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Uterine Glycogen during Pregnancy in Mink (*Neovison vison*): Mediating the Effects of Estradiol

By

Matthew Dean, M.S.

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Dedication

I would like to dedicate this dissertation to my Dad, James “Jim” Allen Dean, who passed away in July 2013. Without his work ethic, I would have never completed this degree.

Acknowledgements

"Anytime you see a turtle up on top of a fence post, you know he had some help."
-Alex Haley

"The length of this document defends it well against the risk of its being read."
-Winston Churchill

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Dissertation Abstract--Idaho State University (2014)

In mammals, the majority of embryos are lost before or during implantation. During this time, embryos are dependent on glucose secreted into the uterine lumen as a component of histotroph, and glucose consumption increases dramatically as implantation approaches. Glycogen stored in the uterus could be used to support the uterus and embryos. Mink embryos undergo delayed implantation, with embryos dependent on histotroph for up to 50 days. Here we show that, at estrus, endometrial glycogen is localized to the epithelial cells, and this glycogen is completely mobilized prior to implantation. During diapause, uterine expression of glucose-6-phosphatase is increased 5-fold compared to estrus, supporting the concept that glucose-6-phosphate is dephosphorylated and secreted into the uterus lumen. Next to understand the mechanism(s) by which estradiol stimulates glycogenesis in the uterine epithelia, we used immortalized cells from the mink uterus (GMMe cells). In the absence of insulin estradiol failed to alter glycogen concentration. In contrast insulin consistently increased glycogen accumulation and this occured through activation of the insulin-like growth factor 1 (Igf1) receptor and the PI3K signaling pathway. In the presence of insulin, 10 nM of estradiol significantly increased glycogen accumulation and the estrogen receptor antagonist ICI 182,780 blocked this effect. Coupled with the well-established fact that estradiol increases uterine Igf1 production, these results indicate that estradiol increases glycogen accumulation by two distinct mechanisms: indirectly by Igf1 signaling and directly in the presence of insulin/Igf. Finally, we analyzed the effect of estradiol and 4-

hydroxyestradiol on uterine glycogen metabolism and determined what effect(s) were mediated by prostaglandins (by using indomethacin). Both steroids equally increased endometrial glycogen content but 4-hydroxyestradiol was less potent. However, both steroids equally increased hexokinase expression (via a prostaglandin-dependent mechanism) and activated glycogen synthase (i.e. decrease phospho-glycogen synthase levels) independently of prostaglandins. Only 4-hydroxyestradiol significantly increased glycogen phosphorylase activity. Thus the increased conversion of estradiol to 4-hydroxyestradiol during diapause may partially explain the decreasing endometrial glycogen concentrations. In conclusion, estradiol increases glycogen content in the epithelia at estrus, directly and via Igf1. Glycogen is subsequently mobilized during diapause, due in part to conversion of estradiol to 4-hydroxyestradiol.

1. Introduction

After the egg is fertilized, the newly formed zygote (1 cell) begins dividing as it moves through the oviduct, arriving in the uterus as a blastocyst composed of hundreds of cells (Boron and Boulpaep, 2005; Senger, 2005). After formation of the placenta, the embryo(s) can exchange nutrients and wastes directly with maternal circulation, but prior to placentation, survival and development of the embryo is dependent on secretions of the epithelial cells that line the lumen of the oviducts and uterus. The importance of these secretions, termed histotroph, has been hypothesized as far back as the third century B.C. by Aristotle (cited in Gray et al., 2001a). Much of the research on storage and release of histotroph has been done in rodents which have a very short (4-5 day) pre-implantation period. In contrast, mink have evolved a mechanism of delayed implantation, which results in parturition occurring during the first week of May, when conditions are favorable for kit survival (Goodman, 1999). Specifically, mink mate during mid-February through March, yet implantation does not occur until the first week of April. Thus mink embryos are dependent on histotroph for up for to 50 days before a placenta begins to develop (reviewed in Sundqvist et al., 1989), and it is likely that mink have evolved more pronounced mechanisms both to store nutrients in the uterus and control their secretion as embryonic development proceeds.

While an interesting model in reproductive biology, many aspects of mink reproduction remain poorly characterized. This dissertation will begin with a brief review of literature pertinent to the reproductive biology of rodents and mink: rodents as a model of spontaneous ovulation, mink as a model for storage and mobilization of nutrients from the endometrium. Then the roles glycogen and glucose in the uterus will be discussed along with possible factors that control uterine glycogen metabolism. Finally, a series of experiments will be presented that were aimed at elucidating the control of uterine glycogen metabolism and factors underlying its control.

2. Literature Review

2.1. Reproduction in Rodents

Estrous Cycles

Rodents have a 4-5 day estrous cycle. Ovulation occurs early on the morning of estrus resulting in a rapid decrease in estradiol concentrations and formation of corpus lutea (CL). Progesterone concentrations rise during diestrus but remain elevated only briefly (12 hrs), due to the actions of 20α-hydroxysteroid dehydrogenase (Ishida et al., 2007). Concentrations of estradiol begin to increase again during metestrus and by proestrus, concentrations are very high, causing females to enter estrus. The high concentrations of estrogen trigger a surge in luteinizing hormone (LH) release, which causes the next ovulation (Butcher et al., 1974).

Luteal Function

In rodents, the brevity of elevated plasma progesterone concentrations during diestrus (Butcher et al., 1974) is due to high expression of 20α-hydroxysteroid dehydrogenase in the CL (Ishida et al., 2007), which metabolizes progesterone to 20α-dihydroprogesterone (Armstrong et al., 1970; Behrman et al., 1970; Choi et al., 2008). However, mating induces twice-daily prolactin surges that decrease activity of this enzyme and allow the CL to release progesterone. These prolactin surges continue for 10-11 days, maintaining progesterone secretion (Gunnet and Freeman, 1983). If mating resulted in fertilization and pregnancy, placenta-produced prolactin and prolactin-like proteins maintain CL function, in place of pituitary prolactin. However, if the mating was not successful (e.g. mating to a vasectomized male), a 10 day pseudopregnancy occurs. During pseudopregnancy, progesterone concentrations increase in a manner similar to pregnancy. However, since there is no placenta to secrete prolactin (and related proteins) after hypothalamic prolactin surges cease, progesterone concentration begin to decease on day 11 (Butcher et al., 1974; Ormandy et al., 1997; Tonkowicz et al., 1983).

Implantation

After mating, fertilization takes place in the oviduct and the embryo develops as it moves toward the uterus. The embryo reaches the uterine lumen on day 4-5 post-insemination (Villalón et al., 1982). After reaching the uterus, the embryos are dispersed equidistant along the length of each uterine horn and implant into the anti-mesometrial side of the endometrium (Hama et al., 2007). Implantation in the rat is very invasive. Trophoblast cells of the placenta digest the lumenal epithelial cells and underlying stroma, reaching maternal arteries. The maternal arteries are breached and maternal blood fills the inter-villous space, resulting in what is known as hemochorial placentation (reviewed in Abrahamsohn and Zorn, 1993; Soares et al., 2012).

In rats and mice, implantation is triggered by increasing estrogen concentrations due to large ovarian follicles developing on days 4 –5 post-mating (Cochrane and Meyer, 1957; Meyer and Nutting, 1964). While the exact actions of estradiol to induce implantation are unclear, there is accumulating evidence that converstion of estradiol to 4-hydroxyestradiol is required for implantation to proceed. Dey et al. (1986) showed that neither 2- nor 4- fluroestradiols could induce implantation in rats. The investigators speculated that the failure of these compounds to induce implantation may be due to their inability to be metabolized into catecholestrogens (i.e. 4-hydroxyestradiol and 2-hydroxyestradiol). Catecholestrogens are estradiol metabolites produced by the hydroxylation of estradiol at the 2- or 4-carbon positions on the A ring, yielding catechol groups (hence catecholestrogens). Hydroxylation at the 2-position yields 2-hydroxyestradiol while hydroxylation at the 4 position produces 4-hydroxyestradiol (see section 2.11.3 Catecholestrogens). Further evidence for support of Dey's hypothesis was that activity of the enzymes which produce 4-hydroxyestradiol (and presumably local 4-hydroxyestradiol concentration) in the uterus increased on the day of implantation in rats (Paria et al., 1990). In a follow up study, Paria et al. (1998) showed that culturing mouse embryos in estradiol, estrone, or

estriol failed to activate the embryos (as determined by binding epidermal growth factor (Egf)). In contrast, 4-hydroxyestradiol induced Egf binding and the effect of 4-hydroxyestradiol was not blocked by addition of ICI 182,780, an estrogen receptor antagonist. They also reported that transferring 4-hydroxyestradiol treated blastocysts into recipient females resulted in implantation. In contrast, incubation of blastocysts with estradiol induced a single implantation site to form in only one recipient. Based on these data, the authors concluded that ovarian estradiol is metabolized to catecholestrogens by the uterus. These catecholestrogens then activate the dormant embryos, inducing implantation.

Decidualization

In rodents, as the embryo implants into the uterus, the underlying endometrium undergoes "rapid remodeling of the uterine stromal compartment resulting in a morphological and functional transformation including stromal cell proliferation and subsequent differentiation…" known as decidualization (Wetendorf and DeMayo, 2012). While physical contact of the endometrium with the embryos is the initiating stimulus for decidualization in rodents, steroid priming is essential for proper decidualization to occur (Kennedy, 1980). Estradiol increases proliferation of the endometrium, in part, by 1) increasing the production of insulin-like growth factor 1 (Igf1) by the uterine stroma (Ogo et al. 2014), and 2) by decreasing local production of Igf binding protein (Hayashi et al., 2005). Progesterone then terminates proliferation and stimulates differentiation by 1) stimulating IGF binding protein 1 expression, which binds and inactivates Igf1 and directly stimulates differentiation via integrins (Matsumoto et al., 2008); 2) directly antagonizing the effects of estradiol on phosphoinositide 3-kinase (PI3K; Chen et al., 2005); and 3) down regulating inhibitors of differentiation (Kodama et al., 2010).

It should be noted that there are major differences in decidualization across species. In women, proliferation of the endometrial stroma is triggered by estradiol during the follicular phase. After ovulation and luteinization, progesterone causes differentiation of this tissue (Teklenburg et al., 2010). In contrast, in rodents physical contact between the lumenal epithelial and implantation embryo is the proximal trigger for decidualization (Lundkvist, 1978), and it has been suggested that mink uteri do not decidualize at all (Das et al., 1999; Vargas, 2011).

2.2. Reproduction in Mink

Mink are anestrus, or reproductively inactive, much of the year. In the northern hemisphere estradiol concentrations begin to rise in early February and remain high through March, causing mink to enter estrus (Pilbeam et al., 1979). Any time during this period mink can mate, and standard ranch practices are for mink to be mated twice during estrus. Each mating results in ovulation and formation of CL (Hansson, 1947; Sundqvist et al., 1989). After ovulation and fertilization, the embryos enter a state of arrested development known as embryonic diapause. The length of diapause is variable, depending on when the females were mated. Regardless of when the mating occurred, progesterone concentrations begin to rise at the end of March and implantation ensues (Figure 1). Near the time of implantation a second increase in estradiol concentrations is observed and progesterone concentrations continue to increase (Lagerkvist et al., 1992). After implantation, the remainder of pregnancy is very consistent in mink at 29-30 days, so that parturition occurs during early May (Figure 1; Concannon et al., 1980).

Induced Ovulation and Superfetation

Unlike humans and rodents, which ovulate spontaneously, ovulation in mink is induced by mating. Through a neural-endocrine reflex, copulation leads to a large (12 fold) increase in plasma LH concentration 2-4 hours after mating (Tauson et al., 2000). This LH surge leads to ovulation of approximately 12 oocytes into the ovarian bursa 30-40 hours after copulation

(Douglas et al., 1994; Hansson, 1947). Fertilization occurs in the bursa or ampulla of the oviduct and development commences as the developing embryo moves toward the uterus. After ovulation, remnants of ovulated follicles form CL. However, the CL secrete very low levels of progesterone until implantation (see below), allowing ovulatory follicles to continue to develop. Approximately seven days after mating, ovulatory follicles are reestablished and estradiol concentrations are sufficient for the female to re-mate with another male (Douglas et al., 1994). This second mating can result in offspring from different ovulation events and different sires to be gestated together, a phenomenon called superfetation (Roellig et al., 2011; Shackelford, 1952). The second mating results in loss of most of the embryos from the first mating, but two matings do increase overall litter size compared to that of a single mating (Adams, 1981).

Delayed Implantation & Embryonic Diapause

In mink delayed implantation is the result of a delay in the signal (i.e. prolactin) triggering implantation; this results in an arrest of embryonic developed (termed embryonic diapause). Collectively these phenomena ensure that parturition occurs in the spring, when conditions are favorable for survival of the offspring (Goodman, 1999; Renfree and Shaw, 2000). Delayed implantation only occurs in a small number of mammalian species and appears to have evolved multiple times, as the species that undergo diapause are diverse (Lopes et al., 2004; Renfree and Shaw, 2000). However, embryos of other species still retain the ability to enter into and survive embryonic diapause. Ptak et al. (2012) found that sheep embryos transferred into mice undergoing experimentally-induced delayed implantation appeared to enter diapause. These embryos experienced less mitosis (i.e. less bromodeoxyuridine (BrdU) incorporation and lower proliferating cell nuclear antigen (Pcna) expression) than control embryos transferred into ewes. Most impressively, 44% of ovine embryos flushed from the murine uterus and then transferred back into sheep uteri resulted in live births, showing the viability of these diapaused embryos.

In mink, approximately 8-9 days after mating the embryos enter the uterine lumen as blastocysts containing approximately 300 cells (Enders, 1952; Hansson, 1947). Upon entering the uterus the blastocysts enter diapause, remaining in the uterine lumen without a placenta for up to 50 days (Figure 1). Diapause in mink is obligatory as it occurs during every gestation and is not due to factors that occur only during some pregnancies (such as lactation), as can occur in mice (Weichert, 1940).

Endocrine Control of Implantation

Implantation in mink is controlled by photoperiod. Specifically, photoperiod lengthening after the vernal equinox results in decreased duration of melatonin secretion (which is secreted at night). The shorter durations of melatonin secretion then increase prolactin secretion (Murphy et al., 1990, 1981). For example, Rose et al. (1986) found that serum prolactin concentrations doubled between March 23rd and 27th. Increased prolactin results in increased progesterone concentrations and termination of diapause (Papke et al., 1980). Unlike rodents, in mink prolactin directly stimulates progesterone production by increased expression of steroidogenic acute regulatory (StAR) protein, P450 side chain cleavage, and 3β-hydroxysteroid dehydrogenase (Douglas et al., 1998). These findings seemed to indicate that increased progesterone concentrations may be the proximal factor in terminating diapause; however, experimental evidence has shown that progesterone is not sufficient to induce implantation (Hammond, 1951). Based on emerging evidence in other species, there are two distinct possibilities as to the factor responsible for implantation in mink: catecholestrogens and phosphoglucoisomerase.

While it is well established that systemic estradiol initiates implantation in rats and mice (See 2.1 Reproduction in Rodents), the role of estradiol in mink implantation is less clear. Murphy et al.

(1982) found that neither medroxyprogesterone (a synthesitic progestin) nor estradiol could trigger implantation in mink. In ferrets (as closely related species that does not undergo delayed implantation), Murphy and Mead (1976) examined the role of estradiol in implantation by using estradiol-antiserum. Showing the validity of their method, treatment of rats with the estradiol antiserum (beginning on day 2 after mating) inhibited implantation in all six animals examined on day 7. The investigators then treated ferrets with antiserum during different time-frames (days 9-13, 10-13, 3-13, or 4-13 after mating) corresponding with the time of implantation. However, all females (control and treated) had observable implantation sites at day 14. In agreement, Mead and McRae (1982) found that treatment of ovariectomized ferrets with progesterone- and estradiol-releasing implants did not induce implantation.

While implantation not does not appear to be induced by estrogens in ferrets (and by extension mink), this is not to say they do not play a role in embryonic development/implantation. Estradiol (either systemic or local in origin) seems to be a common theme in eutherian mammals (reviewed in Levasseur, 1984), and thus estradiol may be facilitating survival and development. In ferrets, Mead and McRae (1982) found that serum estradiol concentrations were significantly increased (2-fold) near the time of implantation (day 8 after mating) compared to day 4 (a similar observation has been made in mink (Lagerkvist et al. 1992). Treatment with an aromatase inhibitor decreased the number of implantation sites per female and estradiol antiserum reduced the number and length of implantation sites, but neither treatment reduced the number of females with implantation sites. Collectively these results indicate that estradiol may play a role in supporting implantation and embryonic growth but cannot induce implantation.

Even catecholestrogen production seems to be common during implantation. In rabbits and rats, uterine production of catecholestrogens increase at implantation (Chakraborty et al., 1990; Paria

et al., 1990) and pig embryos produce increasing amounts of catecholestrogens as implantation approaches (Mondschein et al., 1985). Recently, our laboratory has found that uterine cytochrome P450 1B1 (Cyp1b1; enzyme responsible for 4-hydroxyestradiol production) mRNA expression is increased in the late pre-implantation period compared to estrus in mink (unpublished observations). However, the potential role(s) of catecholestrogens at this time are almost completely unexplored.

The second factor that may induce implantation was found by Mead et al. (1988). These investigators isolated CL from 62 ferrets 6 days after mating (i.e. near time of implantation). The CL were then homogenized and extracts (aqueous or ethanol) were prepared. Interestingly, the ethanol extract was unable to induce implantation in any ferrets (0 of 6); however, the aqueous extract induced implantation in 5 of 6 ferrets treated. Size fractionation of the aqueous extracts revealed the factor inducing implantation to be large (>50 kDa) and pre-treatment with a protease prevented implantation. Taken together these results, strongly suggest the implantation factor to at least contain a peptide. Following up on these studies, the Bahr laboratory purified a 60 kDa protein from the ferret CL which induced implantation (Schulz and Bahr, 2004). This protein was purified, digested with trypsin, and two peptides were sequenced. The sequences aligned perfectly with phosphoglucoisomerase of the pig (*Sus scrofa*). Phosphoglucoisomerase is well established as the second enzyme in glycolysis, isomerizing glucose-6-phosphate to fructose-6-phosphate (or the reverse reactions, depending on intracellular conditions; Voet et al., 2001a). Immunohistochemistry of the ferret CL revealed that the CL produces phosphoglucoisomerase on days 6 and 9 and to a lesser extent on day 12, which corresponds to days in which the CL can induce implantation (Mead et al., 1988). Additionally, immunization against this protein significantly decreased the number of implantation sites in ferrets (0

implantation sites in 2 of 3 ferrets). In a follow up study, Schulz and Bahr (2003) showed that plasma phosphoglucoisomerase activity peaked on day 6 post-mating. Culturing CL collected at different days post mating, the investigators found that day 9 CL secreted more phosphoglucoisomerase into culture media than day 3 CL. Interestingly, the incracellular phosphoglucoisomerase activity was the same in all four collections CL (days 3, 6, 9, and 12), indicating active secretion of the enzyme.

While phosphoglucose isomerase has yet to be linked to implantation in other speices, the two studies from the Bahr laboratory (Schulz and Bahr, 2003; Schulze and Bahr, 2004) provide convincing evidence for an endocrine role of phosphoglucose isomerase, at least in ferrets. Additional evidence supporting this conjecture is that many studies have linked phosphoglucoisomerase to cancer metastasis, where it is known as autocrine motility factor (reviewed in Yanagawa et al. 2004). This is especially interesting considering the many molecular parrellels between implantation and metastasis (reviewed in Murray and Lessey, 1999)

Uterine/Embryonic Interactions at Implantation

In a classic study on regulation of diapause, Chang (1968) performed reciprocal embryo transfers between mink and ferrets. He found that mink embryos began to implant when transferred to ferrets, while ferrets embryos entered diapause in the mink uterus, demonstrating maternal regulation of diapause. In the years since, much research has accumulated showing a role for prostaglandins in embryonic/uterine communications. For example, Song et al. (1998) found that prostaglandin G/H synthase-2 (Ptgs2; also called cyclooxygenase and is required for production of all prostaglandins) mRNA expression was increased on day 2-8 post-expansion of the blastocysts, and this expression was almost exclusively localized to the glandular and luminal epithelium. Furthermore, Desmarais (2007) found that a class of prostaglandin receptors known

as peroxisome proliferator activated receptors (PPARs) are expressed in trophoblast cells of mink embryos and that 15-deoxy-prostaglandin J_2 (natural ligand for PPARs) increase expression of osteopontin (both mRNA and protein), a marker for invading trophoblasts.

The Murphy laboratory has also linked a class of molecules known as polyamines to implantation in mink. Lefèvre and Murphy (2009) used subtractive suppressive hybridization to isolate mRNAs differentially expressed between mink uteri in diapause and activated uteri. One cluster of genes up-regulated by activation collectively encode for increased polyamines, molecules characterized by containing two primary amine groups. Specially, termination of diapause resulted in increased expression of ornithine decarboxylase and a doubling of the tissue concentration of putrescence, a polyamine. And finally, inhibition of ornithine decarboxylase resulted in a reversible arrest of trophoblast cells (Lefèvre et al., 2011). Collectively these studies highlight the complicated and intricate nature of embryonic/uterine interactions at implantation. However, which factors directly cause implantation in mink and which pathways are secondary to that remain to be elucidated.

2.3. Embryonic/Fetal Loss

Loss in Humans

Human fecundity, defined as "the probability to produce a vital term newborn per menstrual cycle during which there was normal sexual activity" is low, typically found to be approximately 30% (Olsen and Rachootin, 2003; van Noord-Zaadstra et al., 1991; Zinaman et al., 1996). This is much lower than many people realize, probably because the rate of spontaneous abortions in clinically recognized pregnancies is also quite low, <10%, suggesting most loss occurs very early. To address this issue, multiple studies have relied upon the presence of human chorionic gonadotropin (hCG) in urine to diagnose early pregnancies and quantify loss. hCG is a hormone produced by trophoblast cells and thus can be used as a marker of pregnancy. Miller et al.

(1980) detected 143 conceptions using this method, and 43% where subsequently lost. Only 14 of the conceptions were lost during clinically recognized spontaneous abortions; 50 of the miscarriages were detected solely through hCG monitoring. Similarly, Wilcox et al. (1988) found 31% of 198 pregnancies detected by urinary hCG were lost (⅔ were lost before being recognized clinically). Wang et al. (2003) found 32% of detected pregnancies were lost, with ¾ of the losses occurring before clinical detection. Furthermore these losses reflect only embryos producing hCG, and undoubtedly, some embryos are lost before hCG becomes detectable. Flushing pre-implantation embryos in the course of assisted reproductive techniques have found that 50% of these embryos appear abnormal (reviewed in Benagiano et al., 2010), suggesting high rates of loss may occur before hCG becomes detectable. While these losses cannot be directly studied due to ethical considerations, the total rate of pregnancy loss in humans is believe to be over 50% (Macklon et al. 2002)

Loss in Livestock

Embryonic and fetal loss is very well studied in livestock species because of their economic importance and minimal ethical issues compared to human research. Fertilization rates tend to be very high in livestock (>95%; Mitchell et al., 1999; Ryan et al., 1993; Wiebold, 1988), and yet only 40-55% of matings result in live birth (reviewed in Diskin and Morris, 2008), indicating high rates of loss. Studies detailing the timing of embryonic/fetal loss have indicated that fetal loss is rare (7%) and most losses occur early in embryonic development (Diskin and Morris, 2008; Silke et al., 2002).

Loss in Mink

In contrast to many other agricultural species, embryonic and/or fetal loss has not been directly studied in mink. Though, insights can still be gained from work not directly addressing the issue. Ovulation rates are much higher in mink than other livestock species. Adams (1981)

found that mating results in approximately 10-11 ovulations. In agreement, exogenous gonadotrophin-releasing hormone (GnRH) also induced ovulation of 11-12 follicles (Douglas et al., 1994). Taking into account the common practice of mating mink twice during the estrous season, there are over 20 potential offspring (Adams, 1973). However, the second mating often results in loss of most, if not all, of the embryos produced by the first mating (Adams, 1981), realistically limiting litter sizes in mink to 10-12, but this is still substantiality higher than observed litter sizes. Bownes (1968) conducted a very large survey of Canadian mink ranchers to address reproductive parameters. The report included 101 mink ranches in Canada, encompassing 12,919 female mink. Only females that had been mated once were used in the final report and the average litter size was calculated to be 4.77 kits per litter. This agrees with more direct measurements made in other reports with litter sizes averaging 4-6 kits (Beard et al., 1997; Tauson, 1993), and seems to agree with observed litter sizes in Southeast Idaho (Ryan Moyle, Moyle Mink and Tannery, Pers. Comm).

Observations on ovulation rate and litter size in mink strongly suggest a very high rate of fertilization failure and/or embryonic/fetal loss. But little work has been done to determine when and where these losses are occurring. A report on the effect of pesticides on mink reproduction (Beard et al., 1997) found that approximately 75% of mink that mated had visible implantation sites at necropsy. However, that paper did not indicate the number of implantations per female, making partial pregnancy losses impossible to estimate. Another study looked at the effect of increases nutrition on reproductive performance (Tauson, 1985). In this investigation, ovulation rate and litter size (12-15 and 5-6, respectively) agreed with other studies. But, in this study implantation rates were reported to be 4.6-8, depending on the particular experiment. This

suggests that the majority of oocyte wastage (90%) occurs before implantation sites become visible.

2.4. Histotroph

Pre-implantation embryos are completely dependent on "extracellular material derived from the endometrium and the uterine glands that accumulated in the space between the maternal and fetal tissues" called histotroph (Burton et al. 2002). And in species with epitheliochorial placentas histotroph remains important throughout pregnancy, due to the high degree of separation between the maternal and fetal blood supplies (Gray et al., 2001a; Senger, 2005). Even in humans, which have invasive placentation, secretion of histotroph into the inter-villous space continues throughout the first trimester of pregnancy (Burton et al., 2002). Many studies have correlated defects in histotroph secretion with unexplained infertility (reviewed in Burton et al., 2007). For example, Dimitriadis et al. (2006) found lower interleukin-11 and leukemia inhibitory factor (*Lif*) expression in the uterine epithelia of infertile women (due to endometriosis) compared to healthy women. Multiple studies have found lower glycogen concentrations in the uterus of infertile or sub-fertile women compare to women of normal fertility (Girish et al., 2012; Maeyama et al., 1977; Zawar et al., 2003).

Uterine Glands

The major source of histotroph is uterine glands, which are invaginations of the lumenal epithelia branching from the uterine lumen. It has long been speculated that uterine glands provide nourishment for developing embryos (Gray et al., 2001a). Indeed, epithelial cells lining uterine glands have high secretory activity during early pregnancy, and these secretions are rich is carbohydrates and lipids (Burton et al., 2002; Hempstock et al., 2004). In addition, endometrial glands are believed to be a major source of growth factors. For example, in women epidermal growth factor (*Egf*) is localized to the glands (Hempstock et al., 2004) and the horse uterus is

characterized by increased *Egf* mRNA in close proximity to trophoblasts on day 35 of pregnancy.

Spencer and Gray (2006) developed a uterine gland knockout sheep by treating ewe lambs post-natally with progesterone, resulting in an endometrium that was histologically normal except for the absence of uterine glands. In these uterine gland knockouts, all embryos were lost before implantation occurred (Gray et al., 2001b), highlighting the importance of glandular secretion in embryonic survival and development Similar results have been shown by the production of uterine gland knock-out mice using transgenic approaches (Dunlap et al., 2011; Jeong et al., 2010). Both groups used Cre-Lox technology to knockout genes involved in uterine adenogenesis: Dunlap et al. (2011) knocked out Wnt7a and Jeong et al. (2010) knocked out Foxa2. Both of these approaches resulted in development of uteri that where histologically normal except for lacking uterine glands. Both of these studies found that transgenic females exhibited normal estrus cycles and would mate. However, the number of implanting embryos was drastically (and significantly) reduced in both studies.

Estradiol induces a transient surge of *Lif* expression by the uterus that is essential for implantation in mice (Chen et al., 2000). As *Lif* is expressed primarily in the glandular epithelia (Kholkute et al., 2000; Passavant et al., 2000; Song et al., 1998), it would be logical to predict that lack of *Lif* is the reason for reduce fertility in uterine gland knockout animals. However, Filant and Spencer (2013) found that intra-uterine treatment with *Lif* did not rescue the decidualization response in uterine gland knockout mice, showing other glandular factors are also required for fertility.

Components of Histotroph

Histotroph is a complex mixture of ions (e.g. K^{+}, Na^{+}, Cl^{-}, PO_4^{-3}, SO_4^{-2}, Mg^{+2}, and Ca^{+2}), nutrients (e.g. glucose, pyruvate, lactate), amino acids, and hormones (e.g. progesterone, estradiol, DHEA) that provides nutrients, regulates development, and mediates maternal-fetal interactions (Hugentobler et al., 2010; Stone et al., 1986; Wales and Edirisinghe, 1989). However, many of these components remain ill-defined, both with respect to how concentrations vary during the pre-implantation period and their precise role (reviewed in Bazer et al., 2011; Burton et al., 2011).

Glucose

One critical component of histotroph is glucose (see 2.5.3 Glucose & Embryonic Development) and as such glucose is present in the uterine fluid (Bazer et al., 1979). But how the concentration changes during the pre-implantation period is unclear. In pseudopregnant mice, Wales and Edirisnghe (1989) found glucose concentrations to be significantly lower on day 1 (0.2 mM) than any other day in the study (days 2-5). Glucose concentrations increased over days 2-4 (0.87, 0.65, and 1.15 mM, respectively) but then started to decrease by day 5 (0.65 mM). It should be noted that the low concentration on day 1 may be due to the large volume of uterine fluid the investigators observed on that day (91 µl) compared to the other days in the study (2.4-6.6 µl). Examining the concentration of glucose in the fluid of the murine reproductive tract at estrus, Harris et al. (2005) found glucose concentration of the uterine fluid to be 0.61±0.06 mM, which was significantly lower than both the concentration in the oviduct (1.65±0.28 mM) and the plasma (11.71±0.76 mM). In women, Gardner et al. (1996) found that glucose concentration in the uterine fluid was constant (3.15 mM) across the menstrual cycle. In cattle, glucose concentrations of the uterine fluid were statistically similar on days 6, 8, and 14 of the estrous

cycle (3.78. 4.01, 4.54 mM, respectively), and glucose concentrations were consistently ~2 mM less than plasma concentrations (Hugentobler et al., 2008).

In contrast, the few studies that have expressed the total amount of glucose in the uterine lumen (in milligram or millimoles) have shown more pronounced and consistent changes. In pigs, Zavy et al. (1982) found significant increases in the amount of recoverable glucose between days 12 to 16 post estrus in both cyclic and pregnant females; however, the magnitude of the increase was 3-fold great in the pregnant group. Similarly, in ewes the nanomoles of glucose in the uterine fluid increased between day 3 and 13 post estrus (Gao et al., 2009b), and pregnancy resulted in a much more dramatic increase. The increased amount of glucose in the uterine lumen corresponds to the rapid elongation of the embryos in both species (reviewed in Blomberg et al., 2008), and as such may represent an energy source for elongation. In support of this, there is a significant correlation between glucose concentration in the uterine fluid and conceptus length on day 13 in cattle (Matsuyama et al., 2012).

2.5. Roles of Glucose in Early Pregnancy

2.5.1. Multiple Pathways of Glucose Metabolism

Discussions of glucose metabolism typically focus on production of ATP. However, glucose can be metabolized by numerous pathways that can have very important effects, particularly in embryonic/uterine physiology (Leese, 2012). After entering a cell, most glucose is phosphorylated by hexokinase producing glucose-6-phosphate. This step is significant in that it traps the glucose inside the cell and allows more glucose to diffuse into the cell. Glucose-6-phosphate can be converted to 6-phosphogluconolactone which is part of the pentose phosphate pathway (PPP). The PPP produces ribulose-5-phosphate, a precursor for nucleic acid synthesis, and NAPDH, which can be used in lipid synthesis (Stanton, 2012). Glucose-6-phosphate can also be converted to fructose-6-phosphate which can be used to glycosylate proteins via the

hexosamine biosynthetic pathway (HBP), an important step in regulating protein function (reviewed in Wells et al., 2003). Or fructose-6-phosphate can be used in glycolysis, producing pyruvate and 2 ATP molecules (net). If the pyruvate is subsequently fed into the TCA cycle and oxidative phosphorylation, an additional 34 ATPs are produced (Voet et al., 2001a). Glucose can also activate transcription factors, such as carbohydrate response element binding protein (ChREBP), which functions as a transcription factor. Interestingly, ChREBP has been shown to bind to ~1,110 sites in the human genome and regulate genes involved in both carbohydrate metabolism and embryonic development (Jeong et al., 2011). Finally, glucose molecules can be stored as part of glycogen, with a single glycogen molecular storing up to 55,000 glucose moieties (Roach et al., 2012).

