increased the level of pAMPK but not the concentration of cAMP. D942, increases intracellular AMP, decreases glycogen in BUTE cells. In conclusion, progesterone acting through the mPRs and AMP stimulated glycogenolysis and may play a crucial role in providing glucose to endometrial tissue or the growing embryo.

Aim 3. Determine if α-acidic glucosidase is present in the bovine uterine epithelium.

We recently showed that progesterone stimulated glycogen catabolism in uterine epithelial cells from the cow and human uterus (aim 2). However, a glycogen phosphorylase inhibitor (GPI) only blocked 50% of the effect of progesterone. Therefore, we hypothesized that progesterone may simulate glycogenolysis in the uterine epithelium via α-acidic glucosidase (GAA). GAA is a lysosomal enzyme that degrades glycogen. GAA is present in the cow endometrium at days 1 and 11 of the estrous cycle. GAA was highly expressed in the stroma on day 1 rather than day 11. GAA was present in the uterine epithelium. However, GAA did not appear to differ in the luminal epithelium on days 1 and 11. BUTE cells were treated with progesterone to determine if progesterone regulates GAA protein level and activity. BUTE cells were treated with different concentrations of progesterone and measured by a western blot. GAA is present in BUTE cells as the full-length inactive protein (110 kDa) and the cleavage peptides inside the lysosome (70 and 76 kDa). Progesterone did not increase the protein level of either the full-length or active peptides. Progesterone did not increase the activity of GAA in BUTE cells. Overall, we confirmed that GAA is present in the cow endometrium and BUTE cells but is not regulated by progesterone.

Aim 4. Determine how progesterone regulates the expression of glucose metabolizing enzymes in the bovine uterine epithelial cells. Progesterone decreases glycogen in BUTE cells through the membrane progesterone receptors (aim 1). RNA sequencing was utilized to determine if progesterone regulates other metabolic pathways in BUTE cells. BUTE cells were treated with progesterone for 24 hours, and RNA was isolated. The Roy J Carver Biotechnology Center conducted RNA sequencing. The RNAseq libraries were prepared with Illumina's 'TruSeq Stranded mRNAseq Sample Prep kit' (Illumina). BUTE cells treated with progesterone had 1,623 of which were upregulated, and 1,449 were downregulated. ShinyGO 0.80 was used to determine the enrichment pathways. GO analysis indicated that steroid biosynthesis had the highest fold enrichment; however, metabolic pathways had the most significant number of genes involved in the pathway. Interestingly, most genes in the pentose phosphate pathway were upregulated. To determine if the pentose phosphate pathway was upregulated, BUTE cells will be treated with progesterone to measure NADPH. Progesterone increased the level of NADPH in BUTE cells but not in the media. Progesterone-treated cells showed a decrease in glucose concentration in the media compared to the controls. Therefore, progesterone increases glucose uptake to increase the production of NADPH.

Chapter 2 Literature Review

2.1 Pregnancy Failure in Cattle

Pregnancy failure occurs around 40-60% of the time in dairy cattle and about 48% in beef cows (Hansen, 2011; Reese *et al.*, 2020). Pregnancy is confirmed in cattle by progesterone level, pregnancy-associated glycoproteins (PAG), rectal palpation, and ultrasounds. The Committee on Bovine Reproduction Nomenclature in 1972 defined the embryonic period from conception until the end of differentiation around day 42 of pregnancy and the fetal period from day 42 to birth (Committee on Bovine Reproductive Nomenclature, 1972). Early embryonic failure is the loss of the pregnancy before day 24, and late embryonic loss is between days 24 and 42 (Santos *et al.*, 2004). In cattle, around 90% of oocytes will be fertilized; however, only 35 to 55% of pregnancies in lactating dairy cows, 70 to 90% of pregnancies in suckled beef cows, and 80 to 90% of pregnancies in beef and dairy heifers will reach term (Ealy and Seekford, 2019). A majority of pregnancy loss occurs within the first 30 days of pregnancy (Reese *et al.*, 2020).

The factors contributing to embryonic loss include genetic, physiological, endocrine, and environmental factors (Diskin and Morris, 2008). Genetic factors include breed, linage, and inbreeding, while environmental factors include nutrition, age, climate, and health status (Ayalon, 1978). The genomic abnormalities that cause embryo death include chromosomal defects, mutations within individual genes, and genetic interactions (VanRaden and Miller, 2006). Another important factor for embryonic loss in cattle is body condition score (BCS). Cows with a lower BCS at calving and artificial insemination (AI) have increased pregnancy loss between days 30 and 58 of gestation (Santos *et al.*, 2009). Over the past several decades, embryonic loss in cattle has increased; therefore, it is critical to understand why the loss is occurring and if it can be prevented (Hansen, 2011). Within the first four weeks after calving, the

energy balance and dry matter intake are essential in determining conception rates when cows are AI at days 70-100 post-calving (Diskin and Morris, 2008). The age of the cow is another contributing factor to early pregnancy loss. The age at breeding does affect the conception rates of dairy heifers, with the maximal conception rate at 15 to 16 months old; however, if a dairy heifer is bred at 26 months or older, the conception rate decreases by 10% compared to the heifer breed at a younger age (Kuhn *et al.*, 2006).

2.2 Pregnancy Failure in Humans

Similarly, early pregnancy loss is common in humans. Pregnancy loss occurs in approximately 15-30% of humans after human chorionic gonadotropin (hCG) becomes detectable in urine (Zinaman *et al.*, 1996; Quenby *et al.*, 2021). However, it has been estimated that 20% of pregnancies are lost before hCG levels are detected; therefore, the predicted early pregnancy loss is closer to 50% in women (Macklon *et al.*, 2002; Rai and Regan, 2006).

In women, pregnancy loss before 20 weeks is defined as a miscarriage, and about ¾ of all miscarriages occur before 12 weeks (Robinson, 2014). There are approximately 130 million births each year and around 23 million miscarriages each year, which is further broken down to 44 miscarriages a minute (Quenby *et al.*, 2021). As women age, the risk of miscarriage increases from 27% in women ages 25-29 to 75% in women over 45 (Hemminki and Forssas, 1999). In addition, women with impaired fertility had an early pregnancy loss of 70% compared to 21% in women without fertility problems (Hakim *et al.*, 1995).

Several risk factors contribute to pregnancy loss in women, including embryonic chromosomal abnormalities, endometrium defects, obesity, age, and lifestyle and environmental factors (Wilcox *et al.*, 1990). Chromosomal abnormalities include numerical (trisomy and

monosomy) and structural abnormalities. In addition, several endometrial defects can occur in women. In women, the endometrial stroma cell must undergo decidualization before the trophoblast cells can invade the endometrium to form the placenta (Gellersen and Brosens, 2014). However, if the decidua is not formed, the placenta cannot correctly attach to the endometrium, and the pregnancy will fail. Another risk factor is obesity and women who are obese have reduced fertility, recurrent miscarriages, pregnancy complications, and impaired decidualization, which can inhibit trophoblast invasion (Antoniotti *et al.*, 2018). Therefore, it is critical to understand if embryonic loss occurring due to the uterus can be prevented.

2.3 Early Pregnancy in Cattle and Humans

Fertilization occurs in the oviduct in mammals. The embryo develops in the oviduct until it reaches the morula stage, which is when it enters the uterus in cattle (Hackett *et al.*, 1993). The final stage in early embryonic development will occur in the uterus. The embryo will implant to continue to develop from an embryo to a fetus. Cattle are an important model for humans during early embryonic development because they share similar biochemical regulatory processes and have similar transcription profiles (Banliat *et al.*, 2022). The cow and human embryos also have similar oocyte diameters, development times, and the stage of zygotic genome activation (Ménézo and Hérubel, 2002). An RNA sequencing comparative study showed that bovine and human embryonic transcriptomes were more similar than mice and humans (Jiang *et al.*, 2014). Therefore, they suggest that bovine embryos are a better model for human embryonic development.

2.4 Glucose Metabolism by the Uterine Epithelium

Glucose enters the uterus through facilitative glucose transporters (GLUTs) and sodium-glucose cotransporters (SGLTs) from maternal circulation and is phosphorylated by hexokinase forming glucose-6-phosphate. Glucose can be metabolized through the pentose phosphate pathway, hexosamine biosynthesis pathway, glycolysis, or the polyol pathway (Chen and Dean, 2023). Glucose can also diffuse into the uterine lumen or be stored as glycogen (Frolova and Moley, 2011a; Sandoval *et al.*, 2021; Chen *et al.*, 2022; Gonzalez *et al.*, 2022). Glucose secretion into the uterine lumen from the glandular epithelium is tightly regulated as the concertation of glucose in uterine fluid is lower than in blood (Wales and Edirisinghe, 1989; Gardner *et al.*, 1996; Hugentobler *et al.*, 2008). Glucose will enter glycolysis to produce pyruvate and ATP (Akram, 2013). Glucose that enters the hexosamine biosynthesis pathway will be used for glycosylation of proteins and other glycoconjugates (Akella *et al.*, 2019). The pentose phosphate pathway will convert glucose into ribose-5-phosphate and is the major source of NADPH for the cell (Ge *et al.*, 2020).

2.5 Glucose Metabolism by the Embryo

Glucose is an essential nutrient for embryo development. After fertilization, pyruvate is the primary energy source; however, by the blastocyst stage, the embryo switches to glucose as the primary energy source in many species (Brinster, 1969; Leese and Barton, 1984; Brown and Whittingham, 1991; Conaghan *et al.*, 1993; Gardner *et al.*, 1993; Martin and Leese, 1995). A recent RNA sequencing study conducted on six species (human, mouse, pig, macaque, marmoset, and opossum) found evidence that embryos have low glycolytic activity during cleavage, then a peak in oxidative phosphorylation when the blastocyte starts to form, and finally, an increase in glycolysis during the embryonic disc formation (Malkowska *et al.*, 2022).

Several studies have looked at the effect of glucose on early embryo development. A study conducted using *in vitro* produced bovine embryos concluded that a glucose concentration above 2.5 mM impairs the embryo's development and shifts the sex ratio of the surviving embryos to more male (Kimura *et al.*, 2005). In mice, embryos cultured without glucose could not develop past the morula stage (Martin and Leese, 1995). *In vivo,* cows given an intravenous infusion of exogenous glucose (750 grams per day) decreased embryo length and area on day 14 of pregnancy compared to controls. Still, they did not affect glucose concentration in the uterine fluid (Leane *et al.*, 2018). Therefore, the embryo must have the correct amount of glucose to survive.

2.6 Uterine Fluid Glucose Concentrations

The endometrium can secrete glucose through glucose transporters into the uterine fluid, and the glucose concentration in the uterine fluid changes throughout the estrous cycle (Figure 1). The glucose concentrations ranged from 3.748 to 4.54 mM in uterine fluid throughout the estrous cycle in cows (Hugentobler *et al.*, 2008). Pregnant heifers have a higher glucose concentration at day 17 in the uterine fluid compared to nonpregnant heifers (Moraes *et al.*, 2020). A recent study on beef heifers determined that glucose concentrations in the uterine fluid were higher in animals with greater antral follicle counts and pregnant heifers (Snider *et al.*, 2022). The amount of glucose (nmol) in the uterine fluid was higher in pregnant ewes than in cyclic ewes from days 10 to 16 of pregnancy (Gao *et al.*, 2009).

In mice, glucose concentrations in the uterine fluid were lower the day after mating and then increased on days 2, 3, 4, and 5 of pseudopregnancy, but glucose could not be detected in unmated mice on day 1 (Wales and Edirisinghe, 1989). In humans, the glucose level changes throughout the menstrual cycle in oviductal fluid but not uterine fluid. The glucose concentration

was 3.11 mM in the follicular phase, decreased to 0.50 mM midcycle, then increased to 2.32 mM in the luteal phase in the oviduct and 3.15 mM in the uterus (Gardner *et al.*, 1996). Overall, glucose in the uterine fluid is likely necessary for pregnancy. The increase of glucose in uterine fluid may come from directly from maternal blood for glycogenolysis in the uterine epithelium.

2.7 Progesterone and Glucose

Progesterone possibly plays an essential role in glucose metabolism and glucose uptake or secretion in the uterus. Heifers with a high progesterone concentration, supplemented with a progesterone-releasing intravaginal device, had an increased uterine fluid glucose level on day 14 of the estrous cycle compared to heifers with a normal progesterone concentration (Simintiras *et al.*, 2019a, b). The authors suggest glucose could be the primary energy source when the embryo elongates in cattle (Leane *et al.*, 2018). In cows, progesterone supplementation increases glucose concentration in the uterine fluid (Hugentobler *et al.*, 2010). Additionally, ewes treated with progesterone (25 mg/day injection) from days 1.5 to 9 of pregnancy increased the amount of glucose in the uterine fluid on day 9 (Satterfield *et al.*, 2010). However, by day 12, the amount of glucose was the same as control animals. Therefore, progesterone increases glucose concentration in uterine fluid.

2.8 Facilitative Glucose Transporters (GLUTs)

GLUTs (Gene family *SLC2A*) transport glucose across the plasma membrane by facilitated diffusion. The *SLC2A* gene family encodes 14 different GLUTs divided into three classes (Deng and Yan, 2016). The GLUTs are divided into three classes based on their similarity in gene sequences. Class I represents GLUTs 1-4, class II represents GLUTs 5, 7, 9, and 11, and class III represents GLUTs 6, 8, 10, 12, and 13 (Navale and Paranjape, 2016).

Glucose transporters are essential in the female reproductive tract so that the glucose can enter the tissue to be stored as glycogen, secrete glucose into the uterine lumen, or be used in metabolic pathways within the tissue. GLUTs 1 (*SLC2A1*), 3 (*SLC2A3*), 4 (*SLC2A4*), 6 (*SLC2A6*), 8 (*SLC2A8*), 9 (*SLC2A9*), 10 (*SLC2A10*), and 12 (*SLC2A12*) are found in the female reproductive tract of several mammalian species (Welch and Gorski, 1999; von Wolff *et al.*, 2003; Mozzanega *et al.*, 2004; Korgun *et al.*, 2005; Frolova *et al.*, 2009; Kim and Moley, 2009; Frolova and Moley, 2011a). Specifically, GLUTs 1, 4, and 8 are found in the whole uterine homogenate, stroma, decidua, luminal epithelium, and glandular epithelium in rats, mice, and humans (Frolova and Moley, 2011b). In addition, GLUTs 3, 6, 9, 10, and 12 are found in the stroma and decidua in the mouse and humans (Frolova and Moley, 2011b).

Several studies have looked at the effect of progesterone on GLUTs in different species. Progesterone upregulates GLUT1 expression in murine and human endometrial stromal cells during decidualization and increases glucose uptake in murine endometrial stromal cells (Frolova *et al.*, 2009). GLUT1 expression was higher during the secretory phase when progesterone is increased than in the proliferative phase in human endometrium tissue but localized in the glandular epithelium (Zhang *et al.*, 2020). However, no difference was found in the expression of GLUT3. To better demonstrate the effect of progesterone on GLUT1, epithelial cells were treated with progesterone. Progesterone increased protein and mRNA expression of GLUT1. The researchers wanted to determine the effect of progesterone *in vivo* in a mouse model. The ovariectomized mice treated with progesterone had higher levels of GLUT1 protein in the uterus compared to the control group. Therefore, GLUTs could transport glucose from epithelial cells into the uterine lumen or cells from circulation.

2.9 Sodium-Glucose Cotransporters (SGLTs)

Sodium-glucose cotransporters (SGLTs, gene family *SLC5A*) use an inward sodium gradient the sodium-potassium ATPase generates to transport glucose into the cell. The *SLC5A* gene family encodes 12 different SGLTs (Vrhovac Madunić *et al.*, 2021).

SGLTs have not been studied extensively in the uterus except for SGLT1 (*SLC5A1*). SGLT1 is present in the small intestine, kidney, heart, liver, pancreas, brain, lung, prostate, and uterus (Vrhovac Madunić *et al.*, 2021). In mice and humans, SGLT1 is present in the endometrium and is essential for glucose accumulation in the uterus. A global SGLT1 knockout mouse (*Slc5a1*$^{-/-}$) decreased endometrial glycogen, litter size, and weight of pups at birth compared to wild-type mice (Salker *et al.*, 2017). Salker et al. (2017) also showed that SGLT1 (SLC5A1) gene expression increased in the primary human endometrial stromal cell after decidualization. In addition, progesterone increases the expression of SGLT1 in the endometrial epithelium of mice (Zhang *et al.*, 2021). In cattle, *SLC5A1* mRNA was detected in the luminal and glandular epithelium on days 13 and 16 in pregnant and open heifers (Forde *et al.*, 2010). Furthermore, on day 16, the expression of *SLC5A1* increased in the pregnant heifers compared to the open heifers. Ewes treated with progesterone had an increase in *SLC5A1* mRNA compared to control on days 9 and 12 of pregnancy (Satterfield *et al.*, 2010). Overall, it is known that SGLT1 is present in the uterus of many species. Therefore, SGLT1 could be an important transporter for free glucose to enter the uterine epithelium.

2.10 Glycogen Structure

Glycogen is a branched macromolecule composed of thousands of glucose residues. Glycogen has multiple tiers or layers, with the first tier holding around 13 glucose residues in

one chain. Hence, as the number of tiers increases, so does the number of glucose residues. For example, if one glycogen molecule has 12 tiers, it will have around 8,178 chains and approximately 53,235 glucose residues (Roach *et al.*, 2012). Glycogen is found in the liver, skeletal muscle, heart, brain, adrenal, adipose, placental tissue, and uterus (Demers *et al.*, 1972; Demers and Jacobs, 1973a; Calder, 1991).

A glucose residue is added to the existing linear chain of the glycogen molecule by an α-1,4 glycosidic bond; however, at a branch point, the two glucose residues are joined together by an α-1,6 glycosidic bond (Jespersen *et al.*, 1993). The liver is the only organ known where the glycogen molecules are comprised of α and β particles. The β particles are composed of thousands of glucose residues. The α particles are formed by connecting multiple β particles (Nawaz *et al.*, 2020). Glycogen within other organs is only composed of β particles. Glycogen molecules in the liver are composed of α and β particles with an average diameter of around 100-150 nm. However, glycogen molecules in skeletal muscle are composed of β particles with an average diameter of around 19-62 nm (Fawcett, 1955; Wanson and Drochmans, 1968).

2.11 Glycogenin

Glycogenin (GYG) is a small glucosyltransferase protein thought to be needed to form a new glycogen molecule (Rodriguez and Whelan, 1985; Pitcher *et al.*, 1988). Glucose residues are transferred from UDP-glucose to glycogenin to form the linear chain of glucose residues. First, glucose is transferred from UDP-glucose to a tyrosine residue within GYG (Roach *et al.*, 2012). α-1,4 glycosidic bonds form this first linear chain of 10 -20 glucose residues. In humans, glycogenin has two isoforms, GYG1 and GYG2 (Curtino and Aon, 2019). GYG1 is ubiquitously expressed, and GYG2 is expressed in the liver, cardiac muscle, and pancreas (Mu *et al.*, 1997). However, mice only express a single GYG gene (Zhai *et al.*, 2001).

Glycogenin knockout mice have high glycogen accumulation in striated muscles, which alters skeletal muscle function and decreases exercise endurance (Testoni *et al.*, 2017). In addition, humans with a mutation in GYG1 did not have GYG1 or GYG2 expressed in skeletal muscle; however, the skeletal muscle was still able to store glycogen (Visuttijai *et al.*, 2020). Therefore, glycogenin may not be necessary for synthesis of new glycogen molecules as previously thought.

2.12 Glycogen Metabolism

Once glucose enters a cell, it is phosphorylated by hexokinase, producing glucose-6-phosphate, and converted to glucose-1-phosphate by phosphoglucomutase (Figure 2). After glucose-1-phosphate is formed, the glucose residue is combined with UTP to form UDP-glucose. Glycogen synthase (GYS) is the enzyme that utilizes UDP-glucose to add glucose to an existing glycogen molecule. Once the elongating glycogen chain has at least 11 glucose residues, glycogen branching enzyme (GBE) transfers seven residues onto a new chain by an α-1,6 glycosidic bond, producing the branched spherical structure of glycogen (Meléndez *et al.*, 1999).

If the glucose level in the cell is low and glucose is needed, one way to provide glucose is to catabolize glycogen molecules. The two essential enzymes for cytosolic glycogen catabolism are glycogen phosphorylase (PYG) and glucose-6-phosphatase (G6PC). First, glycogen phosphorylase breaks a single α-1,4 glycosidic bond from a terminal branch of glycogen to release glucose-1-phosphate (Figure 3). This step will stop around four glucose residues away from the branching glucose residue in the glycogen chain (Ikeda *et al.*, 2022). Afterward, glycogen debranching enzyme (GDE) cleaves the three α-1,4 glycosidic bond glucose residues and the α-1,6 glycosidic bond to release glucose-1-phosphate (Adeva-Andany *et al.*, 2016). Glucose-1-phosphate is then converted back to glucose-6-phosphate by phosphoglucomutase.

Glucose-6-phosphate can be shunted into glycolysis, the pentose phosphate pathway, or the hexosamine biosynthesis pathway (Fothergill-Gilmore and Michels, 1993; Patra and Hay, 2014; Akella *et al.*, 2019). If the glucose residue is to be secreted, glucose-6-phosphatase dephosphorylates glucose-6-phosphate. Glucose formed by this final step can exit the cell through GLUTs.

