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Author
Ma, Wenge

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CARBOHYDRATE RECEPTORS, PRETERM LABOR, AND PERIODONTITIS

by

Wenge Ma

DISSERTATION

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Carbohydrate Receptors, Preterm Labor, and Periodontitis

By Wenge Ma

Thesis Mentor: Susan J. Fisher, Ph.D.

ABSTRACT: Epidemiological studies suggest that pregnant women with periodontitis experience a moderate risk of preterm labor and low-birth-weight infants. However, the molecular mechanisms underlying this association remain unknown. Bacterial infections and host inflammatory responses initiated by invading bacteria, such as those observed in the pathogenesis of periodontitis, are strongly associated with preterm labor. Various carbohydrate structures assembled by an array of glycosyltransferases mediate bacterial attachment and leukocyte trafficking. I hypothesized that mucin-coated oral and uterine cavities present similar carbohydrate motifs that specify bacterial ecology and leukocyte recruitment in both regions. As a corollary to this theory, I proposed that certain individuals express oligosaccharides that make them susceptible to both periodontitis and preterm labor. I chose laboratory mice as a model system in which to test these suppositions. The first part of the project profiled glycosylation-related genes in the mouse uterus and major salivary glands. I conducted a global analysis of glycosylation-related gene expression patterns in these regions. The study design took into consideration potential effects from the ovarian hormones estrogen and progesterone. The results revealed that both the uterus and the salivary glands contained comprehensive glycosylation machinery enabling the construction of a complex glycome. Significantly, I noted about 300

glycosylation-related genes that were differentially regulated by estrogen and/or progesterone in the mouse uterus, but not in major salivary glands. For a subset of genes, I confirmed the glycoarray data at the mRNA level by quantitative PCR and at the protein level by immunolocalization. In the second part of the project, a bank of lectins and antibodies that recognize specific oligosaccharide structures was used to characterize the glycans expressed in mouse uterine and salivary tissues. Specialized carbohydrate structures that govern bacterial and leukocyte adhesion were detected in both regions. Many of these carbohydrate motifs were differentially regulated by estrogen and/or progesterone in the mouse uterus, as well as in major salivary glands. The information obtained from this project serves as an important prerequisite for developing the mouse as a powerful model system to study the association of periodontitis and preterm labor in the context of carbohydrate-mediated cell-cell adhesion.

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PART I. INTRODUCTION AND BACKGROUND

A. INTRODUCTION

For centuries, preterm labor and delivery of low-birth-weight (PLBW) infants has been a leading cause of perinatal morbidity and mortality worldwide (Goldenberg et al., 2008; Hack and Fanaroff, 1993; Han et al., 2010; Philip, 1995; Rush et al., 1976; Shapiro et al., 1980). Due to improvements in neonatal intensive care methods and surfactant therapy, the survival rate of these preterm neonates has improved greatly in the past decades. However, compared with term infants of normal birth weight, PLBW infants are still 40 times more likely to die in the neonatal period (Hack and Fanaroff, 1993; Philip, 1995; Rush et al., 1976; Shapiro et al., 1980). They also face a much higher risk of several neurodevelopmental disturbances, congenital anomalies, and health problems in later life, such as vascular diseases and diabetes (Hack and Fanaroff, 1993; Philip, 1995; Rush et al., 1976; Shapiro et al., 1980). In the United States, the cost of caring for these preterm newborns has been estimated at $5-6 billion annually (Rogowski, 1998). As we do not understand at a fundamental level the processes that lead to birth at term, the etiology of preterm birth is even more enigmatic, one reason that the rate of this devastating pregnancy complication has not changed for the last 40 years (Goldenberg et al., 2008; Hamilton BE, 2006; Rogowski, 1998).

It is now clear that preterm labor is actually a syndrome with many different causal associations (Goldenberg et al., 2008; Romero et al., 1994). Reproductive tract infections are considered to be one of the major initiating factors in preterm labor. Although the mechanism(s) underlying this phenomenon are unknown, infection may be responsible for 30-40% of preterm births (Andrews et al., 2000; Goldenberg et al., 2000; Goncalves et al., 2002; Terzidou and Bennett, 2002). A growing body of evidence clearly indicates an association between periodontal infection and the birth of PLBW infants (Agueda et al., 2008; Alves and Ribeiro, 2006; Boggess et al., 2006; Bosnjak et al., 2006; Goepfert et al., 2004; Jarjoura et al., 2005; Jeffcoat et al., 2001; Konopka et al., 2003; Lopez et al., 2002a; Marin et al., 2005; Mitchell-Lewis et al., 2001; Mokeem et al., 2004; Moreu et al., 2005; Offenbacher et al., 1996; Oittinen et al., 2005; Radnai et al., 2006; Romero et al., 2002; Sembene et al., 2000; Toygar et al., 2007). However, a number of other studies failed to identify an association between periodontal disease and births of PLBW infants (Bassani et al., 2007; Davenport et al., 2002; Gomes et al., 2006; Holbrook et al., 2004; Lunardelli and Peres, 2005; Mitchell-Lewis et al., 2001; Moore et al., 2004; Moore et al., 2005; Rajapakse et al., 2005; Sanchez et al., 2007; Srinivas et al., 2009; Vettore et al., 2008; Wood et al., 2006). Although the standards used to diagnose and categorize periodontal disease varied among these studies, systematic differences in approach did not appear to explain the discrepancies. As yet, no definitive causal relationship at a molecular level has been established between these two conditions. The many similarities between the bacterial ecologies of the oral cavity and the reproductive tract may

provide important clues to explain this correlation (Sections B8 and B9), as well as insights into the mechanisms that underlie a subset of both pathologies.