2.5.2. Glucose Metabolism by the Uterus

Myometrial Metabolism

The myometrium consists of two layers of smooth muscle (circular and longitudinal) located between the endometrium and serosa. Myometrial contractions facilitate sperm transport, embryo spacing, and parturition, thus playing an important role in reproduction (reviewed in Aguilar and Mitchell, 2010). Glucose seems to be the preferred energy substrate for the myometrium during times of contraction such as during implantation and parturition (Steingrímsdóttir et al., 1995; Yochim and Mitchell, 1968a). Yochim and Saldarini (1969) treated rats with U-C^{14}-glucose and found glucose uptake by the myometrium peaked during diestrus in the non-pregnant rat and then peaked again on days 3-4 of pseudo-pregnancy. Steingrímsdóttir et al. (1995) compared the concentrations of various metabolites in the human myometrium at term to rectus abdominis muscle. They found higher concentrations of glucose and a higher lactate/pyruvate ratio in the myometrium, but lower concentrations of triglyceride metabolites, suggesting that the myometrium was preferentially metabolizing glucose compared

to skeletal muscle. Culturing myometrial samples collected at different stages of pregnancy, Smith (1972) found that glucose uptake of the rabbit myometrium increased dramatically at the end of gestation (between day 26 and 30). In rats, Vasilenko et al. (1981) found a 5-fold increase in glycogen concentrations between day 22 and parturition, and notably this increase was more prominent in the myometrium than endometrium. Chew and Rinard (1979) found myometrial glycogen concentration in rats to be 8 times higher at the end of pregnancy than at proestrus. It has been suggested that myometrial glycogen stores serve as an energy reservoir during parturition, when uterine contractions compress arteries, reducing blood flow and avabilablity of glucose and oxygen from maternal circulation (Wray et al., 2001).

Endometrial Metabolism

In non-pregnant rats, endometrial uptake of glucose peaked during diestrus, and in pseudopregnancy, glucose uptake is elevated on days 1-3 (Saldarini and Yochim, 1968). It is important to note, that in that study rats were pseudopregnant and thus the uterus did not decidualize, because decidualization may further increase glucose uptake. *In vitro*, glucose transport into uterine stroma increases during decidualization due to the actions of progesterone (Frolova et al., 2009), and this glucose is required for proper decidualization of the human stroma (von Wolff et al., 2003). While some of this glucose is used to generate ATP, the PPP is also involved in decidualization. First activity of glucose-6-phosphate dehydrogenase (1st enzyme unique to PPP) is increased at the implantation site but not at inter-implantation sites (Moulton, 1974). Progesterone increased this effect and estrogen potentiated the effect of progesterone. Frolova et al. (2011) examined the decidualization of endometrial stromal cells (ESCs) while inhibiting the PPP using dehydropiandrosterone (DHEA), a known inhibitor of glucose-6-phosphate dehydrogenase (Köhler et al., 1970; Raineri and Levy, 1970); 6-aminonicotinadmide (6AN), a more specific G6PDH inhibitor; and short, hairpin RNA (shRNA)

targeted to glucose-6-phosphate dehydrogenase. All three methods resulted in decreased expression of prolactin and insulin-like growth factor binding protein 1 (Igfbp1), markers of decidualization. In order to confirm these observations *in vivo*, the effects of DHEA were examined in ovariectomized mice hormonally primed and subjected to a decidualizing stimulus (intrauterine oil infusion). They found that uteri from DHEA treated-mice were ⅓ the mass of control mice after decidualization. In addition, the uteri of DHEA treated mice had significantly lower expression of bone morphogenetic protein 2 (Bmp2), another marker of decidualization. Histological analysis clearly showed a lack of endometrial thickening in DHEA treatment mice, while such thickening was easily observable in control mice. These results clearly show that flux through the PPP is crucial for decidualization, a process that is itself crucial for implantation and pregnancy.

2.5.3. Glucose & Embryonic Development

Embryos have a complex relationship with glucose. They absolutely depend on glucose for survival; however, the amount of glucose required varies with the stage of development. Additionally, high concentrations of glucose are toxic to embryos, showing that the amount of glucose entering the uterine lumen must be precisely controlled (Jovanovic et al 2005; Leese, 2012).

Cleavage Stage Embryos

Pre-ovulatory oocytes metabolize very limited amounts of glucose directly. Instead much of the energy utilized by oocytes comes from carbohydrate metabolism of cumulus cells (specialized granulosa cells that surround the oocyte) which then transfer pyruvate and lactate directly to the oocyte (Downs and Utecht, 1999; Wang et al., 2012). This inability to metabolize glucose persists after fertilization. Glucose uptake has been shown to be a fraction of pyruvate uptake in multiple species (Gardner et al., 1993; Martin and Leese, 1995). Houghton et al. (1996) found

that cleavage stage embryos consumed no glucose. The lack of glucose metabolism in cleavage stage embryos is thought to be due to a lack of 6-phosphofructokinase (Barbehenn et al., 1974). In support of this, pyruvate and lactate are able to support 94% of zygotes reaching the morula stage and addition of glucose has no significant effect (Brown and Whittingham, 1991). While embryos can become morulas without glucose, they degenerate at this stage in its absence. After blastocoel formation, glucose consumption increases rather dramatically. Houghton et al. (1996) found that early blastocysts consumed glucose at 7.23 pmol/embryo•hour, day 6.5 embryos consumed glucose at 907.76 pmol/embryo•hour and day 7.5 embryos consumed glucose at 3124.61 pmol/embryo•hour. Clearly, glucose usage by embryos increases dramatically as implantation approaches.

Interestingly, glucose is not needed during cavitation (though it is used if available), but prior to it. Martin and Leese (1995) observed that when embryos were collected as 2-cells 64% reached the blastocyst stage without glucose, but when zygotes were collected and cultured without glucose none of the embryos became blastocysts. Chatot et al. (1994) found that just 1 minute of exposure to glucose (27 mM) at the 4-cell stage resulted in 75% of these embryos becoming blastocysts. Continuous exposure to glucose concentrations as low as 0.2 mM also supports development to the blastocysts stage as well as higher concentrations (1-2 mM; Cagnone et al., 2012). These results suggest that glucose is required after the first cleavage event and before cavitation, when metabolism via glycolysis is minimal to non-existent (Figure 2).

There seem to be two possibilities as to why embryos need glucose during cleavage stage development. The pentose phosphate pathway (PPP) and hexoseamine biosynthetic pathway (HBP) have both been shown to occur in pre-implantation embryos. O'Fallon and Wright (1986) found that PPP metabolism peaked at the 2-cell stage and compaction, which coincide with when

mouse embryos need to be exposed to glucose and when they switch to glucose as an energy source. However, the PPP has never been experimentally examined during embryonic development. Investigating the role of the HBP, Pantaleon et al. (2008) found glucosamine could substitute for glucose in supporting development to the blastocyst stage. Since glucosamine cannot enter glycolysis but can be metabolized in the HBP, this suggests that flux through the HBP is a pre-requisite for blastocoel formation. In agreement, inhibition of glutamine-fructose-6-phosphate amidotransferase (GFPT, enzyme in HBP) partially reversed the toxic effects of high glucose concentrations and restored normal expression of solute carrier family 2a3 (Slc2a3; also known as GLUT3; Pantaleon et al., 2008), a glucose transporter localized to the trophoblasts of embryos and implicated in uptake of glucose from the uterine fluid (Pantaleon et al., 1997; Schmidt et al., 2009).

It is also possible that glucose is acting as a signaling molecule in these embryos. Glucose exposure has been linked to peroxisome proliferation-activated receptors (PPAR) expression and Slc16 transport localization, and glucose has also been shown to simulate prostaglandin production by embryonic stem cells (Kim and Han, 2008). Using microarray methodology, Cagnone et al. (2012) analyzed gene expressed in bovine blastocysts produced by in vitro fertilization and cultured in 0.2 mM or 5.0 mM glucose. They found 490 transcripts whose expression was different between treatments, with 63 genes have >1.5 fold change in expression. Carbohydrate response element binding protein (ChREBP) is a glucose activated transcription factor. Upon activation by glucose, ChREBP binds DNA sequences and acts as an activator or inhibitor of transcription. Examining the genes controlled by ChREBP Jeong et al. (2011) found that this transcription factor bound to 1153 sites, potentially regulating over 600 genes, many related to metabolism such as glucose-6-phosphatase. Interestingly, one of the functional

annotation clusters (a group of genes involved in a particular biological process) most regulated by ChREBP is known to be important in embryonic development. Iizuka et al. (2004) found that mating ChREBP$^{+/-}$ mice resulted in +/+, +/-, and -/- offspring in the expected 1:2:1 ratio, indicating no prenatal loss. But since then multiple ChREBP isoforms have been identified (Herman et al., 2012) so that the role of ChREBP in embryonic development remains enigmatic.

Blastocysts & Glucose

Assuming prior exposure to glucose, blastocysts begin to preferentially take up and metabolize glucose (Figure 2; Leese and Barton, 1984; Martin and Leese, 1995). Chi et al. (2002) measured multiple glycolytic and TCA metabolites produced by embryos from 2-cells to blastocysts and found that glycolysis increased during development while flux through the TCA cycle seemed to decrease. Leese and Barton (1984) found that glucose uptake increased 6-fold during cavitation, and Gardner et al. (1993) found that glucose uptake by sheep embryos significantly was significantly increased at every subsequent stage examined after the 8-cell stage. In human embryos, Jones et al. (2001) found higher glucose uptake in blastocysts compared to morulas.

At the same time, oxygen utilization is also significantly increased in blastocysts compared to earlier stages (Houghton et al., 1996), suggesting that the additional glucose is being metabolized through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. In support, Thompson et al. (1996) found a positive correlation between glucose uptake and oxygen uptake of bovine embryos. However, the increased rates of oxygen uptake and oxidative phosphorylation shown in culture studies may be the result of culture conditions and may not be physiologically relevant. Lane and Gardener (1998) found that immediately after flushing, embryos metabolized 28% of glucose to lactate but that percentage increased to 76% at 3 hours and 90% at 6 hours. Flushing of additional embryos at 3 and 6 hours (to serve as controls)

revealed that *in vivo* embryos maintained low levels of glycolysis, so that the increased rate of glycolysis in the cultured embryos was due to culture not development. Additionally, activation of blastocysts in diapause further increases glucose utulization. Spindler et al. (1995) found that tammar wallaby blastocysts used significantly more glucose 8 days after removal of nursing young (with activates embryos). And in mice, blastocysts were taking up more glucose than non-activated blastocysts 16 hours after estradiol treatment, (Spindler et al. 1996).

Uterine Hypoxia

Many investigators have cited hypoxia in the uterine lumen as the reason for the dependence of blastocysts on glycolysis (Leese, 2012). For example, Fischer and Bavister (1993) found the partial pressure of oxygen (pO_2) in the oviduct (35±5 mm Hg) of the rhesus monkey was significantly lower during the mid-follicular phase than post-ovulatory or mid-luteal phases (57±5 and 61±5 mm Hg, respectively). In contrast, pO_2 in the uterine lumen was consistently low, averaging 13±2 mm Hg among all three time points, well below the pO_2 of venous blood (~40 mm Hg). In agreement, oxygen tension the uterus of women undergoing intrauterine insemination, averaged 11.8% (Ottosen et al., 2006), which would translate to approximately 18.8 mm Hg. In contrast, the situation in other species is less clear. The pO_2 in the uterine lumen of rats varies during pregnancy and pseudopregnancy (Yochim and Mitchell, 1968b). Specifically, in pseudopregnant rats, pO_2 was lowest at estrus (22.4 mm Hg) then increased over three days (peaking at 38 mg Hg). Oxygen tension then decreased transiently on day 4 (predicted day of implantation) to 33.8 mm Hg. After day 4, oxygen tension increased to a maximal value of 58.7 mm Hg on day 7. A similar pattern was observed in hamsters, with pO_2 relatively low at estrus (approximately 35 mm Hg), increased over three days, but decreased again on day 4 (Fischer and Bavister, 1993). In rabbits pO_2 was increased on day 1 of pseudopregnancy compared to day 0 but then low again on day 6, the day before implantation

(Fischer and Bavister, 1993). These results indicate that intra-uterine pO_2 varies across species, but a common theme in eutherian mammals is a relatively hypoxic lumen at the time of implantation.

Culture studies suggest that uterine hypoxia might enhance embryonic development. Brison and Leese (1994) found that rat embryos could develop into blastocysts in the presence of inhibitors of oxidative phosphorylation (cyanide, antimycin-A, 2,4-dinitrophenol), and other studies have found increased rates of blastocyst production with transient inhibition of oxidative phosphorylation (Macháty et al., 2001; Thompson et al., 2000). Thompson et al. (1990) cultured bovine and ovine embryos from the 2-cell stage to the morula stage under various O_2 concentrations. Embryos of both species responded to increasing O_2 concentrations in a quadratic manner. Specifically, the percentage of embryos reaching the morula stage increased as O_2 concentration increased, up to 6-8%. At higher O_2 concentrations, the percentage of embryos becoming morula began to decrease. Fujitani et al. (1997) found that culturing bovine embryos at 5% O_2 increased the percentage of embryos reaching the blastocyst stage, expanded blastocyst stage, and hatched blastocyst stage compared to 20% O_2. Interestingly, addition of a free radical generator (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride) decreased the percentage of embryos becoming blastocysts when culture under 5% O_2 but had no effect under 20% O_2. Conversely, addition of superoxide dismutase (converts O_2 to oxygenase and hydrogen peroxide) or hypotaurine (antioxidant) to the media increased the percentage of embryos developing into blastocysts under 20% O_2 but had no effect on embryos under 5% O_2. These results strongly suggest the inhibitory effect of high O_2 concentrations is due to formation of free radicals, and that a hypoxic uterine lumen may be important for reducing embryonic loss.

Warburg Metabolism

Similar to embryos within the uterine lumen, tumors are often hypoxic. Otto Warburg (1956) first proposed that this low pO_2 in tumors reduced flux through the TCA cycle and oxidative phosphorylation. Instead tumors convert the pyruvate resulting from glycoloysis to lactate (i.e. fermentation). This concept, known as Warburg metabolism, is well established today and has become a hallmark of tumor metabolism (reviewed in Vander Heiden et al., 2011). In embryos Manes & Lai (1995) found that 30% of oxygen used by rabbit blastocysts was for non-mitochondrial processes (i.e. not oxidative phosphorylation), and Trimarchi et al. (2000) found that 70% of oxygen was used non-mitochondrially in mouse embryos. It has been shown that 98% of the glucose taken up by cleavage-stage sheep embryos can be accounted for as lactate, thus it is not entering the TCA cycle (Gardner et al., 1993).

These observations have led current investigators to suggest that embryos also rely on Warburg metabolism for ATP generation (Burton et al., 2011; Krisher and Prather, 2012). In support of this, Redel et al. (2012) found that pre-implantation pig embryos expressed both pyruvate kinase M2 (Pkm2) and hexokinase 2 (Hk2), paralogue of glycolytic enzymes associated with Warburg metabolism. In addition, they found that culturing embryos under low O_2 concentrations (5%) increased embryo size and enhanced expression of transaldolase DO1 (TalDO1, an enzyme in the pentose phosphate pathway) and pyruvate dehydrogenase kinase 1 (Pdk1, which inhibits pyruvate dehydrogenase). Together these changes would increase Warburg metabolism in response to decreased oxygen tension. However, low oxygen concentrations is not a prerequisite for Warburg metabolism (review in Vaupel et al., 1989), and in embryos, estradiol may increase the extent of Warburg metabolism. Hamatani et al. (2004) used microarray analysis to show that

estradiol treatment increased expression of glycolytic enzymes, including lactate dehydrogenase (which would result in conversion of pyruvate to lactate).

Collectively, the scientific literature is clear that embryos absolutely need glucose and blastocysts need large and increasing amounts of glucose as implantation approaches. Thus it is critical that the uterus can provide the necessary glucose to the embryos. This means that uterine uptake of glucose from maternal circulation and/or catabolism of uterine glycogen reserves are critical for embryonic survival and development.

Glucose Toxicity

Somewhat paradoxically, high glucose concentrations are also detrimental to pre-blastocyst development. Many studies have found diabetic mothers, even when the diabetes is well controlled, experience higher frequencies of pregnancy loss and fetal malformations than non-diabetic mothers (Buchanan and Kitzmiller, 1994; Casson et al., 1997; Glasgow et al., 1979; Pearson et al., 2006). Jovanovic et al. (2005) found that even non-diabetic women with higher concentrations of fructosamine (indicator plasma glucose concentrations over time) experienced a higher rate of pregnancy loss than women with average fructosamine levels, suggesting that diabetes is not requisite for glucose-mediated embryo-toxicity. And Wiebold (1988) found that cows with morphological abnormal embryos on day 7 also had 2-fold higher glucose concentrations in the uterine fluid than cows with normal embryos.

Numerous studies using pathologic concentrations of glucose (25 mM) have shown ill effects on embryonic development (Barnett et al., 1997; Bermejo-Alvarez et al., 2012; Pampfer et al., 1997). For example, excess glucose has been shown to cause morphological abnormalities (Ellington, 1997), alter protein expression (Ellington, 1997; Moley et al., 1998a), inappropriate paracrine signaling (Leunda-Casi et al., 2001; Wentzel et al., 1999), and increased apoptosis

(Moley et al., 1998b; Pampfer et al., 1997). However, the high glucose concentrations in these studies make it hard to apply these finding to physiological conditions; thus, other studies have tried to determine at what concentrations glucose starts to become toxic. Fraser et al. (2007) cultured embryos at various glucose concentrations from the 2-cell stage and analyzed their development. The investigators found that 15.56 mM of glucose significantly reduced the percentage of embryos expanding and hatching compared to lower concentrations (5.56 and 0.2 mM). However, even 5.56 mM of glucose significantly reduced the number of cells per embryo compared to 0.2 mM of glucose. In agreement, Cagnone et al. (2012) cultured bovine embryos in glucose concentrations ranging from 0.2-10 mM and found that blastocyst viability (determined by cell number per embryo and percentage of embryos becoming blastocysts) began to decrease at 5 mM. Five millimolarglucose also altered expression of genes related to extracellular matrix signaling, calcium signaling, and energy metabolism. Another study with bovine embryos (Kimura et al., 2005) found that 4 mM glucose resulted in significantly fewer embryos reaching the blastocyst stage compared to 1 or 2.5 mM. Interestingly, in that study female embryos seemed to be more susceptible to glucose-toxicity as 4 mM skewed the ratio of surviving embryos toward males. Belkacemi et al. (2005) found that the invasiveness of immortalized trophoblast cells was less when cells were cultured in 5 or 10 mM of glucose compared to lower concentrations, and this reduction in invasiveness was paralleled by reductions in plasminogen activator, active urokinase type PA, and Slc2a1.

These studies suggest that glucose-toxicity is not isolated to diabetic mothers, as even normal plasma glucose concentrations are embryo-toxic. Therefore, simply allowing glucose to diffuse from maternal circulation to the interior of the oviduct/uterine lumen is not sufficient. The uterus

needs to tightly control the amount of glucose reaching the embryos, enough to meet their needs but without reaching toxic concentrations.

Mechanisms of Glucose Toxicity

Comparison of bovine embryos cultured with 5.56 mM of glucose or fructose revealed that glucose, but not fructose, was toxic (Kimura et al. 2005). Frustose can enter the glycoylic pathway, but cannot be metabolized via the hexosamine biosynthetic pathway (HBP; Voet et al. 2001a), indicating that the HBP may be responsible for glucose toxicicity in embryos. Pantaleon et al. (2010) explored this possibility in a series of experiments with mouse embryos. The investigators found that 27 mM of glucose significantly reduced blastocyst formation compared to control (0.2 mM glucose). However, 27 mM of surcrose had no effect on development, showing that the effect of 27 mM glucose was not due to increases osmolarity of the culture media. Evidencing that increased metabolism via the HBP pathway was responsible for the decreased development; addition of benzyl-2-acetamido-2-deoxy-α-D-glcopyranosylidene (BADGP; HBP inhibitor) complete blocked the toxic effects of 27 mM glucose.

2.6. Glucose Transporters in Early Pregnancy

As evidenced above, glucose availability to the uterus and embryos is critical for reproductive success. Glucose can be transported into cells by two distinct classes of transporters. The first class, solute carrier family 2a (Slc2a, also known as GLUTs), transports glucose down its concentration gradient (either into or out of cells, depending on conditions) by facilitated diffusion. The Slc2a family currently contains 14 members, divided into three subclasses (Augustin, 2010). However, only a subset of the Slc2a family members has been found in reproductive tissues. The second class of glucose transporters is Slc5a family members. These glucose transporters are symporters, using the potential energy in the Na^+ concentration gradient

across the cell membrane to transport glucose into cells; this allows Slc5a family members to concentrate glucose inside cells (Wood and Trayhurn, 2003).

2.6.1. Glucose Transporters in the Uterus

Solute Transporter Family 2a (Slc2a)

The uterus has been shown to express multiple Slc2a isoforms, such as Slc2a1, 3, 4, 6, 8, 9, 10, and 12 (reviewed in Frolova and Moley, 2011). But of these, Slc2a1 and Slc2a3 have received most of the attention in the scientific literature. Using immunohistochemistry, Korgun et al. (2001) showed that Slc2a1 was undetectable in the lumenal epithelium on day 1 and 2 of pregnancy of rats. Expression then increased, peaking on day 4. Expression in the stroma was very similar to the lumenal epithelium, undetectable on day 1 and 2, moderate on day 3, and peaking on days 4-5. However, Slc2a1 was never observable in glandular epithelial. Kim and Moley (2009) used immunohistochemistry to localize Slc2a1 in the non-pregnant uterus to the uterine epithelium, particularly to the basolateral surface. This would place Slc2a1 in a position to take up glucose from interstitial fluid before being secreted into the uterine lumen. In the human uterus, Slc2a1 expression in the uterine glands was consistent through the follicular phase, luteal phase, and early pregnancy (weeks 6-9). However, in the stroma Slc2a1 expression was significantly increased during the luteal phase compared to the follicular phase and significantly higher in early pregnancy than the luteal phase (von Wolff et al., 2003)

In the rat uterus, Slc2a3 was moderately expressed in the lumenal epithelium on days 1 of pregnancy. Expression was increased on days 3-4 and then dropped, being undetectable by day 7 (Korgun et al., 2001). In the stroma, Slc2a3 expression was modestly expressed throughout the first 8 days of pregnancy, with no observable changes on days 1-5. In this study, Slc2a3 was never detectable in the glandular epithelium. In women, Slc2a3 expression was consistent and

did not significantly change between the follicular phase, the luteal phase, and early pregnancy (weeks 6-9) in both the glands and stroma (von Wolff et al., 2003).

Uterine expression of Slc2a8 is also starting to receive some research attention. Discovered in 2000, the physiological role of Slc2a8 is still unclear (Doege et al., 2000; Ibberson et al., 2000). Slc2a8 has high affinity for glucose (2mM) but also transports fructose and galactose, leaving its substrate ambiguous. In the cyclic rat uterus Kim and Moley (2009) found Slc2a8 protein expression to change during the reproductive cycle, peaking during estrus. Immunohistochemistry revealed Slc2a8 to be localized to the apical surface of uterine epithelial cells, where it might facilitate glucose secretion into the uterine lumen. Supporting a role for Slc2a8 in pregnancy, $Slc2a8^{-/-}$ mice had reduced litter sizes compared to wildtype females (Adastra et al., 2012). Slc2a8 knockout also impaired decidualization, results in reduced uterine weight and decreased expression of decidualization markers.

Solute Transporter family 5a (Slc5a)

There has been much less research on uterine expression of Slc5a family members, though recent studies have begun to address this issue. Forde et al. (2010) found that Slc5a1 mRNA expression increased approximately 6-fold between day 7 and 13 of the cycle and of pregnancy in the bovine endometrium (implantation day 21). *In situ* hybridization on days 13 and 16 found the gene to be expressed exclusively in the lumenal and glandular epithelium. Similarly, in the cyclic, ovine endometrium Slc5a1 gene expression increased between days 10-14 and then declined (Gao et al., 2009a). Pregnancy resulted in a sharper increase between days 10-12 and expression then remained high through day 18 (implantation day 16). Slc5a11 also had a quadratic expression patter, being modestly higher at day 14 than 12 or 16 in cycle ewes. For this gene, pregnancy resulted in a linear increase in expression from days 10-14 and then

plateaued until day 18. Both of these transporters (mRNA and protein) were primarily isolated to the uterine epithelia, though light staining was observed in conceptus tissue. These results show that Slc5a transporters are well placed to facilitate glucose transport into uterine epithelial cells, at least in ruminants.

Differences between species and laboratories combined with the ability of Slc2a family members to transport glucose into and out of cells make it difficult to reach specific conclusions based on the avaialbe literature. However, the general finding that endometrial glucose transporter expression increases as implantation approachs seems to indicate that more glucose is entering the uterine lumen as implantation approachs. This agrees with studies in livestock which show that the total amount of glucose present in the lumen (i.e. not expressed as a concentration) increases late in the pre-implantatino period (Zavy et al. 1982; Gao et al. 2009b). This conclusion also agrees with a study in rats which found glucose transport into the uterine lumen of rats to be 18.3±28 nmol/hour during proestrus and 61.8±13.7, 48.0±16.8 nmol/hour on days 4 and 5, respectively, of pregnancy (Leese and Gary, 1995; Leese et al. 2008).

2.6.2. Glucose Transporters in the Embryo

Slc2a Family Members

Slc2a1 seems to be ubiquitously expressed in pre-implantation embryos. Pantaleon et al. (2001, 1997) found Slc2a1 protein to be expressed throughout morulas and blastocysts. However, knockdown of expression with anti-sense probes did not affect up-take of 3-O-methyl-D-glucose or the percentage of 2-cell embryos reaching the blastocyst stage (Pantaleon et al., 1997). Interestingly culture media without glucose resulted in increased Slc2a1 expression (Pantaleon et al., 2001). Pantaleon et al. (1997) found that Slc2a3 mRNA expression was not detectable until the 4-cell stage. Immunohistochemistry confirmed that Slc2a3 was not expressed until the 4-cell stage, though the transporter seems to be cytosolicly located at that time. By the morula stage,

Slc2a3 expression was localized to the apical surface of the outer cells, and at the blastocyst stage, Slc2a3 was restricted to the apical surface of the trophoblasts. Knockout down Slc2a3 expression with short-hairpin RNA resulted in significantly less uptake of 3-O-methyl-D-glucose in morulas and blastocysts and fewer embryos survived to the blastocyst stage. In order to access the importance of Slc2a3 in development, Schmidt et al. (2009) produced knockout embryos by crossing $Slc2a3^{+/-}$ adult mice. Similar to Pantaleon (1997), Schmidt et al. (2009) also found Slc2a3 was found to be localized to the apical surface of the wildtype embryos. However, in this study no developmental abnormalities or embryo loss was observed in the $Slc2a3^{-/-}$ pre-implantation embryos. Post-implantation (day 6.5 post-coitum) microscopical abnormalities began to appear and no $Slc2a^{-/-}$ embryos survived until term. Both of these studies show Slc2a3 is localized to the apical surface of trophoblasts, making it ideally locally located for the uptake of glucose from the uterine fluid. However, the absolute importance of this transporter in pre-implantation development is still uncertain.

Insulin stimulates glucose uptake by the blastocyst through activation of both the insulin receptor and the insulin-like growth factor 1 (Igf1) receptor. However, cleavage-stage embryos do not produce insulin, while Igf1 is produced from the 2-cell stage onward (Doherty et al., 1994) making Igf1 a likely ligand for these receptors. It is well established that activation of insulin receptors mediated increased glucose uptake in adult tissue by translocation of Slc2a4 to the plasma membrane. However, it is unclear if pre-implantation embryos express Slc2a4. Tonack et al. (2004) detected Slc2a4 mRNA in mice blastocysts and Santos et al. (2000) detected Slc2a4 in bovine blastocysts using conventional PCR and in situ hybridization. In contrast, Hogan et al. (1991) could not detect Slc2a4 mRNA at any stage of pre-implantation mouse embryos using conventional PCR. Nor could Tonack et al. (2009) could not detect Slc2a4 mRNA in 8-cell,

morula, or blastocysts from hamsters with PCR. The supposed lack of Slc4a in cleavage-stage embryos led some investigators to speculate about the presence of a different Igf/insulin-stimulated Slc2a family member being present in blastocysts. Slc2a8 expression was found to be higher in the placenta than another other tissue from either the mother or fetus, suggesting that Slc2a8 may have a unique role during pregnancy (Carayannopoulos et al., 2000). The same study found that insulin treatment resulted in Slc2a8 protein being translocated from the cytosol to the plasma membrane, and functional knockdown of Slc2a8 expression with anti-sense RNA resulted in inhibited insulin-stimulated glucose uptake (knockdown of Slc2a2 or Slc2a3 did not affect insulin-stimulated glucose uptake), linking Slc2a8 to insulin-stimulated uptake.

Unfortunately, the Slc2a family has never been explored in the pre-implantation mink embryo. However, Wooding et al. (2007) did probe for Slc2a1 and Slc2a3 in the placenta of cats, dogs, and mink. They found only Slc2a, but it was present in the placenta of every species studied. Slc2a1 was on both the basal and apical surfaces of the trophoblasts, making it suitable to take up maternal glucose and transport it to fetal circulation, and evidencing that the carnivore placenta can expresses Slc2a3. Currently the role of Slc2a family members earlier in development is unknown.

Slc5a Family Members

The first evidence of Slc5a family members in pre-implantation embryos came from Wiley et al. (1991) who found the same Na^+/glucose symporter in mouse embryos as had been previously identified in kidneys. Immunofluorescence revealed the protein to be expressed starting at the 4-cell stage, peaking at the morula, and being lower in the blastocysts. Interestingly, by the 16-cell stage, expression was restricted to the apical surface of the outer cells. Further supporting a role for active glucose transport in pre-implantation embryos, Chi et al. (1993) found that both human

and mouse embryos (morulas or blastocysts) were able to concentrate 2-deoxyglucose (2DG) to concentrations 30-fold higher than in the media. However, phlorizin (Slc5a inhibitor) did not prevent the embryos from concentrating glucose, leading the authors to speculate that the active transporter was localized to the inner plasma membrane. Though, a different study (Leppens-Luisier et al., 2001) found that phlorizin did decrease the accumulation of glucose inside embryos, but this effect was transient (lasting <12 min).

2.7. Glycogen Metabolism

The uterus cannot carry out gluconeogenesis (Yánez et al., 2003; Zimmer and Magnuson, 1990), meaning that glucose uptake and/or glycogen catabolism must increase during times of high demand. Glycogen is a macromolecule composed of short chains of glucose residues joined α (1-4) linkages, with individual chains joined to each other via α (1-6) linkages, yielding a highly branched structure (Figure 3). A single, mature glycogen molecule can contain up to 55,000 glucose units (Roach et al., 2012).

Formation of New Glycogen Molecules

Cori (1939) postulated that a carbohydrate precursor was necessary to for new glycogen molecules. However, later studies revealed this role to be carried out by a protein, now termed glycogenin (Pitcher et al., 1987; Rodriguez and Whelan, 1985). Upon dimerization, glycogenin auto-glycosylates, adding 7-10 glucose residues (Lomako et al., 1990). After auto-glycosylation, glycogenin serves as a substrate from glycogen synthase and glycogen branching enzyme, resulting in growth of the glycogen molecules. Studying the role of glycogenin during glycogen re-synthesis in human skeletal muscle, Shearer et al. (2005) found that glycogenin mRNA expression was significantly increased at 120 and 300 minutes post-exercise. However, glycogenin activity and glycogen accumulation was already increased by 30 min post-exercise. Furthermore, only a small amount of apo-glycogenin (ie glycogenin without any glucose

residues attached) was found, further supporting that few glycogen molecules are completly catabolized during intense exercise. These studies indicate that glycogenin is not limiting for glycogen re-synthesis in skeletal muscle after exercise, though it is important to note that glycogenin has not been investigated in the uterus.

Macroglycogen and Proglycogen

As glucose resides are added, the glycogen molecule grows in diameter, first known as proglycogen and then the mature, larger form known as macroglycogen (Lomako et al., 1993). Proglycogen is characterized by being less than 400 kDa in size and still being associated with the glycogenin protein. Macroglycogen is larger than in diameter and the carbohydrate portion has disassociated from the protein. The physiological implications of these two forms is still under debate (James et al., 2008; Katz, 2006), but experimentally this division may be important because some investigators believe the presence of glycogenin makes the carbohydrate portion resistant to acid hydrolysis (Lomako et al., 1991), a technique commonly used to break glycogen down to glucose. However, a more recent study found that if tissue was homogenized before acid hydrolysis, the acid insoluble fraction of glycogen was both very low and extremely consistent during glycogen re-synthesis (Barnes et al., 2009), making the use of hydrolysis combined with homogenization important for measuring glycogen.

Glycogen Synthesis

Glucose can enter cells through two families of glucose transporters, as previously discussed: Slc2a and Slc5a. After glucose enters a cell, it is phosphorylated at the 6C position by hexokinase (Hk), producing glucose-6-phosphate. Hexokinase exists in 4 isoforms (Hk1-3 and glucokinase (Gck) also known as Hk4). Gck expression is restricted to tissues key to systemic glucose homeostasis, such as liver and pancreas. Hk1-3 forms are much more widely expressed, including tissues which also express Gck (Agius, 2008; Voet et al., 2001a). Glucose-6-

phosphate, produced by Hk, is the last common precursor for all possible pathways of glucose. If the glucose-6-phosphate (G6P) is destined to become part of glycogen, it is converted to glucose-1-phosphate by phosphglucomutase. The glucose residue is then transferred to uridine diphosphate (UDP), producing UDP-glucose. Finally, the glucose residue is transferred to the non-reducing end of a preexisting glycogen molecule by the rate-limiting enzyme glycogen synthase (Gys) via α(1-4) linkages (Figure 4).