2.13 Regulation of Glycogen Metabolism

Hexokinase has four isoforms: I, II, III, and IV (Katzen and Schimke, 1965; Ureta, 1982). Hexokinase I is found in most body tissues, such as the brain, fat pad, heart, liver, muscle, kidney, and intestine (Katzen and Schimke, 1965). Hexokinase II is found in insulin-sensitive tissue, including the brain, fat pad, heart, muscle, and intestine (Katzen and Schimke, 1965; Katzen, 1967; Bernstein and Kipnis, 1973). In addition, hexokinase III is present in the liver and kidney, and hexokinase IV, also known as glucokinase, is present in hepatocytes (Katzen and Schimke, 1965; Ureta, 1982; Printz *et al.*, 1993). Estradiol increased hexokinase activity in the uterus of ovariectomized rats (Valadares *et al.*, 1968). Hexokinase I – III can be inhibited by glucose-6-phosphatase, which will allow the regulation of glycogen synthesis (White and Wilson, 1989, 1990; Wilson, 2003).

Glycogen synthase is the rate-limiting enzyme in glycogenesis and has two isoforms, 1 and 2. Glycogen synthase1 is found in the heart, kidney, and muscle, whereas glycogen synthase 2 is located in the liver (Browner *et al.*, 1989; Nuttall *et al.*, 1994). However, glycogen synthase has at least ten residues that can be phosphorylated to reduce the activity of the enzyme in the muscle of the rabbit (Smith *et al.*, 1971; Lomako *et al.*, 1988). Several different ligands initiate the phosphorylation of glycogen synthesis, site 3 being an important site for maintaining the deactivation of the enzyme (Rayasam *et al.*, 2009). Cyclic AMP-dependent protein kinase

phosphorylates sites 1a, 1b, 2, 3a, and 4 (Embi *et al.*, 1981; Sheorain *et al.*, 1985). Site 2 is also phosphorylated by AMP-activated protein kinase (Carling and Hardie, 1989). Glycogen synthase kinase-3 phosphorylates sites 3a, 3b, 3c, and 4 (Rylatt *et al.*, 1980; Fiol *et al.*, 1987). Site 5 is phosphorylated by casein kinase II (Fiol *et al.*, 1987).

The activity of glycogen synthesis is controlled by the phosphorylation of nine serine residues (Jensen and Lai, 2009). Glucose-6-phosphate levels increase the activity of glycogen synthase and its affinity for glucose-6-phosphate. However, phosphorylation of glycogen synthesis leads to a reduction in the affinity for glucose-6-phosphate. The affinity of glycogen synthase for glucose-6-phosphate is increased by insulin and exercise and decreased by high glycogen concentration and adrenaline. Glycogen synthesis is activated by insulin due to the dephosphorylation of glycogen synthesis by inactivating glycogen synthesis kinase 3 (Bouskila *et al.*, 2008). However, it is inactivated by catecholamines and glucagon and increases glycogen levels in the liver (Danforth, 1965; Roach, 1990).

Glycogen phosphorylase (PYG) is the rate-limiting enzyme in glycogenolysis and has three isoforms discovered in the muscle (PYGM), brain (PYGB), and liver (PYGL). In 1938, glycogen phosphorylase activity was shown to be dependent on AMP by phosphorylating glycogen phosphorylase (Cori *et al.*, 1938; Newgard *et al.*, 1989). Glycogen phosphorylase has two interconvertible forms: phosphorylated and dephosphorylated glycogen phosphorylase. Glucose inhibits glycogen phosphorylase by binding to the active site (Newgard *et al.*, 1989). AMP stimulates glycogen phosphorylase activity, while glucose-6-phosphate, glucose, and ATP inhibit glycogen phosphorylase, inhibiting glycogen breakdown (Ercan-Fang *et al.*, 2002). A positive correlation was found between glycogen concentration and glycogen phosphorylase

activity; therefore, as glycogen levels increase within the muscle, so does the activity level of glycogen phosphorylase (Munger *et al.*, 1993).

Glucose-6-phosphatase is a membrane-bound enzyme located on the endoplasmic reticulum, with the active site being in the lumen of the endoplasmic reticulum, and has three isoforms (G6PC 1, 2, and 3) in mice and humans (Pan *et al.*, 1998; Marcolongo *et al.*, 2013). G6PC1 is mainly in the liver, intestine, and kidney (van Schaftingen and Gerin, 2002). G6PC2 is only in the pancreatic islet β-cells (Arden *et al.*, 1999). In contrast, G6PC3 is expressed in every tissue with the highest mRNA expression in the muscle (Martin *et al.*, 2002). The rise in glucose levels in the liver causes an increase in the mRNA expression of glucose-6-phosphatase (Argaud *et al.*, 1997).

2.14 Glycogen breakdown through α-acidic glucosidase

Another mechanism of glycogen breakdown is glycophagy (Figure 3). This process breaks down glycogen in phagolysosomes by the enzyme α-acidic glucosidase (GAA). First, glycogen is encapsulated into the autophagosome and then fused with a lysosome to degrade the glycogen into glucose by GAA (Koutsifeli *et al.*, 2022). GAA is processed from a 110 kDa precursor protein into the active 76 and 70 kDa enzymes in the lysosome (Moreland *et al.*, 2005). GAA breaks the α-1,4 links between two glucose molecules to release glucose from the glycogen molecule (Adeva-Andany *et al.*, 2016). Once glycogen is broken down into glucose, it diffuses out of the lysosome through glucose transporters and into the cytosol. In the cytosol, the cell could use glucose for metabolism or exit through glucose transporters into the lumen of the endometrium. α-glucosidase activity has been detected in the ovine endometrium, but activity did not change throughout the estrous cycle (O'Shea and Murdoch, 1978). This alternative pathway for glycogenolysis has not been well studied in the uterus.

2.15 Glycogen in the Uterus

The endometrium stores glycogen that can be broken down into glucose through glycogenolysis. Our lab has recently shown cows have a lower glycogen level in the luminal and glandular epithelium on day 11 vs. day 1 of the estrus cycle when progesterone levels are high (Sandoval *et al.*, 2021). In mink, progesterone decreases glycogen in the luminal and glandular epithelium in the uterus, while estrogen increases glycogen levels (Bowman and Rose, 2017). Peak endometrial glycogen concentration during the luteal phase correlates with women's fertility (Hughes *et al.*, 1969; Maeyama *et al.*, 1977).

A comparative study determined that estradiol increases glycogen levels in rats, rabbits, and guinea pigs (Demers and Jacobs, 1973a). Immature rats had an increase in uterine glycogen when treated with estradiol compared to the control rats (Demers and Jacobs, 1973b). Glycogen levels in the uterus change throughout the estrus cycle in the rat. Proestrus and estrus have a higher glycogen concentration than diestrus (Greenstreet and Fotherby, 1973).

2.16 Glycogen and Early Pregnancy

A study in rats compared glycogen concentrations in the uterus during the first seven days of pregnancy. They found that glycogen is the highest on day 1, then decreases until day 4, when the levels start to increase again, while glucose is the highest on day 4 (Greenstreet and Fotherby, 1973). In mink, glycogen levels decrease during pregnancy in the endometrium compared to estrus animals (Dean *et al.*, 2014). In addition, in pregnant mink’s endometrium, glycogen levels are undetectable in the glandular epithelium and decreased in the stroma and luminal epithelium compared to animals in estrus. A recent article found relative glycogen content decreased on days 2 and 4 of pregnancy in the glandular epithelium and on day 4 in

luminal epithelium compared to proestrus mice. However, glycogen content in the stroma increases in the implantation site on day 5.5 compared to mice in proestrus (Chen *et al.*, 2022). The authors also showed that glycogen content was higher in the uterine horn stimulated to decidualize than in the unstimulated uterine horn. Therefore, uterine glycogen may play an important role in providing nutrients during early embryo development and decidualization.

2.17 Membrane Progesterone Receptors

Membrane progesterone receptors (mPR) belong to the progestin and adiponectin receptor (PAQR) family and are 7-transmembrane receptors (Tang *et al.*, 2005). There are five membrane progesterone receptors found in mammalian species that include mPRα (*PAQR7*), mPRβ (*PAQR8*), mPRγ (*PAQ5*), mPRδ (*PAQR6*), and mPRε (*PAQR9*). In humans, mPRα, mPRβ, and mPRγ mRNA are expressed in the endometrium throughout the cycle (Fernandes *et al.*, 2005). They are also present in the human myometrium, amnion, chorion, and placenta. In sheep, mRNA expression of mPRα was found in the hypothalamus, pituitary, ovary, corpus luteum, and uterus throughout the estrus cycle (Ashley *et al.*, 2009). In cattle, mPRα, mPRβ, and mPRγ mRNA expression and protein were found throughout the estrous cycle and the first part of pregnancy in the endometrium and myometrium (Kowalik *et al.*, 2019). In canine, mPRα, mPRβ, and mPRγ mRNA expression and protein levels were found throughout gestation in the uterus and placenta, with the predominant location being in the trophoblast cells (Kazemian *et al.*, 2023).

Though they signal through G proteins, mPR are not part of the G-protein coupled receptors (GPCR) (Thomas *et al.*, 2007). mPR α, β, and γ were found to be coupled with inhibitory G proteins, while mPR ε and δ were coupled with stimulatory G proteins (Aickareth *et al.*, 2023). cAMP is a known second messenger of the mPR, which activates the stimulatory G

proteins to increase adenylyl cyclase activity and cause cAMP levels to increase in the cell (Thomas, 2022). Thus, more research is needed to better understand membrane progesterone receptors' function in the uterus.

2.18 Figures

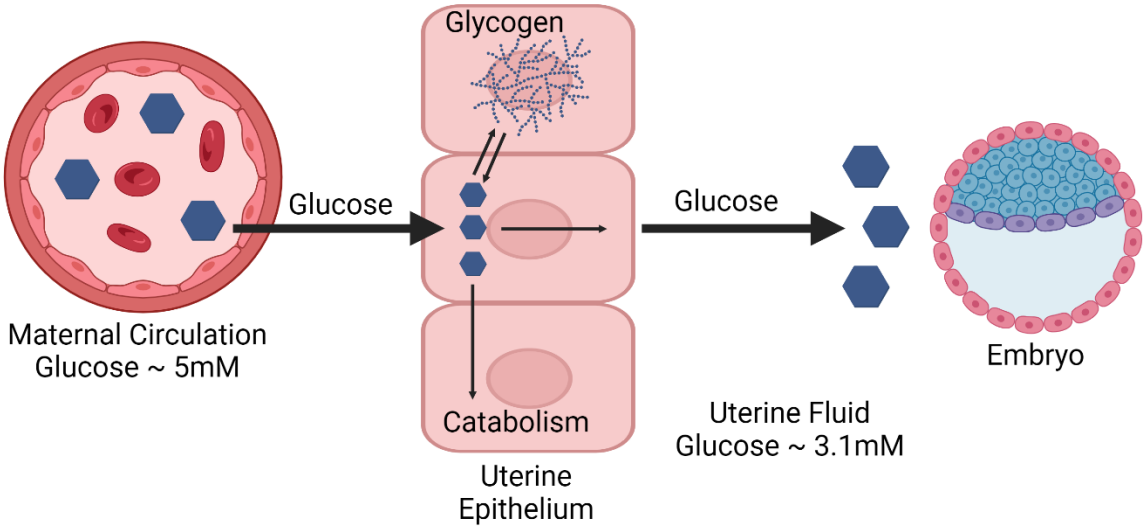


Figure 1. Glucose enters the uterine epithelium from the maternal circulation. The glucose concentration in maternal circulation is about 5 mM, and it enters the uterine epithelium through glucose transporters (GLUTs or SGLTs). Once in the uterine epithelium, glucose can be stored as glycogen, secreted into the lumen, or metabolized. Glucose concentration in the uterine fluid is around 3.1 mM.

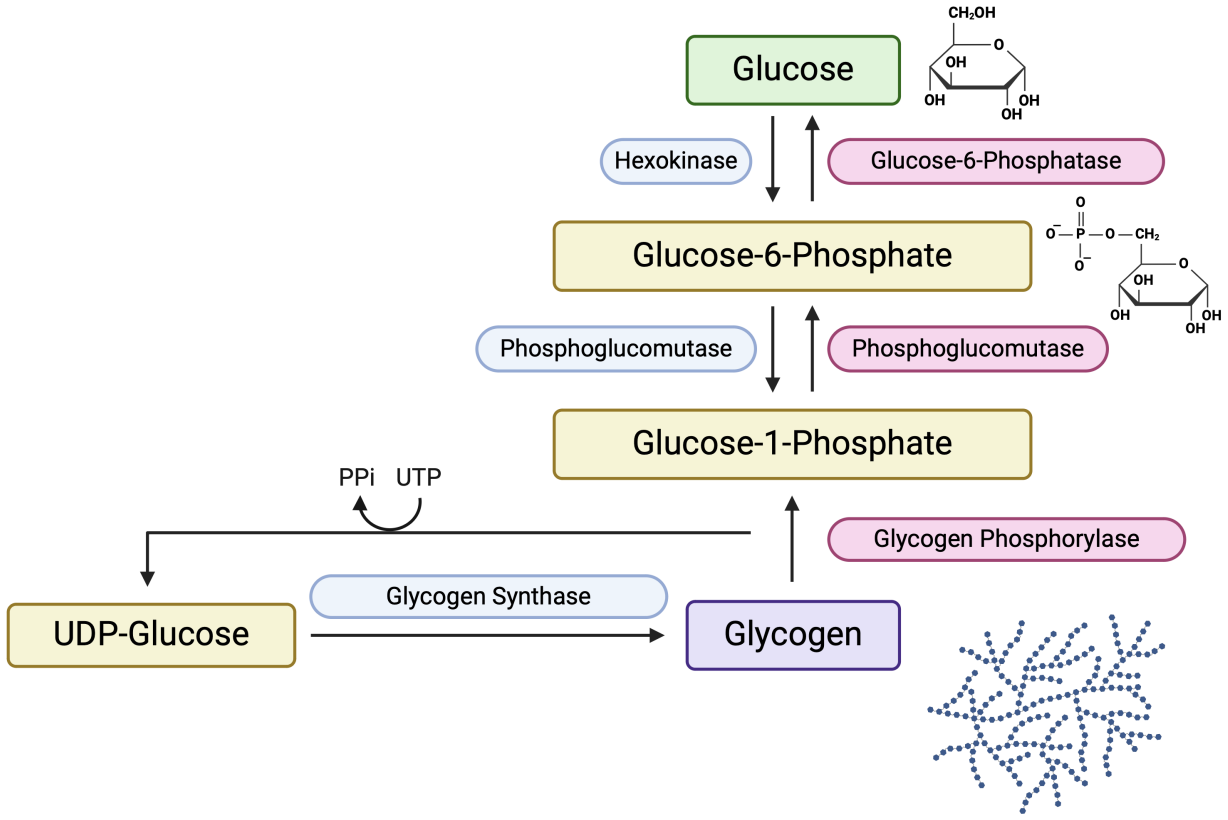


Figure 2. Glycogen metabolism pathway in the cytosol. The left side of the figure shows glycogenesis and the right side shows glycogenolysis. The enzymes important in glycogenesis are shown in blue boxes, and those important in glycogenolysis are demonstrated in pink boxes.

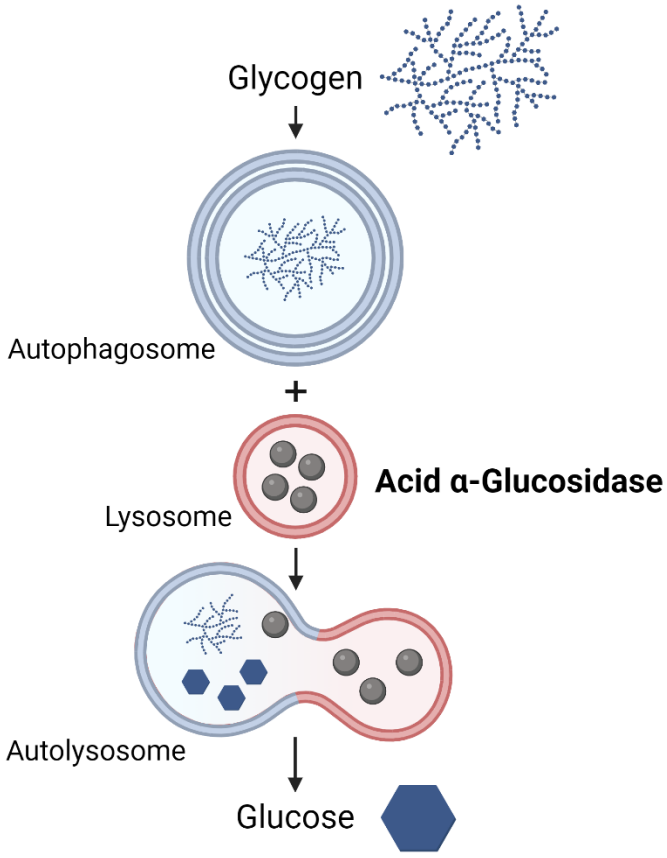


Figure 3. Pathway for glycogen breakdown in the lysosome. Glycogen will be taken into the autophagosome, and then acid α-glucosidase (GAA; shown in bold) in the lysosome will fuse with the autophagosome. This creates the autolysosome, where glycogen is broken down into glucose by GAA.

Chapter 3 Establishment and characterization of epithelial and fibroblast cell lines from the bovine endometrium[1]

3.1 Introduction / Material and Methods / Results / Conclusions

In both livestock and humans, it is estimated that close to 50% of fertilized embryos are lost before or during the implantation period (Wilcox *et al.*, 1988; Diskin *et al.*, 2015). Pregnancy loss in cattle has a major economic impact on producers (Lee and Kim, 2007). Cows can also be a useful biomedical model to understand human pregnancy loss (Ireland *et al.*, 2008). Like humans, cattle typically gestate a single fetus, and the preimplantation period in cattle is more similar to humans than the very short 4-day preimplantation period in mice. Hence, understanding communication between the embryo and the endometrium is critically important in animal agriculture and human medicine.

The uterine endometrium is composed of epithelium and the stromal region, which supports preimplantation development and is the site of implantation. The uterine lumen and the glands that branch from the lumen are lined with epithelial cells, while fibroblasts are the most common cell type found in the surrounding stroma. Communication between the embryo, epithelial cells, and fibroblasts is critical in normal endometrial function. For example, the proliferative effect of estradiol on the uterine epithelium is mediated by insulin-like growth factor secreted by the endometrial fibroblasts (Cooke *et al.*, 1997). In addition, during implantation, the embryo will interact with the uterine epithelium to allow the trophoblast cells

[1] Reproduced with permission from Springer Nature. Berg, M.D., Chen, Z. & Dean, M. Establishment and characterization of epithelial and fibroblast cell lines from the bovine endometrium. *In Vitro Cell.Dev.Biol.-Animal* **58**, 8–13 (2022). https://doi.org/10.1007/s11626-021-00640-z

to attach to the endometrium to help support further development of the embryo (Carson *et al.*, 2000). However, these interactions are difficult to dissect *in vivo*, particularly in species where gene editing is difficult or impractical. Therefore, establishing appropriate *in vitro* models is vital for elucidating the interactions between embryos, epithelial cells, and fibroblasts. The goal of this research was to develop immortalized bovine cell lines from the uterine epithelium and fibroblasts, which could be used to explore cell-to-cell communications within the uterus.

All procedures were approved by the University of Illinois Institution Animal Care and Use Committee (no.19036). The reproductive cycle of Holstein dairy cows was synchronized using the Double-Ovsynch protocol (Souza *et al.*, 2008). Endometrial samples were collected after sacrifice 11 days after behavioral estrus as part of a previous research project (Sandoval et al. 2021). Endometrial samples (~200 mg) were collected in L-15 media (10045CV, Corning) supplemented with penicillin-streptomycin (30001Cl, Corning) and transported to the lab. Samples were moved to digestion solution (1 mg/ml BSA [BP9706100, Thermo Fisher]; 0.5 mg/ml collagenase II [02100502.3, MP Biomedicals, Irvine, CA]; 1x trypsin/EDTA [TRL02, Caisson Labs, Smithfield, UT]; 20 units/ml DNase [FEREN0525, Thermo Fisher]; and 1x penicillin-streptomycin in L-15 media [LVL05, Caisson Labs]) and incubated for 1 hour at 37°C. After digestion, intact tissue was removed with tweezers. The resulting cells were centrifuged at 1,500xg for 10 minutes and plated in T-75 flasks (658175, Greiner Bio-One) in alpha-Minimum Essential Media (αMEM) media supplemented with 10% FBS (12103C-500ML, Sigma-Aldrich), 2 mM L-glutamine (GLL01-500ML, Caisson Labs), 10 mg/ml Insulin-Transferrin-Selenium (ITS; 354350, Corning), 1.8 ng/ml epidermal growth factor (EGF; 20053100UG, Shenandoah Biotechnology Inc), and 18.2 ng/ml estradiol-17β (E2758-1G, Sigma Life Science).