There are two known paralogues of Gys (1 and 2). However, Gys2 has a relatively restricted tissue distribution, while Gys1 is more widely expressed (Echigoya et al., 2011). Harris et al. (1994) found minor differences between the cDNA sequence of uterine Gys and striated muscle Gys but concluded the two genes were "similar if not identical." In agreement, our laboratory has previously shown Gys1 expression in the mink uterus (Rose et al., 2011).

The activity of Gys is controlled in two major ways. First, Gys can be phosphorylated by various kinases (e.g. cAMP-dependent protein kinase). The phosphorylation of Gys is complex, occurring at 9 different serine residues. Overall, phosphorylation either has little effect on or decreases activity of the enzyme, depending on which residue is phosphorylated. Of the 9 potential phosphorylation sites, Serine$^{640/641}$ is believed to be the major inhibitor of activity, making it a frequent target in studies of glycogen metabolism (Lawrence and Roach, 1997; Voet et al. 2001b). The activity of Gys is also regulated allosterically by various substrates and products in glucose metabolism. Of particular importance, G6P allosterically activates phospho-Gys, meaning the the "inactive" form of glycogen synthase can still be activated when intracellular G6P concentrations increase. Exogenous G6P is frequently used in Gys activity assays to activate phospho-Gys, thus reflect activity of total Gys (phosphorylated and

unphosphorylated). The contributions of these two mechanisms (phosphorylation and allosteric regulation) are currently under debate (Bouskila et al. 2010).

Cytosolic Catabolism of Glycogen

Glycogen breakdown (glycogenolysis) can occur via two distinct mechanisms: within the cytosol by glycogen phosphorylase or in the lysosome by acidic α-glucosidase (Gaa). In the cytosol, glycogen phosphorylase (Pyg), first discovered by Cori (1939), liberates glucose residues joined to glycogen via α(1-4) bonds as glucose-1-phosphate. There are currently three known isoforms of Pyg (Johnson, 1992), named for their primary site of expression (liver, muscle, brain termed Pygl, Pygm, Pygb, respectively). However, Pygm has been shown to be expressed in multiple tissue besides muscle (Nam et al., 2011), including the uterus (Rose et al., 2011). After liberation from glycogen, glucose-1-phosphate is isomerized to glucose-6-phosphate (a reversible reaction) by phosphoglucoisomerase. However, Pyg cannot liberate glucose residues within 4-5 residues of a branch point. In order for these residues to be liberated, glycogen debranching enzyme transfers 3-4 of these resides to the reducing end of another branch. The glucose reside at the branch point (joined via an α1-6 bond) is liberated directly as glucose, and the highly branched structure of glycogen means that approximately 10% of the glucose residues in glycogen will be liberated as glucose due to the actions of debranching enzyme (Voet et al., 2001b).

The activity of glycogen phosphorylase (Pyg) is controlled by three mechanisms: phosphorylation, allosterically, and via calmodulin. Control by phosphorylation is much simpler than in case of Gys. Pyg is phosphorylated at only one site (Serine^{14}) which increases activity. The high activity of phospho-Pyg is mostly independent of intracellular AMP concentrations. In contrast, unphosphorylated Pyg has very low activity when intracellular AMP concentrations are

low. When AMP concentrations are high, Pyg activity is increased, providing additionally glucose for the cell (Voet et al. 2001b). This is frequently used in Pyg activity assays: exogenous AMP is added to the reaction to activate non-phosphorylated Pyg and reflect "total" activity (Storety 1987). Pyg can also be regulated by Ca^{+2}. Increased Ca^{+2} concentrations activate calmodulin, which binds to Pyg and increases activity (Voet et al. 2001b).

The glucose-6-phosphate resulting from glycogenolysis can be metabolized by the cell via multiple pathways (see section 2.5.1 Multiple Pathways of Glucose Metabolism). However, if the glucose unit is to be secreted out of the cell, the phosphate group must first be removed. This is accomplished by the enzyme glucose-6-phosphatase catalytic subunit (G6pc). G6pc has a complex enzymology (Foster et al., 1997; Mithieux, 1997) and the currently accepted model consists of four different proteins required for G6pc activity. The active site of G6pc is inside the lumen of the endoplasmic reticulum (ER), thus glucose-6-phosphate must first be transported into the ER lumen by a glucose-6-phosphate transporter. Glucose-6-phosphate is then dephosphorylated, yielding glucose and inorganic phosphate (P_i). Each of these products is then transported out of the ER by separate transporters (reviewed in Hutton and O'Brien, 2009). Though, some investigators have suggested that glucose and P_i are transported out of the ER lumen by the same protein (Chen et al., 2008).

Currently, there are three known isoforms of G6pc (G6pc1-3). G6pc1 expression is restricted primarily to the liver and kidney, organs known to contribute to whole body glucose homeostasis. As such G6pc1 plays a major role in gluconeogenesis and glycogenolysis. G6pc1 knockout mice display hypoglycemia (Lei et al., 1996), and humans with mutations in G6pc1 display hypoglycemia, widespread alterations in metabolism, and growth retardation (Chou and Mansfield, 2008). G6pc2 is 50% identical to G6pc1 at the amino acid level, yet the ability of

G6pc2 to dephosphorylate G6P remains unclear (Arden et al., 1999; Petrolonis et al., 2004). G6pc2 expression is limited to pancreatic β-cells (Arden et al., 1999), and there is some evidence that G6pc2 plays a role in glucose stimulated insulin secretion (Pound et al., 2013; Sanda et al., 2012). However, this remains controversial (Oeser et al., 2011; Shieh et al., 2005). The last isoform, G6pc3, is 36% identical to G6pc1 and is the most ubiquitously expressed isoform of G6pc (Martin et al., 2002); Boustead et al. (2004) identified G6pc3 in every tissue they examined, including the uterus.

Lysosomal Breakdown of Glycogen

Glycogen molecules can also be broken down inside lysosomes by the enzyme α-acidic glucosidase (Gaa). While much less studied that cytosolic catabolism, this method of glycogenolysis is believed to be important in maintaining plasma glucose concentrations immediately after birth (Devos and Hers, 1980; Kalamidas and Kotoulas, 2000). Though little is known about the role of glycogen autophagy in adult animals, the continued occurrence of lysosomal breakdown of glycogen is evidenced by the buildup of glycogen in lysosomes in the skeletal and cardiac muscle of individuals suffering Pompe's disease (glycogen storage disease type II; Hout et al., 2003; Raben et al., 2007).

2.8. Uterine Glycogen

In order to provide glucose to uterine tissues and the developing embryos, the uterus stores glycogen due to the actions of ovarian steroids. However, the way uterine glycogen concentrations change during the reproductive cycle and/or pregnancy has only been studied in a few species.

Uterine Glycogen in Rats

Uterine glycogen has been studied more intensely in rats than any other species. During the estrous cycle, uterine glycogen is highest on proestrus, remains high during estrus, and is

significantly decreased by diestrus (Greenstreet and Fotherby, 1973; Vasilenko et al., 1981). In agreement, estrogen stimulates uterine glycogen accumulation (Bitman et al., 1965; Bo et al., 1973). However, changes in uterine glycogen concentrations of mated rats differ from cyclic rats. Many studies indicate that glycogen concentrations start high on the day of mating and then decreased over the next 3-5 days, reaching basal concentrations on days 4-5, the expected time of implantation (Demers et al., 1972; Greenstreet and Fotherby, 1973; Vasilenko et al., 1981). Glycogen concentrations then increase, at least until days 7-8 (Demers et al., 1972; Greenstreet and Fotherby, 1973). The post-implantation rise in uterine glycogen concentration may be due to glycogen in the decidua. Cecil et al. (1962) found that formation of a decidua in intact mice doubled the glycogen concentration of the uterus. In ovariectomized mice progesterone treatment was required for decidualization to increase glycogen concentration. However, progesterone is required for decidualization to occur (Wetendorf and DeMayo, 2012), so it is unclear if progesterone directly increases glycogen in the decidua or merely primes the uterus, allowing decidualization to occur.

Counter intuitively, activities of both glycogen synthase and glycogen phosphorylase change in a pattern similar to uterine glycogen concentrations after mating. Both Demers et al. (1972) and Greenstreet and Fotherby (1973) found that glycogen synthase activity was high during proestrus and was significantly lower on days 2 and 3, followed by a transient increase over days 4-6. Similarly, glycogen phosphorylase activity (with and without AMP) decreased from proestrus to day 3. There was a prominent increase during days 4-5 before decreasing again on day 6 and increased again between days 6-8. Thus glycogen anabolism and catabolism appears to be increased concomitantly, at a time when glycogen concentrations are decreasing.

Uterine Glycogen in Hamsters

There have also been several studies on uterine glycogen in hamsters. Using histological techniques, Sype and Hillemann (1970) found glycogen to be in the uterine epithelium (lumenal and glandular) and in the uterine lumen at estrus, but the authors noted that much more glycogen appeared in the myometrium. A different study (Thorpe and Connors, 1975) observed minimal glycogen in the endometrium prior to implantation. However, after decidualization and implantation began glycogen started to accumulate in the anti-mesometrial decidua. Similarly, Gregoire and Richardson (1970) found that the glycogen concentration in the uterus of mated hamsters did not change during the pre-implantation period. After implantation, the glycogen concentration increased dramatically (over 4-fold on day 6 and 35-fold by day 7). In agreement with Thorpe and Connors (1975), the increase was localized to the implantation site. Inducing decidualization in pseudo pregnant hamsters revealed that uterine glycogen concentrations were equally high in these uteri as the implantation site of pregnant uteri. This indicates the massive accumulation of glycogen in the pregnant uterus is localized to the decidua around the implanting embryo

Uterine Glycogen in Sheep

In pregnant sheep, glycogen concentration of the endometrium increases modestly during the early pre-implantation period (day 0-8) and then decreases drastically during the latter half of the pre-implantation period (O'Shea and Murdoch, 1978a). Glycogen synthase activity (both with and without exogenous glucose-6-phosphate) increased from day 0-8, explaining the increased glycogen concentration during this time. Glycogen concentrations then decreased by day 15 (implantation day 16). Interestingly, after day 15 glycogen synthase activity without G6P increased to levels seen at day 0 and then remained steady throughout day 44. In contrast, total glycogen synthase plateaued from day 15 to 30 but then decreased sharply by day 44 (O'Shea

and Murdoch, 1978a). Murdoch (1970) found that ovine endometrial glycogen non-significantly increased from 48.9 mg/100 g of tissue at day 0 of pregnancy to 53.8 mg/100 g of tissue on day 8. Glycogen then decreased back to 46.5 mg/100 g of tissue on day 14 and was significantly lower on day 22. Glycogen phosphorylase (with and with AMP) increased significantly on day 8 over day 0. By day 14 activity was back to values not different from day 0, and then remained similar throughout day 44.

Based on these few studies it appears than endometrial glycogen is mobilized in the second half of the pre-implantation period of sheep. Since the ovine uterus does not decidualize (ie there is no massive proliferation of stroma), the glucose is likely to be used for elongation of the embryos. In sheep (and cattle), the embryos elongate prior to imploantation to approixately 20 cm in length (reviewed in Blomberg et al., 2008) and the mobilized glucose probably supported the energic needs of the embryos. In agreemnt with this theory, glucose concentrations and embryo lengths are significantly correctlated in cattle (Matsuyama et al. 2012).

Uterine Glycogen in Primates/Humans

The pattern of uterine glycogen concentrations during the human menstrual cycle is very distinct from other species. Specifically, Milwidsky et al. (1980) found endometrial glycogen concentrations to be lowest during the proliferative phase and then peaked in the early secretory phase. Glycogen concentrations then declined to an intermediate value in the mid- and late-secretory phase. Mimori et al. (1981) also found that endometrialglycogen concentrations were low in the proliferative phase and significantly higher in the secretory phase of the human uterus, and in primates (*Macaca arctoides*) endometrial glycogen concentrations increased during the luteal phase in a pattern very similar to plasma progesterone concentrations (Demers et al., 1973). Showing a causative role for progesterone, treatment with a synthetic progestin (lyndiol)

increased glycogen concentrations in the proliferative phase endometrium of women (Ishihara et al., 1991).

In vitro, Ishihara et al. (1991) found that progesterone treatment approximately doubled the glycogen concentration and estradiol decreased glycogen concentration by 50% of human endometrial cells. Interestingly, co-treatment with progesterone and estradiol resulted in glycogen concentrations not significantly different from treatment with estradiol alone. Further both steroids resulted in changes in glycogen synthase activity that mirrored the changes in glycogen concentration. Addition of an estrogen antagonist (LY 156758) inhibited the actions of estradiol. These results show that progesterone stimulated the accumulation of endometrial glycogen in human and mobilization is stimulated by estradiol. This is interesting because is is opposite of what has been shonw in rats (Paul and Duttagupta, 1973) and mink (Rose et al. 2012) and likely represents an important species difference. Unfortunately, changes in uterine glycogen concentration during embryonic development in the human uterus are unknown.

Uterine Glycogen in Mink

Little is known about changes in uterine glycogen concentrations in mink. Murphy and James (1974) observed diastase-liable periodic-acid Shiff staining (indicative of glycogen) in the uterine epithelia during delayed implantation but not after implantation. Enders et al. (1963) observed a change from large secretory granules to smaller, more numerous secretory granules via electron microscopy, as implantation approach. Combining electron and light microscopy, Gulamhusein and Beck (1973) found similar granules in the estrus and pre-implantation ferret uterus which corresponded with strong periodic acid-Schiff (PAS) staining (indicative of glycogen; Gulamhusein and Beck, 1973). Our laboratory (Rose et al., 2011) recently found that estradiol significantly increased gross uterine glycogen concentrations and glycogen content of

the endometrium, glandular epithelia, and lumenal epithelia, suggesting that these tissues should have high glycogen content during estrus, but this has not been demonstrated.

Potential Uses of Endometrial Glycogen

The mobilization of uterine or endometrial glycogen in a variety of sepcies (see above) strongly suggests that endometrial glycogen is used to support early pregnancy (see 2.9 Uterine Glycogen & Fertility). However, the specific uses of the glycogen likely vary by species. For example, both the human and rodent uterus decidualizes, a process that requires glucose (Frolova et al. 2011). And additionlly the rat embryo is present in the uteine lumen for only hours before implantation (Villalón et al. 1982), reducing the amount of glucose necessary to keep these pre-embryos alive. Therefore, in these two species (rats and human), much of the endometrial glycogen is likely used to support mitosis during decidualization. In contrast, the ovine uterine does not decidualize, but sheep embryos increases in size over 1000-fold during elongation (reviewed in Blomberg et al., 2008) and thus need glucose to provide both energy (ATP) and cellular substrates for mitosis. In contrast, the mink uterus does not decidualize (limiting uterine needs for glucose; Tan et al., 2004) and the embryo does not elongate. However, the embryos do have a long pre-implantation period (up to 50 days; reviewed in Sundqvist et al., 1989) and likely needs a continuous supply of glucose to survive.

2.9. Uterine Glycogen & Fertility

Uterine Glycogen and Reduced Fertility

Changes in uterine glycogen concentration during the reproductive cycle and early pregnancy suggest that this glycogen is an important source of glucose for the uterus and/or developing embryos. While this relationship has never been directly demonstrated, there is suggestive evidence. First, many studies characterizing parameters in infertile women have found that many of those women have abnormall low endometrialglycogen concentrations (Dockery et al.,

1993; Grish et al., 2012; Maeyama et al., 1977; Mimori et al., 1981; Zawar et al., 2003). For example, Gupta et al. (2013) 25% of infertile women have low endometrial glycogen researves compared to women of normal fertility. And progesterone is able to increase uterine glycogen in infertile but not fertile women (Mimori et al., 1981). In addition, endometriosis, a cause of infertility, is associated with decreased uterine glycogen (Ishihara et al., 1991). In hamsters, aged (43–53 weeks) females had both smaller litter sizes and less histologically observable glycogen compared to young (9–12 weeks) females (Xi et al., 1999), and our laboratory has shown that concentrations of uterine glycogen in response to estrogen treatment is higher in parious rats (which have larger litters) than nullparious rats (Rose et al., 2009).

Cytosolic Mobilization of Uterine Glycogen

There are at least three different mechanisms by which uterine glycogen could contribute to histotroph: cytosolic breakdown, lysosomal breakdown, or direct secretion (2.7 Glycogen Metabolism). Cytosolic breakdown would require the actions of both Pyg and G6pc. In human endometrial biopsies, glycogen phosphorylase activity was two-fold higher in glandular epithelia than stroma (Souda et al., 1985). Using histochemical techniques, Burgos and Wislocki (1956) found endometrial G6pc activity to be mostly localized the apical surface epithelial cells in guinea pigs. Similarly, Bhattachary and Saigal (1984) found uterine G6pc activity to be completely localized to the apical surface of the epithelial with no activity detected in the stromal or myometrium. In the human endometrium, G6pc activity is also localized to the epithelial cells (Sawaragi and Wynn, 1969). Thus both Pyg and G6pc are localized to an ideal location to facilitate secretion of glucose liberated from glycogen into the uterine lumen. Furthermore, global knockout of G6pc3 reduced litter size 31% in nullparious mice and 50% during the 2nd and 3rd pregnancies (Jun et al., 2012).

Lysosomal Breakdown of Uterine Glycogen

Glycogen can also be broken down to glucose in lysosomes by acidic α-glucosidase (Gaa; Moreland et al. 2005). Examining the tissue distribution of Gaa in mouse tissue, Ponce et al. (1999) found Gaa to be expressed in the uterine epithelium, though expression was much lower than in many other tissues. Within the ovine endometrium, O'Shea and Murdoch (1978b) found that activity of Gaa did not change during the estrus cycle. Neither was Gaa activity affected by estradiol or progesterone in ovariectomized ewes. In mated ewes, endometrium Gaa activity dropped slightly (but significantly) between estrus and day 8, but then remained constant through day 30. In contrast, Ganguly et al. (1978) found Gaa activity increased steadily from the proliferative phase, through ovulation, and continued to increase in the secretory phase of human endometrial biopsies. That study also found Gaa activity to be increased even more during early pregnancy. However, these are the only two studies to examine uterine Gaa, and whether these differences represent true species differences or methodological artifacts remain unclear.

Secretion of Intact Glycogen into the Uterine Lumen

Several laboratories, including our own, have noted the presence of PAS-positive material inside the uterine lumen, indicating the possibility of intact glycogen (Reinius, 1970; Rose et al., 2011). In a more detailed study of glandular secretions, Burton et al. (2002) observed glycogen along the apical pole of the glandular epithelia and inside the glandular lumen, adjacent to the invading syncytiotrophoblasts using both PAS staining and electron microscopy. Gulamhusein and Beck (1973) observed secretory vesicles in the uterine epithelium of mink through the pre-implantation period. The authors believed these vesicles to correspond to PAS staining observed via light microscopy, and thus may have contained glycogen, though that was not proven. While these results seem to indicate the presence of glycogen in the glandular lumen, it remains

possible that this is a histological artifact. Exocytosis of glycogen into the uterine lumen, while intriguing, remains unproven.

If glycogen is secreted into the uterine lumen, that raises the question as to how that glycogen is utilized, and there seem to be two possibilities. First the embryos may take up intact glycogen molecules. Before hatching, the embryo is encased in a glycoprotein coat called the zona pellucida. However, the zona pellucida is porous, with numerous pores over 200 nm in diameter (Vanroose et al., 2000), meaning glycogen would diffuse through the zona pellucida and reach the embryo. During implantation the embryos became very phagocytic. While phagocytosis of glycogen has never been directly observed, embryos have been observed engulfing entire cells (Schlafke and Enders, 1975). And glycogen has been observed in syncytiotrophoblasts positioned over gland openings (Burton et al., 2011, 2002), leading investigators to theorize that phagocytic uptake of histotroph components is important for pre-implantation development (reviewed in Bevilacqua et al., 2010). The second possible fate of lumenal glycogen is breakdown of glycogen in the lumen. In support of this presupposition, glycogen phosphorylase activity has been observed in rinsings of the uterine lumen (O'Shea and Murdoch, 1978a).

2.10. Hormonal Control of Uterine Glycogen Metabolism

2.10.1. Insulin and Uterine Glycogen

Although it is well known that insulin is the primary stimulator of glycogenesis in many tissues (Voet et al., 2001b), this does not appear to be the case in the uterus. First, uterine glycogen concentrations change in a reproductive-cycle dependent manner in numerous species (Bugalia and Sharma, 1990; Larson et al., 1970; O'Shea and Murdoch, 1978a; Souda et al., 1985; Vasilenko et al., 1981), indicating control by a reproductive hormone(s). Experimentally, Hay et al. (1984) used glucose clamp methodology to investigate the effects of high glucose concentrations on uterine glucose uptake in sheep. While increased glucose concentrations

resulted in higher insulin concentrations and increased glucose extraction by the hind limbs, it had no effect on glucose uptake by the uterus.

Experimentally-induced diabetes resulted in higher uterine glycogen concentrations, not lower, in rats (Jawerbaum et al., 1994). Casalino-Matsuda et al. (2000) investigated the effects of ovariectomy, feeding status (normal or restricted (i.e. food intake reduce 50%)), and insulin on glucose metabolism of isolated rat uteri. In restricted, ovariectomized rats, insulin increased flux through glycolysis. But insulin decreased the rate of glycolysis in intact, ovariectomized rats, and insulin had no effect in normal fed rats (either intact or ovariectomized), so that no clear actions of insulin could be discerned. But the most direct evidence that uterine glycogenesis is regulated by reproductive hormones (primarily estradiol) and not insulin was the finding that neither insulin, glucose, nor both in combination affected uterine glycogen concentration in ovariectomized rats, while these treatments did result in differing glycogen concentrations in liver, skeletal muscle, and the heart. However, treatment with estradiol, independent of any co-treatments, consistently doubled uterine glycogen concentration (Swigart et al., 1962).

While it appears that insulin is not responsible for changes in uterine glycogen concentrations *in vivo*, that is not to say it is unimportant. Only three studies have examined the hormonal control of uterine glycogen in culture. Casimir et al. (1980) isolated endometrial epithelial and stromal cells and cultured each separately. In untreated cells, glycogen content was approximately 3-fold higher in stromal cells than epithelia. In both cells types, both sex steroids (estrogen and progesterone) increased glycogen concentrations numerically and the effects were more prominent in the stromal cells, but it is unclear if these increases were statistically signficant. While this study did not address the issue of insulin in these cells, the treatment media did contain 3% fetal bovine serum (FBS) so the cells were exposed to insulin during treatment, albeit

low concentrations. Ishihara et al. (1988) cultured human endometrial tissue (both epithelium and stroma) and found that progesterone increased glycogen concentration while estradiol decreased the concentration. However, in this study treatment media contained both 5% FBS and 5 μg/ml of exogenous insulin. The only study to directly examine the effects of insulin on endometrial glycogen in culture was Demers et al. (1977). Using guinea pigs, they first showed that progesterone increased endometrial glycogen concentrations both *in vivo* and *in vitro*. Next they showed that priming with estradiol increased the effectiveness of progesterone at increasing glycogen concentrations in the endometrium. Up to that point they had been using Trowell's T8 medium which contains 50 μg/ml insulin, so next they examined the role of insulin. Using Trowell's media (without insulin) supplemented with progesterone, they found that insulin increased uterine glycogen in a dose-dependent manner. Interestingly, they found that the requirement for insulin "could not be overcome by increasing progesterone concentration 10-fold." Thus it appears that insulin may have permissive actions on uterine glycogen metabolism; however, high concentrations of insulin also activate the Igf1 receptor (DeMeyts, 2002; Kim and Accili, 2002). Therefore, the precise roles of insulin and Igf1on endometrial glycogen metabolism remain to be elucidated.

2.10.2. Estradiol Stimulated Uterine Glycogenesis

It has been shown that estradiol stimulates uterine glycogen accumulation in many species, such as rats, hamsters, guinea pigs, and mink, (Demers et al., 1973; Rose et al., 2011). Gregoire et al. (1967) found that a single injection of 25 μg of estradiol benzoate maximally increased uterine glycogen concentration of rats. Looking at the effect of repeated treatments 24 hours apart, Bo et al. (1973) found that a single injection of estradiol dipropionate stimulated maximal glycogen concentrations, but a second injection further increased uterine weight, resulting in significantly more total uterine glycogen. Bo et al. (1952) found that uterine glycogen concentrations reached

maximal values 10 hours after injecting 10 μg of estradiol; similarly, Bitman et al. (1965) found that glycogen concentrations remained unchanged for two hours after treatment and then peaked 12 hours post-injection. Glycogen concentration remained plateaued from 12-24 hours post-injection before beginning to decrease. Since these studies are *in vivo*, it is unknown if the effect of estradiol is direct. Estradiol also stimulates production of Igf1 in the stromal (Ogo et al. 2014), which mediates some of estradiol's actions on the epithelia (Adesanya et al., 1999; Winuthayanon et al. 2010). And in isolated epithelia, estradiol had minimall effects of glycogen concentrations (Casimiri eta l. 1980).

Estradiol Stimulated Glucose Uptake and Phosphorylation

Smith and Gorski (1968) found that the intraperitoneal injection of estradiol (5 μg) before hysterectomy increased uterine glucose uptake and phosphorylation of 2-deoxyglucose during an hour incubation. At the end of the one-hour incubation, uteri from treated rats had 135% more 2-deoxyglucose-6-phosphate than control uteri, and the increase was due to an increased Vmax for hexokinase without any change in Km. Furthermore, it was suggested that the increased phosphorylation was dependent on the transcription of protein because the effect was blocked by cycloheximide (inhibits translation). A more recent study on immature rats (Welch and Gorski, 1999) found that estradiol-stimulated 2-deoxyglucose uptake lasted longer (4 hours) than previously described. Moorthy et al. (2004) also found that estradiol treatment increased hexokinase activity in the uteri of rats. However, these investigators also found that estradiol reduced uterine activity of glucose-6-phosphatase relative to control.

Using western blot, Welch and Gorski (1999) found that of Slc2a1-5, rat uteri expressed only Slc2a1 and Slc2a4. Furthermore, estradiol up-regulated mRNA expression of Slc2a1 only. Microsome and membrane preparations showed that estradiol did not change translocation of

either (Slc2a1 or Slc2a5), suggesting that increased glucose uptake was due to increased Slc2a1 transcription not translocation of existing protein. A different laboratory (Kim and Moley, 2009) found that Slc2a1 protein expression was highest at proestrus, agreeing with results from Welch and Gorski (1999). However, Kim and Moley also found that expression of Slc2a4, 8, and 9b were highest at estrus. The regulation of Slc2a4 may have been due to something other than estradiol because termination of experimental diapause with estradiol did not change Slc2a4 expression but did increase expression of the other 3 isoforms. At estrus, immunohistochemistry revealed Slc2a1 and 9b appearing almost exclusively on the basolateral surface of the uterine epithelia, making these transporters well suited to take up glucose from maternal circulation or glucose liberated from stromal glycogen stores. Interestingly, Slc2a1 appears to be differentially regulated in the decidua; Frolova et al. (2009) found that Slc2a1 expression was 2x higher in the decidualized endometrium than control and almost all Slc2a1 was incorporated in the cell surface. In agreement, progesterone stimulated an increased Slc2a1 expression and uptake of 2-deoxygluce in both primary mouse and immortalized human stroma cells. Opposed to the studies on whole uteri or uterine epithelia, in these cells estradiol caused a non-significant decrease in Slc2a1 expression and a significant reduction in 2-deoxyglucose uptake. While investigating the effects of estradiol (0.1 μg/g body weight) and age on carbohydrate metabolism, Moorthy et al. (2004) found that estradiol treatment consistently doubled the activity of hexokinase in uterine homogenates, regardless of age. Interestingly, progesterone (2.5 μg/g body weight) had no effect alone and did not alter the effects of estradiol. Together these combined effects would result in increased intracellular concentrations glucose-6-phosphate (G6P) which would be metabolized by the cell or stored as glycogen.

Estradiol Stimulated Glycogen Synthase Activity

Estradiol (5 μg/100 g body weight) increased G6P-dependent glycogen synthase activity 6 hours post-injection (Demers and Jacobs, 1973). Activity continued to increase until 24 hours post-injection and remained high for the remainder of the study. G6P-independent activity also increased due to estradiol treatment but a significant difference was not observed until 48 hours post-injection. It appears that the actions of estradiol on uterine glycogen synthase are not unique, as estradiol has been shown to increase hepatic glycogen concentration and increase glycogen synthase activity of skeletal muscle (Beckett et al., 2002; Paul and Duttagupta, 1973).

2.10.3. Hormonal Control of Glycogenolysis

It is clear that in the non-pregnant uteri of most species estradiol stimulates glycogen accumulation (Demers et al., 1973; Rose et al., 2011), but the hormones regulating glycogen breakdown and mobilization are less clear. Of particular interest in rats is the fact that uterine glycogen reaches nadir concentrations on the day of implantation (Demers et al., 1972; Greenstreet and Fotherby, 1973), even though serum estradiol concentrations also peak at this time (Cochrane and Shackelford, 1962; Shaikh, 1971). One possible explanation is the metabolism of estradiol to catecholestrogens at the time of implantation. In support of this uterine production of catecholestrogens increase during the implantation window in rats (Paria et al., 1998). In fact, catecholestrogen production at the time of implantation has been observed in multiple species, sometimes from the uterus (Chakraborty et al., 1990) and sometimes by the embryos (Mondschein et al., 1985). Catecholestrogen have also been shown to mediate metabolism. For example, catecholestrogens have been shown to synergistically increase epinephrine-induced lipolysis in adipocytes (Ackerman et al., 1981).

Our laboratory has recently found that the expression of Cyp1b1 mRNA expression increased in the peri-implantation period of mink (unpublished observations), suggesting that

catecholestrogen production also increases at this time in mink. In a study to investigate the possible roles of catecholestrogen on uterine glycogen metabolism in mink (Rose et al. 2011), our lab found that high doses (400 μg/kg•day) of 4-hydroxyestradiol and 2-hydroxyestradiol stimulated uterine glycogen accumulation equal to estradiol. Looking at mRNA expression of glycogen metabolizing enzymes, they found that catecholestrogens could have effects similar to estradiol or effect very distinct from the parent hormone. For example, all three steroids were equally potent at reducing the expression of glycogen phosphorylase mRNA. But only the catecholestrogens increased glycogen synthase mRNA expression. These results show that catecholestrogen can have effects on uterine glycogen metabolism that are similar and effects that are different from the parent hormone, suggesting that catecholestrogens activate both classical estrogen singlaing pathways and unique, yet unidifientied, pathways.

In a follow up study, using lower doses of each hormone (40 μg/kg•day), Hunt et al. (unpublished observations) found that acolbifene (estrogen receptor α/β antagonist) reduced the glycogenic effect of all three steroids (estradiol, 4-hydroxyestradiol, and 2-hydroxyestradiol) in the mink uterus, suggesting that estrogen-stimulated glycogenesis is mediated by classical estrogen receptors. In contrast, the catecholestrogens stimulated the mRNA expression of glycogenolytic enzymes (glycogen phosphorylase and glucose-6-phosphatase) more than the parent hormone. Indicating that, perhaps, catecholestrogens (independently of estradiol) stimulate uterine glycogenolysis. However, more work needs to be done.

2.11. Direct and Indirect Actions of Estradiol

By definition, estrogen is any compound that produces female-like characteristics (McKean, 2006), with estradiol-17β (commonly referred to as estradiol) being the main estrogen in mammals. Most estradiol is produced by granulosa cells of the ovarian follicles, though many

other tissues can produce estradiol. Once produced, estradiol enters the circulation, where it circulates either free (2%), bound to sex-hormone binding globin (SHBG, 37%), or bound to albumin (61%; Dunn et al., 1981).

2.11.1. Direct Estradiol Signaling

Nuclear Estrogen Receptors

Historically, estrogenic was synonymous with the ability to stimulate increases in uterine size (Koehler et al., 2005). Discovery of the first estrogen receptor (now called estrogen receptor α) provide a molecular bases for the effects of estradiol on the uterus. The discovery of a second estrogen receptor (β) further expanded our ability to understand estradiol's actions, especially in non-reproductive tissues (Koehler et al., 2005; Mosselman et al., 1996).

Estrogen receptor α and β are members of the steroid receptor family of proteins. In the absence of ligand, these receptors reside in the cytoplasm, associated with heat shock proteins, (though there is some evidence of unstimulated estrogen receptors being localized to the nucleus; Baniahmad and Tsai, 1993). After ligand binding, steroid receptors disassociate from their heat shock proteins and form homodimers. Following dimerization, estrogen receptors are translocated to the nucleus where they bind to DNA sequences containing estrogen response elements (ERE; GGTCAxxxTGACC) (Driscoll et al., 1998). Estrogen receptors can both stimulate or inhibit the translation of mRNA depending on the gene and the presence of other transcription factors.

Estrogen receptor α is considered the major estrogen receptor in the uterus. Evidence for this comes from the finding that estrogen receptor α knockout mice are completely infertile with major uterine hypotrophy (Lubahn et al., 1993). In contrast estrogen receptor β knockout mice are fertile, albeit with smaller litters (due to lower ovulation rates, Krege et al., 1998). While

estrogen receptor β may not be essential for murine reproduction, it does affect uterine physiology. In the immature mouse uterus, estrogen receptor β is expressed in both the stroma and epithelium, with estradiol decreasing its expression. Interestingly, in estrogen receptor β knockout mice, the uterus was hyper-responsive to estradiol, showing larger increases in volume, protein content, secretory activity, and expression of insulin-like growth factor 1 compared to wildtype controls (Weihua et al., 2000). Lindberg et al. (2003) used microarray analysis to compare the transcription activity in response to estradiol in wildtype and estrogen receptor β knockout mice. They found that within the genes increased by estradiol, the effect was 85% greater in estrogen receptor β knockout mouse.