Epithelial and fibroblast cell populations were enriched using differential plating times (MacKintosh *et al.*, 2013). First, the mixed cell population was plated in a T-75 flask (658175, Greiner Bio-One) with αMEM media to allow the fibroblasts to attach. After 18 hours of culture the media containing unattached cells was passed to a new T-75 to allow epithelial cells still in the media to attach. These enriched populations were passed to 6-well plates (657160, Greiner Bio-One) where they were transfected with a pW2 plasmid expressing both the large T and small T antigens using lipofectamine 300 (Porrás *et al.*, 1996). Epithelial and fibroblast populations (500 cells) were passed to 10 cm dishes to allow for development of individual cell colonies. Enriched epithelial populations still appeared to contain a large number of fibroblasts. Therefore, these cells were grown in 1% FBS to preferentially support the growth of epithelial cells. After attached cells had grown into colonies of approximately 300-500 cells, individual colonies were selected by coating one end of cloning cylinders with sterile vacuum grease and placing a cylinder over each colony and collecting the colony with trypsin-EDTA. Each colony was then passed to one well of a 24-well plate and expanded (Mortensen *et al.*, 1997). After two rounds of colonial selection, five clones of bovine uterine epithelial (BUTE) cells were established, expanded (clones 1A, 1B, 1C, 1D, and 1E), and tested for expression of keratin (epithelial cell marker) and vimentin (fibroblast cell marker) by western blot as previously described (Sandoval *et al.*, 2021; Ziv-Gal *et al.*, 2021). The mouse uterus was used as a positive control. PVDF membranes were incubated in primary antibody (Table 1) overnight at 4°C. After three washes with TBS-T, the appropriate secondary antibody (Table 1) was added to the membrane and incubated for 30 minutes. All blots were developed using SuperSignal West Pick PLUS chemiluminescent substrate (34577, Thermo Scientific) and imaged using the ImageQuant LAS4000 (GE Healthcare Bio-Science). Of the five BUTE clones, BUTE1A cells were chosen

for further use based on the high expression of keratin, lack of vimentin, and cobblestone appearance (Figure 4A).

Enriched bovine uterine fibroblasts (BFIB) were also passed to 10 cm dishes, and three individual colonies were selected, expanded, and similarly analyzed by western blot. Murine oviductal epithelial cells (MOE) were used as a positive control for kertain (Eddie *et al.*, 2015). Clone BFIB2 were chosen to model uterine fibroblasts due to their lack of keratin expression, high expression of vimentin, and spindle-like morphology (Figure 4B).

The expression of vimentin and keratin was confirmed by immunofluorescence. Cells were cultured on coverslips pretreated with poly-L-lysine (P4707, Sigma-Aldrich), fixed with 4% paraformaldehyde (150146, MP Biomedicals), and permeated with 1% Triton X-100 (TX1568, Millipore). Cells were incubated with 1:250 dilution of the primary antibody against vimentin and 1:500 against cytokeratin at 4°C overnight (Table 1). After 3 washes with PBS, a 1:500 dilution of Alexa-Flour conjugated secondary antibody was added, and coverslips were incubated in the dark for 1 hour at room temperature. Coverslips were mounted with VECTASHIELD Mounting Media containing DAPI (H-1500-10, Vector Laboratories). Fluorescent images were captured with an LSM700 confocal microscope (Zeiss). Negative control slides were processed the same, except that the primary antibody was replaced with an isotype negative control (anti-GFP antibody).

Immunofluorescence confirmed that the BUTE1A cells expressed high levels of cytokeratin and low levels of vimentin, while BFIB2 exhibited high levels of vimentin and lacked detectable cytokeratin (Figure 4C). Confirming successful integration of the plasmid, both BUTE1A and BFIB2 cells expressed SV40 large T antigen as assessed by western blot (Figure 4D; mouse uterus as negative control).

The e-Myco™ plus Mycoplasma PCR Detection kit (2523448, Bulldog Bio) was used to determine if mycoplasma were present. Fifty thousand BUTE1A and BFIB2 cells were lysed by boiling at 95°C. The manufacturer's instructions for protocol II were followed to complete the PCR. Samples were loaded onto a 2% agarose gel and ran for 90 minutes at 100 volts. The gel was imaged using the ImageQuant LAS4000 (GE Healthcare Bio-Science). An internal control (IC) and sample control (SC) were used to confirm successful PCR reactions. Positive and negative controls provided by the manufacturer confirmed the specificity of the primers. Both cell lines tested negative for mycoplasma (Figure 4E).

PCR for the cytochrome c oxidase subunit I gene (*Cox1*) gene was used to confirm the species of origin for BUTE1A and BFIB2 cells (Parodi *et al.*, 2002). Primers (forward: CTACTACTC CTCGCATCCTCTAT and reverse: CGGGTCGAAGAAGGTTGTATT) were designed for *Cox1* (accession number NC_006853.1). DNA was collected from BUTE1A and FIB2 cells using the Quick-DNA Miniprep kit (D3024, Zymo Research). The manufacturer's instructions for cell culture were followed to complete the DNA extraction. PCR was carried out using 100 µg of the resulting gDNA. The thermo-cycling condition were 95 ℃ for two minutes followed by 95 ℃ for one minute, 55 ℃ for one minute and 72 ℃ for 30 seconds for a total of 40 cycles then 72 ℃ for five minutes in the T100 Thermal Cycler (BioRad). The PCR product were loaded onto a 2% agarose gel containing gel red (41008 Biotium) and ran for 90 minutes at 100 volts. The gel was imaged using the FluorChem M imager (Protein simple). The BUTE1A and BFIB2 cells had a band present at the appropriate size of 339 bp (Figure 4F). Therefore, it can be determined that the species of origin for the BUTE1A and BFIB2 cells is *Bos taurus.*

BUTE1A and BFIB2 cells grew well in culture. To date, the BUTE1A cells have been passed up to 45 times and BFIB cells up to 30 times with minimal changes in morphology or

growth. To more accurate assay growth, the doubling time for each cell line was determined. One hundred BUTE1A and BFIB2 cells were plated in 24-well plates (662160, Greiner Bio-One). Starting the next day, cells were imaged every 24 hours. The number of cells per image was counted using ImageJ (n=3). Exponential growth was modeled using GraphPad Prism v 9.0.0 to establish the doubling time for each cell line. BUTE1A cells had a doubling time of 74.99 hours, while the doubling time of BFIB2 cells was only 28.35 hours ($P<0.001$, two-tailed T-test; Figure 5A). Both BUTE1A and BFIB2 cells also displaced contact inhibition. After reaching confluence in a 24-well plate, dead cells did accumulate in the media; however, a confluent monolayer was maintained for at least six days (Figure 5B).

Estrogen and progesterone are important regulators of endometrium function, with direct effects on both the epithelial cells and fibroblasts. To determine if BUTE1A and BFIB2 could respond to these hormones, both cell lines were analyzed by a western blot for the presence of estrogen and progesterone receptors (ER and PR, respectively) using previously validated antibodies (Ziv-Gal *et al.*, 2021). As expected, both BUTE1A and BFIB2 cell lines expressed ERα, PR-A, and PR-B (Figure 6A; positive control mouse uterus).

We have recently shown that both the epithelium and stroma of the bovine endometrium stores glycogen (Sandoval *et al.*, 2021), and glycogen in the uterus is thought to be an important source of glucose for both the embryos and endometrium (Dean, 2019). Therefore, we sought to determine if BUTE1A and BFIB2 cells expressed key glycogen metabolizing enzymes. Western blots using previously validated antibodies (Sandoval *et al.*, 2021) confirmed that both BUTE1A and BFIB2 cells expressed hexokinase 1, glycogen synthase, glycogen phosphorylase, and glucose-6-phosphatase 3 (n=3; Figures 6B; positive control mouse uterus). Our previous work indicated that most endometrial glycogen is localized to the uterine epithelium. To confirm that

BUTE1A cells did in fact store glycogen, we analyzed glycogen content after treatment with insulin (0-100 ng/ml; I2643-25MG, Sigma-Aldrich) as previously described (Dean and Rose, 2018). After 48 hours of treatment, BUTE cells were collected with trypsin-EDTA, counted, resuspended in 400 µl of 30% potassium hydroxide (KOH; 221473-500G, Sigma-Aldrich) to inhibit enzyme activity, and stored at -20°C. Samples were warmed to room temperature, incubated for 30 minutes at 95 °C, and then centrifuged for 1 minute at 21,130xg at room temperature. Next, 1.2 volumes of pure ethanol were added, and they were centrifuged for 20 minutes at 21,130xg at room temperature. The supernatant was decanted and 250 µl of 70% ethanol was added to each sample. The samples were centrifuged for 20 minutes at 21,130 RCF at room temperature and decanted. Samples were dried overnight before adding 50 µl of hydrochloric acid (HCl; A144-500, Fisher Chemical) and incubated at 95 °C for three hours to hydrolyze the glycogen to glucose. Next, 50 µl of sodium hydroxide (NaOH; S318-500, Fisher Chemical) was added to neutralize the acidity. Each sample (40 µl) was mixed with 250 µl of Wako Reagent (99703001, FUJIFILM Medical Systems USA) in a 96-well plate (655180**,** Greiner Bio-One) in duplicate and incubated for 30 minutes at 37°C. Absorbance was read at 505 nm using the µQuant™ (Biotek Instruments). Samples were compared to a standard curve (3-200 µg/ml glycogen) and normalized to cell number.

Insulin (50 ng/ml) increased glycogen content 2-fold ($P<0.001$), while 100 ng/ml of insulin increased glycogen content 58% ($P=0.084$ via one way-ANOVA followed by Dunnett's test; n=5; Figure 6C). These results confirm the ability of BUTE1A cells to store glycogen and indicate that BUTE1A and BFIB cells may make a useful model to study cell-to-cell commutation in the bovine uterus.

Communication between the cells of the uterus and embryo is critical in establishing a successful pregnancy. Animal embryos can be generated in a relatively straightforward manner, though they can only be maintained in culture for a certain number of days before requiring transfer to a recipient female. In mice, genetic engineering has allowed researchers to probe this communication by knocking out genes in a tissue-specific manner. However, in other species, these types of experiments are difficult to impossible. Primary cells are the preferred method for studying cell communication in vitro. However, that requires collecting tissue for a local abattoir, which may not be available for all investigators. Additionally, the isolation and expansion of primary cells can be time-consuming and may not yield pure cell populations. This has led to the development of uterine epithelial and fibroblast cell lines from the pig and mink (Moreau *et al.*, 1995; Wang *et al.*, 2000). However, we are unaware of any cell lines produced from the bovine uterus until now.

Many studies on uterine function use mice, since murine models are economical, have short pregnancies and can be easily genetically modified. However, the reproduction of mice is different from other mammals in important ways. For example, the preimplantation period in mice is only four days long, and during most of that time the embryos are in the oviduct not the uterus (Potts and Wilson, 1967). In contrast, the preimplantation period in humans and cows is 12 and 20 days, respectively. In both cows and humans, uterine glands are more numerous than in mice, highlighting a role for glandular secretions in those species (Gray *et al.*, 2001). However, other aspects of human uterine biology are better mimicked by mice. For example, the uteri of humans and mice undergo decidualization and both species have endotheliochorial placentas. Therefore, it is important to understand cell communication in the uterus of a wide variety of mammalian species.

In conclusion, we have developed and validated cell lines to model uterine epithelial and fibroblast cells from cows. These cells express the appropriate cell markers and steroid receptors, indicative of their cell type. These cells will allow researchers to better model the cow uterus in vitro and lead to insights on normal cell communication between the epithelium and fibroblasts.

3.2 Table and Figures

Table 1. Primary and secondary antibodies with conditions used for western blot (WB) and immunofluorescence (IF) analysis. Block (BSA or milk) was diluted to 5% in TBS-T.

Antigen	Catalog No	Technique	Dilution	Block
Primary Antibodies				
Glycogen Synthase	3886 Cell Signaling	WB	1:100	BSA
Hexokinase 1	2024 Cell Signaling	WB	1:1000	BSA
Glycogen Phosphorylase	Ab231963 Abcam	WB	1:500	BSA
Glucose-6-phosphatase 3	PA5-70653 Invitrogen	WB	1:500	BSA
Pan-keratin	4545 Cell Signaling	WB	1:250	BSA
		IF	1:250	Goat Serum
Vimentin	5741 Cell Signaling	WB	1:500	BSA
		IF	1:250	Goat Serum
Large T antigen	15729 Cell Signaling	WB	1:1000	BSA
Progesterone Receptor	A0321 ABclonal	WB	1:500	Milk
Estrogen Receptor alpha	A0296 ABclonal	WB	1:500	Milk
GFP	2956 Cell Signaling	IF		Goat Serum
α tubulin	2144 Cell Signaling	WB	1:500	BSA
β actin	A2066 Sigma-Aldrich	WB	1:1000	Milk
Secondary Antibodies				
Rabbit	7074S Cell Signaling	WB	1:1000	BSA or Milk
Mouse	7076S Cell Signaling	WB	1:1000	BSA or Milk
Rabbit	A11037 Thermo Scientific	IF	1:500	Goat Serum
Mouse	A11001 Thermo Scientific	IF	1:500	Goat Serum

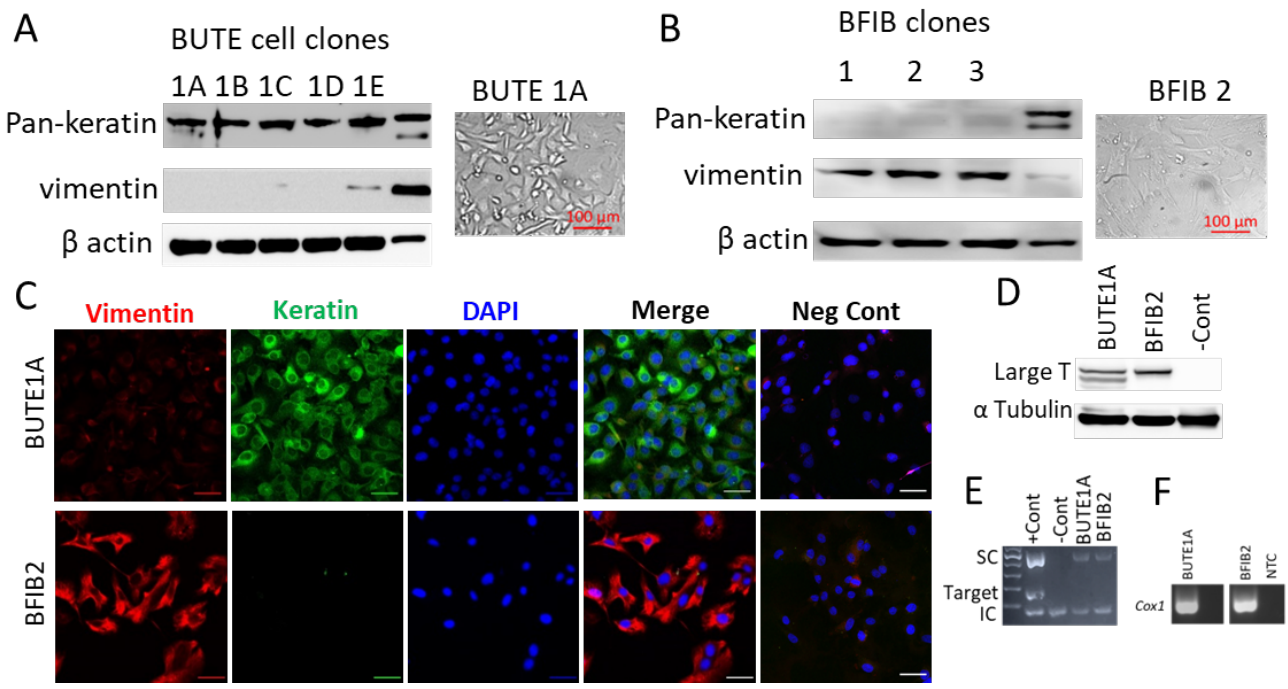


Figure 4. BUTE1A and BFIB2 are valid models of the endometrial epithelium and fibroblasts. A) Five clones of BUTE cells were analyzed for expression of keratin and vimentin by western blot. +Cont, mouse uterus. B) Keratin and vimentin expression was assessed in three BFIB clones. +Con, murine oviductal epithelial (MOE) cells. A-B Representative of 3 western blots. C) BUTE1A and BFIB cells were analyzed for expression of keratin and vimentin by immunofluorescence (scale bar is 50 µm). Neg Cont, primary antibody replaced with anti-GFP antibody. n=3. D) BUTE1A and BFIB2 cells express large T antigen. -Cont, mouse uterus. E) BUTE 1A and BFIB2 cells are negative for mycoplasma (sample control, SC; internal control, IC). +Cont and -Cont, templates provided with kit. F) PCR bands generated in BUTE1A and BFIB cells using bovine specific primers for the *Cox1* gene. NTC, no target control.

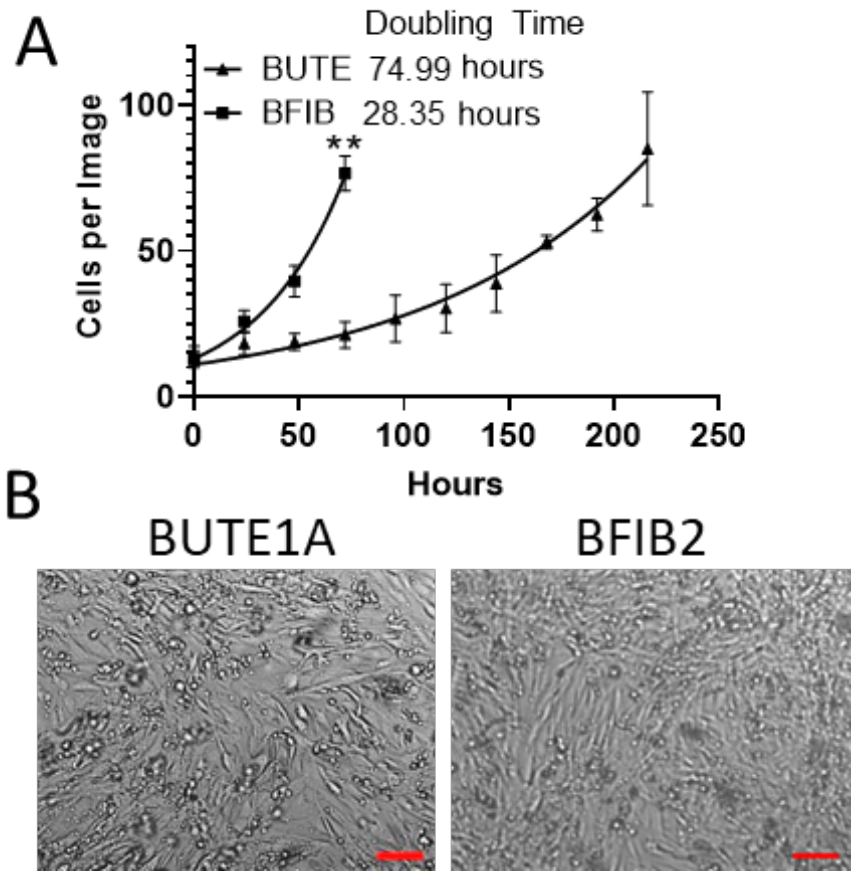


Figure 5. Growth characteristics of BUTE1A and BFIB2 cells. A) One hundred BUTE1A or BFIB2 cells per well were plated in 24-well plates in complete media. The center of each well was photographed every 24 hours for 4 or 14 days. The number of cells was counted in each image using ImageJ. Doubling rate was calculated by modeling exponential growth using GraphPad PRISM version 9.0.0. B) BUTE1A and BFIB2 cells display contact inhibition (scale bar is 100 μm). Upon reaching confluence, BUTE1A and BFIB2 cells maintain a confluent monolayer for at least six days. n=3. **P<0.01.

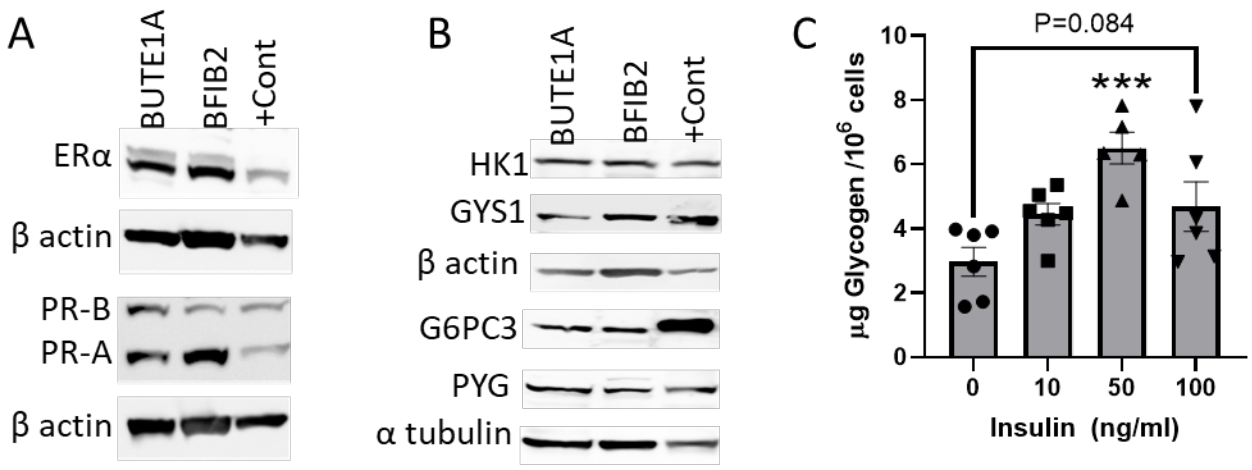


Figure 6. BUTE1A and BFIB2 cells express steroid receptors and glycogen metabolizing enzymes. A and B) Proteins from BUTE1A and BFIB2 cells were isolated with RIPA and separated with a 10% SDS-PAGE gel. Proteins were transferred to PVDF membrane and probed overnight with primary antibodies against estrogen receptor alpha (ERα) progesterone receptor A and B (PR-A and PR-B) (A) and glycogen metabolizing enzymes (HK1, hexokinase 1; GYS1, glycogen synthase; G6PC3, glucose-6-phosphatase 3 and PYG, glycogen phosphorylase) (B) A-B +Cont, mouse uterus. Representative of 3 western blots. C) BUTE1A cells were treated with insulin for 24 hours and assessed for glycogen. n=5. ***P<0.001.