Membrane Bround Estrogen Receptor

Razandi et al. (1999) transfected CHO cells (originally isolated from Chinese Hamster Ovary) with either estrogen receptor α or β and determined that the genes that encode nuclear estrogen receptors also give rise to plasma membrane bound receptors. Using these transfected cells, the investigators showed that estradiol treatment increased intracellular cAMP and inositol trisphosphate (IP3) concentrations. To test the hypothesis that dimerization of membrane bound estrogen receptors are required for activation (similar to nuclear activities), the same laboratory (Levin, 2009) transfected CHO cells with wildtype estrogen receptors or estrogen receptors modified to prevent dimerization. Only the wildtype receptors (which could dimerize) were able to increase cAMP concentrations in response to estradiol, showing that dimerization is required for activity, even in membrane-localized estrogen receptors.

Originally identified as on orphan receptor (Carmeci et al., 1997), GPR30 was later discovered to bind estradiol and has been renamed G protein-coupled estrogen receptor 1 (Gper1; Filardo et al., 2002; Maggiolini and Picard, 2010). Gper1 has been localized both to the outer plasma

membrane and the endoplasmic reticulum membrane. Gper1 signaling is complex; it has been shown to activate acetylate cyclase/cAMP signaling, increase intracellular Ca^{++} concentrations, stimulate PI3K/Akt, and activate Erk (reviewed in Maggiolini and Picard, 2010). Gao et al. (2011) found uterine Gper1 localized to epithelial cells in the mouse uterus, where it inhibited estradiol-stimulated uterine growth via inhibition of estrogen receptor α/ERK signaling. Evidencing that Gper1 has a role in carbohydrdate metabolism, *Gper1*$^{-/-}$ mice exhibit impaired estradiol-stimulated insulin release and hyperglycemia (Mårtensson et al., 2009).

2.11.2. Role of Insulin-Like Growth Factor 1

One well characterized effect of estradiol is to induce mitosis of uterine epithelia (Clark, 1971; Zhang et al., 1998). Using Cre-Lox technology, Winuthayanona et al. (2010) created a mouse lacking estrogen receptor α (*Esr1*) in the uterine epithelial, and lack of *Esr1* blocked to ability of estrogen to induce leukemia inhibitory factor (*Lif*) expression but did not alter the mitotic response. However, in global *Igf1* knockout mice, estradiol-stimulated mitosis is reduced by over 66% (Adesanya et al., 1999), indicating that Igf1 mediates estradiol's effect. It is well established that estradiol increases the production of *Igf1* by uterine stromal cells (Kapur et al., 1992; Ogo et al., 2014), and in the cow uterus, *Igf1* production by the sup-epithelial stroma peaks at estrus when estradiol concentrations are high (Robinson et al., 2000). This has lead most authors to conclude that stromal *Igf1* mediates the mitotic effect of estradiol on the uterine epithelia. To directly test the role of uterine derived Igf1 in epithelial proliferation, Cooke et al. (1997) isolated epithelia and stroma tissue for wildtype and Igf1 knockout mice and prepared various tissue grafts (stroma $^{-/-}$/epithelial$^{+/+}$, for example) and transplanted these grafts into wildtype, nude female mice. As expected, the transplanted epithelial cells responded to estradiol treatment with increased mitosis in the stroma$^{+/+}$/epithelial$^{+/+}$ and stroma$^{+/+}$/epithelial$^{-/-}$ mice.

Confirming a need for stromal Igf1 in epithelial proliferation, the transplanted epithelia in stroma$^{-/-}$/epithelial$^{+/+}$ mice did not respond to estradiol treatment.

While the above results seem conclusive, there is evidence to suggest that stromal-produced Igf1 is not necessary for epithelial cell mitosis. Sato et al. (2013) transplant ~40 mg uterine cross-sections from both Igf$^{+/+}$ and Igf1$^{-/-}$ mice to athymic recipients. In this study, the investigators found that Igf1$^{-/-}$ uteri increased in weight after transplant. In response to estradiol treatment, both types of uteri (wildtype and knockout) had equal responses in epithelial cell height and rate of mitosis. These results led the authors to conclude that systemic Igf1 was sufficient to mediate estradiol-induce epithelial proliferation and locally produced Igf1 was not needed.

2.11.3. Catecholestrogens

Production of Catecholestrogens

Estradiol can be metabolized through a variety of pathways. Most of these conversions results in metabolites with lower affinity for transport proteins and increased water solubility by oxidation (typically hydroxylation), glucouronidation, sulfonation, and/or O-methylation. These changes result in most excretion (90%) occurring via urine [304].

However, modification of estradiol can also result in bioactive metabolites. Two hydroxylated metabolites of estradiol that have received much attention in scientific research are 4-hydroxyestradiol and 2-hydroxyestradiol (Figure 5). These metabolites, collectively termed catecholestrogens, due to the A ring now being a catechol group, have a half-life considerably shorter than the parent hormone (Emons et al., 1982; Ginsburg et al., 1998) and likely work in an autocrine/paracrine manner to mediate some of the actions of estradiol. One of the most well established, and pertinent, biological roles for catecholestrogens is their absolute requirement for activation of blastocysts and implantation in rodents (Paria et al. 1998, 1990).

The short half-life of catecholestradiols is due to the multiple pathways by which they can be metabolized. The catecholestradiols can be metabolized to catecholestriols, methylated by catechol-O-methyl transferase (COMT), or be converted into semiquinones. Metabolism by peroxidases into semiquinones and then quinones is a frequent target of catecholestrogen research because of the role of these metabolites in oncogenesis through redox cycling and production of DNA adducts (Cavalieri et al. 2000). However, it must be noted that catecholestrogens have important physiological actions.

Cytochrome P450 Enzymes

Catecholestrogens are produced by cytochrome P450 (Cyp) enzymes. Cyp is a large family of enzymes involved in metabolism and detoxification of many xenobiotics and steroids (reviewed in Oesch-Bartlomowicz and Oesch, 2008). Cyp1a1 and cyp1b1 are primarily responsible for catecholestrogen production, yielding 2-hydroxyestradiol and 4-hydroxyestradiol, respectively. While the uterus expresses both Cyp1a1 and Cyp1b1, Paria et al. (1990) found the activity of Cyp1b1 was 90% higher than that of Cyp1a1, indicating a predominance of 4-hydroxyestradiol in uterine tissue.

Hormonal Control of Cyp1a1 and Cyp1b1

It appears that estradiol at least partially controls its metabolism to catecholestrogens via modulation of Cyp1a1/1b1 expression. In MCF-7 cells, Tsuchiya et al. (2004) showed that estradiol increased Cyp1b1 expression via binding of ERα to an estrogen response element (ERE). Using the same cell line, Madak-Erdogan and Katzenellenbogen (2011) found that estradiol increased Cyp1b1 expression, though to a lesser extent than Cyp1a1 expression. DuSell et al. (2010) analyzed a pre-existing dataset (GSE848) and found that 4-hydroxytamoxifen (selective estrogen receptor modulator) increased Cyp1a1 and Cyp1b1 expression 57 and 20-fold, respectively in MCF-7 cells. Confirming these findings, they showed

that 4-hydroxytamoxifen increased both Cyp1a1 and Cyp1b1 in MCR-7, MDA-MB-231, and SKBR cells, though two of those cell lines lack ERα (MDA-MB-231 and SKBR3). Even in MCF-7 cells knockdown of ERα did not alter tamoxifen's effects, showing this effect was independent of ERα.

Specific to the uterus, Kretzschmar et al. (2010) found that estradiol (both 0.5 and 4.0 μg) decreased Cyp1a1 expression in rats. Rataj et al. (2012) found that estradiol treatment reduced Cyp1a1 expression and increased Cyp1b1 expression. An ERα agonist (16α-LE2) had similar effects, decreasing Cyp1a1 expression and non-significantly increases expression of Cyp1b1. Interestingly the effects of the ERβ agonist 8β-VE2 were opposite that of E2, increasing Cyp1a1 expression 4-fold and decreasing Cyp1b1.

Potential Involvement of Prostaglandins

One well defined action of catecholestrogens is their ability to stimulate prostaglandin production. For example, catecholestrogens stimulate prostaglandin production in the ovaries of catfish (Chourasia and Joy, 2012), and Ho et al. (2008) found that a mixture of 2- and 4-hydroxyestradiol stimulated prostaglandin G/H synthase (cyclooxygenase) expression and prostaglandin E2 production by bronchial epithelial cells. More relevant to the current research, there are several reports that catecholestrogens stimulate uterine prostaglandin production more than the parent hormone (Kelly et al., 1983; Kelly and Abel, 1980; Pakrasi and Dey, 1983). Kelly and Abel (1980) found that 2-hydroxyestradiol increased prostaglandin E production in the human endometrium by 200%; estradiol only increased production by 30%. Another study using rabbit endometrium (Pakrasi and Dey, 1983) found that 4-hydroxyestradiol increased prostaglandin F production 3-fold over controls and estradiol had no effect; 2-hydroxyestradiol was less effective, only increasing concentration in the media 2-fold. In regards to prostaglandin

E, 4-hydroxyestradiol increased prostaglandin production 2.5-fold over controls and 2-hydroxyestradiol was similarly effective. Estradiol either had no effect or a significantly reduced effect on prostaglandin production in the media, depending on the dose used. Supporting not only that catecholestrogens simulate prostaglandin production but that this is biological important, the Paria et al. (1998, 1990) laboratory carried out a series of experiments to look at the role of catecholestrogens on implantation in rodents. In one study, it was found that estrogens with reduced ability to be metabolized to catecholestrogens fail to stimulate implantation (Dey et al., 1986). This indirectly links prostaglandins to catecholestrogens because it is well established that prostaglandins are a required mediator of implantation (reviewed in Kennedy et al., 2007).

More directly linking catecholestrogen-stimulated prostaglandin production to implantation, Paria et al. (1998) flushed embryos from pregnant mice, treated these embryos with various compounds, and then transferred the embryos into pseudopregnant mice. Incubation with estradiol only induced implantation in 1 of 9 mice, and that mouse only had 1 implantation site. In contrast, 4-hydroxyestradiol resulted in implantation in 9 of 9 mice (5.9±1.0 implantation sites per mouse). The results with prostaglandin E2 were similar, 100% of females had implantation sites. When embryos were treated with 4-hydroxyestradiol and DuP (a selective Ptgs2 inhibitor) implantation was prevented in every female; however, treatment with 4-hydroxyestradiol, DuP, and prostaglandin E2 rescued implantation (implantation sites in 5 of 5 females). These results indicate that blastocyst produced prostaglandins, resulting from 4-hydroxyestradiol stimulation, are requisite for implantation in mice.

2.12. Prostaglandins

Prostaglandins are paracrine/autocrine signaling molecules present in most bodily tissues. First isolated from semen by Swedish scientist U.S. von Euler, they were named from the mistaken belief they were derived from the prostate. The first two subtypes of prostaglandins purified (prostaglandin E_2 and prostaglandin $F_{2\alpha}$) were named based on their solubility: prostaglandin E_2 in ether and prostaglandin $F_{2\alpha}$ in phosphate buffer (which is spelled with an F in Swedish). Later prostaglandins were named alphabetically. The numeral in each name indicates the degree of unsaturation of the side chains; all known naturally occurring, biologically active prostaglandins have two double bonds (Carsten and Miller, 1990).

Synthesis of prostaglandins begins with the liberation of arachidonic acid from membrane phospholipids by phospholipase A2. In the cytosol, it is converted to prostaglandin H_2 by prostaglandin G/H synthase (Ptgs, formerly known as cyclooxygenase) 1 or 2. Ptgs 1 is constitutively expressed in most tissues; whereas, Ptgs 2 is considered inducible (Helliwell et al., 2004). Prostaglandin H2 is converted to one of five major prostaglandin-like moleculaes (D_2, E_2, $F_{2\alpha}$, I_2, or thromboxane A_2) by synthases specific for each type (Figure 6; Hata and Breyer, 2004). Prostaglandins mediate their effects by signaling through one of two types of receptors. The classical prostaglandin receptors are G-protein coupled receptors (GPCRs). Prostaglandins I_2, $F_{2\alpha}$, and thromboxane A_2, each have one GPCR to bind (IP, FP, and TP, respectively). Prostaglandin D_2 can bind two different GCPRs (DP and CRTH2), and prostaglandin E2 can bind to four different GPCRs (EP1-4). The GPCRs for each receptor can signal through $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha q}$ pathways, depending on the receptor, tissue, and effect being examined (reviewed in Hata and Breyer, 2004).

Prostaglandins can also signal via peroxisome proliferator-activated receptors (PPAR), which are nuclear transcription factors (Lim and Dey, 2002). PPARs, first discovered in 1992 (Dreyer et al., 1992), have three isoforms (α, β/δ, and γ) that have been shown to mediate a variety of actions including energy metabolism (Arck et al., 2010). Upon binding a ligand, PPARs dimerize with retinoid X receptor (RXR). The complex is then translocated to the nucleus where it can stimulate or inhibit transcription of genes with PPAR response elements (Kliewer et al., 1992). Linking PPARs to mink reproduction, Desmarais et al. (2007) showed that PPARγ mRNA expression was upregulated in invading trophoblasts of the mink. Treatment of isolated trophoblasts from mink with 15-deoxy-PGJ2 (natural PPAR ligand) stopped proliferation and triggered differentiated into an invasive phenotype.

Prostaglandins in the Uterus

The first evidence linking prostaglandins to pregnancy came from Horan (1971) who found that sodium salicylate reduced litter size in rat, though at the time it was unknown that sodium salicylate was a Ptgs inhibitor. As more direct evidence, *Ptgs2* knockout mice are infertile with a complete lack of embryo implantation and decidualization (Lim et al. 1997b). While *Ptgs1* knockout mice (mated to wildtype males) have normal sized litters (Langenbach et al. 1995), Reese et al. (1999) found a compensatory increase of Ptgs2 in the uterus of *Ptgs1* knockout mice. And even with compensatory Ptgs2 expression, Ptgs1 knockout mice had lower uterine prostaglandin production and lower vascular permeability than wildtype mice. In mink uteri *Ptgs2* is undetectable until after implantation sites are observed. After implantation, Ptgs2 was highly expressed in the uterine epithelia surrounding the implantation embryos (Song et al., 1998). In skunks (another species which undergo delayed implantation) a similar pattern was observed, Ptgs2 was unobservable until after implantation had started. But this study also found Ptgs1 to be expressed steadily throughout the pre-implantation period. After implantation was

initiated, expression of both Ptgs1 and Ptgs2 was greatly increased. Similarly, Ptgs1 is expressed throughout the pre-implantation in mice and Ptgs2 was only detected after implantation, leading the authors to speculate that Ptgs1 is important for the uterus prior to implantation (Chakraborty et al., 1996).

While it is clear that uterine prostaglandins are important (Reese et al. 1999; Lim et al. 1997b), the specific prostaglandins required for implantation remains elusive. In rats, Kennedy (1986) found that prostaglandin E and prostaglandin F are elevated at the implantation site. In contrast, Lim et al. (1999) found the concentrations prostaglandin E_2 and prostaglandin I_2 to be increased at the implantation site, with no difference in prostaglandin $F_{2\alpha}$ concentrations. In rabbits, one study (Sharma, 1979) only found uterine prostaglandin E2 concentrations to be increased at the implantation site; while another study (Pakrasi and Dey, 1982) found increased concentration of both prostaglandin E_2 and prostaglandin $F_{2\alpha}$ were found to be elevated during implantation. Decidualization in mice has been associated with increased prostaglandin $F_{2\alpha}$ concentrations (Rankin et al., 1979) or prostaglandin E_2 and prostaglandin $F_{2\alpha}$ (Jonsson Jr. et al., 1979), while Milligan and Lytton (1983) found prostaglandin E_2 and prostaglandin I_2 to be unchanged.

More recent studies have utilized *in situ* hybridization and immunohistochemistry to localize the enzyme and receptors required for prostaglandins synthesis and action to specific tissue types. In the rat, Cong et al. (2006) found Ptgs1 to be present in the luminal epithelium. Ptgs2 was found in the sub-luminal stroma; microsomal PGES1 (mPGES1, mRNA and protein) were induced on day 6 of pregnancy by the blastocyst. Ni et al. (2002) found basal expression of mPGES in the luminal epithelium before implantation and intense expression in the stroma surrounding the implanting blastocyst. At day 5 of pregnancy (one day after implantation) cytostolic PGE2

(cPGES) was strongly expressed in the luminal epithelium and weakly expressed in the stroma; expression increased substantially in the stroma after implantation (Ni et al., 2003).

In the mink, Song et al. (1998) found Ptgs2 to be present in the epithelium, stroma, and necks of uterine glands; expression was increased by the blastocyst. A Similar study (Lim et al. 1997a) probing for prostaglandin receptors in rat uterus have found EP2 to be undetectable on days 1-4 of pregnancy (pre-implantation), whereas by day 6 EP2 mRNA and protein was abundantly expressed in the luminal epithelium of the implantation site. However, expression had decreased by day 7 and was undetectable by days 8 and 9. Utilizing a delayed implantation model, Shi et al. (2005) found EP2 to be induced during implantation, before declining to undetectable levels, similar to the findings during normal pregnancy. Utilizing pseudopregnant rats, Papay and Kennedy (2000) found EP2 to be undetectable on day 4 by *in situ* hydridization. By days 5 and 6, it was strongly expressed in the luminal epithelium, with weak expression in the glandular epithelium. By day 4, EP3 was undetectable, but became apparent in the glandular epithelium by day 5. Expression of EP3 in the glandular epithelium on day 6 was intensified if the animals received a deciduogenic stimulus on day 5. EP4 was highly expressed in the luminal epithelium and surrounding stroma on day 4 of pseudopregnancy. Expression was similar on day 5 but was less apparent on day 6, regardless if a deciduogenic stimulus had been applied. Lim and Dey (1997) found EP2 to be expressed only in the luminal epithelium on days 4 and 5 of pregnancy, and expression was relatively unaffected by the blastocyst; implying estradiol and progesterone as likely candidates for its control. Indeed, Lim and Dey (1997) found estradiol to negatively regulate expression, and progesterone to induce epithelial expression.

The above results show that prostaglandins are absolutely required for pregnancy to occur (Reese et al. 1999; Lim et al. 1997b), and the specific expression of the various prostaglandin-

synthesizing enzymes and receptors (Lim and Dey 1997; Song et al. 1998; Shi et al. 2005) suggests that prostaglandin play diverse and important roles during pregnancy. Yet specific actions of specific prostaglandins are difficult to identify. This is likely due to species differences, overlapping actions of different prostaglandins, and multiple enzymes with the same function.

2.13 Prostaglandins & Glycogen Metabolism

Hepatic Glycogen

While the potential role of prostaglandins on uterine glycogen metabolism has only been modestly studied (see below), the role of prostaglandins on hepatic glycogen metabolism has been studied more intensively. Many studies have found that prostaglandins stimulate hepatic glycogenolysis (Casteleijn et al. 1988; Okumura and Saito, 1990). For example, Kanemaki et al. (1993) used insulin to stimulate the storage of radiolabeled glucose and then examined the effects of prostaglandin E_2 on glycogenolysis in vivo. They found that addition of prostaglandin E_2 (10^{-7}M) significantly decreased the amount of glycogen after 1 hour of incubation. Additionally there was a corresponding increase in radiolabeled glucose released into the media, showing that the liberated glucose was not catabolized by the cells. In agreement with glycogenolytic actions of prostaglandins, Gómez-Foix et al. (1989) found that both prostaglandin E_2 and $F_{2\alpha}$ increase the activity of glycogen phosphorylase and decrease the activity of glycogen synthase in isolated hepatocytes.

In contrast to studies showing prostaglandins stimulated hepatic glycogenolysis (Kanemaki et al. (1993, for example), some investigators have found that prostaglandins prevented glucagon-stimulated glycogenolysis (Brass et al., 1984; Hespeling et al., 1995). To elucidate the signaling mechanisms underlygint divergent responses, Püshcel et al. (1993) treated isolated hepatic cells with prostaglandin E_2, glucagon, or both. Prostaglandin E_2, without glucagon, increased

glycogen phosphorylase activity 60% and glucose output 20% relative to control, similar to previous studies (Kanemaki et al. 1993). Glucagon had stronger effects, increasing glycogen phosphorylase activity 160% and glucose output by 50%. However, glucagon and prostaglandin E_2 together had less potent effects than either hormone alone. Investigating this further, the investigators pre-treated hepatocytes with phorbol ester 4β-phorbol 12- myristate 13-acetate (PMA; inhibitor of phospholipase C) or pertussis toxin (inhibitor of $G_{\alpha i}$) and the repeated the experiment. PMA reduced the glycogenolyic effects of prostaglandin E_2, while pertussis toxin completely blocked the anti-glycogenolyic actions of prostaglandin E_2. Next, to determine which prostaglandin E_2 receptors are mediating the glycogenolytic effect, they used various agonist and antagonists for EP1-3 (since then a fourth prostaglandin E2 receptor have been identified; Regan et al., 1994). Interestingly, sulproston (EP1 and EP3 agonist) stimulated glycogen phosphorylase at a much lower concentration than misoprostol (EP3 agonist). In contrast, both agonists equally inhibited glucagon-stimulated glycogenolysis. These results lead the authors to conclude that the glycogenolyic effect of prostaglandin E_2 is mediated via EP1 signaling through a $G_{\alpha i}$/phospholipase C pathway, while the anti-glycogenolyic effects are mediated by EP3 signaling through a $G_{\alpha i}$ pathway.

The study by Püshcel et al. (1993) provides an explaination for the differing actions of prostaglandins alone compared to the actions of prostaglandins in the presence of glucagon on liver cells. It also indicates that the major action of prostaglandins in the liver (absent glucagon) is to stimulate the breakdown of glycogen. This conclusion agrees with studies in other species, such as Busby et al. (2002) who found that prostaglandin E_2 stimulated the breakdown of glycogen in fish hepatocytes.

Prostaglandins & Uterine Glycogen

There have only been a few reports concerning prostaglandins and uterine glycogen (Beatty and Bocek, 1972; López et al., 1991). Gonzalez et al. (1994) found that prostaglandin E_2 significantly increased glucose uptake in cultured uteri; however, indomethacin had no effect. Inhibition of Ptgs in uteri incubated in media containing glucose resulted in significantly lowered glycogen after 60 minutes (López et al., 1991). Inclusion of prostaglandins E_1, E_2, or $F_{2\alpha}$ was unable to reverse the effect of the Ptgs inhibitors. If the uteri were incubated in media without glucose, glycogen concentrations decreased after 60 min, regardless of treatment. In cultured myometrium, from pregnant rhesus monkeys, prostaglandin E_1 increased the rate of glucose uptake and glycolysis compared to controls (Beatty and Bocek, 1972). Prostaglandin E_1 also increased lactate produced by glycogenolysis, so that the percentage of lactate arising from glycogen remained the same as controls (30%). Interestingly, glycogen concentrations did not differ in response to prostaglandin E_1, so presumably glycogen was being formed at the same rate as breakdown. When looking at the effects of prostaglandin E_1 and epinephrine, they found that neither hormone affected uterine glycogen concentrations by themselves, but when both were added to the culture media, glycogen concentrations were significantly reduced. While these studies do show that prostaglandins do modulate uterine glycogen metabolism the overall effect of prostaglandins remains unclear.

3. Statement of Problem

In couples trying to conceive, human fecundity is maximal during the first two menstrual cycles at only 30% per cycle (Zinaman et al., 1996). Approximately 10% of US women (6.7 million) have impaired fecundity and another 6% of women (1.5 million) are clinically infertile (Chandra et al., 2013). While difficult to characterize in humans, it is known that most pregnancy losses occur before or during implantation (Benagiano et al., 2010). Mink undergo obligatory embryonic diapause, meaning that mink embryos do not implant until up to 50 days post coitum, making them a unique model for pre-implantation embryo survival. Additionally, mink are an economically important species ($292 million in US in 2011; National Agriculture Statistics Service, 2012). However, oocyte wastage exceeds 90% in mink (Lefèvre and Murphy, 2008; Tauson, 1985), indicating that reproductive efficiency of mink could be greatly increased. Thus a better understanding of factors contributing to pregnancy loss will have important implications for both human medicine and animal agriculture.

Glucose is an essential nutrient for both the uterus and pre-implantation embryos (Chatot et al., 1994; Frolova et al., 2011). In both rodents and sheep, uterine glycogen is catabolized during the pre-implantation period and likely contributes to the energetic demands of the uterus and/or embryos. However, this has never been confirmed in a species which undergoes obligatory embryonic diapause. Therefore, **our first hypothesis is that endometrial glycogen is mobilized after mating in mink (*Neovison vison*)**.

While estradiol stimulates uterine glycogen accumulation *in vivo* (Rose et al., 2011; Swigart et al., 1962), Casimiri et al. (1980) found minimal effects of estradiol on the glycogen concentration of uterine epithelium isolated from the rodent uterus. It is clear that estradiol can

have both direct and indirect actions on the uterine (Adesanya et al., 1999). Thus **our second hypothesis was that estradiol directly stimulates glycogenesis in the uterine epithelia**.

It is clear that estradiol stimulates uterine glycogenesis *in vivo* (Demers et al., 1973; Rose et al., 2011). However, as implantation approaches the uterus converts more estradiol to 4-hydroxyestradiol (Paria et al., 1990; Hunt et al. unpublished observations) and 4-hyrdoxyestradiol has been shown to stimulate uterine prostaglandin synthesis (Kelly and Abel, 1980; Pakrasi and Dey, 1983). Thus conversion to 4-hydroxyestradiol may alter uterine responses to estradiol. **Our final hypothesis is that 4-hydroxyestradiol has distinct effects on uterine glycogen content and metabolism which are mediated through prostaglandins**.

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Figures for Literature Review

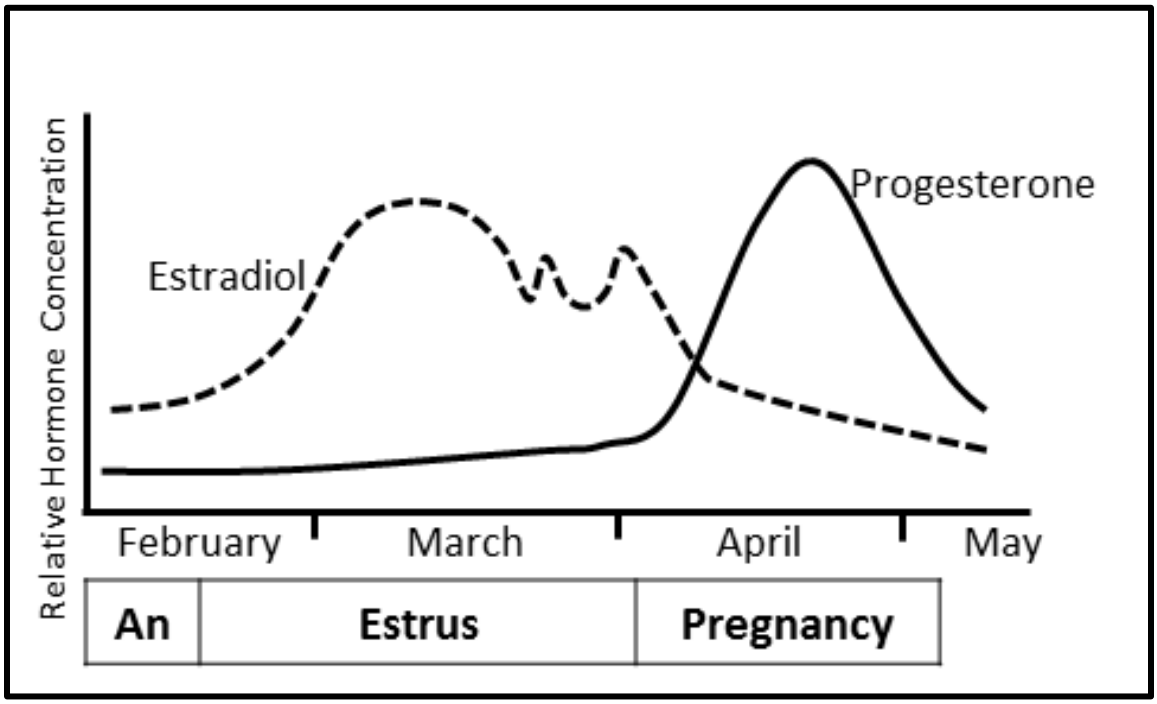

Figure 1. Realtive change in serum estradiol and progesterone concentrations in mink. Mink are anestrus (An) much of the year but come into estrus during mid-Feburary in the Northern Hemisphere due to rising estradiol concentrations (dashed line). Estradiol concentrations begin to decline in late March but there are two brief increases, one corresponding to mating and one corresponding to implantation. Progesterone concentrations (solid line) increase slightly after mating then increases more dramaicaly approimately one week before implantation (first week of April). Progesterone concentrations are high through the remainder pregnancy, with parturition occuring the first week of May.

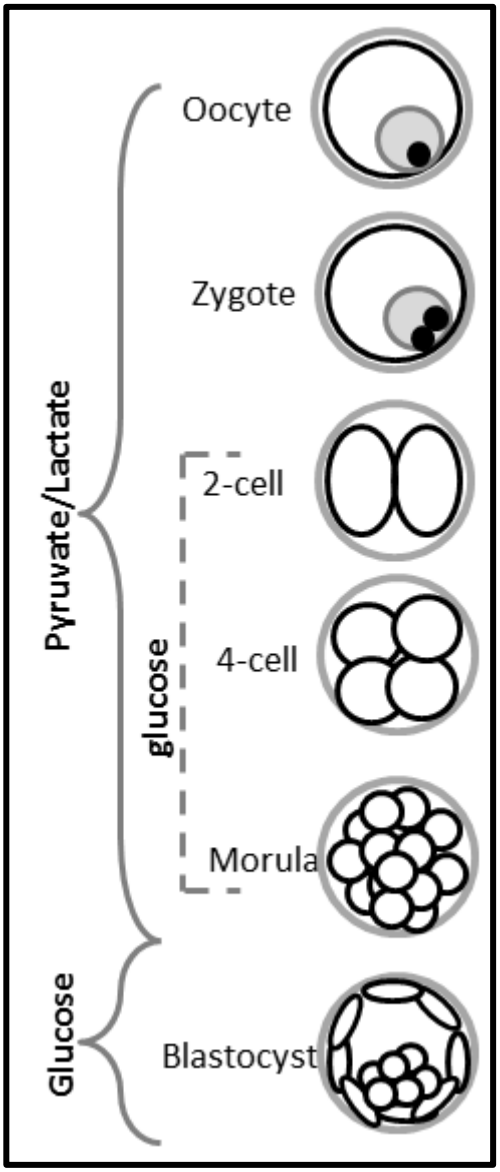

Figure 2. Energy requirements for developing embryos. Development from the oocyte through the morula is supported by pyruvate and lactate. During cavitation embryos switch to preferentially metabolizing glucose. However, to become blastocysts transient exposure to glucose must occur after the first cleavage and before the blastocyst stage (dashed line). Grey circle, zone pellicuda; black circles, cell membrane; filled grey circle, nucleus.

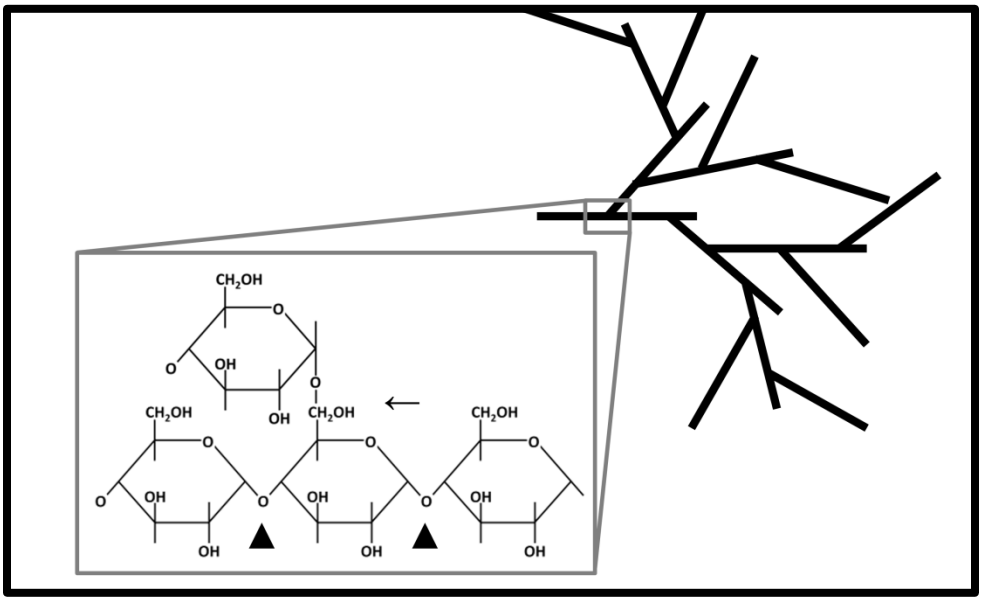

Figure 3. Diagram of glycogen's structure. Glycogen is composed of 12-14 glucose residues joined into linear chains (black lines). Each short, linear chain has approximately 2 chains branching from it, giving glycogen a highly branched structure. Individual glucose residues (insert) in a linear chain are joined by α1-4 bonds (▲), with α1-6 bonds (←) giving rise to branches.

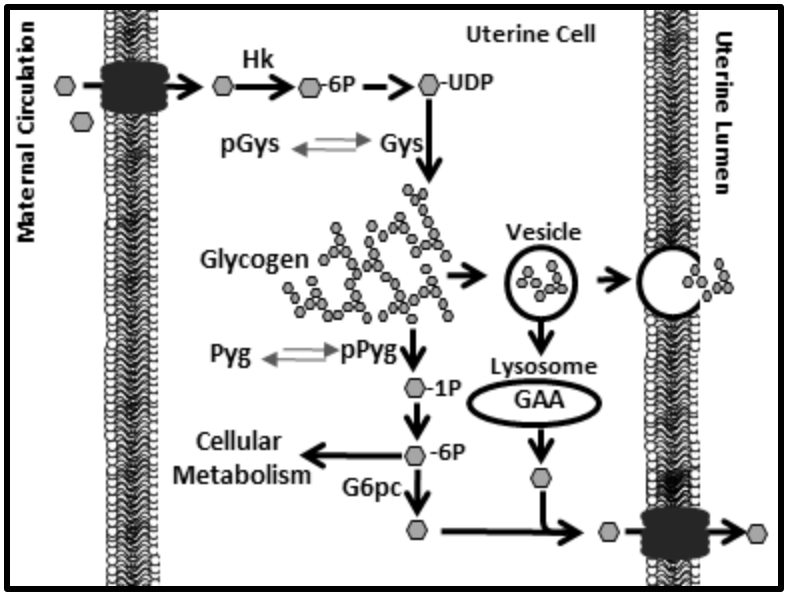

Figure 4. Diagram of glycogen metabolism. Glucose (⬡) enters cells through transporters termed solute carrier family 2a (Slc2a) and is then phosphorylated by hexokinase (Hk), yielding glucose-6-phosphate (⬡-6P). ⬡-6P is isomerized to ⬡-1P before the glucose residue is transferred to uridine diphosphate. Glycogen synthase (Gys) can then transfer the glucose residue to a preexisting glycogen molecule and is the rate-limiting enzyme in glycogenesis. Glycogen can be mobilized by 3 distinct mechanisms. Glycogen can be taken into vesicles and secreted via apocrine and/or merocrine secretion into the uterine lumen, or the vesicles can fuse with lysosomes where acidic α-glucosidase (GAA) liberates glucose, which then diffuses out of the lysosome. Finally, glycogen phosphorylase (pPgy) liberates ⬡-1P, which is isomerized to ⬡-6P. ⬡-6P can be used by the cell or it can be dephosphorylated by glucose-6-phosphatase (G6pc) and transported out of the cell.

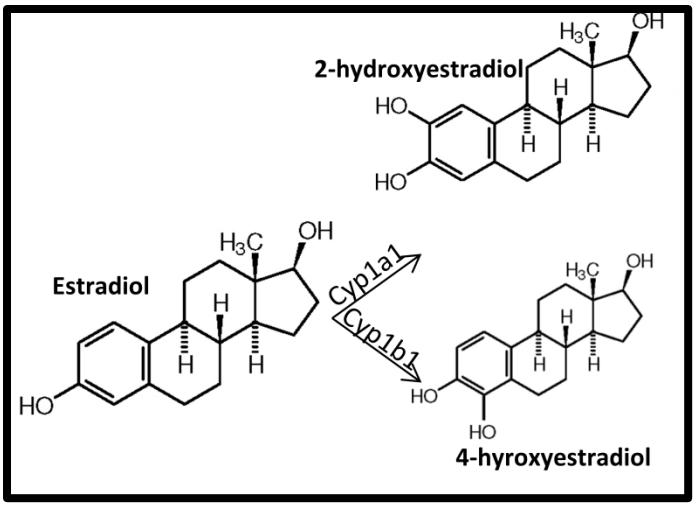

Figure 5. Conversion of estradiol to 2-hydroxyestradiol and 4-hydroxyestradiol. Estradiol can be hydroxylated at the 2-carbon position by cytochrome P450 (Cyp) 1a1 producing 2-hydroxyestradiol or hydroxylated at the 4-carbon position by the Cyp1b1 producing 4-hydroxyestradiol.

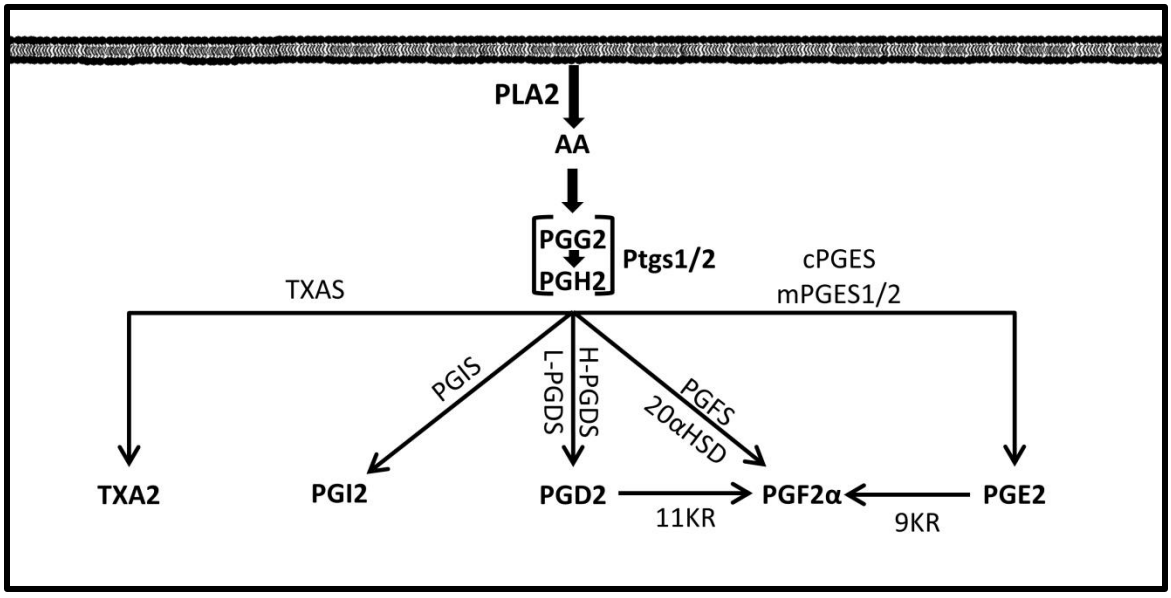

Figure 6. Pathways of prostanoid synthesis. Phospholipase A2 (PLA2) liberates arachidonic acid (AA) from lipids in the plasma membrane. AA is then converted to prostaglandin (PG) G2 and then PGH2 by prostaglandin G/H synthase 1 or 2 (Ptgs1/2; also knows as COX). PGH2 can then be converted to one of five prostanoids: thromboxane A2 (TXA2), prostacyclin (PGI2), prostaglandin D2 (PGD2), prostaglandin F2α (PGF2α), or prostaglandin E2 (PGE2). TXA2 and PGI2 are produced by thromboxane A synthase (TXAS) and prostaglandin I synthase (PGIS), respectively. PGD2 is produced by both hematopoietic prostaglandin D synthase (H-PGDS) and lipocalin- prostaglandin D synthase (L-PGDS). PGF2α is produced by prostaglandin F synthase (PGFS) or by the enzyme 20α-hydroxysteroid dehydrogenase (2αHSD), the same enzyme that inactivates progesterone. PGE2 is produced by cyctolic prostaglandin E synthase (cPGES) or membrane-bound prostaglandin E synthase 1 or 2 (mPGES1/2). Additionally, PGF2α can be produced from PGD2 by 11-ketoreductase (11KR) or from PGE2 from 9-ketoreductase (9KR).

4. Chapter 1

Uterine Glycogen Metabolism in Mink During Estrus, Embryonic Diapause and Pregnancy[#]

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Abstract

We have determined uterine glycogen content, metabolizing enzyme expression and activity in the mink, a species that exhibits obligatory embryonic diapause, resulting in delayed implantation. Gross uterine glycogen concentrations were highest in estrus, decreased 50% by diapause and 90% in pregnancy ($P \leq 0.05$). Endometrial glycogen deposits, which localized primarily to glandular and luminal epithelia, decreased 99% between estrus and diapause ($P \leq 0.05$) and were nearly undetectable in pregnancy. Glycogen synthase and phosphorylase proteins were most abundant in the glandular epithelia. Glycogen phosphorylase activity (total) in uterine homogenates was higher during estrus and diapause, than pregnancy. While glycogen phosphorylase protein was detected during estrus and diapause, glycogen synthase was almost undetectable after estrus, which probably contributed to a higher glycogenolysis/glycogenesis ratio during diapause. Uterine glucose-6-phosphatase 3 gene expression was greater during diapause, when compared to estrus ($P \leq 0.05$) and supports the hypothesis that glucose-6-phosphate resulting from phosphorylase activity was dephosphorylated in preparation for export into the uterine lumen. The relatively high amount of hexokinase-1 protein detected in the luminal epithelia during estrus and diapause may have contributed to glucose trapping after endometrial glycogen reserves were depleted. Collectively, our findings suggest to us that endometrial glycogen reserves may be an important source of energy, supporting uterine and conceptus metabolism up to the diapausing blastocyst stage. As a result, the size of uterine glycogen reserves accumulated prior to mating may in part, determine the number of embryos that survive to the blastocyst stage, and ultimately litter size.

Keywords: mink, embryonic diapause, uterus, glycogen

1. Introduction

Pre-embryonic growth prior to implantation is supported by nutrients in uterine glandular secretions (histotroph) that include glucose, glycogen, proteins, amino acids and fats [1–3]. Glucose uptake and metabolism by pre-embryos is essential for blastulation [4,5] and hatching [6]. Moreover, endometrial decidualization is dependent upon glucose metabolism [7], and glucose is the preferred energy substrate of the myometrium [8,9].

Although gluconeogenesis does not occur in the uterus [10,11], glucose is stored as glycogen [12,13]. In rodents, gross uterine glycogen concentrations peak during proestrus-estrus, then decrease during implantation and early pregnancy [12,13]. The human uterus accumulates massive amounts of glycogen within glandular and luminal epithelia during the late-proliferative to early-secretory phases, that is mobilized during the late secretory phase [14–16]. While the importance of uterine glycogen reserves to a successful pregnancy remains to be determined, women with reduced fertility frequently have extremely low endometrial glycogen concentrations [17].

Storage of glucose as glycogen, begins with phosphorylation of the sugar by hexokinase (Hk), producing glucose-6-phosphate, that is isomerized to glucose-1-phosphate and converted to uridine diphosphate glucose. Glucosyl units from uridine diphosphate glucose are subsequently transferred to the non-reducing ends of glycogen molecules by glycogen synthase (Gys). Glycogen is catabolized by glycogen phosphorylase (Pyg), releasing glucose-1-phosphate, which may be isomerized to glucose-6-phosphate. Subsequently, glucose-6-phosphate may enter the glycolytic pathway as a fuel source, or be dephosphorylated by glucose-6-phosphatase (G6pc), yielding free glucose for potential export into the systemic circulation and/or uterine lumen.

Mink exhibit obligatory embryonic diapause, with as many as 17 blastocysts in a state of arrested development for as long as 50-60 days *post coitum* resulting in delayed implantation [18,19]. It is likely that uterine glycogen reserves are an important source of energy for pre-embryonic growth and implantation. Murphy and James [20] detected glycogen deposits in uterine epithelia of mink during diapause but not post-implantation. However, there have been no subsequent reports on glycogen metabolism in the mink uterus, especially between estrus, embryonic diapause and pregnancy. Therefore, our objectives were to determine: (1): glycogen content of the uterine endometrium, glandular and luminal epithelia, stroma and myometrium, (2): the cellular localization of Gys, Pyg and Hk proteins and (3): Pyg activity in gross uterine homogenates during estrus, embryonic diapause and pregnancy in mink.

2. Materials and methods

2.1 Animals

Mink were maintained outdoors under ranch conditions, fed a mixture of chicken and fish by-products and received water *ad libitum*. Initially, uteri were collected on March 4, from unmated mink (n=3) in estrus. Additional mink (n=6) were bred according to standard farm practices which involved mating on March 3 and 4, followed by re-mating to a different male on March 12 and 13. Subsequently, uteri were obtained from mink (n=3) on March 23, flushed with 1.0 ml saline and examined for the presence of un-implanted blastocysts. Because implantation sites are not visible until the first week of April [21], and all recovered blastocysts had an intact zona pellucida, we concluded that embryos were in a state of diapause on March 23. Finally, uteri were collected from mink (n=3) on April 14 with pregnancy verified by the presence of implanted embryos. Because pregnancy (implantation to parturition) in mink is approximately 30 days, with peak parturition occurring in early May [21], we judged these uteri to be from mink in mid-pregnancy, henceforth referred to as pregnant/pregnancy.

Each mink was anesthetized with ketamine hydrochloride (20mg/kg body weight; 45-290, Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and the uterus exteriorized by mid-ventral laparotomy. Uteri were flushed with saline and/or quick-frozen on dry ice, and stored at -80.0°C. Each animal then received a lethal dose of Euthasol (011355, MWI Veterinary Supply, Boise ID). For the validation of western blot analysis (WBA), uteri were collected from anestrus rats immediately after euthanasia by CO_2 asphyxiation, quick frozen in liquid nitrogen and stored at -80.0°C. Animal care and research procedures were approved by the Institutional Animal Care and Use Committee of Idaho State University and complied with the Guide for the Care and Use of Laboratory Animals.

2.2 Glycogen concentrations in uterine homogenates

Glycogen concentrations were determined following a modified procedure of Good et al. [22]. Uterine tissue (50 mg) from each mink was lyophilized for 3 days, homogenized in 20 vol 30% KOH, and heated at 100°C for 30 min, to inactivate enzymes and destroy free glucose. To isolate glycogen, samples were diluted with 1.2 vol 95% ethanol, frozen at -80°C for 60 min, then thawed and centrifuged at 9,600 x g for 10 min. The supernatant was discarded and the pellets dried overnight. To breakdown glycogen to glucose, 100 µl 1.0 N HCl was added to each tube and heated at 90-100°C for 2.5 h. Glucose concentrations, as a measure of glycogen content, were detected using Glucose Auto Kit (439-90901, Wako Chemicals Inc., Richmond, VA) and quantified spectrophotometrically (λ=505nm), by comparing unknowns against a standard curve of increasing glucose concentrations. Intra-assay coefficient of variation was 1.3% and inter-assay coefficient of variation was 8.3%.

2.3 Glycogen content of uterine tissues

Uterine samples were fixed in 10% neutral buffered formalin, dehydrated and mounted in paraffin blocks. Transverse uterine sections (4 µm) were incubated with Periodic Acid Schiff

(PAS) reagent, and counter-stained with hematoxylin. Images were captured digitally at 25, 200 and 400x, and analyzed in triplicate for each mink using ImageJ software [23]. Because PAS stains glycogen as well as other carbohydrates, a consecutive section was pre-treated with diastase (A8220, Sigma Chemical Co., St. Louis, MO), to digest glycogen prior to PAS staining and served as a negative control. Glycogen content of each tissue was quantified by subtracting negative control values from those of sections stained with PAS without diastase and then expressed as a percentage of the area that stained positive for glycogen.

To ensure that glycogen measurements for glandular epithelia were accurate approximations of total glandular glycogen content and not affected by differences in gland number, we quantified uterine glands during estrus, diapause and pregnancy. For each mink, the total number of glands was counted in three independent cross sections at 25x, using Cell Counter in ImageJ software. To be included, a gland had to be completely separate from neighboring glands and luminal epithelium.

2.4 RNA isolation and q-PCR analysis

Total RNA was isolated from 25 to 50 mg uterine tissue from each mink using QIAGEN RNeasy Fibrous Tissue Mini Kit (74704, QIAGEN, Valencia, CA, USA) as previously described [24]. Quantitative PCR (qPCR) was performed in triplicate, using Fast Start SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN) containing forward and reverse primers (4 μM each) plus 5 μl of cDNA template (100-200 ng) per reaction. Each sample was subjected to 40 alternating cycles of a three-segment amplification program: (1). 15s denaturation at 95°C, (2). Annealing for 1 min at 55°C (*Actb, Gapdh*) or 60°C (*G6pc3*), and (3). Elongation at 72°C for 1 min. The PCR products (amplicons) were detected in real time, by measuring SYBR-Green fluorescence during the annealing stage, with the Applied Biosystems 7300 Real-Time PCR

System (Applied Biosystems, Foster City, CA, USA). Efficiency of amplicon doubling during each PCR cycle was, *Actb*: 100%, *Gapdh*: 99%, and *G6pc3*: 88%. Negative controls contained no template and amplification was never above background. Primer specificity as determined by melt-curve analyses yielded a single melting temperature for each amplicon (Table 1).

Fold changes in gene expression were calculated by the relative standard curve method. Standard curves for each amplicon, were generated from 3 pooled uteri collected from anestrus mink in November. The cDNA from these uteri was diluted (1:10, 1:100, 1:1,000 and 1:10,000), or undiluted and used to construct standard curves (cDNA ng/ml versus quantification cycle, Cq) for each gene product (R^2 = 0.98 to 0.99). We chose *Gapdh* with which to normalize our data, as it showed the least variation in expression (*Gapdh* Cq for estrus = 14.19 ±0.03, for diapause, Cq = 14.20 ± 0.04 and for pregnancy, Cq = 14.29 ± 0.02), as compared to Beta-Actin (*Actb* Cq for estrus = 22.1 ± 3.2, for diapause Cq = 20 ± 1.58 and for pregnancy Cq =19.6 ± 3.3).

2.5 Western blotting analysis

As a verification of antibody specificity for immunohistochemistry, we conducted western blot analyses (WBA) for Gys and Hk1 proteins in mink and rat uterine tissues collected during anestrus. We were unable to develop WBA for the Pyg protein. Although all primary antibodies were produced against human proteins they were validated against mouse proteins by the manufacturer (Cell Signaling Technology, Danvers, MA, USA). Antibodies against Gys were not isoform specific and could therefore bind to Gys1 (muscle) and Gys2 (liver).

In brief, uterine proteins were isolated in RIPA Lysis buffer (sc-2448, Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions. Protein concentrations were determined using Pierce BCA protein assay kit (23225, Thermo Scientific, Rockford, IL, USA). Proteins were resolved by molecular weight using SDS-PAGE and

transferred onto nitrocellulose membranes, blocked for 1 h in a 5% milk buffer containing Tris Buffered Saline (TBS) + Tween 20 (BP337-100; Fisher Scientific) to reduce non-specific binding [26]. Membranes were incubated with primary antibodies specific for Hk1 (2024S, Cell Signaling Technology, Danvers, MA, USA 1:1000 dilution) and Gys (3886S, 1:1000 dilution) overnight (4°C) in blocking solution. Subsequently, membranes were incubated with secondary antibody (anti-rabbit IgG-HRP; 7074S; Cell Signaling Technology) at 1:1000 at room temperature for 2h. Lastly, HRP substrate (WBKLS0100, Millipore, Billerica, MA) was added to the membranes and the resulting blots visualized by chemiluminescence.

2.6 Immunohistochemistry

Uterine samples were fixed in 10% neutral buffered formalin, dehydrated and mounted in paraffin blocks. To determine the uterine cell types that expressed Gys, Pyg and Hk1 proteins we analyzed tissue sections (4 μm) with immunohistochemistry according to Cell Signaling Technologies protocol (Danvers, MA) with conditions optimized for the endometrium. In brief, uterine cross sections were incubated with sodium citrate at 90°C for 20 min to unmask antigens. Sections were permeabilized with 0.3% Triton X-100 and blocked for 1 hr in 10% donkey serum (50-230-7396, Fisher Scientific), followed by a 30 min incubation at room temperature in Levamisole (196142; Sigma Aldrich) to inactivate endogenous alkaline phosphatase. Subsequently, primary antibodies for Hk1 (rabbit anti-human; 2024S, Cell Signaling Technology), total Gys (rabbit anti-human, 3886, Cell Signaling Technology), and total Pyg (goat anti-human, sc-46347, Santa Cruz Biotechnology, CA) were added to sections in 10% donkey serum, and incubated overnight at 4°C. Slides were then washed 3 times in TBS and secondary antibody (goat anti-rabbit, # T2191, Life Technologies, Grand Island, NY or donkey anti-goat; sc-2020 Santa Cruz) added in 10% donkey serum and incubated at room temperature for 2 hours. Sections were then washed in TBS, and incubated in the dark in the presence of

color substrate (SK-5300, Vector Labs, Burlingame, CA.), until the development of a blue color was detected. While the time required for color development varied between antibodies, all sections from mink collected during estrus, diapause and pregnancy that were probed with a specific antibody were each incubated for the same duration of time, to allow for a semi-quantitative analysis of the protein. After color development, sections were washed in TBS to stop the reaction and counterstained with nuclear fast red. Sections were then dehydrated through a graded ethanol series and mounted on slides with Permount (4112; Richard Allan Scientific, Kalamozoo, MI). Negative controls were similarly treated, except primary antibodies were omitted and there was no observable staining for any of the enzymes.

2.7 Glycogen Phosphorylase Activity

Mink uterine Pyg activity was measured according to Storey [25]. In brief, uterine samples (15-20 mg) were homogenized in 39 volumes of homogenization buffer (15 mM imidazole, 5mM EGTA, 100mM NaF, 0.1mM PMSF and 15mM beta mercaptoethanol) at 30°C. Samples were centrifuged at 2,000 x g for 30 seconds and 80 µl of the resulting supernatant added to the reaction buffer (final volume 1.0 ml) consisting of: 50 mM KH_2PO_4, 2 mg/ml oyster glycogen type 2, 0.4 mM NADP, 10 µM glucose-1-6-bisphosphate, 0.25 mM EDTA, and 15mM $MgCl_2$ final concentrations), with phosphoglucomutase, and glucose-6-phosphate dehydrogenase (G7877-1KU and P3397-1KU; Sigma Aldrich) in amounts that exceeded Pyg activity. Because phosphoglucomutase and glucose-6-phosphate dehydrogenase were shipped in ammonium sulfate solution, that has been shown to activate glycogen phosphorylase [26], enzyme mixtures were washed three times with KH_2PO_4 using centrifugal filter units (UFC801024, Millipore, Billerica, MA, USA) before adding them to the reaction buffer.

To measure total Pyg (phosphorylated and non-phosphorylated) activity, we added adenosine monophosphate (AMP, 25 mM final concentration) to duplicate tubes to activate any non-phosphorylated Pyg. The resulting NADPH from the reactions was detected spectrophotometrically (λ= 340 nm every 30 sec for 10 minutes). Absorption values were multiplied by the extinction coefficient of NADPH (6220/M • cm) to obtain enzyme units (U), where 1 U = amount of enzyme required to metabolize 1μM of substrate per minute. Phosphorylase activities were then normalized per milligram protein for each sample with protein concentrations determined using the Bradford assay procedure (23236, Pierce Biotechnology, Rockford, IL). To validate the assay we measured total (pPyg) and unstimulated (Pyg) activities in 20, 40, 80 and 160 μl of homogenized tissue. As expected, each successive doubling in homogenate volume resulted in an approximate doubling in NADPH produced. Because glucose exerts feedback inhibition on Pyg activity [27], we added 5.0 mM glucose to duplicate reaction mixtures, which reduced total enzyme activity 65%.

2.8 Statistical Analysis

Gross uterine glycogen concentrations, endometrial and myometrial glycogen content, mRNA, gland number, and Pyg activity were analyzed by one-way ANOVA, followed by Tukey's post- hoc test using R (version 2.13.2; http://www.r-project.org). ImageJ data for stroma, glandular and luminal epithelia were analyzed by Nested ANOVA with tissue type nested within each collection period. Differences were considered significant at $P \leq 0.05$.

3.0 Results

3.1 Total Uterine Gland Number

Total gland number per uterine cross-section (mean ± S.E) did not differ among estrus (344±22.2), diapause (269.5±34.5), and pregnancy (249.0±29.0). There was a tendency toward a greater gland number during estrus, compared to diapause and pregnancy ($P = 0.127$).

3.2 Uterine Glycogen Content

For pregnant animals, we initially dissected embryos free from the uterus and measured glycogen concentrations for inter-implantation (3.31 ± 0.99 mg/g) and implantation (3.97±0.49 mg/g) tissues to determine if the presence of embryos affected uterine glycogen content. Because there was no difference in glycogen concentrations between the two sites, all subsequent analyses for pregnant mink are for inter-implantation tissues.

Gross uterine glycogen concentrations were highest during estrus, decreased 50% by diapause, and 90% during pregnancy (Table 2; $P \leq 0.05$). ImageJ analyses revealed that glycogen content decreased 50% in the myometrium and 80% in the endometrium between estrus and diapause (Table 2, Figure 2; $P \leq 0.05$). Glycogen content of both myometrium and endometrium during pregnancy was reduced 99% compared to estrus ($P \leq 0.05$).

Endometrial glycogen deposits during estrus, were localized primarily to glandular (32% PAS +) and luminal (35% PAS +) epithelia, with only 1% of the stroma staining positive with PAS (Figure 2, Table 3). Glycogen content of the stroma, glandular and luminal epithelia was over 99% lower during diapause when compared to estrus (Table 3; Figure 2, $P \leq 0.05$), with the nutrient virtually undetectable in pregnancy. Glycogen deposits were sporadically detected within the lumen of uterine glands (Figure 2; 400x).

3.3 Uterine Glycogen Metabolizing Enzyme Expression and Activity

The validation of primary antibodies for Hk1 and Gys was achieved by WBA which revealed only a single band corresponding to the correct molecular weight for each protein in mink and rat uterine tissues (Figure 1).

During estrus, total Gys staining by immunohistochemistry was strong in the glandular epithelia and moderate in luminal epithelia (Figure 3). Signal strength for Gys protein was very

low in both epithelia during diapause and pregnancy. No Gys staining was observed in endometrial stroma during estrus, diapause or pregnancy. A strong signal for Pyg protein was detected in glandular epithelia during estrus that was reduced during diapause and undetectable in pregnancy (Figure 3). We could detect no Pyg staining in endometrial stroma during estrus, diapause or pregnancy. Total Pyg activity (+AMP), was highest during estrus and diapause when compared to pregnancy ($P \leq 0.05$; Figure 4). There was no difference in activity of unstimulated Pyg (-AMP) among estrus, diapause and pregnancy, although activity tended to be reduced in pregnancy when compared to diapause ($P = 0.10$). Immunohistochemical staining for Hk1 protein was strongest in luminal epithelia and weaker in glandular epithelia during estrus and diapause and was undetectable in both cell types during pregnancy (Figure 3). No Hk1 protein staining was observed in endometrial stroma during estrus, diapause or pregnancy. Although we were unable to detect G6pc3 by immunohistochemistry, uterine *G6pc3* mRNA expression was 5-fold higher during diapause and 3.5 fold higher during pregnancy, compared to estrus (Figure 5, $P \leq 0.05$).

4.0 Discussion

4.1 Gross and Endometrial Glycogen

Glycogen content (gross, endometrial, myometrial, epithelial and stromal) decreased from estrus, through diapause ($P<0.05$) and pregnancy ($P<0.05$; Tables 2, 3; Figure 2). These findings agree with those for animals that do not exhibit obligatory embryonic diapause such as rodents [12], cats [28], ferrets [29], and sheep [30], illustrating that uterine glycogen reserves are a potential source of energy for uterine and pre-embryonic growth through implantation and early pregnancy.

Endometrial glycogen deposits localized almost exclusively to glandular and luminal epithelia, supporting the hypothesis that glycogen is a source of glucose for uterine histotroph.

Interestingly, glycogen reserves in both epithelia were almost completely mobilized by diapause. In support of this conclusion, it requires 8-11 days, after mating induced ovulation, for the mink conceptus to develop to the dormant blastocyst stage as it enters the uterine lumen [18,19]. This suggests to us that the blastocysts recovered from mink on March 23, as a result of matings on March 12 & 13, had resided in the uterine lumen for only 2-3 days, with perhaps some still in the oviducts. While blastocysts from the first matings (March 3 & 4) probably were in the uterine lumen for a longer duration of time, all of them still had an intact zona pellucida. Desmarias et al., [31] showed that it required 10 days after blastocyst re-activation in late March to early April, before implantation occurred in mink. Moreover, circulating progesterone concentrations in mink are low until approximately March 22, then rapidly increase with implantation taking place 5-10 days later [32,33]. Collectively, these findings strongly suggest to us that mink endometrial glycogen reserves were an important source of energy for uterine and conceptus growth through the dormant blastocyst stage. As further evidence, Brown and Whittingham [34] showed that 1-cell mouse conceptuses in vitro failed to develop to the blastocyst stage unless glucose was added to the incubation media. This is consistent with the finding that as morulas develop into blastocysts, they switch from pyruvate and lactate, to glucose as the preferred energy substrate [35]. Although blastulation occurs in the oviducts, Enders [18] showed that the utero-tubal junction in mink did not present a barrier to the movement of fluids between oviductal and uterine compartments.

It is possible that mink pre-embryonic metabolism to the dormant blastocyst stage depends largely on uterine glycogen reserves as an energy source, whereas subsequent blastocyst reactivation and implantation is supported by glucose transported from the maternal circulation. Such a phenomenon could represent a distinguishing feature of the reproductive cycle of species

exhibiting obligatory embryonic diapause. Because mink pre-embryos may reside in a relatively hypoxic uterine lumen for much longer durations of time than species that do not exhibit diapause, a continuous source of glucose for anaerobic metabolism could be critical to survival, and influence the number of embryos that undergo implantation and therefore litter size at birth.

As evidence of glucose transport into the uterine lumen, many types of glucose transporters (GLUTs 1, 3, 4, and 8) have been detected in the endometrium of rat [36], sheep [37], and humans [38]. Das et al. [39] detected Glut1 protein in apical and basolateral membranes of sheep uterine glandular epithelial cells during late gestation. Korgun et al. [36] detected Glut3 protein in apical membranes of rat uterine luminal epithelia on days 1-6 of gestation only, which would include implantation around day 5. In mice, Glut1 protein expression by stromal cells was low on day 4 *post coitus* (just before implantation), increased on day 5 and was greatest on day 7 [40]. Sheep endometrial Glut1 mRNA expression increased from day 10 to 14 of pregnancy, prior to implantation (day 16), and remained elevated through day 20 [39].

Low glycogen content in the uterine stroma of mink compared to the epithelia (Table 3, Figure 2) was not surprising. The mink endometrium is reported to not undergo decidualization in preparation for implantation [41], and may have lower energy requirements than the stroma of species that decidualize. For example, rodent endometrium, which decidualizes prior to implantation, had a stromal cell glycogen content greater than the uterine epithelia [42]. Therefore, because the uterine stroma of mink delineated with ImageJ software included a considerable amount of extracellular matrix, in addition to cells, it is possible that our estimates of stromal cell glycogen content were low.

Interestingly, we detected glycogen deposits in what appeared to be the lumen of mink uterine glands (Figure 2), which agrees with previous findings [24]. Burton et al., [1] reported glycogen deposits in the lumen of human uterine glands as well the inter-vellus space separating maternal and fetal membranes at the end of the first trimester. It is possible that glycogen, secreted intact by uterine epithelia is taken up through phagocytosis by pre-embryos. The carnivore trophoblast becomes extremely phagocytic as implantation progresses [43]. Glycogen may also be degraded intra-luminally, as glycogen granules contain anabolic and catabolic enzymes. As evidence, glycogen phosphorylase activity in sheep uterine luminal fluids increased during pre-implantation, peaking near the expected time of implantation [30].

4.2 Myometrial Glycogen

Myometrial glycogen content in mink decreased 50% between estrus and diapause ($P<0.05$; Table 2). We suspect that the mobilized glycogen supported myometrial contractions facilitating sperm transport and embryo spacing. Mating induces myometrial contractions [44] and it is reasonable to predict that part of the mobilized glycogen was used to support these contractions. However, myometrial glycogen content in the rat increased the week before birth [45], presumably to support contractions during parturition, and we cannot rule out this possibility in mink.

It is possible that glycogen reserves of the myometrium contribute to endometrial and/or embryonic energy requirements, since blood vessels penetrate through the myometrium and pass into the endometrium. The immediate product of glycogen catabolism by Pyg is glucose-1-phosphate which may be converted to glucose-6-phosphate by phosphoglucomutase. This is a reversible reaction allowing for interconversion as the concentration of one or the other molecules increase. Phosphoglucomutase activity was detected in human myometrium [46].

Moreover, glucose-1-phosphatase was detected in the myometrium of the Indian desert hedgehog, and the level of enzyme activity was comparable to glandular epithelia during pro-estrus and estrus [47].

For the glucose component of glucose-6-phosphate to be exported by smooth muscle cells would require the expression of glucose-6-phosphatase. This enzyme was detected in human [46], but not goat myometrium [48]. Unfortunately, even after experimenting with a wide range of incubation times for each antibody (primary and secondary) and color development, we were unable to detect any of the enzyme proteins within the myometrium. We speculate that because the tissues had been fixed for a prolonged period of time prior to immunohistochemistry, that continued cross linking of proteins altered their conformation, reducing antigenicity. Nevertheless, our finding of increased *G6pc3* gene expression in whole uterine homogenates during diapause and pregnancy, when compared to estrus ($P<0.05$; Figure 5) agrees with the rapid reduction in myometrial glycogen content observed at these times ($P<0.05$; Table 2). The potential role of myometrial glycogen as a source of energy for endometrium and embryos, deserves further investigation.