Chapter 4 Membrane progesterone receptors mediate progesterone-stimulated glycogenolysis in the uterine epithelium

4.1 Abstract

Abnormal glucose concentrations in the uterine lumen can contribute to pregnancy loss. The demand for glucose increases dramatically as the morula enters the uterus. Yet, how glucose diffusion into the uterine lumen is regulated remains unclear. We have previously shown glycogen levels in the bovine uterine epithelium decrease in the luteal phase. The aim was to elucidate the role of progesterone in glycogen breakdown in uterine epithelial cells. Progesterone decreased glycogen levels in bovine uterine epithelium (BUTE) cells. RU486, a nuclear progesterone receptor (nPR) antagonist, did not block progesterone's effect. RT-PCR confirmed that BUTE cells express all five membrane progesterone receptors (mPR). A specific mPR agonist (Org OD 02-0) reduced glycogen levels in BUTE cells. Immunohistochemistry showed that the bovine uterine epithelium expresses mPRs. These results suggest that progesterone acts through mPRs to stimulate glycogenolysis. Neither progesterone nor Org OD 02-0 changed intracellular cAMP concentrations. BUTE cells treated with progesterone had an increase in phospho-AMPK levels at 24 hours. However, experiments with the AMPK activator (A-769662) and the AMPK inhibitor (dorsomorphin) indicated that AMPK did not mediate glycogenolysis. D942 treatment, which increases intracellular AMP concentrations, decreased glycogen levels. Glycogen phosphorylase inhibitor (GPI) was only able to partially block the effect of progesterone on BUTE cells. Confirming these results in humans, progesterone and Org OD 02-0 had similar effects in Ishikawa (human uterine epithetical) cells. In conclusion, progesterone stimulates glycogen breakdown in the uterine epithelium via mPR/AMP signaling. Glucose

released from glycogen could support embryonic development or be metabolized by the uterine epithelium.

4.2 Introduction

In cattle, around 90% of oocytes will be fertilized; however, early embryonic loss occurs in about 40-60% of pregnancies in both beef and dairy cows (Hansen, 2011; Ealy and Seekford, 2019; Reese *et al.*, 2020). In cattle, most of these pregnancy failures occur before day 32 of pregnancy (Reese *et al.*, 2020). In humans, early pregnancy loss occurs in 15-30% of pregnancies after human chorionic gonadotropin (hCG) becomes detectable in urine (Zinaman *et al.*, 1996; Quenby *et al.*, 2021). It has been estimated that around 20% of pregnancies are lost before hCG levels are detectable; therefore, the predicted early pregnancy loss is close to 50% in women (Macklon *et al.*, 2002; Rai and Regan, 2006). In women, about 75% of all pregnancy losses occur before 12 weeks (Robinson, 2014). Hence, in both species a majority of pregnancy losses occur before or during implantation.

Several factors contribute to early embryonic loss in cattle and humans, such as embryonic abnormalities and inadequate uterine support. An essential nutrient for the preimplantation embryo is glucose, which must diffuse into the lumen by facilitate diffusion. After fertilization, lactate is the primary energy source as the embryo moves through the oviduct (Leese and Barton, 1984; Krisher and Prather, 2012). As the morula enters the uterus, the embryo switches to glucose as the primary energy source (Brinster, 1969; Leese and Barton, 1984; Brown and Whittingham, 1991; Conaghan *et al.*, 1993; Gardner *et al.*, 1993; Martin and Leese, 1995). The demand for glucose increases by 50-fold by the blastocyst stage compared to the 2-cell stage, but glucose becomes toxic if the concentration is too high (Gardner *et al.*, 1993;

Khurana and Niemann, 2000; Uhde *et al.*, 2018). How the uterus regulates glucose concentrations in uterine fluid to match the changing needs of the embryo is unclear.

Progesterone may play an essential role in glucose uptake and metabolism in the uterus. In heifers, progesterone concentrations on day 14 of the estrus cycle and glucose concentrations in uterine fluid were positively correlated (Simintiras *et al.*, 2019a). Similarly, multiple studies in cattle have shown that progesterone supplementation increased the glucose concentration in uterine fluid (Hugentobler *et al.*, 2010; Satterfield *et al.*, 2010; Simintiras *et al.*, 2019b).

A potential source of glucose for the embryo is from glycogen stored in the uterine epithelium. Each glycogen molecule is composed of tens of thousands of glucose residues joined by α(1→4) and α(1→6) linkages. Glycogen is present in the endometrium of many species, including cattle, mice, rats, and humans (Dean, 2019). Our lab has recently shown that in mice, glycogen levels are decreased in the uterine epithelium during the preimplantation period relative to proestrus (Chen *et al.*, 2022). We have also shown that cows have a lower glycogen levels in the luminal and glandular epithelium on day 11, when progesterone levels are high, compared to day 1 of the estrus cycle (Sandoval *et al.*, 2021). Hence, progesterone-stimulated glycogenolysis may play a role in providing glucose to endometrial tissue or the growing embryo.

Membrane progesterone receptors (mPRs) belong to the progestin and adiponectin receptor (PAQR) family and are 7-transmembrane receptors (Tang *et al.*, 2005). cAMP is a known second messenger of the mPRs by activation of the stimulatory G proteins which increase adenylyl cyclase activity to cause cAMP concentrations to increase inside the cell (Thomas, 2022). There are five membrane progesterone receptors found in mammals—mPRα (*PAQR7*), mPRβ (*PAQR8*), mPRγ (*PAQR5*), mPRd (*PAQR6*), and mPRe (*PAQR9*). In humans, mPRα, mPRβ, and mPRγ mRNA are expressed in the endometrium during all stages of the cycle

(Fernandes *et al.*, 2005). In bovine endometrium, mPRα, mPRβ, and mPRγ mRNA and protein were found throughout the estrus cycle and the first part of pregnancy (Kowalik *et al.*, 2019). Thus, mPRs are clearly expressed in the endometrium, but no functional role for them has been discovered in the uterus. The aim of this study was to determine if progesterone decreases glycogen levels in the uterine epithelium and elucidate the pathways activated by progesterone that stimulates glycogenolysis.

4.3 Material and Methods

Cell Culture

Bovine uterine epithelial (BUTE) cells were generated from the endometrium of a Holstein dairy cow on the University of Illinois Dairy Farm by our laboratory. They have been previously validated and described (Berg *et al.*, 2022; Gonzalez *et al.*, 2022). BUTE cells were grown in a-Minimum Essential Media (αMEM) media containing 5.55 mM glucose (complete composition in Supplemental Table 1) supplemented with 10% FBS (12103C-500ML, Sigma-Aldrich), 2 mM L-glutamine (GLL01-100ML, Caisson Labs), 10 mg/ml ITS (25-800-CR, Corning) 1.8 ng/ml EGF (20053100UG, Shenandoah Biotechnology), and 18.2 ng/ml estradiol-17β (E2758-1G, Sigma). Ishikawa cells were grown in DMEM/F12 media with 14.5 mM glucose (complete composition in Supplemental Table 2) supplemented with 10% charcoal-stripped FBS. Both cell lines were maintained at 37°C in a humidified incubator with 5% CO_2. Cell lines were passed every 3-4 days.

Cell Culture Experiments

BUTE and Ishikawa cells were plated and allowed to grow until 80% confluent. Then the media was removed, rinsed twice with PBS, and the cells were serum and steroid starved in media without phenol red and treated with insulin-like growth factor 1 (IGF1, 50 ng/ml;

2000550UG, Shenandoah Biotechnology) for 24 hours to stimulate glycogenesis (Gonzalez *et al.*, 2022). The media was removed and replaced with serum and steroid-free media containing 50 ng/ml of IGF1 and appropriate treatments for indicated times. Controls were treated with vehicle (DMSO) for all experiments.

Hormone, Activators, and Inhibitors

IGF1 (2000550UG, Shenandoah Biotechnology) was dissolved in sterile PBS at 100 µg/ml. Progesterone (P8783-5G, Sigma Aldrich) and Org OD02-0 (mPR agonist; 2085, Axon Med Chem) was dissolved in DMSO at 100 mM and then diluted to 0.1, 1, and 10 mM in DMSO. RU486 (nPR antagonist; 10006317, Cayman Chemical Company), forskolin (adenyl cyclase activator; 11018, Cayman Chemical Company), D942 (increases intracellular AMP; 14741, Cayman Chemical Company), A-769662 (AMPK activator; 11900, Cayman Chemical Company), dorsomorphin (AMPK inhibitor; 14741, Cayman Chemical Company), and glycogen phosphorylase inhibitor (GPI; 17578, Cayman Chemical Company) were dissolved in DMSO. The final concentration of compounds in media consisted of 0, 0.1, 1, and 10 µM for progesterone and Org OD02-0; 10 µM for RU486, forskolin, D942, A-769662, and GPI; and 5 µM for dorsomorphin. DMSO was used as the vehicle control for all experiments.

Western Blot

BUTE and Ishikawa cells were plated in a 10 cm dish and treated as indicated. After the conclusion of the treatment, media was removed from each 10 cm plate, rinsed once with PBS, lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (P0044-1ML, SigmaAldrich and A32953, Thermo Scientific) and stored in at -20°C. Protein concentration was determined with Pierce™ BCA Protein Assay Kit (23227, Thermo Scientific). Western blots were carried out as previously described (Sandoval *et al.*, 2021; Ziv-

Gal *et al.*, 2021; Gonzalez *et al.*, 2022). Protein (25-35 µg) was loaded on 10% SDS-PAGE gel and ran until adequate separation was achieved at 120 volts. The gel was then transferred onto a PVDF membrane. Membranes were blocked with 5% BSA in tri-buffers saline (TBS) with 0.1% tween (TBS-T). The primary antibodies (Supplemental Table 3) were diluted in their respective block, added to the membranes, and incubated overnight at 4°C. After three washes with TBS-T, the appropriate secondary antibody (anti-rabbit; 7074S, Cell Signaling) was added to the membrane and incubated for 30 minutes. All blots were developed using SuperSignal West Pick PLUS chemiluminescent substrate (34577, Thermo Scientific) and imaged using the FluorChem M (Protein Simple).

Immunohistochemistry

Uteri from cows were collected on days 1 and 11 of the estrus cycle, fixed, and placed into paraffin blocks as part of previous project (Sandoval *et al.*, 2021). Paraffin blocks were sectioned at 5 µm, and two to three sections were placed on a slide and allowed to dry overnight. Slides were deparaffinized and rehydrated. Slides were placed in sodium citrate and microwaved to retrieve the antigen. The slides were briefly placed in TBS to ensure they were at room temperature, followed by 3% hydrogen peroxide (H325-500, Fisher Scientific) for 15 minutes. The slides were placed into a hydrated chamber and a species-specific serum block (3% BSA, 10% rabbit or goat serum, in TBS) was added to the slides for 1 hour at room temperature.

The primary antibodies are listed in Supplemental Table 3. The antibody against mPRα was previously validated (Kowalik *et al.*, 2019). Here the antibodies for mPRδ and mPRε were validated using a blocking peptide (Supplemental Figure 1A). Antibodies were added to the slides and incubated at 4°C overnight. The slides were washed in TBS-T then a species-specific secondary antibody (BA-1000 or BA-5000, Vector Laboratories) was added for thirty minutes at

room temperature, and the ABC complex (PK-4000, Vector Laboratories) was prepared. The slides were washed, and the ABC complex was added to the slides for thirty minutes. After the final wash, the slides were placed on white bench paper to witness the DAB reaction (SK-4100, Vector Laboratories). Then the slides were counterstained, dehydrated, and placed in xylene overnight to be mounted with Permount (SP15100, Fisher Scientific) the next day. Images were captured with a Zeiss Axioskop upright microscope with an Axiocam 305 color camera. Negative control slides were processed the same except that the primary antibody was replaced with an isotype specific negative control (anti-GFP antibody; 2956 Cell Signaling).

Glycogen Assay

Intracellular glycogen was measured as previously validated by our laboratory (Berg *et al.*, 2022; Gonzalez *et al.*, 2022). At the end of treatment, BUTE and Ishikawa cells were collected with trypsin, counted, centrifuged, resuspended in 30% potassium hydroxide (KOH; 221473-500G, Sigma-Aldrich), and stored in at -20°C. Samples were thawed to room temperature and the standard curve (0-200 µg/ml glycogen) was made. All samples including the standard curve and blank were incubated for 30 minutes at 95°C then centrifuged for 1 minute at 18,213 x g. After, 480 µl of 100% ethanol was added to all samples and centrifuged for 20 minutes at 18,213 x g to precipitate glycogen. The supernatant was removed and 250 µl of 70% ethanol was added. The samples were centrifuged for 20 minutes at 18,213 x g to remove any remaining glucose, the supernatant was decanted, and samples dried overnight. The next day 50 µl of hydrochloric acid (HCl; A144-500, Fisher Chemical) was added to each sample and incubated at 95°C for three hours to hydrolyze glycogen. Once an hour all samples were vortexed and centrifuged. After the three-hour incubation, 50 µl of sodium hydroxide (NaOH; S318-500, Fisher Chemical) was added to the samples and mixed to neutralize acidity. Next, 40

µl of the sample was placed into a 96 well plate in duplicate. Wako Reagent (250 µl; 99703001, FUJIFILM Medical Systems USA) was added to each well and incubated for 30 minutes at 37°C. The plate was read at 505 nm using the µQuant™ (Biotek Instruments). The standard curve was used to determine the amount of glycogen in each sample, which was normalized to the number of cells.

RNA Isolation and Reverse-Transcriptase Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to determine if BUTE and Ishikawa cells expressed the mPRs. Total RNA was extracted from BUTE and Ishikawa cells using TRIzol (15-596-018, Thermo Fisher Scientific). Iscript™ Reverse Transcription Supermix (1708841, Bio-Rad) was used to transcribe RNA into cDNA for PCR. Promega GoTaq™ DNA Polymerase (PRM3005, Thermo Fisher Scientific) kit was used for the PCR reaction and no-template controls for each mPR. Primers were designed specifically for each mPR from the *Bos taurus* and *Homo sapiens* genome (Supplemental Table 4). The thermo-cycling conditions were 95°C for two minutes followed by 95°C for one minute, 55°C for one minute and 72°C for 30 seconds for a total of 40 cycles then 72°C for five minutes in the T100 Thermal Cycler (BioRad). The PCR products (5 µl) were loaded onto a 4% agarose gel and ran for 90 minutes at 100 volts. The gel was imaged using FluorChem M (Protein Simple).

cAMP ELISA

Cyclic AMP (cAMP) ELISA kit (581001, Cayman Chemical Company) was used to determine the level of cAMP in BUTE cells. The manufacturer instructions were followed. Briefly, media was aspirated, 1.6 mL of 0.1 M HCl was added to the plate and incubated at room temperature for 20 minutes. The cells were scraped off the plate with a cell scraper and placed into a 2 ml microcentrifuge tube. Next, the tubes were centrifuged at 1,000 x g for 10 minutes

and the supernatant was transferred into a new 2 ml tube and stored at -80°C until all replicates were collected. The samples were thawed and 50 µl of the sample was transferred to a new tube and diluted with 100 µl of ELISA buffer. A standard curve (0.3-750 pmol/ml) was prepared and 50 µl of standard, sample, and buffer for blank as well as 100 µl of buffer to the non-specific binding (NSB) wells. All wells were added to the plate in duplicate. Next, 50 µl of cAMP AChE tracer was added to each well except for the total activity (TA) and blank wells and 50 µl of cAMP ELISA antiserum was added to each well except for the TA, NSB and blank wells. The plate was covered and incubated for 18 hours at 4°C. The next day the plate was washed five times with wash buffer then 200 µl of Ellman's Reagent was added to each well and 5 µl of tracer was added to the TA wells. The plate was covered with a plastic film then placed on a shaker with a box covering the plate to allow it to develop in the dark for 120 minutes at room temperature. The plate was read at 415 nm using the µQuant™ (Biotek Instruments). The cAMP concentration was calculated using the spreadsheet provided by Cayman Chemical Company.

Statistics

One-way ANOVA followed by a Dunnett's multiple comparisons test or Tukey's multiple comparison test was used for all experiments with >2 treatments. Experiments with two treatments were analyzed by t-test. Statistical analyses were conducted in GraphPad PRISM (Version 9.0.0), and the significance criterion alpha was $P<0.05$.

3.4 Results

Progesterone stimulates glycogen breakdown independently of nuclear progesterone receptors (nPR)

BUTE cells were treated with range of progesterone concentrations (0-10 µM) for 48 hours. Surprisingly, 0.1 and 1 µM had no effect, but 10 µM significantly decreased glycogen

levels in BUTE cells by 99% compared to the control (Figure 7A; P<0.001). We have previously shown that BUTE cells express nPRs (PR-A and PR-B) (Berg *et al.*, 2022). PCR showed that BUTE cells expressed both progesterone receptor membrane component (PGRMC) genes (*PGRMC1* and *PGRMC2*) (Supplemental Figure 1B). To determine if progesterone was acting through the nPRs or PRGMCs, BUTE cells were treated with RU486, as it blocks both the nPR and PGRMC receptors . As expected, progesterone dramatically decreased glycogen levels (P<0.001) and RU486 had no effect by itself. In cells treated with progesterone + RU486, glycogen decreased by 86%, similar to the effect of progesterone alone (Figure 7B; P<0.001), indicating progesterone does not act through nPRs or PRGMCs to reduce the glycogen levels.

Progesterone stimulates glycogenolysis in BUTE cells by activating the mPRs

RU486 does not block membrane progesterone receptors (mPRs) (Thomas *et al.*, 2007; Kelder *et al.*, 2010; Dressing *et al.*, 2012), leading us to hypothesize that the mPRs were mediating the effect of progesterone. Immunohistochemistry confirmed that mPRα, mPRd, and mPRe are present in the bovine endometrium. All three receptors were more highly expressed in the epithelium than in the stroma. mPRα and mPRδ were consistently expressed on day 1 and 11 (Figure 8A). In contrast, mPRε immunostaining was notably higher on day 1 than on day 11 (Figure 8A). Previous research showed that the bovine uterine epithelium expresses mPRα, mPRβ, and mPRγ (Kowalik *et al.*, 2019), indicating that the bovine uterine epithelium expresses all 5 mPR isoforms. PCR confirmed that all five mPRs are also expressed in BUTE cells (Supplemental Figure 1B).

To further determine the role of progesterone in glycogen metabolism, BUTE cells were treated with 0, 0.1, 1, and 10 mM of the mPR agonist Org OD 02-0 for 48 hours (Kelder *et al.*, 2010). BUTE cells treated with 10 μM Org OD 02-0 had a 94% decrease in glycogen levels

compared to the control (Figure 8B; P<0.0001). These results indicate that progesterone stimulates the breakdown of glycogen in BUTE cells by activating the mPRs.

cAMP is not activated by mPRs

Glycogen phosphorylase (PYG) is the rate-limiting enzyme in cytosolic glycogenolysis. Therefore, we analyzed the effect of progesterone on PYG levels. However, progesterone did not increase PYG, as determined by western blot (Supplemental Figure 1C). Therefore, we next explored intracellular pathways that would increase PYG activity.

Research in other models has shown that mPRs can increase cAMP concentrations (Valadez-Cosmes *et al.*, 2016). However, neither progesterone nor Org OD 02-2 changed cAMP levels in BUTE cell after 0.5, 1, or 24 hours of treatments (Figure 9A-C). Additionally, BUTE cells treated with 10 μM forskolin (adenylyl cyclase activator) had a 25-fold increase in cAMP concentrations at 0.5 and 1 hour (P<0.0001), but 10 μM forskolin did not decrease glycogen levels (Figure 9C-D).

AMPK is activated downstream of the mPR but does not decrease glycogen in BUTE cells

Progesterone/mPR signaling can also activate AMPK, a regulator of glycogenolysis (Nie *et al.*, 2022). IHC confirmed that pAMPK is expressed in the cow endometrium, specifically in the glandular and luminal epithelium on days 1 and 11 and stroma on day 11 of the estrus cycle (Figure 10A). The immunostaining was darker on day 11 than day 1 in the glandular and luminal epithelium as well as the stroma, suggesting that phosphorylation of AMPK could be regulated by progesterone. Confirming this, BUTE cells treated with 10 μM of progesterone for 24 hours had an 18% increase in pAMPK levels determined by western blot (Figure 10B-C; P<0.001). To determine if the increase of pAMPK led to the decrease in glycogen levels, BUTE cells were treated with a vehicle, progesterone, AMPK activator (A-769662; 10 μM) or progesterone +

AMPK inhibitor (dorsomphorin; 5μM). However, the AMPK inhibitor was not able to counteract the effect of progesterone on glycogen levels in the BUTE cells, as progesterone decreased glycogen levels by 92% in the presence of dorsomphorin (Figure 10D). In agreement, BUTE cells treated with the AMPK activator had similar glycogen levels to control (Figure 10E). These results show that progesterone increases pAMPK levels, but this increase in pAMPK is not responsible for the decrease in glycogen.