4.3 Uterine Glycogen Metabolizing Enzyme Expression and Activity

The large reduction in glycogen content of the glandular and luminal epithelia between estrus and diapause (Table 3) occurred in parallel with a pronounced reduction in the amount of Gys protein (Figure 3). Uterine Pyg protein staining was strongest in the glandular epithelia during estrus, it decreased by diapause and was undetectable in pregnancy (Figure 3). Consistent with these findings, mink uterine Pyg activity (total) was significantly greater during estrus and diapause when compared to pregnancy (Figure 4). We interpret these findings to mean that as

Gys protein levels (and presumably activity) decreased between estrus and diapause, the relatively constant level of Pyg activity resulted in uterine glycogen mobilization.

While uterine glycogen synthesis in mink appears to be predominantly under estrogenic regulation, the influence of hormones on glycogen catabolism is poorly understood. Because circulating progesterone concentrations in mink increase following mating induced ovulation [33], it is reasonable to expect that the hormone might be involved in uterine glycogen catabolism. Paul and Duttagupta [49] showed that in bilaterally ovariectomized rats, exogenous estradiol increased uterine glycogen content, whereas concomitant treatment with progesterone, significantly inhibited glycogen synthesis in a dose-dependent pattern. While progesterone might inhibit uterine glycogen synthesis in mink, perhaps by reducing Gys expression, it is also possible that the hormone promotes glycogen catabolism. Preliminary findings, from our laboratory show that in ovariectomized mink pre-treated for 3 days with estradiol, followed by progesterone for 3 days, uterine glycogen content was significantly lower than in mink treated with estradiol alone (unpublished data). This suggests to us that progesterone was glycogenolytic in the mink uterus. Because prolactin is luteotropic in mink and circulating concentrations of the hormone increase before progesterone, it is also possible that prolactin affects uterine glycogen metabolism. Prolactin receptors have been detected in the mink uterus and ovaries [50,51], and the hormone has been shown to stimulate glycogenolysis in the rat epididymis [52].

Immunohistochemical detection of Hk1 protein was strongest in luminal epithelia during estrus and diapause and undetectable in pregnancy (Figure 3). In rats, uterine Hk activity was higher during estrus than any other stage of the estrous cycle [54]. In pregnant rats, uterine Hk activity peaked on day 1, days 5-6 (peri-implantation) and again on day 12, during mid-gestation [13]. Spellman et al. [53] showed that Hk activity in human endometrium increased

progressively throughout the menstrual cycle, being significantly higher during secretory than proliferative phases. Species differences probably account for some of these inconsistencies. Nevertheless, hexokinase activity during diapause in mink could contribute to glucose trapping by the epithelia, after glycogen stores were depleted.

In summary, our findings indicate to us that glycogen, synthesized by uterine glandular and luminal epithelia of the mink is likely to be a significant source of glucose for histotrophic secretions. Both Gys and Pyg proteins were most evident in glandular epithelia. While Pyg protein was detected during estrus and diapause, Gys protein was almost undetectable post-estrus. Uterine Pyg activity (total) was higher during estrus and diapause than pregnancy. We interpret these findings to mean that as uterine glycogen synthesis decreases between estrus and diapause, this unmasks the glycogenolytic activity of Pyg. Consistent with our hypothesis that the resulting glucose from glycogen breakdown and/or Hk1 trapping, may be exported into the uterine lumen, *G6pc3* mRNA expression was 5-fold greater during diapause, compared to estrus. The near complete mobilization of glycogen from uterine luminal and glandular epithelia, by the diapausing blastocyst stage, suggests that this nutrient may in part, determine the number of embryos that survive to the peri-implantation stage and perhaps litter size at birth.

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Table 1. Primers used for qPCR of mink uterine gene transcripts and amplicon characteristics.

Genes	Forward & reverse primers	Amplicon	Melt Temp (°C)	Accession No.*
G6pc3	ATCAGCCTAGCCTTCAAGTGGTGT AGG CTATCTTCTGGCCATTTCCCA	187 bp	87.78 ± 0.22	NM_138387.3
Gapdh	TTGTCAGCAATGCCTCCTGTACCA ACCAGTGGAAGCAGGGATGATGTT	200 bp	85.03 ± 0.48	AF076283.1
Actb	GATGACC CAGATCATGTTCGAG CCATCTCCTGCTCGAAGTCC	321 bp	86.0 ± 0.03	AF076283.1

Glucose-6-phosphatase-3 (*G6pc3*); Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*); beta-actin (*Actb*).

*http:/www.ncbi.nlm.nih.gov.

Table 2. Mean (±S.E.) glycogen concentrations in whole uteri, and percent glycogen content of uterine myometrium and endometrium from mink collected during estrus, diapause and pregnancy.

	Gross Glycogen (mg/ g tissue)	Myometrial Glycogen (% Area PAS+)	Endometrial Glycogen (% Area PAS+)
Estrus	25.67±1.44^{A}	31.64 ± 1.2^{A}	36.07 ± 2.01^{A}
Diapause	11.51±2.08^{B}	17.45 ± 0.60^{B}	7.79 ± 1.51^{B}
Pregnancy	3.31±0.99^{C}	0.21 ± 0.08^{C}	0.44 ± 0.44^{C}

$^{A-C}$Within a column, means with different superscripts differ ($P \leq 0.05$).

Table 3. Mean (±S.E.) percent glycogen content of uterine glandular epithelia, luminal epithelia, and stroma for mink collected during estrus, diapause and pregnancy.

	Glandular Epithelia (% Area PAS+)	Luminal Epithelia (% Area PAS+)	Stroma (% Area PAS+)
Estrus	$31.92 \pm 6.90^{A,a}$	$34.90 \pm 1.58^{A,a}$	$1.079 \pm 0.21^{A,b}$
Diapause	$0.096 \pm 0.077^{B,a}$	$0.26 \pm 0.18^{B,a}$	$0.015 \pm 0.015^{B,a}$
Pregnancy	undetectable^{B,a}	$0.11 \pm 0.06^{B,a}$	$0.003 \pm 0.002^{B,a}$

$^{A-C}$Within a column, means with different superscripts differ ($P \leq 0.05$).

^{a,b}Within a row, means with differ superscripts differ ($P \leq 0.05$).

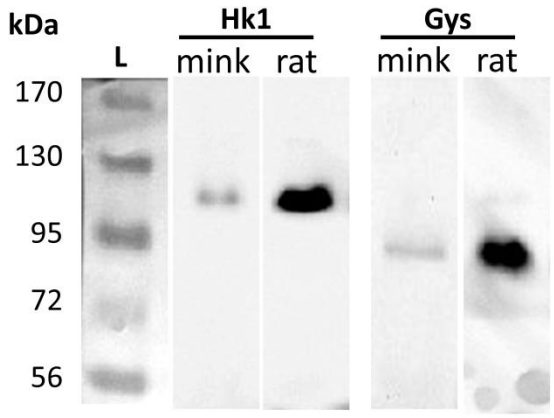


Figure 1. Western blot analyses for hexokinase 1 (Hk1) and glycogen synthase (Gys) proteins in mink and rat uterine tissues collected during anestrus. Ladder (L) with molecular weights in kilodaltons (kDa).

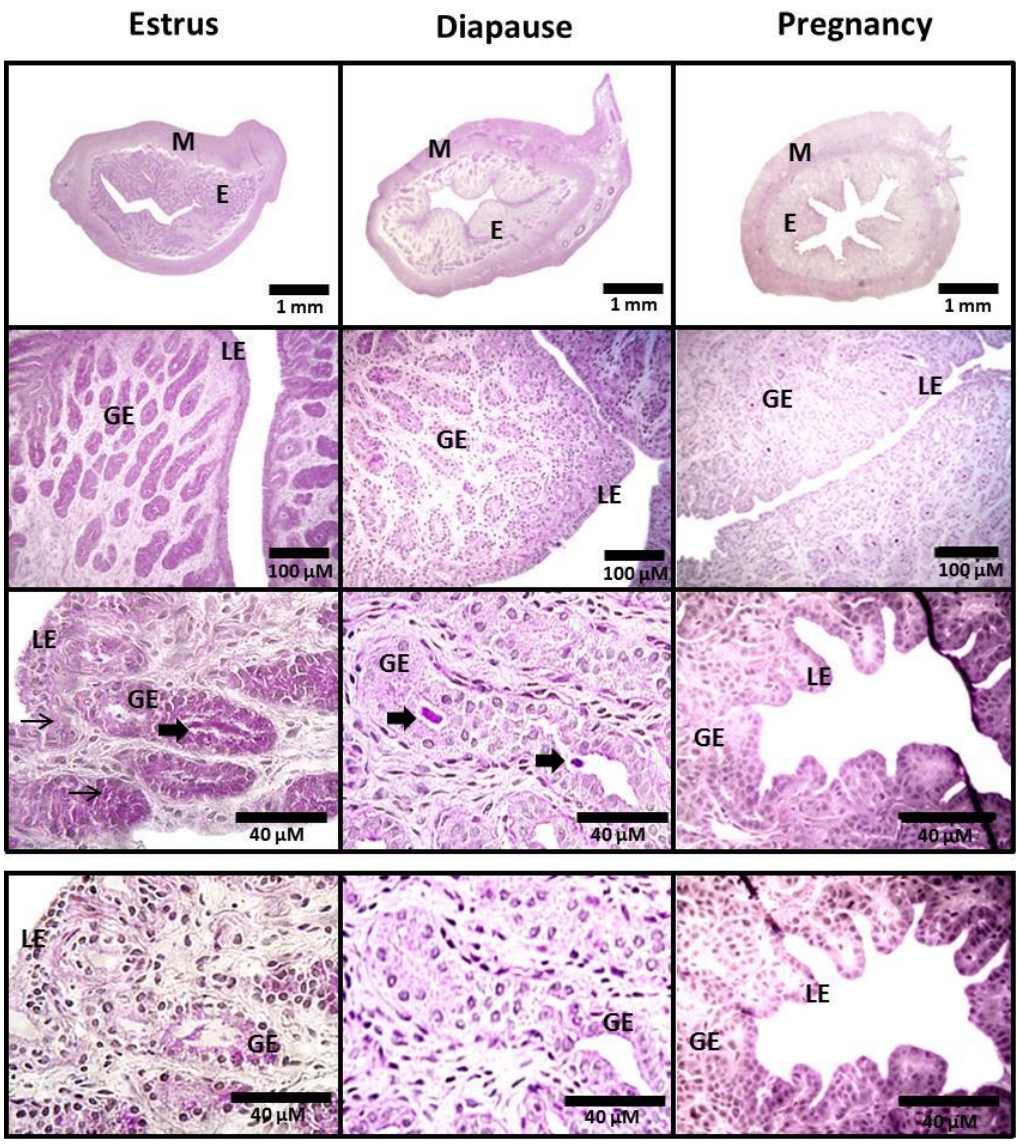
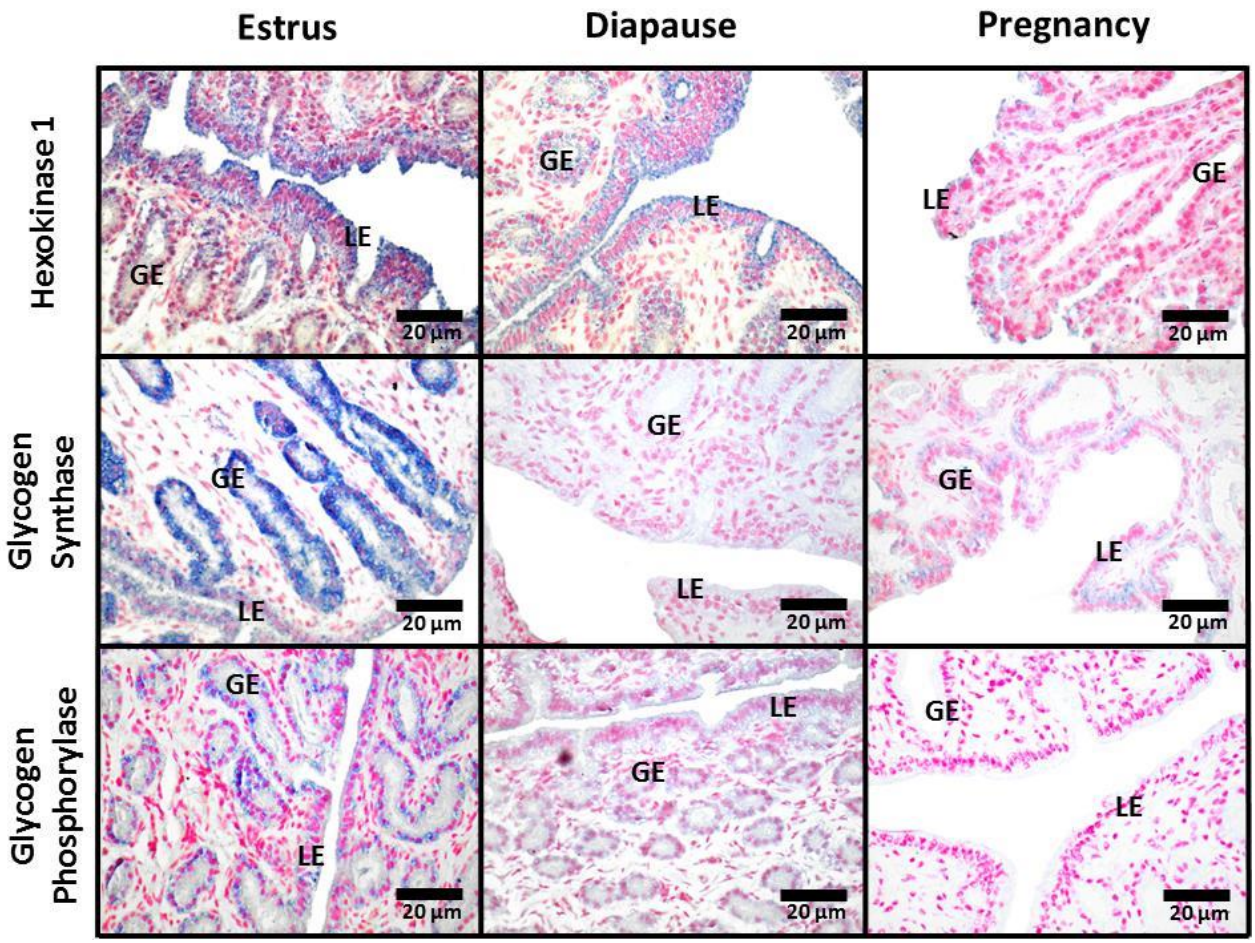


Figure 2. TOP PANEL: Uterine cross sectional images of mink collected during estrus, diapause and pregnancy. All sections were stained with Periodic Acid Schiff (PAS) and counterstained with hematoxylin. Images were captured at 25x (top row), 200x (middle row), or 400x (bottom row). BOTTOM PANEL: Sections (400x) were pre-treated with diastase to digest glycogen, prior to PAS and hematoxylin staining to illustrate a negative control. Small arrows identify areas of positive PAS staining in epithelial cells. Large arrows identify positive PAS staining within the glandular lumen.



M, myometrium; E, endometrium; GE, glandular epithelium; LE, luminal epithelium.

Figure 3. Immunohistochemical localization of hexokinase-1 (Hk1), glycogen synthase (Gys) and glycogen phosphorylase (Pyg) proteins in uterine cross sections from mink collected during estrus, diapause and pregnancy. Images were captured at 400x. GE, glandular epithelium; LE, luminal epithelium.

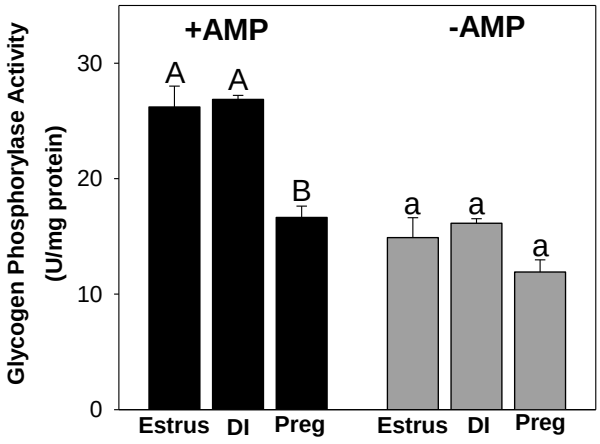


Figure 4. Total (+AMP) and un-stimulated (-AMP) glycogen phosphorylase activities in mink uterine homogenates from animals collected during estrus, diapause (DI) and pregnancy (Preg). $^{A,B;a,b}$ Groups without a common superscript differ ($P \leq 0.05$).

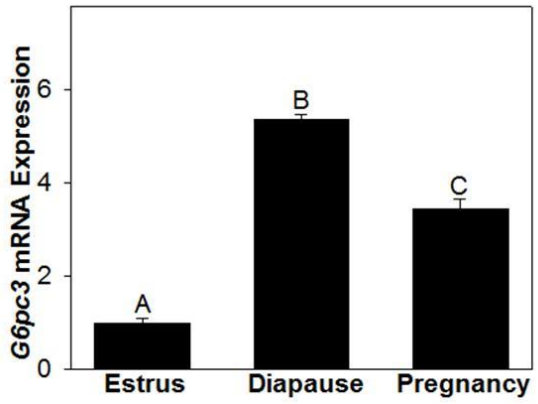


Figure 5. Relative (mean ±S.E.) mRNA expression levels for glucose-6-phosphatase 3 (*G6pc3*) by the uteri of mink collected during estrus, diapause and pregnancy.

[a-c] For each enzyme, groups without a common letter differ (P≤0.05)

5. Chapter 2

Evidence that Estradiol Stimulates Glycogen Accumulation in Uterine Epithelial Cells from Mink both Directly and through IGF-1

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Abstract

Estradiol stimulates accumulation of uterine glycogen during estrus, which is then used to support embryonic/uterine glucose needs after mating. However, the underlying mechanism is unknown. Here we use immortalized epithelial cells from the mink uterus (GMMe) to elucidate the process. First, GMMe cells displayed an epithelial phenotype and expressed several epithelial cell markers (cytokeratin 18, mucin 1, and insulin-like growth factor binding protein 3 (*Igfbp3*). GMMe cells also expressed the glycogen metabolizing enzymes hexokinase 1, glycogen synthase 1, glycogen phosphorylase- muscle, and glucose-6-phosphatase 3. Estradiol failed to stimulate glycogenesis in chemically defined media. In contrast, high concentration of insulin (16.6 and 50.0 μm/ml) significantly increased glycogen concentrations ($P \leq 0.05$), an effect inhibited by the insulin-like growth factor 1 (Igf1) receptor antagonist PPPT. Next, the PI3K inhibitor LY294002 also inhibited insulin-stimulated glycogen synthesis. In the presence of insulin, 10 nM estradiol significantly increased the glycogen concentration ($P \leq 0.05$). GMMe cells did not express estrogen receptor β or G-protein estrogen receptor (Gper) 1. However, GMMe cells did express estrogen receptor α and the glycogenic effect of estradiol was blocked by inclusion of ICI 182,780 (estrogen receptor α/β antagonist). Combined with previous reports that estradiol stimulates uterine Igf1 production, these results suggest that estradiol indirectly stimulates glycogen formation via stromal production of Igf1. Activation of the Igf1 receptor then stimulates glycogen synthesis through the PI3K/AKT pathway. In the presence of insulin/Igf estradiol can also directly increase glycogen storage.

Keywords: insulin-like growth factor, estrogen, glycogenesis, GMMe, histotroph

Introduction

Prior to implantation, embryos (termed pre-embryos) are completely dependent on uterine secretions into the lumen, termed histotroph, which includes growth factors, hormones, and nutrients such as glucose (Burton et al., 2002; Gao et al., 2009). Preembryos absolutely require glucose during cleavage development, and after cavitation, glucose uptake increases exponentially (Gardner et al., 1993; Leese, 2012). As the uterus cannot produce glucose (Yánez et al., 2003a; Zimmer and Magnuson, 1990a), glucose secreted into the uterine lumen must come directly from maternal circulation (Leese et al., 2007) and/or catabolism of uterine glycogen reserves (Demers et al., 1972). Evidencing that endometrial glycogen contributes to fertility, multiple studies have found that a subset of infertile women have diminished endometrial glycogen reserves (Gupta et al., 2013; Maeyama et al., 1977). Additionally, endometrial glycogen is mobilized during the pre-implantation period in mice and sheep (Demers et al., 1972; O'Shea and Murdoch, 1970). In mink, we have recently shown that endometrial glycogen is localized specifically to the epithelia at estrus and this glycogen is completely mobilized prior to implantation (Dean et al., 2014).

Sex steroids, not insulin, stimulate uterine glycogenesis *in vivo* (Hay et al., 1984; Rose et al., 2011; Swigart et al., 1962), but the steroid responsible for uterine glycogen accumulation varies with species. In some species (guinea pigs and humans) progesterone stimulates endometrial glycogen accumulation (Casimiri et al., 1980; Ishihara et al., 1988), while in other species (rats and mink) estradiol stimulates endometrial glycogen synthesis (Paul and Duttagupta, 1973; Rose et al., 2011; respectively). Currently it is unclear if the effects of ovarian steroids on epithelial glycogen are direct. In endometrial biopsies (containing epithelium and stroma) from humans and guinea pigs, progesterone increased the glycogen concentration (Demers et al., 1977; Ishihara et al., 1988). However, culturing rodent epithelia and stroma separately, Casimiri et al.

(1980) found that estradiol increased glycogen content of stroma but had minimal effects on the epithelia. Complicating interpretation, the media used by Demers et al. (1977) and Ishihara (1988) contained exogenous insulin, and while the media used by Casimir (1980) did not contain insulin, it did contain 10% fetal bovine serum (FBS).

Estradiol has direct effects of uterine epithelia, such as increasing expression of leukemia inhibitory factor (*Lif* Winuthayanon et al., 2010); however, effects of estradiol on the epithelia can also be mediated indirectly by insulin like growth factor-1 (Igf1). As evidence, estradiol increases epithelial mitosis in mice lacking *Esr1* (also called estrogen receptor α) in uterine epithelia (Winuthayanon et al., 2010) but not in Igf1 knockout mice (Adesanya et al., 1999). In agreement with this conjecture, estradiol also increases the production of Igf1 by uterine stroma (Ogo et al., 2014) and decreases expression of insulin-like growth factor binding protein 3 in the ovine uterus (Hayashi et al., 2005), which collectively results in more biologically active Igf1. These finding raise the question of how estradiol stimulates epithelial glycogensis, directly or through Igf1.

Mink embryos undergo embryonic diapause for up to 50 days before implantation (Sundqvist et al., 1989), making mink a useful model of histotrophic biology. Paracrine communication can be difficult to study *in vivo*, but Moreau *et al.* (1995) produced an immortalized cell line from the epithelia of the mink uterus (GMMe cells). These cells might prove useful for dissecting control of glycogen metabolism in the uterine epithelium, but it has not been shown that they metabolize glycogen. Therefore, our objectives were to determine if GMMe are a suitable model for the study of mink uterine physiology, if estradiol simulates glycogenesis in these cells directly, and what role(s) insulin or Igf1 play in epithelial glycogenesis using this in vitro model.

2. Materials and Methods

Cells

Epithelial cells from the mink uterus that were immortalized by transfection with a plasmid encoding the SV40 large T antigen (GMMe cells; Moreau et al., 1995) were purchased from American Tissue and Culture Collection (CRL-2674). Cells were maintained in T-75 flasks (430641, Corning) at 37°C with a humidified mixture of 5% CO_2 and 95% air. Cells were grown in complete media consisting of 47.5% DMEM (11966-025, Life Technologies), 47.5% Ham's F12 (11765-054, Life Technologies), 5% FBS (03-600-511, Fisher Scientific) and 1x penicillin-streptomycin (30-002-CI , Cellgro). Complete media was replaced at least once per week, and experiments were conducted at pass numbers <20 (pass 1= delivered from ATCC).

Cell Images

Images of confluent layers of GMMe cells in complete media were captured with a Leica DMIL inverted microscope using a Leica DFC295 digital camera. Images were captured at 200x.

RNA Isolation and RT-PCR

Confluent cells in complete media were collected via trypsin (which cleaves proteins holding cells to the flask; 25-052-CI, Cellgro) and counted with a Coulter Z2 cell counter (Beckman Coulter, Brea, CA). Total RNA was isolated from $5x10^6$ cells using the Qiagen RNeasy MiniKit (75144) per the manufacturer's instructions. Purity was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific), and only RNA with 260:280 and 260:230 ratios >1.9 was used. RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (205313, Qiagen), and the resulting cDNA was diluted 10-fold prior RT-PCR using DreamTaq Green PCR Mater Mix (K1081, Thermo Scientific). Each reaction contained 12.5 µl of DreamTaq Master Mix, 5 µl of diluted cDNA, forward and reverse primers (450 nM final concentration, from Integrated DNA Technologies), and enough water to bring the total volume to 25 µl. The PCR reaction was carried out with a Dyad DNA Engine thermocycler (BioRad)

and started with an initial denaturation (95°C for 3 min). Cycling consisted of 40 cycles of denaturation (95°C for 30 seconds), annealing (54°C for 30 seconds), and extension (72°C for 1 minute) phases. PCR products were separated on a 1.5% agrose gel containing ethidium bromide next to a ladder of known molecular sizes. Images were captured using a VersaDoc imaging machine (Bio-Rad). No template and no reverse transcriptase (RT) negative controls were used in all reactions (not shown) and cDNA from anestrus mink uteri was used as a positive control (shown).

To confirm primer specificity, amplicons from mink uterine tissue was purified from a 1% agrose gel using the QIAquck Gel Extraction Kit (28704, Qiagen), following the manufacturer's instructions. Purified amplicons were submitted to the Idaho State University Molecular Research Core Facility for sequencing. Obtained sequences were aligned against corresponding sequences in the mink, ferret, or rat using BLAST (Table 1).

Primers for glycogen phosphorylase- muscle (*Pygm*), hexokinase 1 (*Hk1*), and glycogen synthase 1 (*Gys1*) were designed using mink sequences. Primers for cytokeratin 18 (*Krt18*), mucin 1 (*Muc1*), insulin-like growth factor binding protein 3 (*Igfbp3*), vimentin (*Vim*), Igf1 receptor (*Igf1r*), estrogen receptor α (*Esr1*), estrogen receptor β (*Esr2*), and G-protein estrogen receptor (*Gper1*) were based on conserved regions of ferret sequences. Primers for the insulin receptor (*Insr*) were based on conserved regions of the rat sequence. Conserved regions were determined by aligning sequences from multiple mammalians species (e.g. ferret, rat, human, cow, and dog) as available using BLAST, and all primers were order of Integrated DNA technologies.

Cell Viability Assay

To determine cell viability in response to dimethyl soulfoxide (DMSO, used a vehicle for steroids and inhibitors) and acetic acid (vehicle for insulin), $1x10^5$ GMMe cells per well were

seeded into a 96-well plate (165306, ThermoScientific) in complete media. Approximately 12 hours later, complete media was removed, the cells were rinsed 2x with PBS, and 100 µl of treatment media with either DMSO (0.0%, 0.1%, 0.2%, or 0.4%) or acetic acid (0.000% or 0.0046%) was added to each well. Cells were incubated with treatment for 48 hours, after which, one volume of CellTiter-Glo Substrate reconstituted in CellTiter-Glo Buffer (G7570, Promega) was added to each well. The plate was incubated with shaking for 20 minutes and relative luminescence (which reflects total ATP content), as a proxy for number of metabolic active cells, was measured with a Synergy HT Multi-Mode Microplate Reader (Bio-Tek).

Treatment media was composed of 1:1 mixture of the same F12 media used in the complete media, but the DMEM (A14430-01, Life Technologies) contained no glucose or phenol red. Thus the final concentration of glucose was 5 mM and phenol red was 1.59 µM. This concentration of phenol red is 50% lower than the lowest concentration that Berthois et al. (1986) found to have detectable estrogenic effects. Additionally, treatment media was supplemented with glutamine (final concentration 2.5 mM; 25030-081, Life Technologies) and 1x penicillin-streptomycin (30-002-CI, Cellgro).

Glycogen Experiments

GMMe cells ($1.5x10^6$) were seeded into T-75 flasks in complete media. Two days after reaching confluence, complete media was removed, the cells were rinsed 2x with PBS, and treatment media plus appropriate treatment was added to each flask. After 48 hours of treatment, media was removed, cells were collected with trypsin, and the number of cells was counted by a Coulter Z2 cell counter (Beckman Coulter, Brea, CA). 10^7 cells were pipetted into 1.5 ml microfuge tubes, centrifuged at 2,000xg, washed with PBS, and stored at -80°C until analysis.

Glycogen was measured according to the method of Good et al. (Good et al., 1933) and previously described for uterine tissue (Dean et al., 2014) adapted for cultured cells. Cells were resuspended in 500 µl 30% KOH and then snap frozen, thawed at 100°C, and vortexed three times to ensure lysis. After heating to 100°C for 30 minutes, 600 µl of 95% ethanol was added to each sample. Samples were then frozen at -80°C for 1 hour, thawed, and centrifuged at 16,000 x g for 10 min. Supernatant was removed and the pellet dried overnight. The glycogen was hydrolyzed by incubation in 50 µl 1.0 M HCl at 100°C for 2.5 hours. The resulting glucose, as a proxy for glycogen, was measured against a standard curve of increasing glucose concentrations (0.0195-10.0 mM) using the Wako Autokit (Wako Diagnostics, Richmond, VA; #439-90901) per the manufacturer's instructions. The maximal intra-assay CV was 2.0% and the inter-assay CV as 2.3%.

Hormones and Inhibitors

Estradiol (E2; 194565, MP Biomedical), picropodophyllotoxin (PPPT; Igf1r antagonist; ab144623, Abcam), LY294002 (LY; PI3K inhibitor; 70920,Cayman Chemical), and ICI 182,870 (ICI; estrogen receptor antagonist; 100112691, Caymen Chemical) were dissolved in DMSO prior to being added to the media. Recombinant human insulin (91077C, Sigma Aldrich) was dissolved in 1% acetic acid. All drugs were filter sterilized using a 0.22 µm filter (SLGP033RS , Millipore), diluted as necessary, and added to media to achieve indicated concentrations. Vehicle (acetic acid and/or DMSO) was then added to controls. The concentration of acetic acid in all experiments with insulin was 0.0046%. In experiments with one compound dissolved in DMSO, the final concentration of DMSO was 0.1% and in experiments with 2 compounds in DMSO (estradiol and ICI, for example) the final concentration of DMSO was 0.2%. In experiments utilizing inhibitors (PPPT, LY294002, PD18416, ICI), the inhibitors were added to cells 1 hour before hormones.

Statistical Analysis

Data from experiments with two groups were analyzed by a Student's T test. Data with more than two treatment groups were analyzed by ANOVA and then the Bonferroni method, with each treatment group compared to control as indicated. Analysis was conducted using Sigma Plot 11.0. All experiments were replicated ≥ 4 times and data are presented as mean ± SEM.

3. Results

GMMe Validation

Histologically GMMe cells displayed a cobblestone-like appearance (Figure 1A). RT-PCR revealed that GMMe cells express the epithelial cell markers *Krt18* and *Muc1*, (Abe and Oshima, 1990; Brayman et al., 2004), and the cells expressed *Igfbp3* (Figure 1B), which has been shown to be localized to the uterine epithelia in sheep (Satterfield et al., 2008). While the cells expressed the known stromal cell marker *Vim* (data not shown; Leader et al., 1987), this agrees with their original characterization (Moreau et al., 1995). The cells also expressed glycogen mertabolizing enzymes (*Hk1*, *Gys1*, *Pygm*, and *G6pc3*; Figure 1C).

Next we wished to determine the effect of vehicle (DMSO and acetic acid) on GMMe viability. 0.4% DMSO decreased cell viability by 16% relative to 0.0% DMSO ($P \leq 0.05$), but lower concentrations (0.1% and 0.2%) had no effect (data not shown). 0.0046% acetic acid had no effect compared to treatment media without acetic acid (data not shown). Subsequent experiments using DMSO as vehicle were conducted with 0.1% or 0.2% DMSO and experiments with insulin used 0.0046% acetic acid.

Insulin Increases GMMe Glycogen via the IGF-1 Receptor

Estradiol alone failed to alter the glycogen concentration in GMMe cells in chemically defined media (Figure 2). Earlier *in vitro* studies which found that ovarian steroids altered endomemtrial glycogen (Demers et al. 1977; Ishihara et al. 1980) had exogenous insulin in the culture media.

Therefore we tested the ability of insulin to affect glycogen metabolism. The lower concentrations of insulin used (1.8 and 5.5 μg/ml) tended to increase glycogen concentrations ($P \leq 0.1$), and increased significantly at 16.6 and 50.0 μg/ml ($P \leq 0.05$, Figure 4A). Interestingly, all four concentrations tested had similar effects, approximately doubling glycogen content compared to controls (control: 0.21±0.04 $\mu g/10^6$ cells; all treatments combined: 0.40±0.03 $\mu g/10^6$ cells).

Using RT-PCR, the insulin receptor (*Insr*) was barely detectable, but the insulin like growth factor 1 receptor (*Igf1r)* was readily detected (Figure 3B), indicating that the Igf1 receptor may be mediating the increased glycogen concentration in response to insulin (Baudry et al., 2001). To test this hypothesis, we tested the ability of PPPT (Igf1r antagonist) to block insulin's actions. Insulin approximately tripled glycogen content of these cells ($P \leq 0.05$), and inclusion of PPPT abolished this effect (Figure 3C).