Increased AMP decrease glycogen in BUTE cells

The above results suggest that progesterone increases AMP concentrations via mPRs. AMP allosterically activates and increases glycogen phosphorylase activity to breakdown glycogen (Agius, 2015; Zois and Harris, 2016). To test this hypothesis, BUTE cells were treated with D942, which increases intracellular AMP concentrations (Kosaka *et al.*, 2005), for 48 hours. BUTE cells treated with D942 (10 μM) had a 92% decrease in glycogen (Figure 11A; $P<0.01$). These results confirm that the increase in the concentration of AMP induces glycogenolysis in BUTE cells.

To determine if PYG mediated the decrease in glycogen due to progesterone, BUTE cells were treated with vehicle, progesterone (10 μM), GPI (glycogen phosphorylase inhibition; 10 μM), or progesterone + GPI. BUTE cells treated with PYG inhibitor alone had a 77% increase in glycogen levels compared to the control, indicating cells have a basal level of glycogen synthesis and catabolism. BUTE cells treated with progesterone had a decrease in glycogen levels by 97% (Figure 11B; $P<0.01$). GPI could only partially block the effect of progesterone in BUTE cells treated with progesterone + GPI. Thus, the glycogen level in BUTE cells treated with progesterone and GPI was decreased by 53% compared to control treated cells (Figure 11B;

P<0.01). This suggests that progesterone activates PYG and other, yet to be determined, pathways to decrease glycogen levels in the uterine epithelium.

Progesterone acting through the mPR stimulates glycogen breakdown in Ishikawa cells lacking nPRs

To confirm that progesterone stimulates glycogen breakdown in other species, glycogen was measured in Ishikawa cells (human uterine epithelial cancer cell line) that lack the nPR as confirmed by western blot (Figure 12A) (Dean *et al.*, 2018). Ishikawa cells expressed mPRα, mPRβ, mPRγ, PGRMC1, and PGRMC2 at the mRNA level (Supplemental Fig. 1D). Ishikawa cells were treated with 10 μM progesterone or 10 μM Org OD 02-0 showed similar results to the BUTE cells. Both compounds significantly decreased glycogen levels (96% and 97%, respectively; Figure 12B-C; P<0.01). Therefore, progesterone acts through the mPR in uterine epithelial cells to stimulate glycogenolysis in both human and bovine models.

4.5 Discussion

We have shown that glycogen levels in the uterine epithelium were high at estrus and low during the luteal phase of the cow (Sandoval *et al.*, 2021). Additionally, we found that estradiol indirectly stimulates glycogenesis in uterine epithelium via IGF1 (Gonzalez *et al.*, 2022). Here, we show that a high concentration of progesterone decreased glycogen by acting through the membrane progesterone receptor and AMP in BUTE and Ishikawa cells. Collectively, this suggests that cyclic changes in glycogen content of the uterine epithelium are regulated by ovarian steroids. In the follicular phase, glycogen is stored in response to the actions of estradiol/IGF1. This glycogen is mobilized after ovulation due to the direct effects of progesterone on the uterine epithelium.

In our in vitro models, it took a progesterone concentration (10μM) much higher than serum concentration to decrease glycogen and increase pAMPK levels. Supporting the use of these concentrations, these changes mirror the changes in glycogen content and phospho-AMPK we observed in vivo during the luteal phase. The necessity of these high concentrations could be due to several reasons. First, the concentration of progesterone in uterine tissue is much higher than serum, ranging from 169-297 ng/g (around 1 μM) (Weems *et al.*, 1988, 1989). Concentrations of progesterone in uterine tissue are also higher in the uterine horn ipsilateral to the CL (Pope *et al.*, 1982). Secondly, BUTE and Ishikawa cells were treated with high IGF1 concentrations (50 ng/ml) while simultaneously being treated with progesterone. In vivo, IGF1 production would decrease while progesterone concentrations increase (McCarthy *et al.*, 2012; Gonzalez *et al.*, 2022). Thus, it may take higher concentrations of progesterone to overcome the effects of IGF1 used in our study. Supporting our use of 10 μM in vitro, many published articles use the same concentration (Harduf *et al.*, 2009; Jiang *et al.*, 2017; Kasubuchi *et al.*, 2017; Dean *et al.*, 2018; Park *et al.*, 2020; Long *et al.*, 2021).

Previous research suggested that progesterone reduces glycogen in the uterine epithelium of other species (Nie *et al.*, 2022). Yet the mechanism of how progesterone decreases glycogen has not been explored. There are several ways to decrease intracellular glycogen levels. The first is to break down glycogen into glucose-6-phosphate (G6P) by PYG, and then G6P is dephosphorylated by glucose-6-phosphatase. We have previously shown that both of these enzymes are present in the uterine epithelium (Sandoval *et al.*, 2021). Here, we found that progesterone/mPR signaling increased AMP concentrations, and PYG is allosterically activated by AMP (Ercan-Fang *et al.*, 2002). However, our results using GPI indicate that PYG is only responsible for about 50% of progesterone-stimulated glycogenolysis.

Another way to catabolize glycogen is inside lysosomes via α-acid glucosidase (GAA). First, glycogen is encapsulated into the autophagosome and then fused with a lysosome to degrade the glycogen into free glucose by GAA (Koutsifeli *et al.*, 2022). GAA is processed from a 110 kDa precursor protein into the active 76 and 70 kDa enzymes in the lysosome (Moreland *et al.*, 2005). GAA breaks the α-1,4 links between two glucose molecules to release glucose from the glycogen molecule (Adeva-Andany *et al.*, 2016). Once glycogen is broken down into glucose, it diffuses out of the lysosome through glucose transporters and into the cytosol. In the cytosol, glucose could be used by the cell for metabolism or exit through glucose transporters into the lumen of the endometrium. α-glucosidase activity has been detected in the ovine endometrium, but activity did not change throughout the estrous cycle (O'Shea and Murdoch, 1978). This alternative pathway for glycogenolysis has not been well studied in the uterus.

Finally, glycogen could be released from the cells by extracellular vesicles (EV). Several studies in the uterus have shown that EVs are produced by the endometrial epithelium and secreted into the uterine lumen to aid in the embryo endometrium cross-talk (O'Neil *et al.*, 2020; Sui *et al.*, 2023). Glycogen levels in hepatic cells treated with EV isolated from plasma in obese patients were reduced compared to the control (Afrisham *et al.*, 2020). Enzymes often physically associated with glycogen molecules have been found in EV isolated from the uterus during pregnancy in cattle (Kusama *et al.*, 2018). Two TEM studies appear to show glycogen being encapsulated into vesicles and secreted into the glandular lumens in humans (Cornillie *et al.*, 1985; Demir *et al.*, 2002). More research is needed to determine if the uterus can secrete EVs containing glycogen.

One main limitation of the study was primarily relying on an in vitro model. Cell lines cannot fully recapitulate in vivo conditions. However, this model allowed us to examine the

direct effects of progesterone on the uterine epithelium and work out intracellular signaling pathways that could not be done in vivo. Our major results, such as progesterone decreasing glycogen in the uterine epithelium, progesterone increasing pAMPK levels, and the uterine epithelium expressing mPRs, all agree with our in vivo observations (Sandoval *et al.*, 2021).

In conclusion**,** we show that progesterone stimulates glycogenolysis in uterine epithelial cells, explaining the decrease in epithelial glycogen that has been observed in the luteal phase and during pregnancy of various species (Bowman and Rose, 2017; Dean, 2019; Sandoval *et al.*, 2021). Importantly, this effect of progesterone was mediated by the mPRs, the first functional effect of mPRs found in the uterus. Glucose liberated from glycogen could diffuse into the uterine lumen to support embryo development. It could also be converted to other nutrients, such as fructose or lactate, used by the embryo before diffusion (Moses *et al.*, 2022; Chen and Dean, 2023). Finally, the epithelial cells could use glucose to meet their energy requirements or to produce glycans that mediate embryo-epithelium attachment at the beginning of implantation (Gonzalez *et al.*, 2022; Sun *et al.*, 2023). Further research needs to determine the importance of glycogen in the uterus and the fate of the liberated glucose during early embryo development.

4.6 Tables and Figures

Supplemental Table 1. Composition of αMEM media.

Component	Concentration (mg/L)
2'-Deoxyadenosine	10
2'-Deoxycytidine	11
2'-Deoxyguanosine	10
Adenosine	10
Biotin	0.1
Calcium Chloride	200
Choline Chloride	1
Cytidine	10
D-Calcium Pantothenate	1
D-Glucose	1,000
Folic Acid	1
Glycine	50
Guanosine	10
L-Alanine	25
L-Arginine	127
L-Ascorbic Acid	50
L-Asparagine	50
L-Aspartic Acid	30
L-Cysteine	100
L-Cystine	31
L-Glutamine	292
L-Histidine	42
L-Isoleucine	52
L-Leucine	52
L-Lysine	73
L-Methionine	15
L-Phenylalanine	32
L-Proline	40
L-Serine	25
L-Threonine	48
L-Tryptophan	10
L-Tyrosine	52
L-Valine	46
Lipoic Acid	0.2
Magnesium Sulfate	98
Myo-Inositol	2
Niacinamide	1
Phenol Red	10
Potassium Chloride	400
Pyridoxal	1
Pyruvic Acid	110
Riboflavin	0.1
Sodium Chloride	6,800
Sodium Phosphate	140
Thiamine	1
Thymidine	10
Uridine	10
Vitamin B12	1.4
Sodium Bicarbonate	2,200

Supplemental Table 2. Composition of DMEM/F12 media.

Component	Concentration (mg/L)
Biotin	0.00365
Calcium Chloride	116.65
Choline Chloride	8.98
Cupric Sulfate, Pentahydrate	0.00125
D-Calcium Pantothenate	2.24
D-Glucose	2,700
Ferric Nitrate	0.05
Ferrous Sulfate, Heptahydrate	0.417
Folic Acid	2.66
Glycine	18.75
Hypoxanthine Sodium	2.385
L-Alanine	4.45
L-Arginine	147.5
L-Asparagine	7.5
L-Aspartic Acid	6.65
L-Cysteine	17.56
L-Cystine	31.285
L-Glutamine	365.1
L-Glutamic Acid	7.35
L-Histidine	31.48
L-Isoleucine	54.37
L-Leucine	58.95
L-Lysine	91.35
L-Methionine	17.24
L-Phenylalanine	35.48
L-Proline	17.25
L-Serine	26.25
L-Threonine	53.55
L-Tryptophan	9.02
L-Tyrosine	55.815
L-Valine	52.85
Linoleic Acid	0.044
Lipoic Acid	0.105
Magnesium Chloride	28.61
Magnesium Sulfate	48.85
Myo-Inositol	12.61
Niacinamide	2.0185
Phenol Red	8.1
Potassium Chloride	311.8
Putrescine, Dihydrochloride	0.08
Pyridoxine	2.031
Pyruvic Acid	110
Riboflavin	0.219
Sodium Chloride	6999.5
Sodium Phosphate, Dibasic	71
Sodium Phosphate, Monobasic	62.5
Thiamine	2.1685
Thymidine	0.365
Vitamin B12	0.68
Zinc Sulfate, Heptahydrate	0.4315

Supplemental Table 3. Primary antibodies and conditions used for western blot (WB) and immunohistochemistry (IHC) analysis.

Antigen	Catalog No	Technique	Dilution	Block
mPRα (PAQR7)	HPA046936-100UL Sigma-Aldrich	IHC	1:100	Goat Serum
mPRδ (PAQR6)	HPA073505-100UL Sigma-Aldrich	IHC	1:100	Goat Serum
mPRε (PAQR9)	HPA052798-100UL Sigma-Aldrich	IHC	1:100	Goat Serum
pAMPKα	2535S Cell Signaling	WB	1:1000	BSA
		IHC	1:50	Goat Serum
AMPKα	5831S Cell Signaling	WB	1:1000	BSA
PYG	Ab231963 abcam	WB	1:500	BSA
GFP	2956S Cell Signaling	IHC	1:100/1:50	Goat Serum
Rabbit IgG	I-1000-5 Vector Laboratories	IHC	1:50	Goat Serum
α tubulin	2144S Cell Signaling	WB	1:500	BSA

Supplemental Table 4. Primer sequences used for mPRs and PGRMCs analysis in BUTE and Ishikawa cells.

Species	Gene Name	Gene Symbol	Primer (5'→3')	Accession Number
Cow	Membrane Progesterone Receptor α	*PAQR7*	F-GGCGCAAACTTGATCTTCATC	NM_001038553.1
			R-CTATTCCCATCTGTTCCCATCC	
	Membrane Progesterone Receptor β	*PAQR8*	F-TCTATGTCCTGTCCTCCATCA	NM_001101135.2
			R-CCACGAAGTAGAAGGTGTAGTG	
	Membrane Progesterone Receptor γ	*PAQR5*	F-CAAGACTCTGAGGAAGAAATGG	XM_002690486.5
			R-GAGGCTGAAGATGAGGCATAG	
	Membrane Progesterone Receptor δ	*PAQR6*	F-GGGAAGATGGTATCACTGG	NM_001046225.2
			R-CACATGTTGACGGTCTCGTT	
	Membrane Progesterone Receptor ε	*PAQR9*	F-CGATCAGGTGTACTACGTTGAG	NM_198504.4
			R-GACCAGCAGCAGCATGTA	
	Progesterone Receptor Membrane Component 1	*PGRMC1*	F-CTGGGACTCTCAGTTCACTTTC	NM_001075133.1
			R-CTTCCGAGTGCTCTCATCTTT	
	Progesterone Receptor Membrane Component 2	*PGRMC2*	F-CGTATGAAGAAGCGGGACTT	NM_001099060.1
			R-TGGTCACGTCGAAGACTTTC	
Human	Membrane Progesterone Receptor α	*PAQR7*	F-CTAGGCAGGAGGTGAAACTTAG	NM_178422.6
			R-CCGCATCCAGCAATGAAATC	
	Membrane Progesterone Receptor β	*PAQR8*	F-GCAGAAGGGAAGAAGGTGTAAG	NM_133367.5
			R-CTCTACCTCCGGTTCTCTCTTT	
	Membrane Progesterone Receptor γ	*PAQR5*	F-TGTCCTCGCCTTTTGCTTATC	NM_017705.4
			R-GGTACGAGCTGGCTTCATTT	
	Progesterone Receptor Membrane Component 1	*PGRMC1*	F-TGAGGGAGGGACATACAGAATAG	NM_006667.5
			R-GTACTGTTTCCTCGTGGTTCAG	
	Progesterone Receptor Membrane Component 2	*PGRMC2*	F-AGTCTTCGACGTGACCAAAG	NM_006320.6
			R-GGCATCCCTACCAGCAAATA	

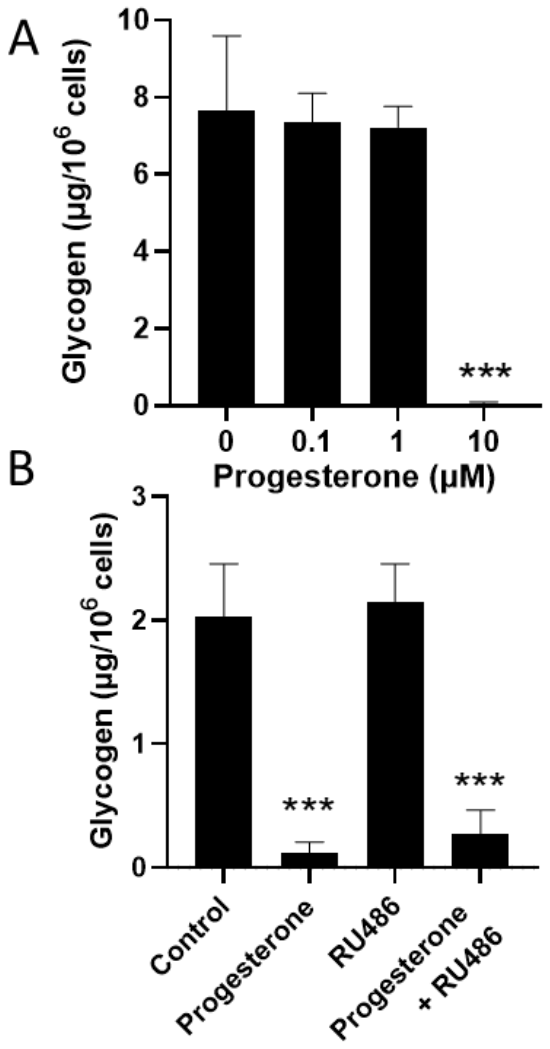


Figure 7. Progesterone stimulates glycogen breakdown in BUTE independently of the nuclear progesterone receptor. A-B) Glycogen levels in BUTE cells treated with progesterone (0, 0.1, 1, and 10 µM; A) or 10 µM progesterone and 10 µM RU486 (B) as indicated. n=6. ***P<0.001 relative to 0 µM or control.

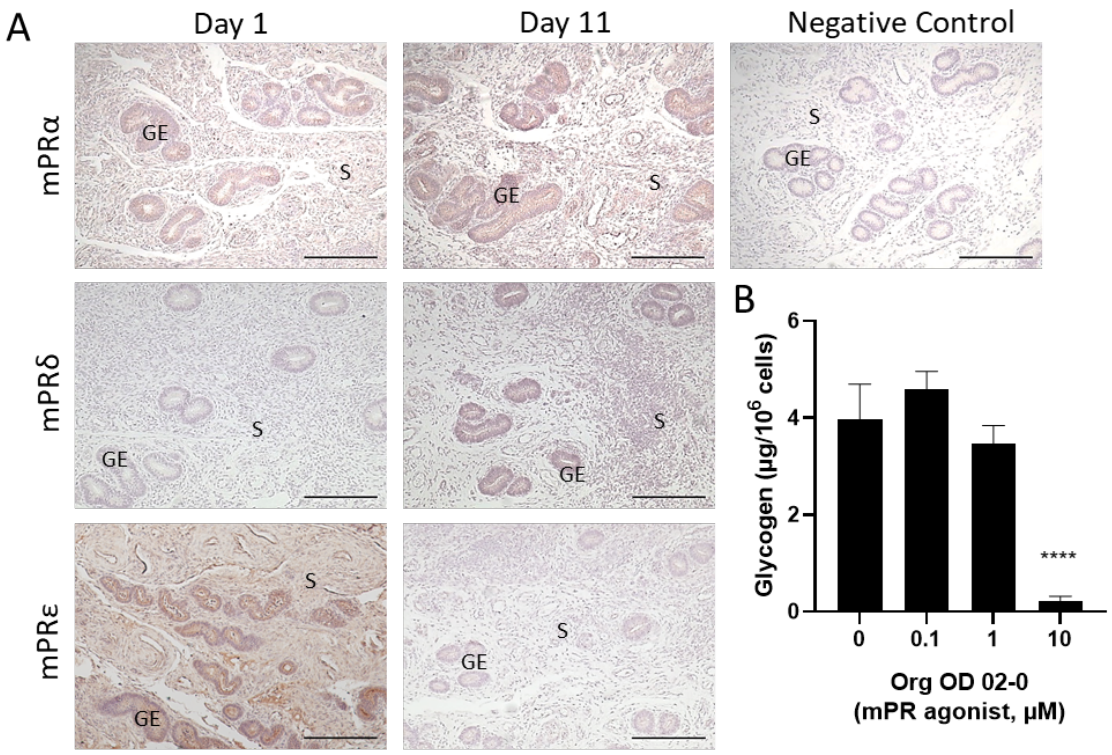


Figure 8. Progesterone acts through the membrane progesterone receptor (mPR) stimulates glycogen breakdown in BUTE cells. A) Immunohistochemistry for mPRα, mPRδ and mPRε is in the cow endometrium on Days 1 and 11 of the cycle. B) Glycogen levels in BUTE cells treated with the specific mPR agonist Org OD 02-0 (0, 0.1, 1, and 10 µM) for 48 hours. GE: Glandular Epithelium S: Stroma. n=4-6. Scale bar = 200 µm. ****P<0.0001 relative to 0 µM.