In myoblasts, the Igf1 stimualtes glycogen synthesis via the PI3K/AKT pathway (Sasaoka et al., 2001). To confirm that the PI3K/AKT pathway mediates the insulin/Igf1r induced stimulation of glycogen accumulation in GMMe cells we used LY294002 (PI3K inhibitor; Vlahos et al., 1994). Insulin increased the glycogen concentration of GMMe cells approximately 4-fold ($P \leq 0.05$), and LY294002 completely abolished this effect (Figure 3D).

Estradiol Stimulates Epithelial Glycogenesis in the Presence of Insulin

While estradiol could not increase glycogen concentration without insulin (Figure 1), it was still possible that estradiol could augment glycogenesis in the presence of insulin. We next tested the ability of estradiol to increase the glycogen concentrations of GMMe cells in the presence of 50 μg/ml of insulin. In the presence of insulin, 0.1 and 1.0 nM estradiol had no effect on glycogen concentration, but 10 nM estradiol significantly increased the glycogen concentration of GMMe

cells (P≤0.05) over 0 nM estradiol. At 100 nM estradiol, glycogen concentrations tended to be higher than 0 nM estradiol (P≤0.10, Figure 4A).

RT-PCR revealed that GMMe cells express estrogen receptor α (*Esr1*), but not estrogen receptor β (*Esr2*) or the G-protein coupled receptor (*Gper1*, Figure 4B). To confirm that estradiol was increasing glycogenesis via Esr1 we used ICI to block estradiol's actions. As previously shown, 10 nM of E2 increase glycogen concentrations in GMMe cells, in the presence of insulin (P≤0.05), and this effect was completely abrogated in the presence of ICI (Figure 4C).

4. Discussion

Our findings indicate that GMMe cells exhibit an epithelial morphology and express epithelial cell markers such as *Krt18*, *Muc1*, and *Igfbp3* (Figure 1; Abe and Oshima, 1990; Brayman et al., 2004; Satterfield et al., 2008). The cells also expressed *Vim*, which is considered a stromal cell marker (Leader et al., 1987). However, this may not limit their usefulness because GMMe cells expressed Vim during their initial characterization (Moreau et al., 1995), in culture most cell types (regardless of origin) express Vim (Franke et al, 1979) and Vim is not known to affect glycogen metabolism. GMMe cells also express G*ys1*, *Hk1*, *Pygm*, and *G6pc3* (Figure 1) showing they can synthesis and catabolize glycogen, and we have previously localized three of these proteins (Gys, Hk1, and Pyg) to the epithelia of the mink uterus (Dean et al., 2014). Collectively, these results support the use of GMMe cells as a model for glycogen metabolism in epithelia of the mink uterus.

It is clear that estradiol stimulates gross glycogen accumulation in the uterus of rats (Bitman et al., 1965; Bo et al., 1973), and we have shown that estradiol increases both the gross and epithelial glycogen content in the uterus of mink (Rose et al., 2011). The lack of effect of estradiol, in the absence of insulin, may seem surprising, but this agrees with Demers et al.

(1977) who found that progesterone had no effect on glycogen concentration in the guinea pig endometrium if insulin was absent from the media. It is well established that estradiol can have both direct and indirect actions on uterine epithelia (Adesanya et al., 1999). This led us to hypothesize that the glycogenic action of estradiol is indirect, perhaps similar to the mitotic actions of estradiol on the uterine epithelia (Adesanya et al., 1999).

Our finding that insulin increases the glycogen concentration of GMMe cells (Figure 3) may suggest that systemic insulin stimulates epithelial glycogen synthesis *in vivo*, but we believe this to be unlikely. First, pharmacological concentrations of insulin were required to achieve a significant effect (admittedly the insulin was from a different species and may have low affinity for the mink insulin receptor), and secondly, it is well established that high concentration of insulin can activate Igf1r (Baudry et al., 2001; De Meyts, 2002). More directly, we found that GMMe cells express very low levels of *Insr* mRNA, and PPPT, a specific Igf1r antagonist (Girnita et al., 2004), blocked the glycogenic effect of insulin. While we did not stimulate GMMe cells with Igf1, the production of Igfbp3 by GMMe cells (Figure 1) coupled to species differences would make such an experiment difficult to interpret.

In agreement with our conclusion that Igf1, not insulin, simulates epithelial glycogenesis, Swigart et al. (1962) found that neither insulin nor glucose gavage increased uterine glycogen concentration in rats. In contrast, estradiol doubled the glycogen concentration in the uterus, regardless of other co-treatments (insulin or glucose). Using glucose clamp methodology, Hay et al. (1984) found that high glucose concentrations in sheep, which increased plasma insulin concentrations, did not affect the uptake of glucose by the uterus.

This is the first study to link Igf1 to uterine glycogen metabolism, but Igf1 has been shown to stimulate glycogenesis in other tissues. For example, Park et al. (1999) found that activation of

both the Insr and the Igf1r stimulated glycogenesis in murine hepatocytes, albeit insulin was more potent. Yamamoto-Honda et al. (1995), however, demonstrated Igf1 to be more potent than insulin in activation of glycogen synthase in PC12 cells (orginially isolated from the rat adrenal medulla). And Igf1 knockout reduced the glycogen stores in neurons of mice (Cheng et al., 2000). Further evidence that Igf1, not insulin, stimulates uterine epithleial glycogen accumulation is that the epithleia of many species express the Igf1r (Hayashi et al., 2005; Tang et al., 1994; Zhu and Pollard, 2007).

Igf1 stimulating epithelial glycogenesis could come from two distinct sources: systemic circulation or uterine stroma. Many studies have shown that estradiol stimulates the uterine stroma to produced Igf1. Maekawa et al. (2009) found that Igf1 expression was highest at proestrus in mice, an effect caused by estradiol. And in cultured murine stromal cells, Ogo (2014) showed estradiol increased Igf1 via Esr1. In the bovine uterus, Igf1 expression peaked at estrus in the sub-epithelial stroma (Robinson et al., 2000). In the neonatal ovine uterus, chronic treatment with estradiol-17b valerate resulted in increased Igf1 expression (Hayashi et al., 2005).

In contrast, Sato et al. (2013) transplanted *Igf1*$^{-/-}$ uteri into the kidney capsule of wildtype mice and found that estradiol stimulated normal proliferation in the model, indicating that systemic, not local, Igf1 mediates the actions of estradiol. In line with this, estradiol decreases uterine *Igfbp3* expression and increases expression of pregnancy associated plasma protein A (*Pappa*, an Igfpb protease; Hayashi et al., 2005; Maekawa et al., 2009). Collectively these results indicate that estradiol increases the levels of biologically-active Igf1 in the uterus through a variety of mechanisms.

In the presence of insulin, estradiol did increase glycogen concentrations, indicating that estradiol can directly increase glycogen concentrations of the epithelia. Gper1 is a membrane-

bound estrogen receptor that has been localized to the uterus (Gao et al., 2011) and been shown to be important in plasma glucose homeostasis (Mårtensson et al., 2009). We rule out its involvement because of the lack of mRNA expression in GMMe cells (Figure 5B) and because the estrogen receptor antagonist ICI does not antagonize Gper1 (Chen et al., 2014).

In contrast, we detected *Esr1* in GMMe cells and ICI inhibited the glycogenic action of estradiol (Figure 5). Esr1 is well established as a ligand-activated transcription factor, but Esr1 has also been localized to the plasma membrane (reviewed in Levin, 2009). In MCF-7 cells, estradiol and BSA-conjugated estradiol increased IRS1 protein levels and the effect was blocked by ICI, indicating involvement of Esr1 or Esr2 expressed on the cell surface (Bernard et al., 2006). In the uterus, estradiol has been shown to increase expression of the Igf1r (Murphy and Ghahary, 1990). Collectively these results suggest that the estradiol induced increased glycogen concentrations (in the presence of insulin) may be due to increased sensitivity to insulin/Igf.

Conclusion

In conclusion, GMMe cells are useful model for delineating paracrine interactions controlling glycogen metabolism in the endometrium of mink. Using these cells we show that, estradiol has no effect on glycogen concentration in the absence of insulin. However, estradiol can directly stimulate glycogen accumulation in GMMe cells via Esr1 in the presence of insulin. In addition, insulin stimulates glycogenesis is through the Igf1r and the PI3K pathway. These results suggest that *in vivo*, estradiol increases epithelial glycogen concentrations both indirectly (through local Igf1) and directly via the Esr1.

Acknowledgements

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Table 1. Gene, common name, primer sequences, accession numbers, amplicons size, and BLAST results for RT-PCR primers used to characterize GMMe cells.

Gene	Common Name	Primer Sequence (5'→3')	Accession Number*	Amplicon Size (bp)	Percent Identical*	E Value*
Krt18	Cytokeratin 18	f-CCACCTTCTCTTCCAACTATC r-GTTCCGTCTCATACTTGACTC	XM_004818919.1	507	90%	$5x10^{-65}$
Muc1	Mucin 1	f-GAGCTGCAGAGGAACATTT r-TGCAGAGACCTCCTCATAG	XM_004778649.1	591	97%	0.0
Vim	Vimentin	f-CAGTCTTTCAGACAGGATGTT r-GCCAATAGTGTCTTGGTAGTT	XM_004757575.1	480	98%	0.0
Igfbp3	IGF Binding Protein 3	f-GCACACCAAGATAGACATCAT r-CCAAGTGCCTGAGACAATC	XM_004793933.1	513	98%	0.0
Igf1	Insulin-Like Growth Factor 1	f-CAGGATCATTTGCTCTGTAGG r-TGATGAAGTGGAGGGTAGAG	XM_004787155.1	483	98%	$2x10^{-148}$
Hk1	Hexokinase 1	f-AATGCCAAAGAAATCCTGACCCGC r-TGGGTGTGCCCTTGTTATCTCGAA	KM026508	163	100%	0.0
Gys1	Glycogen Synthase 1	f-AAGATGGCAGCCACGGACAAATTC r-TCAATAGTAGTGCCGACAGGGTCA	KM084858	110	100%	0.0
Pygm	Glycogen Phosphorylase- muscle	f-TCATCACCCTGTACAACCGCA r-AGCCTTCCCTCCAATCATCAC	KM084856	80	100%	0.0
G6pc3	Glucose-6-phosphatase 3	f-CCCTACTGCCTCGCCCAGGT r-TGGGCTTGTGAGCTGGCCCTA	NM_001270397.1	610	94%	0.0
Insr	Insulin Receptor	f-TTGGCCCTGTGACCCATCAAAT r-TTGGCATTGCCTTCGTACACCA	NM_017071.2	568	99%	0.0

Table 1 continued.

Igf1r	Insulin-Like Growth Factor 1 Receptor	f-TCTACTACAGCGAGGAGAAC r-GGGTTTGGGTAGGGAAATAAA	XM_004803447.1	488	97%	0.00
Esr1	Estrogen Receptor α	f-GCCAGGCTTTGTTGATTTG r-CTTGCACTTCATGCTGTAGA	XM_004753574.1	502	96%	0.0
Esr2	Estrogen Receptors β	f-GGCAGTGAGTGGTTACATAC r-GTTCAAGATGGCTTCCCTT	XM_004738913.1	503	94%	0.0
Gper1	G-protein Estrogen Receptor	f-CAGCAGCGTCTTCTTTCTC r-TTGACAATGTGGCCTGTTAG	XM_004776319.1	504	96%	0.0

*refers to sequence used to design primers and sequence that results were compared against

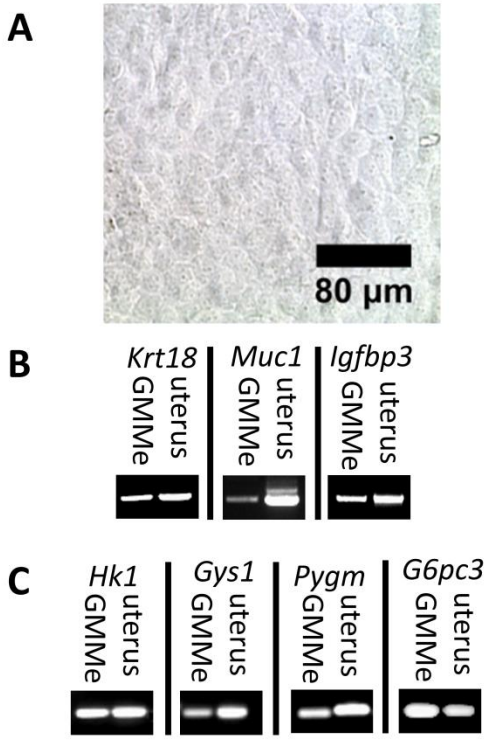

Figure 1. GMMe cells retain an epithelial phenotype and express glycogen metabolizing enzymes in complete media. A) Confluent monolayer of GMMe cells displaying a cuboidal phenotype. B) Results of RT-PCR showing that GMMe cells express cytokeratin 18 (*Krt18*) and mucin 1 (*Muc1*), epithelial cell markers. GMMe cells also express Igfbp3 (IGF binding protein 3), which is expressed exclusively in the uterine epithelia. C) RT-PCR revealed that GMMe cells express hexokinase 1 (*Hk1*), glycogen synthase 1 (*Gys1*), glycogen phosphorylase-muscle (*Pygm*), and glucose-6-phosphatase 3 (*G6pc3*), indicating they can to metabolize glycogen.

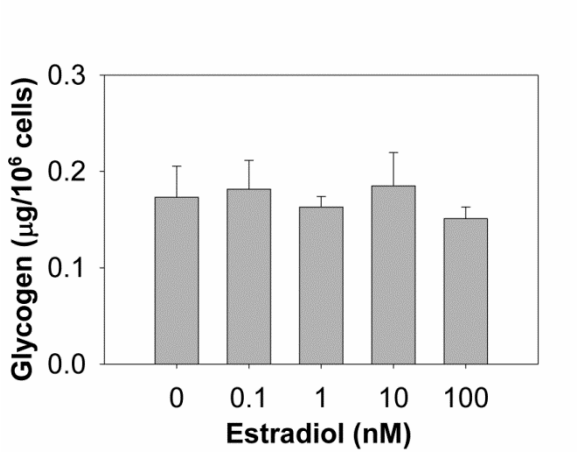

Figure 2. Glycogen concentration (mean±SEM) in GMMe cells incubated with estradiol (0-100 nM) for 48 hours in chemically defined media without insulin. Estradiol (0.1-100 nM) had no effect on glycogen concentration of GMMe cells compared to control (0 nM estradiol). $n \leq 4$.

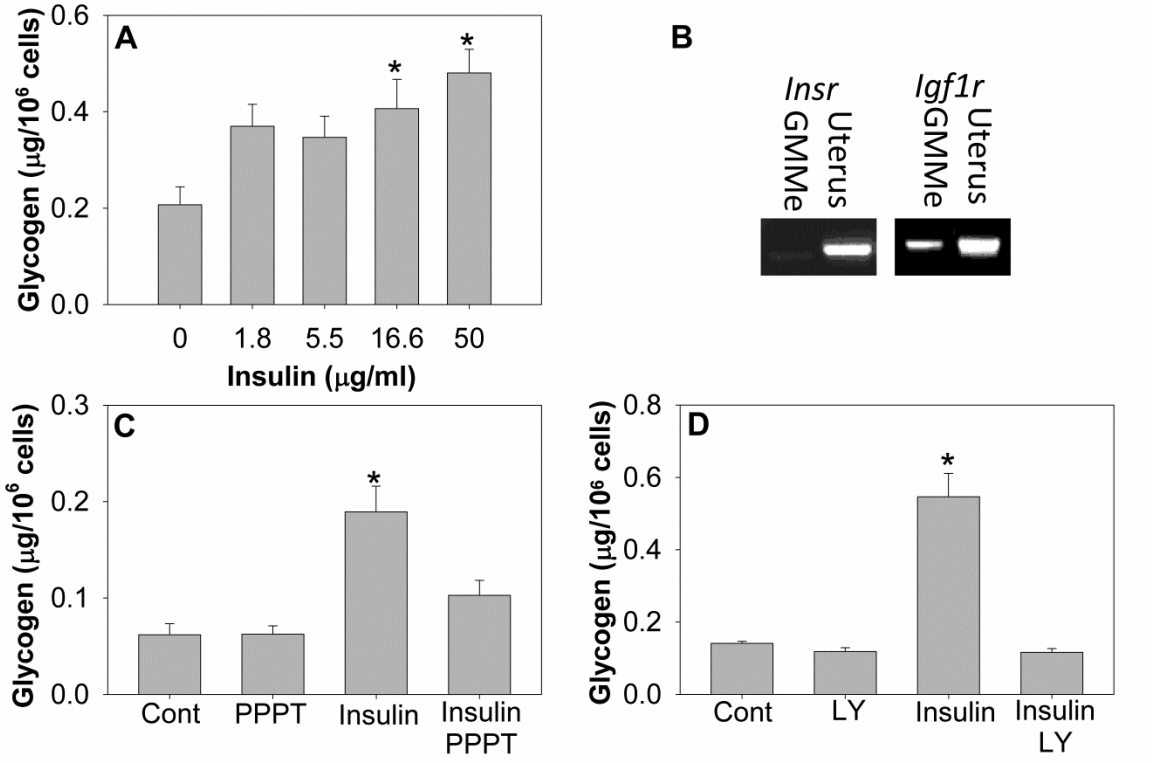

Figure 3. Insulin stimulates glycogenesis through the Igf1. A) Low concentrations of insulin (1.8 and 5.5 μg/ml) tended to increase glycogen concentration over controls (0 μg/ml; P≤0.1) and this effect was significant at higher concentrations (16.6 and 50 μg/ml; P≤0.05). B) RT-PCR revealed a very faint band for the insulin receptor (Insr) in GMMe cells; however, the insulin-like growth factor 1 receptor (Igfr1) was readily detected. C) Insulin (50 μg/ml) increased glycogen concentration (P≤0.05) over control (cont) and 1 μM PPPT (Igf1r antagonist) abolished this effect. Mean±SEM. n ≤4. *P≤0.05 relative to control. D) Insulin increased the glycogen concentration almost 4-fold relative to control (P<0.05) and 50 μM LY294002 (LY; PI3K inhibitor) completely blocked insulin's actions. Mean±SEM. n≤4. *P≤0.05 relative to control or 0 μg/ml insulin.

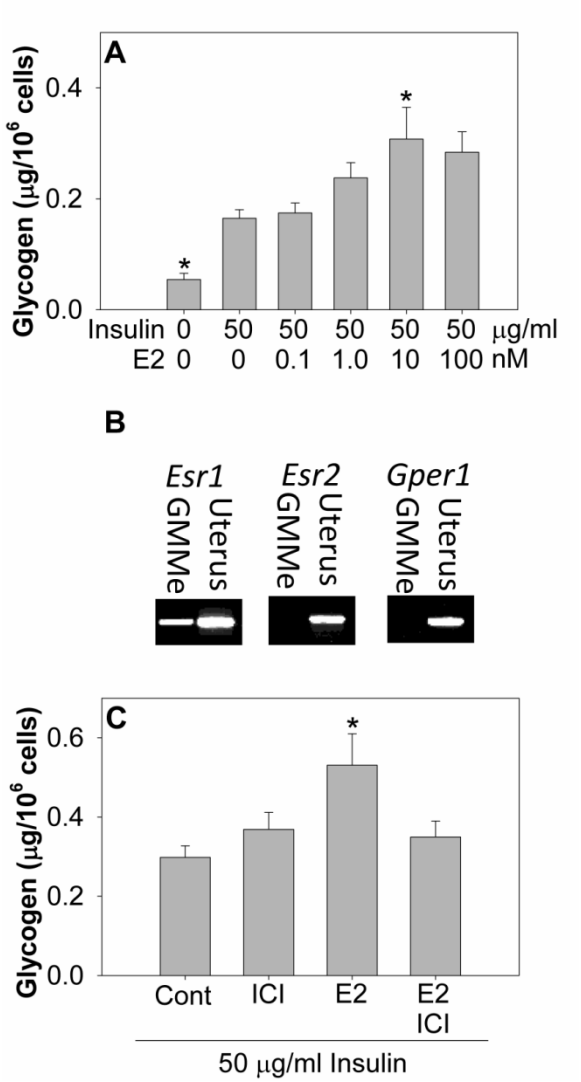

Figure 4. In the presence of insulin, estradiol (E2) stimulates glycogen accumulation via estrogen receptor α (*Esr1*). A) In the presence of insulin (50 μg/ml), 10 nM E2 significantly in increased glycogen content compared to control (0 nM E2 and 50 μg/ml; P≤0.05) and 100 nM E2 tended to increase glycogen concentrations (P≤0.10). B) RT-PCR showing that GMMe cells express estrogen receptor α (*Esr1*) but not estrogen receptor β (*Esr2*) or G-protein estrogen receptor 1 (*Gper1*). C) In the presence of 50 μg/ml of insulin, 10 nM E2 increased glycogen concentrations compared to control (P≤0.05) and the effect was blocked by 5 μM of the estrogen receptor α/β antagonist ICI 182,780 (ICI). Mean±SEM. n≤4. *P≤0.05 relative to control.

6. Chapter 3

Comparing the Effects of Estradiol and 4-Hydroxyestradiol on Uterine Glycogen Metabolism and the Role of Prostaglandins

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Abstract

Estradiol (E2) stimulates accumulation of glycogen in the uterus, which is an important reservoir of glucose after mating. However, the uterus can convert estradiol to 4-hydroxyestradiol (4OHE2), and 4OHE2, not E2, stimulates uterine prostaglandin production. In the liver prostaglandins mediate glycogenolyic signals, but their role in uterine glycogen is unexplored. Here we investigate the effect of E2 and 4OHE2 on uterine glycogen metabolism in mink and determine which effects are mediated by prostaglandins. Both steroids increased the gross glycogen concentration over control ($P \leq 0.05$), but 4OHE2 increased endometrial glycogen content to a lesser extent than E2 ($P \leq 0.05$). 4OHE2 + indomethacin (inhibits prostaglandin production) resulted in higher endometrial glycogen than 4OHE2 alone ($P < 0.05$). Both steroids increased hexokinase expression 2-fold ($P \leq 0.05$), an effect that was blocked by indomethacin ($P \leq 0.05$). And both steroids reduced the levels of phospho-glycogen synthase (pGys) by 66% ($P \leq 0.05$), which would increase glycogen synthase activity. Indomethacin did not alter the effect of E2 on pGys, but 4OHE2 + indomethacin resulted in pGys levels higher than 4OHE2 alone ($P \leq 0.05$). Interestingly, 4OHE2, but not E2, significantly increased glycogen phosphorylase activity, and indomethacin did not alter the effect of either steroid. Collectively, these results indicate that both steroids equally stimulate endometrial glycogenesis, and 4OHE2 also stimulates glycogen catabolism. However, the role of prostaglandins is unclear. 4OHE2 + indomethacin resulted in higher endometrial glycogen than 4OHE2 alone, but indomethacin did not alter the effect of 4OHE2 on glycogen phosphorylase activity. Further investigation is warranted.

Keywords: endometrium, mink, diapause, glycogen synthase, glycogen phosphorylase, hexokinase

1. Introduction

After fertilization, mammalian embryos rely on secretions into the uterine lumen (i.e. histrotroph) which contain hormones, growth factors, and nutrients. Late in the pre-implantation period, the amount of glucose in the uterine lumen increases sharply (Gao et al., 2009; Zavy et al., 1982), coinciding with increased demand for glucose by the developing blastocysts (Gardner et al., 1993). As the uterus cannot produce glucose (Yánez et al., 2003b; Zimmer and Magnuson, 1990b), the uterus catabolizes uterine glycogen reserves during the pre-implantation period (Dean et al., 2014; Greenstreet and Fotherby, 1973a). In humans, up to 25% of infertile women have abnormally low endometrial glycogen concentrations (Gupta et al., 2013; Maeyama et al., 1977). These results indicated that uterine glycogen is an important reservoir of glucose during early pregnancy and may be critical for fertility in mammals.

Estradiol stimulates glycogen accumulation in the uterus of both rats and mink (Demers et al., 1973; Rose et al., 2011), explaining the high glycogen concentration during proestrus/estrus (Dean et al., 2014; Greenstreet and Fotherby, 1973b). However, the uterus expresses cytochrome P450 1B1 (Cyp1b1), which hydroxylates estradiol at the 4-carbon position yielding 4-hydroxyestradiol (Murray et al., 2001). 4-hydroxyestradiol has actions that overlap with estradiol (van Aswegen et al., 1989), but it also has important biological actions, distinct from the parent hormone (Pakrasi and Dey, 1983; Paria et al., 1998). Paria et al. (1990) found that uterine estradiol-4-hydroxylase activity peaked on day 4 in mice, and our laboratory recently found significantly higher *Cyp1b1* expression in the mink uterus during diapause compared to estrus (unpublished observations). In both cases, this coincides with low or decreasing glycogen concentrations (Dean et al., 2014; Greenstreet and Fotherby, 1973a). This suggests that

uterine conversion of estradiol to 4-hydroxyestradiol may alter uterine responses to estrogen, and an understanding of the relationships between estrogens and uterine glycogen metabolism may have implications for human medicine and animal husbandry.

It is well established that estrogens stimulate production of prostaglandins in the uterus (Castracane and Jordan, 1976; Ham et al., 1975). However, Pakrasi and Dey (1983) found that 4-hydroxyestradiol, not estradiol itself, simulated the production of prostaglandin E and F in endometrial tissue of rabbits. Prostaglandins are critical mediators of embryo/uterine crosstalk and defective prostaglandin production has been linked to human infertility (Achache et al., 2010). In the liver, prostaglandins are principally glycogenolyic. For example, prostaglandins E2 and F2α increased glycogen breakdown by increasing glycogen phosphorylase activity (Gómez-Foix et al., 1989; Okumura and Saito, 1990), but the role of prostaglandins in uterine glycogen metabolism is unexplored.

The objectives of this study were to determine the effects of estradiol and 4-hydroxyestradiol on uterine glycogen metabolism (Figure 1) and determine if these actions were mediated by prostaglandins. To do this, mink were treated with vehicle, estradiol, estradiol + indomethacin (inhibits prostaglandin synthesis), 4-hydroxyestradiol, or 4-hydroxyestradiol + indomethacin. We determined the gross uterine glycogen concentration and used histological analysis to determine the glycogen content within the endometrium, stroma, glandular epithelia, and lumenal epithelia. We also analyzed levels of hexokinase 1, phospho-glycogen synthase, and total glycogen synthase by western blot. Finally, we determined the activity of glycogen phosphorylase (with and without exogenous AMP).

2. Materials and methods

Animals

Mink (black variety from Moyle Mink and Tannery, Heyburn, ID) were ovariectomized in the Idaho State University animal facility and returned to cages on November 20th as previously described (Rose et al., 2011). Starting 9 days later, mink were treated once daily for three consecutive days with vehicle, estradiol (50 μg/kg•day), estradiol + indomethacin (50 μg/kg•day and 10 mg/kg•day, respectively), 4-hydroxyestradiol (50 μg/kg•day), or 4-hydroxyestradiol + indomethacin (50 μg/kg•day and 10 mg/kg•day, respectively). Sesame seed oil was used as vehicle (Rose et al., 2011), and n=5-6 for each treatment group. Estradiol (E8875), 4-hydroxyestradiol (H4637), and indomethacin (I8280) were purchased from Sigma-Aldrich. Twenty-four hours after last treatment, mink were anesthetized with ketamine hydrochloride (20mg/kg; Ketaset, 45-290, Fort Dodge Animal Health) and the uterus exteriorized by mid-ventral laparotomy. Uteri were subsequently removed, quick-frozen on dry ice, and stored at -80.0°C until analyzed. Immediately after removal of the uterus, each animal was administered a lethal dose of Euthasol (011355 MWI Veterinary Supply). Animal care and research procedures were approved by the Institutional Animal Care and Use Committee of ISU, and complied with the Guide for the Care and Use of Laboratory Animals.

Plasma Glucose Concentrations

At the time of hysterectomy, a sample of blood was collected via cardiac puncture. Samples were allowed to clot at 4°C overnight and were then centrifuged at 2,000xg for 20 min. Serum was aspirated and stored at -20°C until analysis. Serum glucose concentrations were determined using the Glucose Auto Kit (439-90901, Wako

Chemicals Inc., Richmond, VA) and quantified spectrophotometrically (λ=505nm), by comparing unknowns against a standard curve of increasing glucose concentrations.

Glycogen concentrations in uterine homogenates

Uterine glycogen concentrations were determined as by the method of Good et al. (1933) and previously used in our laboratory (Dean et al., 2014). Approximately 50 mg uterine tissue (a cross section representing all uterine tissues) from each animal was lyophilized for 3 days, homogenized in 20 volumes 30% KOH, and heated at 100°C for 30 min to inactivate enzymes and destroy free glucose. To isolate glycogen, samples were diluted with 1.2 volumes 95% ethanol, frozen at -80°C for 60 min, thawed, and centrifuged at 9,600 x g for 10 min. The supernatant was discarded and the pellets dried overnight. To hydrolyze glycogen to glucose, 100 µl 1.0 N HCl was added to each tube and heated at 100°C for 2.5 hours. Glucose concentrations, as a measure of glycogen content, were determined using Glucose Auto Kit (439-90901, Wako Chemicals) and quantified spectrophotometrically (λ=505nm), by comparing unknowns against a standard curve of increasing glucose concentrations.

Histological Analysis

Uteri were fixed overnight in 10% neutral buffered formalin before being embedded in paraffin. Transverse sections (4 µm) were incubated in Periodic Acid Schiff (PAS) reagent. Since PAS stains all polysaccharides, not just glycogen, a consecutive section was pre-treated with diastase (A8220, Sigma Aldrich), to digest glycogen prior to PAS staining, and served as a negative control. Digital images were captured at 25 and 400x, and analyzed in triplicate for each animal using ImageJ software as described (Rose et al., 2011). Each tissue was delineated and the area stained positive with PAS was divided by total area. To calculate percent area positive for glycogen within each tissue, the area

positive for PAS staining in negative controls was subtracted from the area positive for PAS in sections not treated with diastase.

Western blotting

Western blot was carried out as previously described (Dean et al., 2014). Proteins were isolated from ~50 mg cross-section of uterus in RIPA Lysis Buffer containing protease and phosphatase inhibitors (sc-2448, Santa Cruz Biotechnology) according to the manufacturer's instructions. Protein concentrations were determined using Pierce BCA protein assay kit (Thermo Scientific, # 23225, Rockford, IL, USA). Twenty five micrograms of protein were resolved by molecular weights using SDS-PAGE and transferred onto nitrocellulose membranes and blocked for 1 hour in a 5% milk buffer containing TBS-Tween 20 (BP337-100; Fisher Scientific) to reduce non-specific binding.

Primary antibodies specific for hexokinase 1 (2024S, 1:1000 dilution), glycogen synthase (3886S, 1:1000 dilution), phospho-glycogen synthase (3891S, $Ser^{640/641}$, 1:500 dilution), and β-actin (4970S, 1:1,000 dilution) were purchased from Cell Signaling. All antibodies were produced against human epitopes and produced a single band of correct size with mink uterine proteins (not shown).

Membranes were incubated with primary antibodies overnight (4°C) in blocking solution. Membranes were subsequently incubated with secondary antibody conjugated to horseradish peroxidase (7074S; Cell Signaling Technology) at 1:1000 for 2 hours at room temperature. Blots were visualized by chemiluminescence using HRP substrate (WBKLS0100, Millipore). Bands were photographed with a VersaDoc imaging machine (Bio-rad), and relative band density, in pixels, was determined using ImageJ

software ver. 144o (Abramoff et al., 2004). Band density for each protein of interest was normalized to the band density of the loading control (β-Actin), with all samples measured in triplicate. Antibodies against glycogen synthase were not isoform specific and thus measured combined glycogen synthase 1 and 2.

Glycogen Phosphorylase Activity

Glycogen phosphorylase activity was measured according to Storey (1987) and had previously been validated in our laboratory (Dean et al., 2014). In brief, 15-20 mg uterine cross-sections were homogenized in 39 volumes of homogenization buffer (15 mM imidazole, 5mM EGTA, 100mM NaF, 0.1mM PMSF and 15 mM beta-mercaptothanol). Samples were centrifuged at 2,000 x g for 30 seconds and 80 µl of the resulting supernatant was added to the reaction buffer (final volume 1.0 ml, temperature 30°C) consisting of: 50 mM KH_2PO_4, 2 mg/ml oyster glycogen type 2, 0.4 mM NADP, 10 µM glucose-1-6-bisphosphate, 0.25 mM EDTA, and 15 mM $MgCl_2$), with phosphoglucomutase, and glucose-6-phosphate dehydrogenase (G7877-1KU and P3397-1KU; Sigma Aldrich) in excess. Exogenous enzymes were supplied from the manufacturer in ammonium sulfate suspension, which has been shown to activated glycogen phosphorylase (Sprang et al., 1991); therefore, enzymes were washed three times with KH_2PO_4 buffer, prior to being added to the reaction buffer.

To determine activity of phosphorylated and total glycogen phosphorylase, activity was determined both without exogenous adenosine monophosphate (-AMP) and with 25 mM AMP exogenous (+AMP). The resulting NADPH from the reactions was detected spectrophotometrically (340 nm every 30 sec for 10 minutes). Absorption values were converted to enzyme units using the extinction coefficient of NADPH (6220/M • cm).

Phosphorylase activities were then normalized to milligram of protein with protein concentrations determined using the Bradford assay procedure (23236, Pierce Biotechnology).

Statistical Analysis

In order to minimize type II error, data were analyzed via two separate posthoc analyses. After ANOVA, each treated group was compared to control (vehicle) values using Dunnett's correction. Then individual pairwise contrasts (estradiol vs estradiol+ indomethacin; 4-hydroxyestradiol vs 4-hydroxyestradiol+ indomethacin; estradiol vs 4-hydroxyestradiol) were made using Bonferroni's analysis. All analyses were made using the asbio package in R (version 3.0.2). Data are presented as mean ± SEM and significance was set at $P \leq 0.05$.

3. Results

Plasma Glucose, Endometrial Area, and Uterine Glycogen Content

None of the four treatments altered plasma glucose concentrations compared to control. At the gross glycogen level, all four treatments increased concentrations over control ($P \leq 0.05$). There were no significant differences between any of the treatment groups contrasted with Bonferroni analysis (Table 1), though the glycogen concentration in 4-hydroxyestradiol treated mink was 5.5 mg/g lower than in estradiol treated mink ($P=0.30$).

Histological analysis indicated that all four treatments increased endometrial area over control (4.5-5.5 fold; $P \leq 0.05$) with no detectable difference between treated groups (Table 1). All four treatment groups also resulted in significantly higher endometrial glycogen content compared to control (8-18 fold; Dunnett's $P \leq 0.05$). However, 4-hydroxyestradiol increased endometrial glycogen accumulation to a lesser extent than

estradiol ($P \leq 0.05$). Interestingly, indomethacin did not alter the effect of estradiol, but 4-hydroxyestradiol + indomethacin resulted in significantly higher endometrial glycogen than 4-hydroxyestradiol alone ($P \leq 0.05$), with values comparable to estradiol treatment (Figure 2).

In control uteri, the glycogen content of the glandular epithelia, lumenal epithelia, and stroma was extremely low. Dunnett's analysis revealed that all four treatments increased glycogen content over control in both types of epithelia ($P \leq 0.05$) but had no effect on stroma. Similar to endometrial glycogen content, Bonferroni analysis indicated that 4-hydroxyestradiol + indomethacin resulted in higher glycogen content than 4-hydroxyestradiol in all three endometrial tissues ($P \leq 0.05$) but indomethacin did not alter the effect of E2 (Table 2).

Glycogen Metabolizing Enzyme Expression

Both steroids increased hexokinase-1 protein expression compared to control ($P \leq 0.05$), and in both cases this effect was inhibited by inclusion of indomethacin ($P \leq 0.05$), indicating the increased hexokinase-1 in response to both estrogens was mediated by prostaglandins. Expression of total glycogen synthase (un-phosphorylated and phosphorylated) was unaffected by any treatment. However, levels of phospho-glycogen synthase were reduced 66% by both estradiol and 4-hydroxyestradiol ($P \leq 0.05$), explaining the increased uterine glycogen concentration in treated mink. Inclusion of indomethacin did not alter the effect of estradiol, but indomethacin partially blocked the effect of 4-hydroxyestradiol on phospho-glycogen synthase ($P \leq 0.05$; Figure 3).

The activity of total glycogen phosphorylase (+AMP) was unaffected by any treatment. Without exogenous AMP (-AMP; analogous to phospho-glycogen phosphorylase),

estradiol tended to increase glycogen phosphorylase activity compared to control ($P \leq 0.1$), and inclusion of indomethacin had not effect. In contrast, 4-hydroxyestradiol increased activity 3.7 fold over control ($P \leq 0.05$), and again indomethacin did not alter this effect (Figure 4).

4. Discussion

Glycogenesis

We found that both estradiol and 4-hydroxyestradiol (50 μg/kg•day) increased gross uterine glycogen concentrations in the mink uterus (Table 1), which agrees with a previous study from our laboratory using 400 μg/kg•day (Rose et al., 2011). Similarly, Gregoire (1967) found that estradiol benzoate increased gross glycogen concentrations in rat uterus, and Demers et al. (1973) found that estradiol increased glycogen concentrations in the uterus of rats, rabbits, and guinea pigs. Interestingly, the glycogenic effect of estrogens does not appear to be limited to the uterus. Estradiol also increased the glycogen concentration of the liver (Paul and Duttagupta, 1973), though the effect was less dramatic.

Both the current study (Table 2) and Rose et al. (2011) show that these estrogens specifically increase glycogen content of the epithelia *in vivo*. In agreement, Demers et al. (1977) and Ishihara et al. (1988) found endometrial glycogen content was increased by ovarian steroids (progesterone in the case of humans and guinea pigs) using tissue biopsies which contained both epithelia and stroma. In contrast, Casimiri et al. (1980) found minimal effects of estradiol on epithelia from the rat uterus cultured in isolation. Collectively these results raise the possibility that the glycogenic effect of estrogens on the uterine epithelia may be indirect.

Explaining the increase in uterine glycogen, both estradiol and 4-hydroxyestradiol equally increased hexokinase-1 protein expression and decreased phospho-glycogen synthase levels (Figure 3; which would result in increased glycogen synthase activity). This agrees with studies in rats which have shown that estradiol increases hexokinase expression and activity (Moorthy et al., 2004; Smith and Gorski, 1968). In addition, we found that indomethacin blocked the effect of both estrogens on hexokinase-1 (Figure 3), indicating that the effect is mediated by prostaglandins. To our knowledge, this is the first work linking prostaglandins to uterine hexokinase.

Moorthy et al. (2004) and Demers et al. (1973) found that estradiol increased the uterine glycogen synthase activity in the rat uterus, which agrees with our results. Indomethacin did not alter the effect of estradiol on glycogen synthase, indicating that prostaglandins are not involved with this effect. In contrast, indomethacin partially abrogated the effect of 4-hydroxyestradiol (Figure 3). Thus 4-hydroxyestradiol appears to increase glycogen synthase activity via two separate pathways, one that involves prostaglandins and one that does not.

Glycogenolysis

Our finding that 4-hydroxyestradiol resulted in lower endometrial glycogen content than estradiol might indicate that 4-hydroxyestradiol is a "weaker" estrogen. However, our data do not support this contention. Instead, we suggest that both 4-hydroxyestradiol and estradiol equally stimulate glycogenesis, but 4-hydroxyestradiol independently stimulates glycogenolysis. In support of this idea, endometrial glycogen content was 50% lower in response to 4-hydroxyestradiol compared to estradiol (Figure 2), both steroids equally increased hexokinase expression and decreased levels of phospho-glycogen synthase

(Figure 3), and 4-hydroxyestradiol significantly increased glycogen phosphorylase activity (-AMP) but estradiol did not (Figure 4). In agreement with this hypothesis, 4-hydroxyestradiol binds to classical estrogen receptors with a similar affinity as estradiol (Martucci and Fishman, 1976; van Aswegen et al., 1989). And accumulating evidence suggests that 4-hydroyxestradiol can mediate actions independently of estrogen receptors. Philips et al. (2004) showed that 4-hydroxyestradiol bound to proteins isolated for estrogen receptor α knockout mice, and 4-hydroxyestradiol could not be displaced by estradiol. Jobe et al. (2011) found that 4-hydroxyestradiol stimulated the proliferation of endothelial cells collected from the pregnant ovine uterus via β-adrenergic receptors. And Lee et al. (2003) showed that 4-hydroxyestradiol (but not estradiol) acted through the transcription factor NF-ER-like 2 (Nrf2), leading to activation of the antioxidant responsive element (ARE) in mouse astrocytes. Therefore, there are a number of potential mechanisms by which 4-hydroxyestradiol could increase glycogen breakdown in the uterus, independently of estradiol. And future work will be aimed at identifying these "receptors".

We have recently shown that endometrial glycogen is high at estrus and is mobilized before implantation (Dean et al., 2014), indicating that endometrial glycogen may be an important source of glucose for the uterus and embryos. Increased expression of *Cyp1b1* during diapause (unpublished observations) coupled with our data showing that 4-hydroxyestradiol (but not estradiol) significantly increases glycogen phosphorylase activity (-AMP; Figure 4) suggests that uterine conversion of estradiol to 4-hydroxyestradiol may contribute to the mobilization of uterine glycogen during diapause. Currently the role of prostaglandins in this process remains enigmatic. 4-

hydroxyestradiol stimulates production of prostaglandins E and F form rabbit endometrium (Pakrasi and Dey, 1983), and the same prostaglandins stimulates glycogen breakdown in the liver (Okumura and Saito, 1990). Our finding, that 4-hydroxyestradiol + indomethacin results in higher endometrial glycogen content than 4-hydroxyestradiol (Figure 2), agrees with the conjecture that prostaglandins mediate 4-hydroxyestradiol-stimualted glycogenolysis in the uterus. However, our inability to detect differences in glycogen phosphorylase activity between 4-hydroxyestradiol and 4-hydroxyestradiol + indomethacin (Figure 4) compromises this interpretation.

Conclusion

In conclusion, both estradiol and 4-hydroxyestradiol increased endometrial glycogen content. The effect of 4-hydroxyestradiol was less than the effect of estradiol, but indomethacin increased the glycogenic effect of 4-hydroxyestradiol. Both steroids stimulate glycogenesis via increased hexokinase expression and activation of glycogen synthase. While prostaglandins mediate the actions of both steroids on hexokinase, the effect of estradiol on phospho-glycogen synthase is independent of prostaglandins and the effect of 4-hydroxyestradiol is only partially mediated by prostaglandins. 4-hydroxyestradiol also significantly increased glycogen phosphorylase activity, explaining the lower endometrial glycogen in response to 4-hydroxestradiol compared to estradiol. However, indomethacin did not alter the effect of 4-hydroxyestradiol on glycogen phosphorylase activity. Therefore, the role of prostaglandins in 4-hydroxyestradiol-stimulated glycogenolysis is unclear.

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Table 1. Plasma glucose concentration (mM), gross uterine glycogen concentration (mg/g of tissue), and endometrial area (mm^2) in mink treated with vehicle (control), estradiol, 4-hydroxyestradiol (4OHE2), or indomethacin (INDO) as indicated for 3 consecutive days before hysterectomy and blood collection.

Treatment	Plasma Glucose Concentration (mM)	Endometrial Area (mm^2)	Gross Glycogen (mg/g)
Control	4.02±0.21	0.70±0.12	2.67±0.34
Estradiol	4.97±0.49	2.99±0.34*	23.44±2.53*
Estradiol + INDO	3.84±0.29	3.08±0.40*	18.69±2.72*
4OHE2	4.29±0.28	3.86±0.23*	17.92±2.40*
4OHE2 + INDO	4.15±0.27	3.21±0.58*	16.67±1.60*

* indicates significant difference from control ($P \leq 0.05$). mean±SEM. $n \leq 5$.

Table 2. Glycogen content (mean ± SEM) of the glandular epithelia, lumenal epithelia, and stroma in mink treated with vehicle (Control), estradiol, 4-hydroxyestradiol (4OHE2), or indomethacin (INDO) as indicated for 3 consecutive days before hysterectomy. Glycogen content of each tissue was determined using Periodic Acid Shiff (PAS) reagent (with and without diastase pre-treatment) and analyzing the resulting images with ImageJ.

Treatment	Glandular Epithelial Glycogen (% Area)	Lumenal Epithelial Glycogen (% Area)	Stromal Glycogen (% Area)
Control	0.0048±0.0011	0.0002±0.0002	0.0012±0.0005
Estradiol	0.193±0.095*	0.044±0.029*	0.0010±0.0003
Estradiol + INDO	0.155±0.023*	0.017±0.0003*	0.0004±0.0002
4OHE2	0.223±0.041*	0.016±0.003*	0.0008±0.0003
4OHE2 + INDO	0.685±0.0043*^{B}	0.309±0.082*^{B}	0.0026±0.0007^{B}

*significantly different from control; ^{B}significantly different from 4OHE2 ($P<0.05$). $n\leq5$.

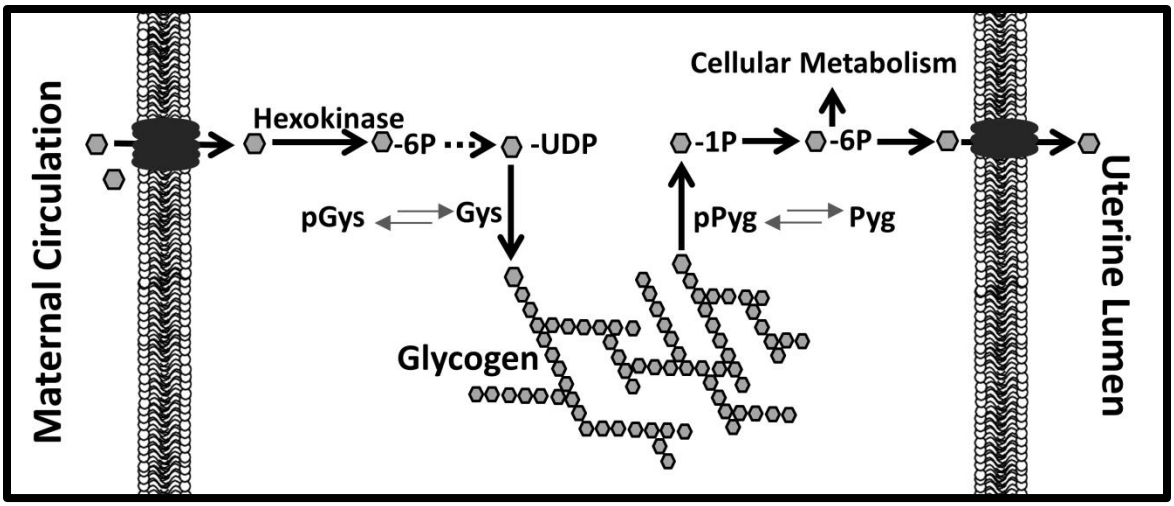

Figure 1. Schematic of glycogen metabolism. After glucose (⬡) enters a cells, it is phosphorylated by hexokinase, producing glucose-6-phosphate (⬡-6P). ⬡-6P is converted to uridine diphosphate glucose (⬡-UDP), and then the glucose residue is transferred to a preexisting glycogen molecule by glycogen synthase (Gys), the activity of which is reduced by phosphorylation (pGys). Phospho-glycogen phosphorylase (pPyg) liberates glucose-1-phosphate (⬡-1P) from glycogen, and the activity of glycogen phosphorylase (Pyg) is reduced by dephosphorylation. ⬡-1P is isomerized to ⬡-6P, which can be metabolized by the cell or dephosphorylated and secreted.

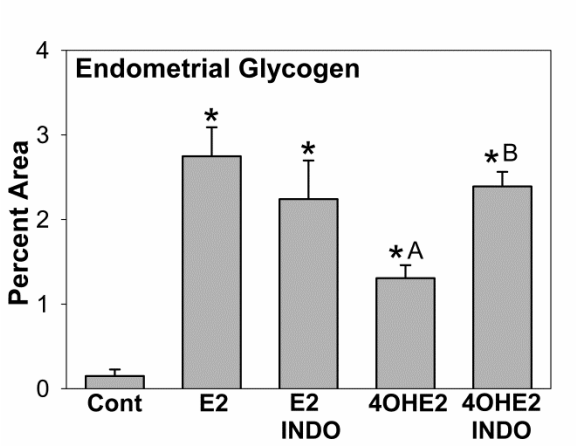

Figure 2. Glycogen content (mean ± SEM) of the endometrium in mink treated with vehicle (Cont), estradiol (E2), 4-hydroxyestradiol (4OHE2), or indomethacin (INDO) as indicated for 3 consecutive days before hysterectomy. Endometrial glycogen content was determined using Periodic Acid Shiff (PAS) reagent (with and without diastase pre-treatment) and analyzing the resulting images with ImageJ.

*indicates significant difference from control ($P \leq 0.05$). [A]indicates a significant difference from E2 ($P \leq 0.05$). [B]indicates a significant difference from 4OHE2 ($P \leq 0.05$). $n \leq 5$.

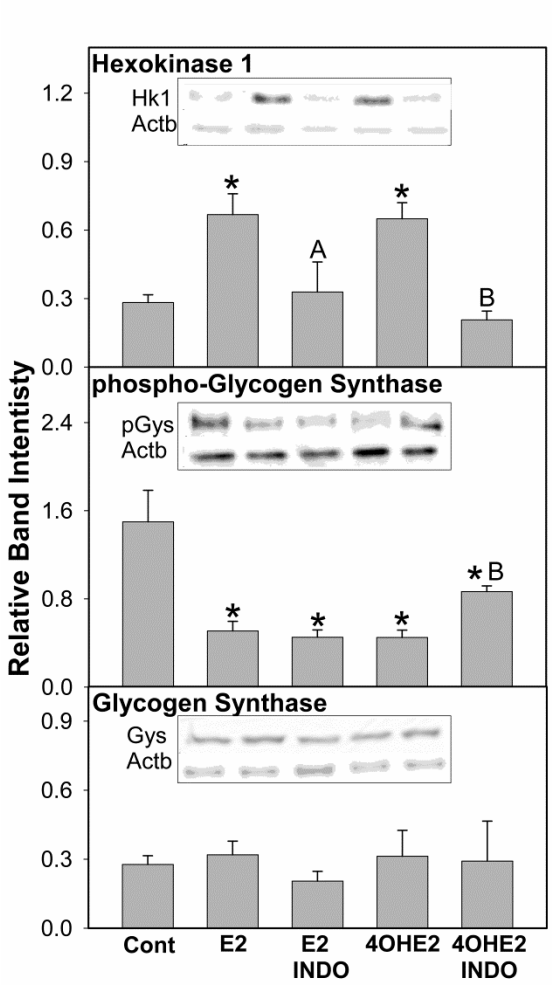

Figure 3. Protein levels (mean ± SEM) of glycogenic enzymes in the uteri of mink treated with vehicle (Cont), estradiol (E2), 4-hydroxyestradiol (4OHE2), or indomethacin (INDO) as indicated for 3 consecutive days before hysterectomy. Levels of each protein were determined by western blot and normalized to β-actin (Actb).

* indicates significant difference from control ($P \leq 0.05$). ^{A}indicates a significant difference from E2 ($P \leq 0.05$). ^{B}indicates a significant difference from 4OHE2 ($P \leq 0.05$). $n \leq 5$.

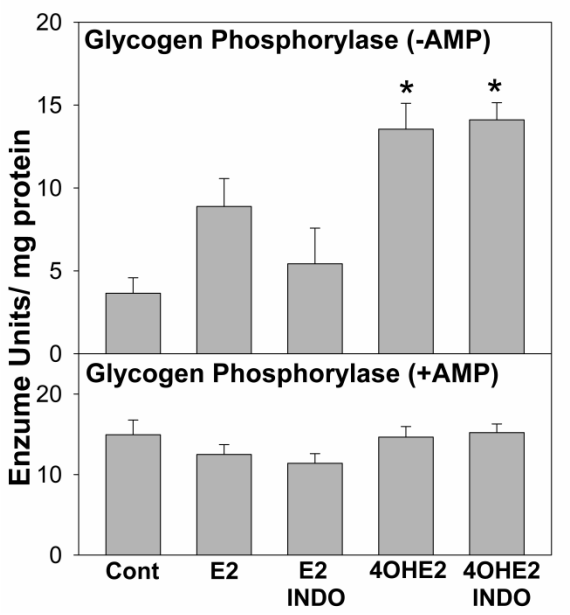

Figure 4. Analysis of glycogen phosphorylase activity (mean ± SEM) in the uteri of mink treated with vehicle (Cont), estradiol (E2), 4-hydroxyestradiol (4OHE2), or indomethacin (INDO) as indicated for 3 consecutive days before hysterectomy. Phosphorylase activity was determined without exogenous AMP (-AMP) and with 25 mM of AMP (+AMP; which activates non-phosphorylated glycogen phosphorylase). Activity was normalized to milligram of protein.

*indicates significant difference from control ($P \leq 0.05$). $n \leq 5$.

7. Implications & Future Research

7.1. Need for Causative Link between Uterine Glycogen and Fertility

The current finding that uterine glycogen is mobilized during pre-implantation in mink agrees with studies in rats and sheep (Greenstreet and Fotherby, 1973; Murdoch, 1970), and suggests that endometrial glycogen is an important source of glucose for the uterus and/or embryos. While correlative evidence supports this conjecture (Demers et al., 1972; Gardner et al., 1993; Girish et al., 2012), a direct need for uterine glycogen during pregnancy has never been demonstrated. Historically, such an experiment would have been difficult to perform. Systemically inhibiting glycogen synthase or phosphorylase would alter plasma glucose concentrations (Parker et al., 2004) which would compromise fertility independently of uterine glycogen concentrations (Jovanovic et al., 2005). However, two transgenic mouse lines have recently been developed that would circumvent these problems (Duran et al., 2013; Pederson et al., 2004).

Pederson (2004) obtained mice with a heterozygous deletion of *Gys1* from Lexicon Genetics Incorporated. By crossings these mice, the investigators generated $Gys1^{-/-}$, $Gys1^{-/+}$, and $Gys1^{+/+}$ mice. However, the number of $Gys1^{-/-}$ mice born was much lower than predicted and further investigation revealed that many knockout embryos died *in utero* due to abnormal cardiac development, though 10% of $Gys1^{-/-}$ mice were able to survive with no overt phenotype. In a subsequent study (Pederson et al., 2005), the investigators found that while *Gys1* knockout did inhibit glucose uptake by muscle tissue, the mice had normal plasma glucose concentrations and were relatively healthy. These results indicate that these mice may be a useful model for studying glycogen and fertility; however, their reproduction has never been characterized.

A second transgenic mouse line has been developed that would allow for more direct investigations of uterine glycogen. Duran et al. (2013) produced mice in which exons 6, 7, and 8 of *Gys1* are flanked by LoxP sites (*Gys1*^{LoxP} mice). By crossing these mice with Nestin$^{cre/+}$ mice, the investigators produced a mouse line lacking *Gys1* in neurons. For our purposes, crossing *Gys1*^{Loxp} mice with *Pgr*$^{cre/+}$ mice (Cre recombinase is driven by the progesterone receptor promoter) would produce mice lacking *Gys1* in reproductive tissues (Daikoku et al., 2008; Dunlap et al., 2011). Such a model would be very useful for studying the role of glycogen at the gross uterus level.

Two other Cre mouse lines have been generated that would allow for the knockout of *Gys1* specifically in the uterine epithelium. Winuthayanon et al. (2010) develop a Cre mouse using the *Wnt7a* promoter, and Daikoku et al. (2014) developed a mouse line with Cre expression driven by lactoferrin. Both methods result in uterine expression of Cre being restricted to epithelia. Crossing either of these mice with *Gys1*^{LoxP} mice, would generate mice lacking *Gys1* in the uterine epithelial. The role(s) of epithelial glycogen could then be examined independently of glycogen the stroma or myometrium. This may be particularly important since stroma cells of rats (and presumably mice) store large amounts of glycogen and decidualization of stromal cells requires large amounts of glucose (Casimiri et al., 1980; Frolova et al., 2011).

7.2 Glycogen as a Source of Histotroph

The localization of endometrial glycogen to the epithelia at estrus, its catabolism between estrus and implantation, and the high expression of glucose-6-phosphatase 3 during diapause, indicates that epithelial glycogen is being catabolized, dephosphorylated, and secreted into the uterine lumen. In agreement with the conjecture, glucose-6-phosphatase

activity has been localization to the uterine epithelia in both the guinea pig and human uterus (Burgos and Wislocki, 1956; Sawaragi and Wynn, 1969), and glucose-6-phosphatase 3 knockout reduces litter size by up to 50% in mice (Jun et al., 2012). However, in the current research we did not determine the glucose concentration in the uterus fluid due the technical limitations of working with a small species (Leese et al., 2007).

GMMe may represent a useful model for determining the fate of the glycogen stored in epithelial cells. Here we show that insulin and estradiol stimulates the formation of glycogen in these cells, making it possible to next determine what hormone(s) stimulates glycogen breakdown and the fate of the resulting glucose. Our laboratory has *in vivo* evidence that progesterone stimulates the breakdown of glycogen in the uterine epithelia of mink (unpublished observations). First, we would need to determine the effects of progesterone on the glycogen concentration in GMMe cells. Assuming progesterone stimulates glycogenolysis, we would need to optimize both the concentration and time necessary to decrease glycogen concentrations. After optimizing these variables, we could determine if progesterone results in secretion of glucose in the media. To confirm the glucose being secreted into the media is coming from glycogen catabolism, we could block the effect with the glycogen phosphorylase inhibitor CP-91149 (PZ0104, SigmaAldrich). Collectively, if progesterone stimulates glycogenolysis in GMMe cells, increased secretion of glucose into the culture media, and both of the effects were blocked by CP-91149, that would be compelling evidence that epithelial cells can secreted glucose that was stored as glycogen.

7.3 Is there a 4-Hydroxyestradiol Receptor?

Here we show that 4-hydroxyestradiol, not estradiol, significantly increased glycogen phosphorylase activity, and previously our laboratory found that 4-hydroxyestadiol increased glucose-6-phosphatase 3 and glycogen phosphorylase- muscle mRNA expression more than estradiol (unpublished observations). Collectively these results suggests that some actions of 4-hydroxyestradiol may be mediated by a unique '4-hydroxyestradiol receptor.' Such a receptor would be difficult to identifying *in vivo* due to interconversion of estradiol and 4-hydroxyestradiol after treatment (reviewed in Zhu and Conney, 1998), GMMe cells represent an intriguing opportunity to study the effects of 4-hydroxyestradiol that are distinct from the effects of estradiol on uterine epithelia. First a clear, unique action of 4-hydroxyestradiol would need to be demonstrated. While GMMe cells do express Cyp1b1 activity (unpublished observations), the activity of this enzyme could be blocked with inhibitors (e.g. TMS; 10038, Cayman Chemical) or knockdown techniques (e.g. siRNA) *in vitro*. After a unique action of 4-hydoryxestradiol is identified, there are several potential 4-hydroxyestradiol receptors that could be explored.

Classical Estrogen Receptors

Catecholestrogens can bind to and activate classical estrogen receptors (Schütze et al., 1994). 2-hydroxyestradiol has a lower affinity for the estrogen receptor and disassociates from the estrogen receptor fasters than estradiol (Barnea et al., 1983; Davies et al., 1975), explaining it being a "weaker" estrogen (Zhu and Conney, 1998). In contrast, 4-hydroxyestradiol disassociates from the estrogen receptors at an equal rate to estradiol (Barnea et al., 1983). Martucci and Fishman (1976) found that 4-hydroxyestradiol had 60% lower affinity for estrogen receptors isolated for the mouse uterus compared to

estradiol, but van Aswegen et al (1989) found that 4-hydroxyestradiol had 50% higher affinity for estradiol receptors isolated from breast cancer tissue than estradiol. Thus 4-hydroxyestradiol activation of classical estrogen receptors seems more likely to explain the overlapping effects of estradiol and 4-hydroxyestradiol than any unique actions of 4-hydroxyestradiol.

Philips et al. (2004) used [^{3}H]-labeled 4-hydroxyestradiol to probe for 4-hydroxyestradiol binding in estrogen receptor α knockout mice. Scatchard plot analysis indicated binding to a single protein with high affinity (similar to 4-hydroxyestradiol's affinity for estrogen receptor α). Using proteins isolated from the lung, the investigators found estradiol could not displace 4-hdyroxyestradiol binding. Interestingly, norepinephrine displaced 40% of the 4-hydroxyestradiol, more than any of the other compounds tested (excluding other catecholestrogens).

Adrenergic Receptors

Adding a second hydroxyl group to the A ring of estradiol results in production of a catechol group, hence the name catecholestrogens. Catechol groups are a prominent feature of catecholamines, leading some investigators to hypothesize that catecholestrogens bind to adrenergic receptors. In MCF-7 cells Ciesa et al. (2008) found that catecholestrogens stimulated mitosis via the α2-adrenegeric receptor. Within the uterus, Jobe et al. (2011) found that endothelial cells collected from pregnant sheep expressed α2, β2, and β3 adrenergic receptors. Using specific agonists and antagonists they showed that catecholestrogens stimulate proliferation through β2, and less potentially β3, receptors. Strengthening the possibility that catecholestrogens mediate actions on uterine glycogen metabolism, radiographic imaging reveal high concentration

of β-adrenergic receptors in the rat uterus (Brauer and Burnstocks, 1998). Binding sites were particularly abundant in the uterine epithelia, the cells which contain high glycogen content in mink (Dean et al., 2014; Rose et al., 2011).

Fortier et al. (1988) found that isoproterenol (β-adrenergic agonist) stimulated adenylyl cyclase activity and prostaglandin E_2 production in isolated, bovine uterine epithelial cells. In sheep, infusion of isoproterenol results in increased prostaglandin E_2, but not prostaglandin F_{2a}, concentrations in uterine veins (Lye et al., 1987). Norepinephrine has also been show to increase prostaglandin E_2 production in isolated rat uteri (Chaud et al., 1986). These observations are interesting because catecholestrogens have been shown to stimulate uterine prostaglandin production through an unknown pathway (Kelly and Abel, 1980; Pakrasi and Dey, 1983) and because prostaglandin E_2 stimulates glycogen breakdown in the liver (Okumura and Saito, 1990; Püschel et al., 1993).

Estrogen Related Receptors (ERR)

Estrogen related receptors (ERRs) are orphan nuclear receptors identified based on their similarity with the classical estrogen receptors. These receptors are well-known to have major effects on metabolism (Luo et al., 2003). For instance, in ERRs increase expression of genes involved in multiple metabolic pathways (i.e. fatty acid transport, mitochondrial oxidation, and respiration) in both skeletal muscle and cardiac cells (Huss et al., 2004). Strengthening the possibility that EERs may mediate changes in uterine glycogen metabolism, Bombail (2010) found EERα expression to increase in human stromal cells in response to decidualizing stimuli. And this change was associated with changes in many metabolic genes, which the authors believed may link endometrial

ERRα expression to uterine bioenergetics. However, to date there is no direct evidence that 4-hydroxyestradiol activates ERRs.

Nuclear Factor κB (NFκB)

NFκB is an inducible transcription factor best known for its role in the immune response. However, NFκB has much broader actions including epithelial cell differentiation, cell survival, and metabolism (reviewed in Hayden and Ghosh, 2012). In the inactive state, dimers of NFκB are held in the cytoplasm due to an association with the IκB protein. Signals that induce NFκB activity do so by activating IκB kinase, which phosphorylates IκB, resulting in its breakdown. Free NFκB dimerizes, which is then translocated to the nucleus and acts as a transcription factors.

In order to investigate the role of NFκB in catecholestrogen stimulation of prostaglandin production, Ho et al. (2008) treated bronchial epithelial cells with a mixture of both 2- and 4-hydroxyestradiol of various concentrations. They found that both 1nM and 10 nM significantly increased cyclooxygenase expression and NFκB expression. Interestingly, 10 nM estradiol had little effect on its own but estradiol and catecholestrogen synergistically increased NFκB activity, reactive oxygen species production, cyclooxygenase expression, and prostaglandin E_2 production. Kimura et al. (2004) found that activated NFκB changed in a reproductive cycle dependent manner, being highest at proestrus and estrus. In mated mice, activated NFκB started to increase slowly at 1.5 days post-coitum but increased dramatically near the time of implantation, and the activated NFκB was localized to the lumenal and glandular epithelium.

Again these observations are interesting because, as state before, catecholestrogens have been shown to stimulate uterine prostaglandin production (Kelly and Abel, 1980; Pakrasi

and Dey, 1983) and because prostaglandins stimulate glycogen breakdown in the liver (Okumura and Saito, 1990; Püschel et al., 1993). In addition, the study by Ho et al. (2008) found synergistic actions of estradiol with catecholestrogens, a possibility not address in most studies.

Adenylyl Cyclase

One intriguing potential mechanism-of-action for catecholestrogens is via inhibition of adenylyl cyclase. In vitro, Paul and Skolnick (1977) found that catecholestrogens decreased the concentration of cAMP in the rat hypothalamus. Following up on this observation, Steegborn (2005) incubated purified catalytic domains of adenylyl cyclase with increasing concentrations of 2-hydroxyestradiol and 4-hydroxyestradiol. Analysis revealed that 2-hydroxyestradiol inhibited adenylyl cyclase activity in a non-competitive fashion. In agreement, modeling of adenylyl cyclase showed that 2-hydroxyestradiol bound to a novel site on adenylyl, resulting in a conformational change of the active site. The physiological relevance of these observations are unknown.

NF-E2-Related Factor 2 (Nrf2)

Nrf2 is a basic leucine zipper transcription factor known to increase transcription of antioxidant responsive element (ARE) driven genes (Moi et al., 1994; Thimmulappa et al., 2002). Investigating the possibility that Nrf2 mediated the neuroprotective effect of estrogens, Lee et al. (2003) stimulated IMR-32 cells with estradiol, 2-hydroxyestradoil, and 4-hydroxyestradiol. Both 4-hydroxyestradiol and 2-hydroxyestradiol (but not estradiol) increased ARE activation in a dose-dependent manner. Additionally, 4-hydroxyestradiol, not estradiol, increased the amount of Nrf localized to the nucleus and increased expression of *NQO1* (a ARE driven gene). In primary astrocytes from wildtype

mice, 4-hydroxyestradiol also increased ARE activation, but in Nrf2$^{-/-}$ pups the effect was absent. Collectively, this is compelling evidence that 4-hdyroxyestradiol activates Nrf2.

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