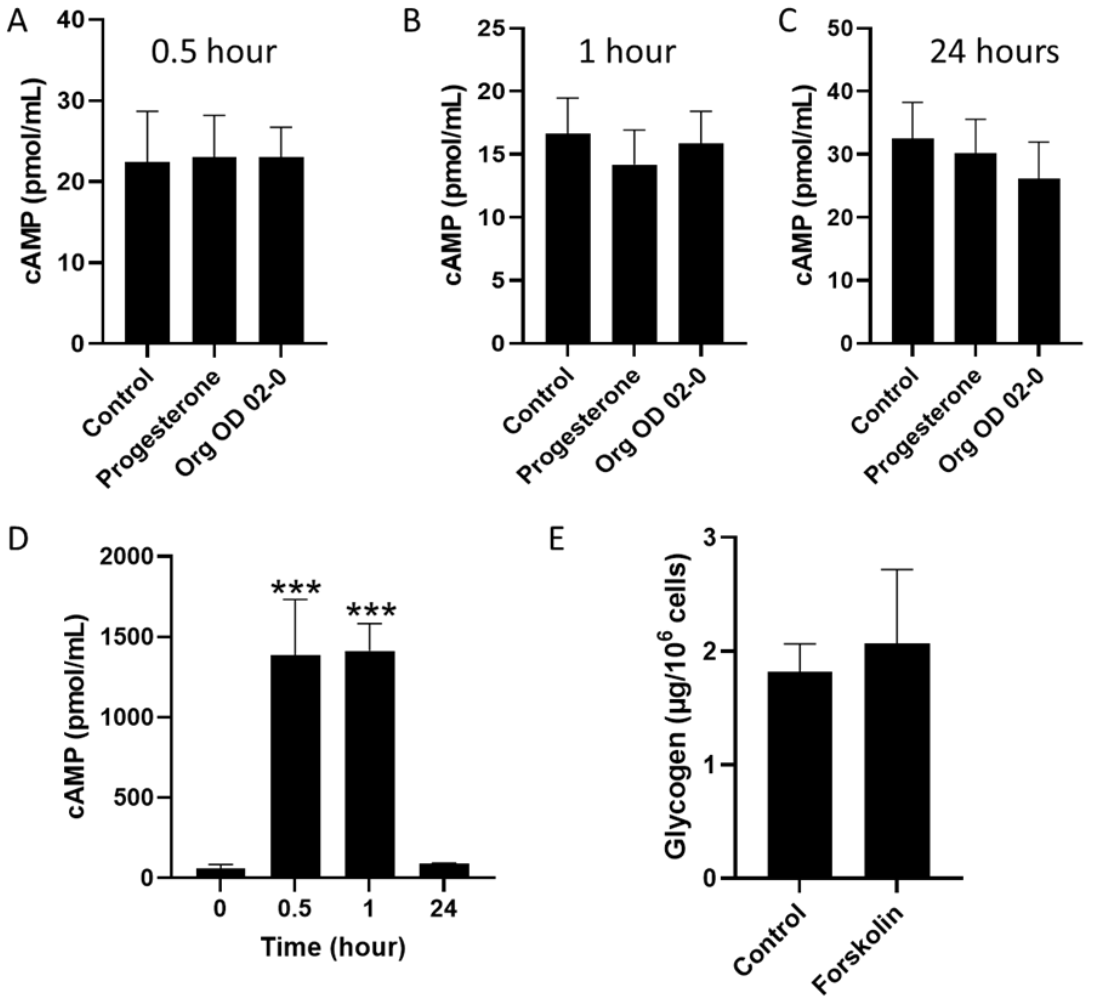


Figure 9. Progesterone does not regulate glycogen via cAMP. A-C) cAMP concentrations in BUTE cells treated with 10 µM of progesterone or 10 µM of Org OD 02-0 at 0.5 (A), 1 (B), and 24 hours (C). D) cAMP levels in BUTE cells treated with 10 µM of forskolin (adenylyl cyclase activator) at 0, 0.5, 1 and 24 hours. E) Glycogen levels in BUTE cells treated with forskolin (10 µM) for 48 hours. n=5-6. ***P< 0.001 relative to control or 0 hour.

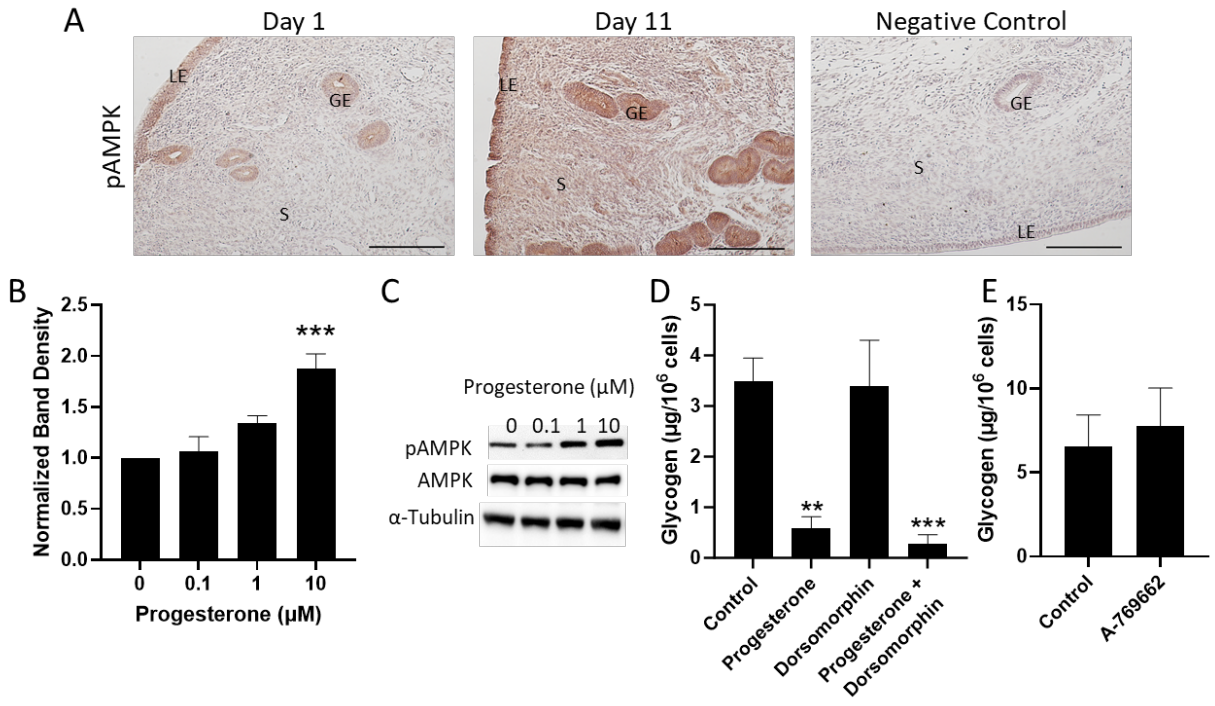


Figure 10. Activation of AMPK does not stimulate glycogen breakdown in BUTE cells. A) Immunohistochemistry for pAMPK in the cow endometrium on Days 1 and 11 of the cycle. B-C) Western blot showing the amount of AMPK and pAMPK protein in BUTE cells treated with various concentrations of progesterone as indicated D-E) Glycogen levels in BUTE cells treated with an AMPK inhibitor dorsomorphin (5 μM; D) or AMPK activator A-769662 (10 μM; E) for 48 hours**.** n=4-6. ***P<0.001 **P<0.01 relative to control or 0 μM. GE: Glandular Epithelium LE: Luminal Epithelium S: Stroma. Scale bar = 200 μm.

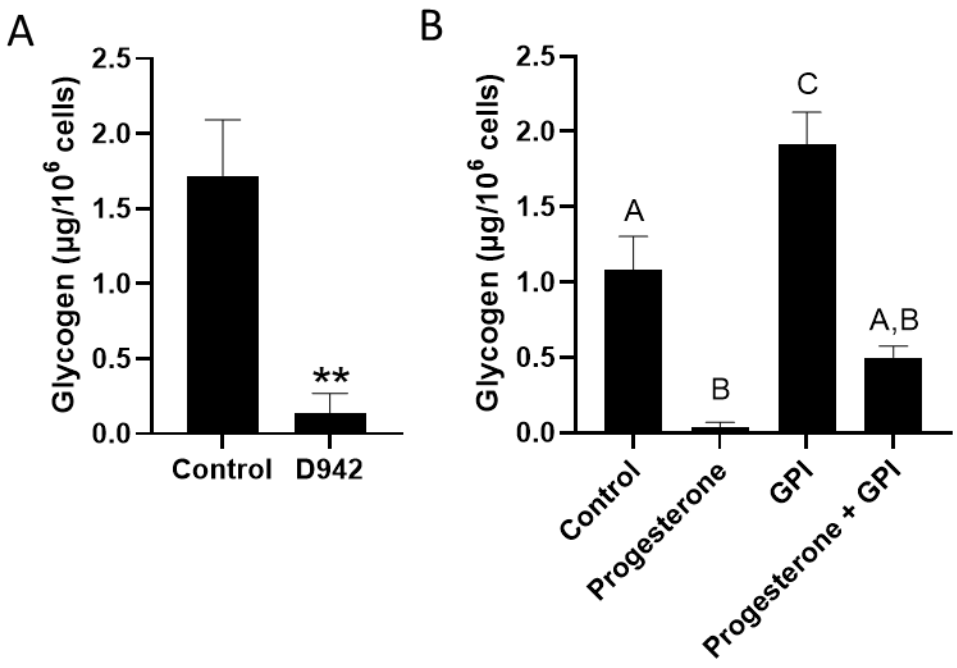


Figure 11. An increase in AMP stimulates glycogenolysis in BUTE cells. A-B) Glycogen levels in BUTE cells treated with D942 (increases intracellular AMP, 10 µM; A) or progesterone (10 µM) and glycogen phosphorylase inhibitor (GPI, 10 µM; B) for 48 hours**.** n=6. **P<0.01 relative to control.

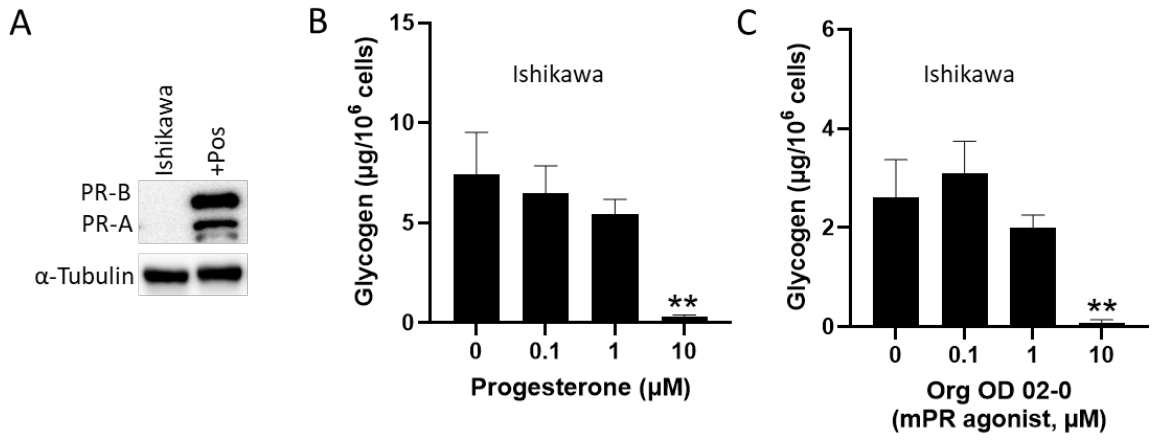
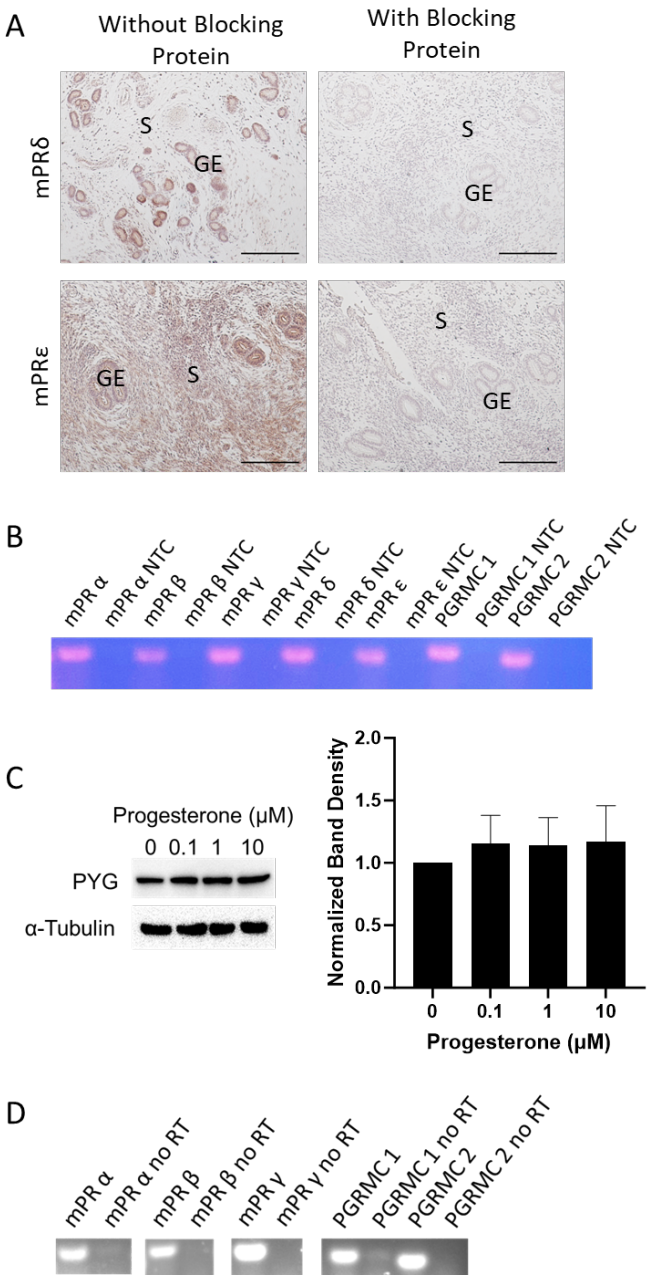


Figure 12. mPR stimulates glycogenolysis in human cells. A) Western blot to show Ishikawa cell lack the nuclear progesterone receptors. B-C) Glycogen levels in Ishikawa cells that lack the nuclear progesterone receptor treated with progesterone (0, 0.1, 1, and 10 µM; B) or the specific mPR agonist Org OD 02-0 (0, 0.1, 1, and 10 µM; C) for 48 hours. n=6 **P<0.01 relative to 0 µM.



Supplemental Figure 1. A) Validate for mPRδ and mPRε antibodies using blocking proteins. B) RT-PCR shown all 5 mPRs are expressed in BUTE cells. C) Western blots showing no difference in PYG levels in BUTE cells following progesterone treatment. D) RT-PCR showing mPRα, mPRβ, mPRγ, PGRMC1, and PGRMC2 expression in Ishikawa cells.

Chapter 5 The glycogenolytic enzyme acid α-glucosidase is expressed in the bovine uterine endometrium[2]

5.1 Abstract

Progesterone has been shown to stimulate glycogen catabolism in uterine epithelial cells. Acid α-glucosidase (GAA) is an enzyme that breaks down glycogen within lysosomes. We hypothesized that progesterone may stimulate glycogenolysis in the uterine epithelium via GAA. We found that GAA was more highly expressed in the stroma on day 1 than on day 11. However, GAA did not appear to differ in the epithelium on days 1 and 11. Progesterone (0-10μM) had no effect on the full-length inactive protein (110 kDa) or the cleavage (active) peptides present inside the lysosome (70 and 76 kDa) in bovine uterine epithelial (BUTE) cells. Furthermore, the activity of GAA did not differ between the BUTE cells treated with 10 μM progesterone or control. Overall, we confirmed that GAA is present in the cow endometrium and BUTE cells. However, progesterone did not affect protein levels or enzyme activity.

5.2 Introduction

As bovine embryos enter the hypoxic environment of the uterine lumen, glucose uptake increases dramatically (Absalón-Medina *et al.*, 2014; Chi *et al.*, 2020). However, if the glucose concentration is too high, it becomes toxic to the embryo (Uhde *et al.*, 2018). Glycogen, the storage form of glucose, is present in the endometrium of many species (Dean, 2019; Sandoval *et al.*, 2021; Chen *et al.*, 2022; Gonzalez *et al.*, 2022). We have shown cows have higher glycogen

[2] Reproduced with permission from Wiley. Berg M.D. and Dean M. The glycogenolytic enzyme acid α-glucosidase is expressed in the bovine uterine endometrium. Reprod Domest Anim. 2024 Jun;59(6):e14643. doi: 10.1111/rda.14643. PMID: 38877774.

content in the luminal and glandular epithelium on day 1, relative to day 11 of the estrous cycle (Sandoval *et al.*, 2021).

There are two main mechanisms for glycogen catabolism in cells, one in cytosol and the other in lysosomes. The first mechanism breaks down glycogen through glycogen phosphorylase in the cytosol of the cell. Glycogen phosphorylase cleaves an α-1,4 glycosidic bond from a terminal branch of glycogen to release glucose-1-phosphate. Glucose-1-phosphate is isomerized to glucose-6-phosphate, which can be used by the cells. Glucose-6-phosphate can also be dephosphorylated and diffused out of the cell. This pathway has been well-established in many tissues (Brody and Westman, 1958; Nie *et al.*, 2022). We have shown that glycogen phosphorylase and glucose-6-phosphatase are expressed in the uterine epithelium of mice and cows (Sandoval *et al.*, 2021; Chen *et al.*, 2022).

The second mechanism of glycogen breakdown is called glycophagy. This process breaks down glycogen in the phagolysosomes by the enzyme acid α-glucosidase (GAA). First, glycogen is encapsulated into the autophagosome, which then fuses with a lysosome carrying the enzyme GAA to degrade the glycogen into glucose (Koutsifeli *et al.*, 2022). GAA breaks the 1,4 linkage between the glucose residues (Adeva-Andany *et al.*, 2016). Once the glycogen is catabolized into glucose, it diffuses out of the lysosome through glucose transporters. This alternative pathway for glycogenolysis has not been extensively studied in the uterus. Therefore, we hypothesized that progesterone stimulates glycogenolysis in the uterine epithelium via GAA.

5.3 Material and Methods

Bovine uterine epithelium (BUTE) cells were plated and allowed to grow until 80% confluent. Then the media was removed, rinsed twice with PBS, and the cells were serum and

steroid starved in media without phenol red and treated with insulin-like growth factor 1 (IGF1, 50 ng/ml; 2000550UG, Shenandoah Biotechnology) for 24 hours to stimulate glycogenesis (Gonzalez *et al.*, 2022). The media was removed and replaced with serum and steroid-free media containing 50 ng/ml of IGF1 and appropriate treatments for 24 hours. Controls were treated with vehicle (DMSO) for all experiments.

Western blots were performed as previously described (Sandoval *et al.*, 2021; Gonzalez *et al.*, 2022). Protein was separated on a 10% SDS-PAGE gel and was transferred onto a PVDF membrane, and membranes were blocked with 5% BSA in TBS-T. Anti-GAA (1:1000; ab137068, Abcam) or α-tubulin (1:500; 2144, Cell Signaling) antibodies were diluted in 5% BSA, added to the membranes, and incubated overnight at 4°C. The secondary antibody (anti-rabbit; 7074S, Cell Signaling) was added to the membrane and incubated for 30 minutes. Blots were developed and imaged.

Immunohistochemistry was carried out as previously described on samples collected on days 1 and 11 of the estrous cycle (Sandoval *et al.*, 2021; Gonzalez *et al.*, 2022). The anti-GAA antibody was diluted 1:50 (ab137068, Abcam) and incubated at 4°C overnight. Images were attained with a Zeiss Axioskop upright microscope with an Axiocam 305 color camera. In negative controls, the primary antibody was replaced with an isotype antibody (IgG antibody; I-1000-5, Vector Laboratories).

GAA activity (ab174093, Abcam) was measured per manufacturer instructions. The plate was incubated at room temperature overnight, and the final absorbance was measured the next day. The activity was determined by the standard curve and then normalized to the amount of protein as determined by BCA.

One-way ANOVA followed by Dunnett's multiple comparisons test or Tukey's multiple comparison test was used for all experiments with >2 treatments. Experiments with two treatments were analyzed using a t-test. Statistical analyses were conducted in GraphPad PRISM (Version 9.0.0), and the significance criterion alpha was $P<0.05$.

5.4 Results

GAA is present in the glandular epithelium, luminal epithelium, and stroma of the cow endometrium at days 1 and 11 of the estrus cycle (Figure 13A). GAA was more highly expressed in the stroma on day 1 than on day 11. Immunostaining for GAA did not appear to differ in the epithelium on days 1 and 11. Previous literature indicated that progesterone decreased glycogen levels in the uterus (Nie *et al.*, 2022). Therefore, BUTE cells were treated with 10 μM progesterone. However, progesterone did not change the activity level of GAA in BUTE cells (Figure 13B). In agreement, the full-length inactive protein (110 kDa) nor the active, cleaved peptides (70 and 76 kDa) differed across treatments (0, 0.1, 1, and 10 μM) (Figure 13C-E). Overall, this study confirmed that GAA is present in the cow endometrium and BUTE cells, but GAA is not regulated by progesterone.

5.5 Discussion

Glycogen in the cow uterus is lower on day 11 than on day 1 of the estrus cycle, suggesting that progesterone may decrease glycogen in the uterus. We have now shown that both glycogen phosphorylase and GAA are present in the uterine epithelium (Sandoval *et al.*, 2021). Here, we show that GAA is not regulated by progesterone. An *in situ* hybridization study showed

that GAA is expressed in the mouse endometrium, with higher staining in the stroma (Ponce *et al.*, 1999). This data agrees with our analysis of GAA within the cow endometrium.

We determined that the active GAA proteins are present in the BUTE cells, but progesterone did not affect GAA abundance. In vitro, cells can lack responses to hormones. One potential explanation for our results is that BUTE cells do not have a functional progesterone receptor. However, we have previously shown that BUTE cells express the progesterone receptor (Berg *et al.*, 2022). Additionally, we have found that progesterone affects gene expression in BUTE cells via RNAseq, affecting expression of genes known to be regulated by progesterone such as SERPINE2, SERPING1, SULT1A1, and PRDX6 (unpublished observations).

A study conducted in ewes determined that the activity level of GAA does not change through the estrous cycle in the endometrium (O'Shea and Murdoch, 1978). The activity of GAA did not increase in BUTE cells treated with progesterone, supporting the results previously published in ewes. In conclusion, GAA is present in the cow uterus but is not regulated by progesterone.

5.6 Figure

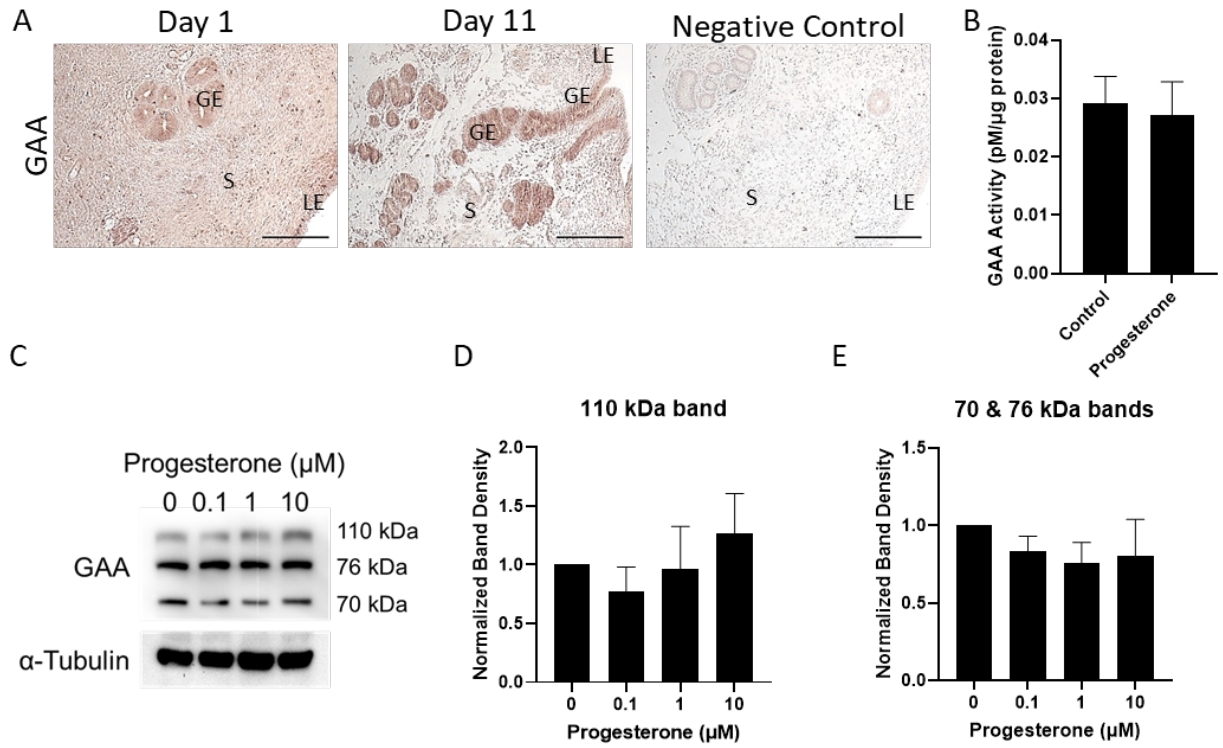


Figure 13. Acid α-glucosidase (GAA) is expressed in BUTE cells and the bovine uterine endometrium but not regulated by progesterone. A) Immunohistochemistry for GAA is in the cow endometrium on Days 1 and 11 of the cycle. B) GAA activity in BUTE cells. C-E) Western blot for GAA in BUTE cells. GE: Glandular Epithelium LE: Luminal Epithelium S: Stroma. n=4. scale bar=200 µm.

Chapter 6 Progesterone increases genes associated with glucose catabolism in the bovine uterine epithelium

6.1 Abstract

Early embryonic loss occurs in about 50% of pregnancies. How progesterone regulates glucose catabolism has been studied very little in the uterus. Therefore, our objective was to determine how progesterone regulates the transcriptome of bovine uterine epithelial (BUTE) cells, focusing on genes related to glucose metabolism. BUTE cells were treated with 10 μM progesterone for 24 hours and RNA was isolated and analyzed via RNAseq. A total of 13,354 genes were detected, 1,623 of which were upregulated, 1,449 were downregulated, and 10,282 showed no change. ShinyGO 0.80 analysis determined there were 6 significantly enhanced pathways. Further analysis determined genes regulating glucose catabolism showed a significant change in the progesterone-treated BUTE cells. Specifically, three of the seven genes that regulate the pentose phosphate pathway were upregulated in BUTE cells treated with progesterone. The glucose concentration remaining in the media was measured to quantify glucose uptake. Progesterone treatment reduced glucose concentrations, suggesting increased glucose uptake. BUTE cells treated with progesterone also had an increase in NADPH levels within the cells but not in the media. In conclusion, progesterone increases genes associated with glucose catabolism, decreases glucose concentration within the media, and increases NADPH in BUTE cells. The uterine epithelium could use the NADPH for cell proliferation, fatty acid synthesis, or cholesterol synthesis.

6.2 Introduction

Pregnancy loss occurs in 50% of pregnancies. A critical nutrient for the uterus is glucose, yet how glucose is regulated is not very well understood. Glucose enters the uterus through glucose transporters from the maternal circulation. Then, it is phosphorylated by hexokinase to glucose-6-phosphate. Glucose can be metabolized through the pentose phosphate pathway, hexosamine biosynthesis pathway, glycolysis, the polyol pathway, or the glycogenesis pathway (Chen and Dean, 2023). In the mouse, progesterone increased the expression of GLUT1, glucose uptake, and glucose-6-phosphate dehydrogenase (G6PD) uterine epithelial cells (Zhang *et al.*, 2020). They suggested that progesterone was increasing the localization of GLUT on the cell membrane to increase glucose in the cell. Once in the cell, glucose would be catabolized by glycolysis and the pentose phosphate pathways to prepare the uterus for implantation. The pentose phosphate pathway produces NADPH and ribose-5-phosphate (Stincone *et al.*, 2015). As the uterus prepares for implantation, the epithelial cell will proliferate, requiring two products made by the pentose phosphate pathway, NADPH and ribose-5-phosphate. However, how progesterone affects glucose metabolism in the bovine uterine epithelium has not been studied. Therefore, the aim of this study was to determine how progesterone regulates the expression of glucose metabolizing enzymes in the bovine uterine epithelial cells.

6.3 Material and Methods

Cell Culture

BUTE cells were generated from a Holstein dairy cow and validated as described (Berg *et al.*, 2022; Gonzalez *et al.*, 2022). BUTE cells were maintained in a-Minimum Essential Media

(αMEM) media containing 5.55 mM glucose supplemented with 10% FBS (12103C-500ML, Sigma-Aldrich), 2 mM L-glutamine (GLL01-100ML, Caisson Labs), 10 mg/ml ITS (25-800-CR, Corning) 1.8 ng/ml EGF (20053100UG, Shenandoah Biotechnology), and 18.2 ng/ml estradiol-17β (E2758-1G, Sigma). BUTE cells were maintained at 37°C in a humidified incubator with 5% CO_2 and were passed every 3-4 days.

Cell Culture Experiments

BUTE cells were plated and allowed to grow until 80% confluent. Then the media was removed, rinsed twice with PBS, and the cells were serum and steroid starved in media without phenol red and treated with insulin-like growth factor 1 (IGF1, 50 ng/ml; 2000550UG, Shenandoah Biotechnology) for 24 hours to stimulate glycogenesis (Gonzalez *et al.*, 2022). The media was removed and replaced with serum and steroid-free media containing 50 ng/ml of IGF1 and appropriate treatments for 24 hours. Controls were treated with a vehicle (DMSO) for all experiments.

RNA Isolation and Reverse Transcription

Total RNA was extracted from BUTE cells using the Quick-RNA MiniPrep Plus Kit (R1058, Zymo Research) per the manufacturer's instructions. BUTE cells were plated in a 10 cm dish and treated with vehicle or 10 µM progesterone for 24 hours. Media was removed, rinsed 2 times with PBS, and lysed. The samples were transferred into the Spin-Away™ Filter and centrifuged. Ethanol (300 µl) was added to the flow-through. The sample was transferred into a Zymo-Spin IIICG Column in a collection tube and centrifuged, the flow-through was discarded. The filter was treated with A DNase I and incubated at room temperature for 15 minutes. Next, the column was washed with RNA wash buffer two times, and the RNA was eluted in 100 µl of

DNase/RNase-Free water. The RNA samples were stored at -80°C. The RNA samples were run on a 1% agarose gel with 1% bleach at 100V for 60 minutes to determine the quality of the RNA.

RNA Sequencing and Quality Check

The RNAseq libraries were prepared with Illumina's 'TruSeq Stranded mRNAseq Sample Prep kit' (Illumina, San Diego, CA) at the Roy J. Carver Biotechnology Center, University of Illinois Urbana-Champaign. The libraries were pooled and quantitated by qPCR and sequenced on one SP lane for 101 cycles on a NovaSeq 6000 sequencing platform, generating 100 pb single-end reads (Illumina, San Diego, CA). Fastq files were generated and demultiplexed with the bcl2fastq Conversion Software (version 2.20, Illumina). Adaptors were trimmed from the 3'-end of the reads.

Quality check of raw reads was performed using FastQC (version 0.11.8, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) on individual samples. Average per-base read quality scores were over 30 in all samples, and no adapter sequences were found, indicating those reads are high in quality.

Alignment and Gene-Level Quantification

Salmon version 1.4.0 was used to quasi-map reads to the transcriptome of the bovine reference genome (ARS-UCD1.2, NCBI) and quantify the abundance of each transcript (Patro *et al.*, 2017). Then quasi-mapping was performed with the additional arguments --seqBias and --gcBias to correct sequence-specific and GC content biases, --numBootstraps=30 to compute bootstrap transcript abundance estimates, and --validateMappings to help improve the accuracy of mappings. Gene-level counts were then estimated based on transcript-level counts using the "bias corrected counts without an offset" method from the "tximport" R package (Soneson *et al.*,

2016). The percentage of reads mapped to the transcriptome ranged from 81.4 to 86.2%. The unmapped reads were discarded, and the remaining reads, varying from 23.8 to 32.5 million per sample, were kept for further analysis.

Normalization and Differential Gene Expression Analysis

The TMM (trimmed mean of M-values) normalization (Robinson *et al.*, 2010) in the edgeR package (Robinson and Oshlack, 2010) was performed to calculate a normalization factor for each sample to adjust for biases in RNA composition. In total, 13,353 expressed genes with at least 0.5 cpm (counts per million) in at least 4 samples were considered for differential expression analysis. Multidimensional scaling in the limma package (Risso *et al.*, 2014) was used as a sample quality control step to check for outliers or batch effects. The normalized logCPM values of the topmost 5,000 variable genes were chosen to construct the multidimensional scaling plot. The Remove Unwanted Variation (RUV) method (Risso *et al.*, 2014) was used to estimate extra factors that are spurious technical variations in the samples and not biologically related.

Differential gene expression (DE) analysis between the DMSO and Progesterone was performed using the limma-trend method (Chen *et al.*, 2016) with treatment and 1 RUV factor included in the model. The statistical tests were corrected for multiple testing, and only genes with a False Discovery Rate (FDR) < 0.05 were considered significant.

Functional Enrichment Analysis

Pathway analysis of genes regulated by progesterone was done with ShinyGO 0.80 (http://bioinformatics.sdstate.edu/go/) from South Dakota State University, accessed in February 2024. The species was *bos taurus*, and the 4,541 genes significantly altered by progesterone were

entered for analysis. The 13,682 genes detected were used as background. The FDR cutoff was 0.05, and the total number of pathways to show was 500. The minimum pathway size was 2, and the maximum was 2,000. Pathways significantly overrepresented were determined by using KEGG (Kanehisa and Goto, 2000).

Glucose Assay

BUTE cells were plated in a 10 cm dish and treated with vehicle or progesterone for 24 hours. Media was collected from each 10 cm plate at 24 hours and aliquoted into 1 ml microcentrifuge tubes. All samples were stored at -20°C. Add 10 µl of standards and samples to the appropriate well on the plate. Next, add 200 µl of Wako Reagent to each well and incubate at 37 °C for 30 minutes (250 µl; 99703001, FUJIFILM Medical Systems USA). The plate was read at 505 nm using the Biotek Synergy H1 Multimode Reader (Agilent). The standard curve was used to determine the level of glucose in each sample.

NADPH Assay

BUTE cells were plated in a 10 cm dish and treated with vehicle or progesterone for 24 hours. At the end of the experiment, media was collected from each 10 cm plate, aliquoted into 1 ml microcentrifuge tubes, rinsed twice with PBS, and collected in 200 µl of lysis buffer. Samples were centrifuged at 254 x g for 5 minutes. All samples were stored at -20°C. The level of NADPH in each sample was determined with an NADPH assay kit (ab186031, Abcam) per manufacturer instructions. 50 µl of standards and samples were added to the appropriate well on the plate. 50 µl of NADPH reaction mix was added to the wells and mixed. The plate was incubated for 2 hours at room temperature and then read at OD 460 nm using the Biotek Synergy H1 Multimode Reader (Agilent). The standard curve was used to determine the level of NADPH

in each sample. NADPH level in the cell was then normalized to the amount of protein as determined by BCA assay.

Immunohistochemistry

Uteri from cows were collected on days 1 and 11 of the estrus cycle, fixed, and placed into paraffin blocks as part of a previous project and immunohistochemistry was carried out as done previously (Sandoval *et al.*, 2021; Chen *et al.*, 2022). Paraffin sections (5 μm) were deparaffinized and rehydrated. Slides were boiled in sodium citrate to retrieve antigens. The slides were placed in 3% hydrogen peroxide (H325-500, Fisher Scientific) for 15 minutes and then placed into a hydrated chamber with block for 1 hour (3% BSA, 10% goat serum, in TBS). Primary antibody was added to each slide (G6PD; ab76598, Abcam) and **i**ncubated at 4°C overnight. The slides were washed in TBS-T and the secondary antibody (BA-1000, Vector Laboratories) was added for thirty minutes at room temperature. The slides were washed, and the ABC complex was added. After the final wash, the DAB substrate was added (SK-4100, Vector Laboratories). Then the slides were counterstained, dehydrated, and mounted with Permount (SP15100, Fisher Scientific). Images were captured with a Zeiss Axioskop upright microscope with an Axiocam 305 color camera. Negative control slides were processed the same, except that the primary antibody was replaced with an isotype specific negative control (IgG antibody; I-1000-5 Vector Laboratories).

Statistics

RNAseq data were analyzed as described above. All other data were analyzed by one-way ANOVA followed by a Dunnett's multiple comparisons test or Tukey's multiple comparison test, which was used for all experiments with >2 treatments. Experiments with two treatments

were analyzed using a t-test. Statistical analyses were conducted in GraphPad PRISM (Version 9.0.0). Every treatment was replicated 4 times, and the significance criterion alpha was $P<0.05$.

6.4 Results

BUTE cells treated with progesterone (10 μM) or vehicle (DMSO) for 24 hours were collected, and RNA was extracted and sent for sequencing. After quality control, 13,354 gene transcripts were detected. The principal components analysis shows a complete separation of treatment along PC1 (Figure 14A). Of the 13,354 genes detected, 1,623 were upregulated, 1,449 were downregulated, and 10,282 showed no change (Figure 14B). ShinyGO 0.80 determined the enrichment pathway in the 3,072 significant genes. The analysis indicated that 6 pathways were significant, including metabolic pathway, glutathione metabolism, and arginine and proline metabolism (Figure 14C).

Progesterone significantly increased GLUT 4 in BUTE cells (Figure 15A). There was no difference in gene expression in the three hexokinase isoforms (Figure 15B). The hexosamine biosynthesis pathway had 1 gene that trended to be increased: GFPT2 (Figure 15C). For glycolysis, progesterone significantly decreased ENO3 (Figure 15D). Progesterone significantly alters 1 of 2 genes in the polyol pathway (SORD) (Figure 15E). Progesterone downregulated 4 out of 12 genes (PGM2, GYG1, PYGL, and AGL) in the glycogen metabolism pathway (Figure 15F). The pentose phosphate pathway was mostly altered by progesterone. Four 3 out of 7 unique genes to the PPP were significantly upregulated (G6PD and TKT), and one was downregulated (RPE) (Figure 15G).

Glucose concentration was measured to determine if progesterone increased glucose uptake in BUTE cells. BUTE cells treated with progesterone had a decrease in glucose

concentration in the media, indicating increased glucose uptake (Figure 16A). A major product of the pentose phosphate pathway is NADPH. BUTE cells treated with progesterone had an increase in NADPH levels within the cell. NADPH concentrations in the media were low and did not change due to treatment (Figure 16B-C). Immunohistochemistry showed that the cow endometrium had a higher expression of G6PD (the rate limiting enzyme in the PPP) on day 11 vs day 1 in the glandular and luminal epithelium (Figure 17).

6.5 Discussion

We found a significant change in the expression of genes associated with glucose metabolism in progesterone-treated BUTE cells. transcription of several genes in the pentose phosphate pathway were increased, suggesting that the abundance of NADPH might be affected. Progesterone increased the level of NADPH within the cells after 24 hours of treatment. G6PD was highly expressed in the uterine epithelium on day 11 vs. day 1 when progesterone levels were high. NADPH is a major product of the pentose phosphate pathway. Cholesterol synthesis requires NADPH as a reducing agent. The steroid (cholesterol) synthesis enrichment pathway was significantly increased in progesterone treated BUTE cells. The increase in NADPH could also be utilized for fatty acid synthesis. Fatty acids can be secreted by the uterine glandular epithelium in histotroph, which plays an important role in embryo elongation (Ribeiro *et al.*, 2016). Both cholesterol and fatty acids are essential for creating new cell membranes to support cell proliferation (Ye and DeBose-Boyd, 2011). NADPH and ribose-5-phosphate are also needed for cell proliferation (Cho *et al.*, 2018). Therefore, as the uterus prepares for implantation, the epithelium will require an increase in NADPH. Progesterone increases cell proliferation in human uterine epithelial cells (Zhang *et al.*, 2020). We have shown that progesterone increases

NADPH levels within the cell, decreases glucose concentration in the media, and increases genes associated with the pentose phosphate pathway. Therefore, we need to determine if progesterone increases cell proliferation to prepare the uterus for implantation.

In conclusion, progesterone increases genes associated with glucose metabolism, which can prepare the uterus for embryo implantation. Further research is needed to determine the downstream product from the increase in NADPH.

6.6 Figures

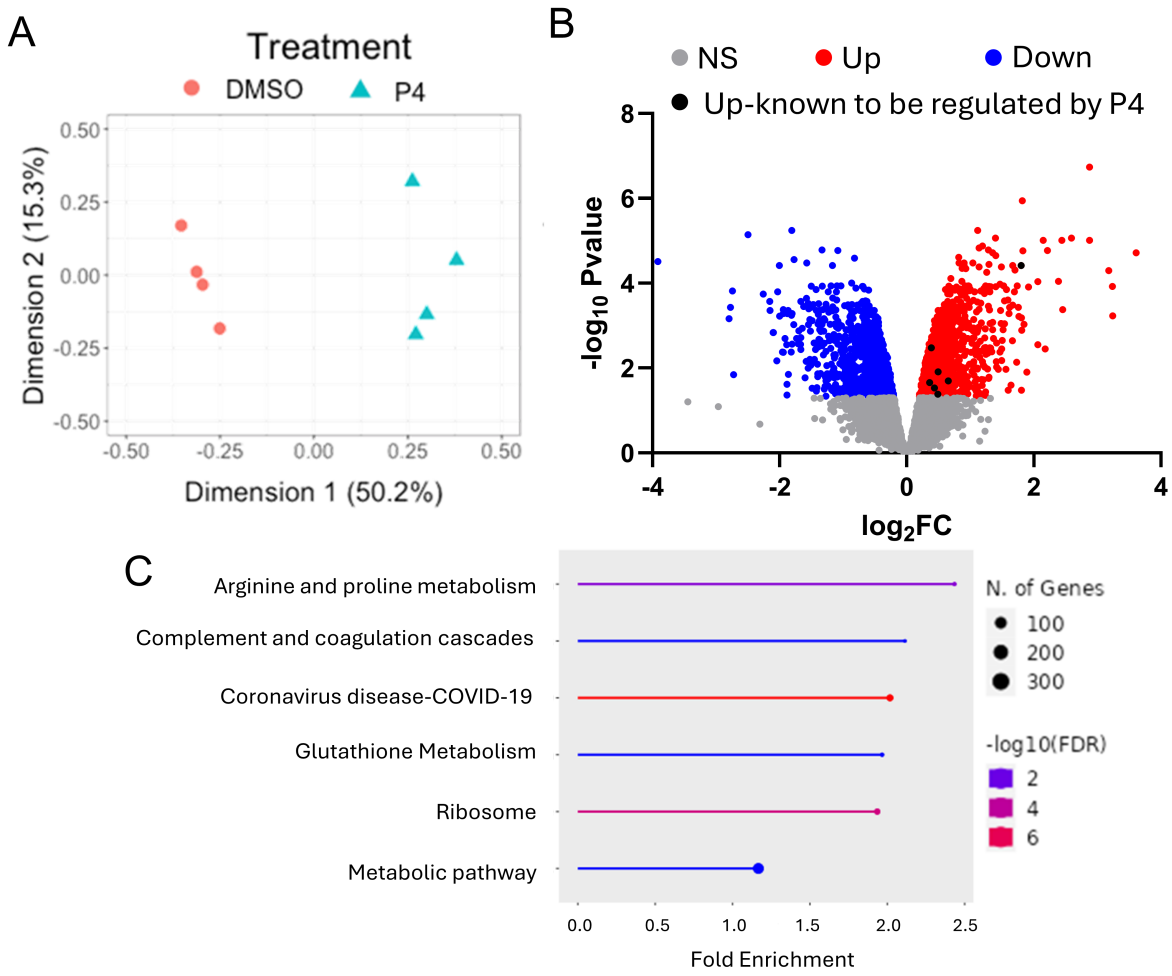


Figure 14. Differential expression of genes and associated pathways in progesterone treated BUTE cells. A) PCA plot for genes in progesterone (P4) and DMSO treated cells. B) Significant genes in progesterone vs DMSO treated BUTE cells. The blue represents downregulated genes, the red represents upregulated genes, and the grey is not significant genes. C) Significant enrichment pathways in progesterone-treated cells.

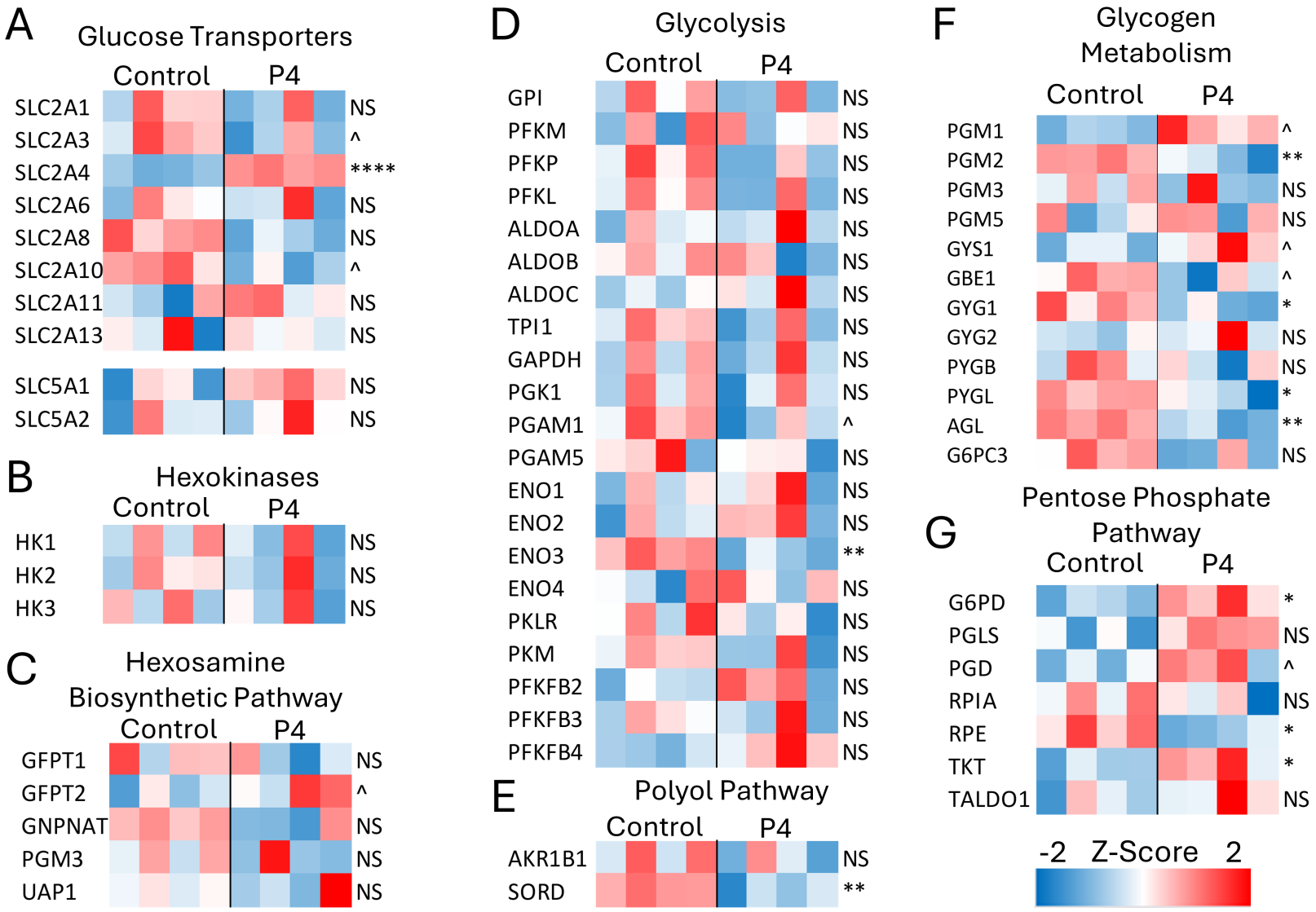


Figure 15. Regulation of genes involved in glucose metabolism or transport in BUTE cells.

Figure 15 (continued). Regulation of genes involved in glucose metabolism or transport in BUTE cells. The genes for glucose transportation were A) Genes for facilitative glucose transporters (GLUTs) and sodium-glucose cotransporters (SGLTs). Genes involved in glucose catabolism pathway were Hexokinases (B), Hexosamine Biosynthesis Pathway (C), Glycolysis (D), Polyol Pathway (E), Glycogen Metabolism (F), and Pentose Phosphate Pathway (G). Gene expressed in the RNA sequencing data whereas red is upregulated and blue is downregulated. Each box represented an individual sample. n=4. ****FDR<0.0001 **FDR<0.01 *FDR< 0.05 ^ 0.05<FDR<0.1.

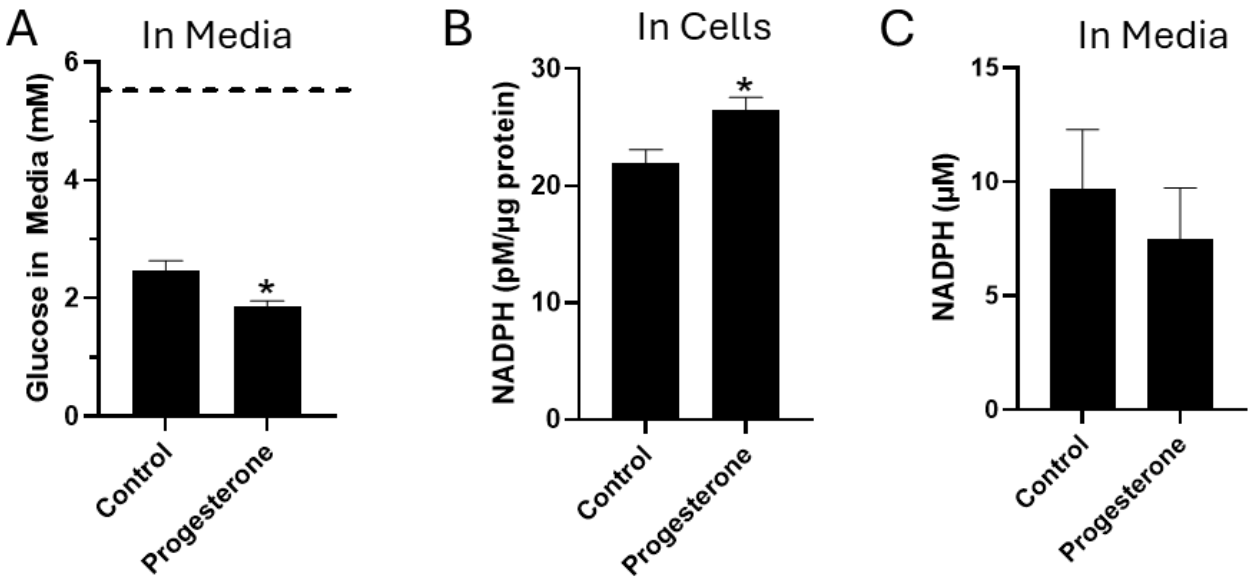


Figure 16. Progesterone decreases glucose in the media and increases NADPH levels within the cell. A) Glucose concentration in media in BUTE cells treated with 10 µM of progesterone for 24 hours. B) NADPH concentration in BUTE cells (B) and media (C) treated with 10 µM of progesterone for 24 hours. n=4. *P< 0.05 relative to control.

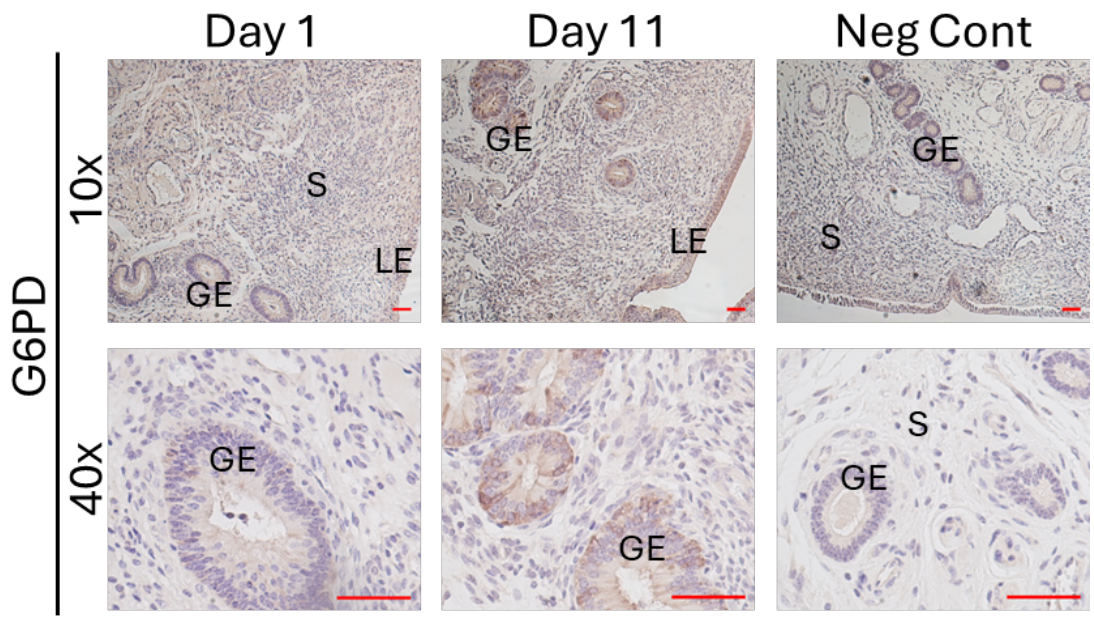


Figure 17. Glucose-6-phosphate dehydrogenase (G6PD) is expressed in the bovine uterine endometrium. Immunohistochemistry for G6PD is in the cow endometrium on Days 1 and 11 of the cycle. GE: Glandular Epithelium LE: Luminal Epithelium S: Stroma. n=4. Scale bar = 50 μm.

Chapter 7 Conclusion and Future Directions

7.1 Conclusion

We have shown that the BUTE and BFIB cells are a good model for glucose metabolism in the uterine endometrium of cows. Progesterone regulates glycogen breakdown in the uterine epithelial cells through membrane progesterone receptors (mPR). Using various activators and inhibitors, we found that progesterone decreases glycogen levels by acting through mPRs. Activation of mPRs increased intracellular AMP concentrations. AMP increases levels of phospho-AMPK and the activation of glycogen phosphorylase, as AMP is an allosteric regulator of glycogen phosphorylase (Barford *et al.*, 1991). A glycogen phosphorylase inhibitor could only partially block the effect of progesterone on glycogen breakdown. This led us to investigate and determine if glycogen was being broken down in lysosomes. BUTE cells were treated with progesterone or control. We determined that GAA is present in the uterine epithelium but is not regulated by progesterone in BUTE cells.

Progesterone increases genes in pathways related to glucose catabolism and redox metabolism. As we have also shown, progesterone increases the transcript level of genes in the pentose phosphate pathway and increases the level of NADPH in the cell. However, several questions remain as to why the uterine epithelium produces more NADPH in progesterone-treated cells. The BUTE cells could use NADPH to synthesize cholesterol, fatty acids, or DNA. The RNA sequencing data showed an increase in genes regulating cholesterol synthesis. Therefore, further research needs to be conducted to determine if the increase in NADPH leads to an increase in cholesterol synthesis. Glucose concentration in the media was decreased by progesterone treatment. Surprisingly, the increase in glucose uptake and the breakdown of glycogen to glucose could be shunted into the pentose phosphate pathway to increase the level of

NADPH in BUTE cells. This leads us to speculate that progesterone increases glucose catabolism in BUTE cells. A better understanding of how hormones within the uterus regulate glycogen and glucose metabolism is needed. Hence, it provides valuable information to better understand how a disease can affect glycogen and glucose metabolism.

Overall, my research significantly advances our understanding of the role of progesterone in uterine glycogen and glucose metabolism. In addition, future research can use these identified changes to help decrease early embryonic loss in dairy cattle and humans.

7.2 Future Directions

Our research has shown that hormones acting on the uterus regulate glycogen, but how various diseases uterine glycogen metabolism is unknown. In the future, I would study how the effect of a disease state changes the regulation of glycogen by hormones in the uterus. Uterine diseases in cattle and women could play a role in glycogen metabolism.

Aim 1A Develop a model to study endometriosis in a mouse.

I would develop a mouse model to study endometriosis, a common disease in humans. In the mouse model, I would induce endometriosis by injecting decidualized endometrium from a donor mouse into the peritoneal cavity. First, a donor will be artificially decidualized in both uterine horns, as previously described (Chen *et al.*, 2022). All mice will be ovariectomized to remove estrogen and progesterone then rest the mice for two weeks. After the two-week period has ended, mice will be treated with estrogen for three days, rest for two days, a combination of estrogen and progesterone for three days, and two to four hours after the last estrogen and progesterone injection, 15-20 μl of corn oil will be injected into both uterine horns followed by four days of progesterone injections. The uteri will be collected two to four hours after the last progesterone injection. After collection, the decidualized endometrium would be disassociated

into a single cell suspension in saline and injected into the peritoneal cavity (Burns *et al.*, 2022). The control mice would receive an injection of saline in the peritoneal cavity. The mice would be collected 7, 10, and 14 days after injection to determine the best time for the lesions to develop. Once the lesion timeline is determined, the next step is to measure uterine glycogen levels by PAS staining and enzymes crucial for glycogen metabolism by IHC and western blot.

Aim 1B. Determine if endometriosis affects glycogen levels during pregnancy.

The estrous cycle will be monitored every day for two weeks by vaginal lavage to confirm the endometriosis mice (ENDO) cycle regularly compared to control mice. To determine if fertility is affected, ENDO mice will be bred to wild-type males to determine if litter size, pups born alive vs. dead, and pup weight are different in ENDO mice compared to controls for six months. The breeding pair will contain one female ENDO mouse with a wild-type male. After determining if endometriosis affects fertility, I will determine if the glycogen levels are affected in mice with endometriosis during pregnancy. ENDO mice and control mice will be bred to wild-type males. The uteri will be collected on days 3.5 and 5.5 to count the number of implantation sites in the mice. The uteri will also be used to measure glycogen levels by PAS staining and IHC for glycogen metabolism enzymes.

Another future direction would be a comparative study to determine how wildlife compares to domestic animals in glycogen storage. I would compare the cow, horse, pig, dog, and cat for the domestic animals and the okapi, rhinos, wild dogs, wild cats (tiger and lion), and bears for the wild animals. It would be very challenging to collect wild species on a specific day, therefore finding more domestic animals that could be used to model the wild species. I would compare the gestation length and estrous cycle to determine how glycogen levels change. I would mainly focus on glycogen levels during the pre and post-implantation periods.

Aim 1A. Determine the glycogen level over the estrous cycle in domestic and wild animals.

Uterine samples would be collected from domestic animals on different days of the cycle, with at least one collection on the day of ovulation and one during the mid-luteal phase. Since wildlife samples cannot be collected on specific days of the cycle, I would try to find a close domestic relative to model the glycogen levels over the cycle. PAS staining is used to stain glycoprotein, glycolipid, and glycogen. Since PAS stains all glycol molecules, diastase (PAS+D) is used to digest glycogen in the sample; therefore, subtracting the positive staining/total area in the PAS image from the positive staining/total area in the PAS+D image, the relative glycogen content can be determined. I would also compare the enzymes involved in glycogen metabolism (hexokinase, glycogen synthase, glycogen phosphorylase, and glucose-6-phosphatase) on the same collection days as glycogen levels.

Aim 1B. Determine the level of glycogen during pregnancy in domestic and wild animals.

I would determine how glycogen levels and metabolism enzymes change throughout pregnancy and if the glycogen levels change based on the placenta type and length of gestation. I would collect samples in domestic animals before implantation, during implantation, mid- and late-gestation, and on the day of delivery. I would measure glycogen by PAS staining and enzymes crucial for glycogen metabolism by IHC and western blot.

7.3 Figure

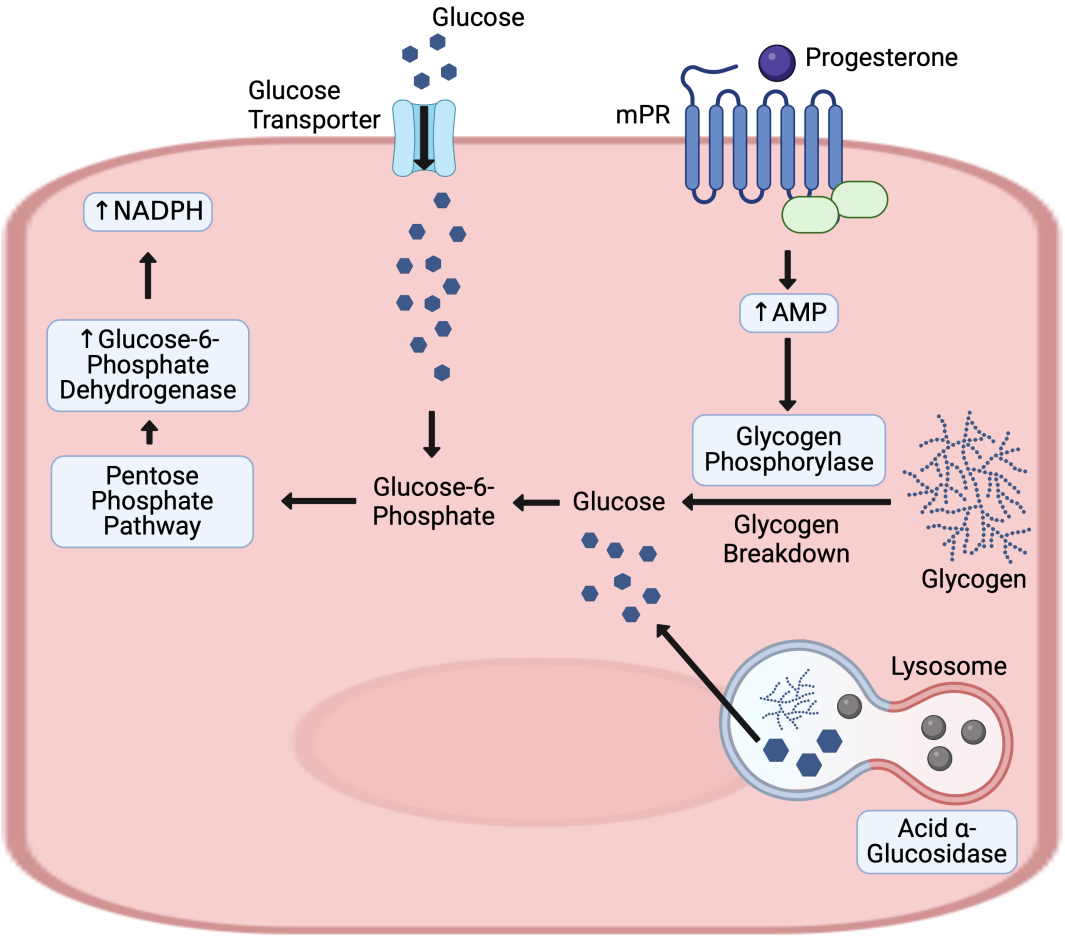


Figure 18. Summary figure. Progesterone acts through membrane progesterone receptors to stimulate the breakdown of glycogen into glucose. Glucose enters the cell through glucose transporters. Once in the cell glucose can be stored as glycogen. Glycogen can be broken down by acid α-glucosidase in lysosomes or by glycogen phosphorylase in the cytosol. The increase in glucose enters the pentose phosphate pathway to increase NADPH within the cell.

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