With regard to the oral cavity, previous studies demonstrated that adhesion of bacteria that normally colonize this region (*e.g., Actinomyces naeslundii* and several *streptococcal* strains) and certain periodontal pathogens (*e.g., Fusobacterium nucleatum*), is mediated by carbohydrate receptors that are carried by salivary mucins (Section B4) (Gillece-Castro et al., 1991; Murray et al., 1982; Prakobphol et al., 1999; Prakobphol et al., 1998; Prakobphol et al., 2000). For example, salivary mucin MG2 carries different sets of carbohydrate receptors that can mediate adhesion with both bacteria and neutrophils (Prakobphol et al., 1999; Prakobphol et al., 1998). As these and other carbohydrate structures are critical for the maintenance of a healthy oral environment, the glycosyltransferases that synthesize them have been well studied in oral mucosa and salivary glands under normal and malignant conditions (Section B4) (Dabelsteen, 2002; Dabelsteen and Gao, 2005; Dabelsteen and Jacobsen, 1991; Liu et al., 1999; Liu et al., 1998; Nita-Lazar et al., 2009). As for the human reproductive tract, ABH blood group antigens and related carbohydrate structures are detected in cervical and uterine endometrium (Stubbe Teglbjaerg et al., 1991), and change under malignant conditions (Skovlund, 1997). Moreover, the cancer-related glycan alterations are related to the ovarian hormones estrogen and progesterone (Skovlund, 1997). It has been shown that special fucosylated and sulfated carbohydrates that facilitate rolling and tethering

of leukocytes along vasculature, the first event in leukocyte extravasation, were also detected on human uterine epithelial cells, human salivary mucins and mouse major salivary glands (Genbacev et al., 2003; Prakobphol et al., 2005) (Ma and Fisher, unpublished data). **The principal goal of this thesis project is to test the hypothesis that the mucin-coated oral and uterine cavities present similar carbohydrate structures that specify the bacterial ecology and leukocyte recruitment of both regions.** This theory also suggests that certain individuals express carbohydrate motifs that make them susceptible to both periodontal disease and preterm labor. **The approach is to develop a mouse model for testing this hypothesis.** The research plan consists of the following specific aims:

Aim 1. Profile the glycosylation machinery expressed by the mouse major salivary glands and uterus. In contrast to humans, little is known about glycosylation-related gene expression patterns in the mouse oral and uterine cavities. A combination of microarray and immunolocalization approaches will be utilized to describe the expression of glycosyltransferases and related enzymes involved in carbohydrate synthesis. As sulfation of certain carbohydrate structures plays critical role in many biological events, sulfotransferases will also be studied. The effects of the ovarian hormones estrogen and progesterone on the expression pattern of these genes will also be investigated.

Aim 2. Characterize the oligosaccharide structures that are carried by mouse salivary and uterine glycoproteins and glycolipids. The carbohydrate structures presented by the major human salivary glycoproteins have already

been described (Gillece-Castro et al., 1991; Prakobphol et al., 2005; Prakobphol et al., 1993; Prakobphol et al., 1999; Prakobphol et al., 1998; Thomsson et al., 2002). In addition to presenting oligosaccharide bacterial receptors, human salivary mucins carry a different set of structures that serve as the carbohydrate ligands for L-selectin (Prakobphol et al., 2005; Prakobphol et al., 1999; Prakobphol et al., 1998), a molecule that initiates leukocyte-endothelial cell interactions. We already know that reagents specifically recognizing these highly specialized structures react with human salivary and uterine mucins (Genbacev et al., 2003; Prakobphol et al., 2005). Many of these reagents also react with oligosaccharides carried by mouse vascular endomucins, the sialomucins expressed in high endothelial venules (Bistrup et al., 1999; Mitoma et al., 2009; Tu et al., 1999b). A bank of antibodies and lectins that specifically react with these and other carbohydrate structures will be used to determine whether similar structures are presented by mouse oral and uterine cavities. The effects of estrogen and progesterone on the oligosaccharides will also be examined.

In summary, these experiments will test the relationship between carbohydrate features expressed in the murine oral cavity and reproductive tract as one way to gain insights into the bacterial colonization of both regions, as well as leukocyte recruitment. This important information will improve our understanding of the pathogenesis of both periodontal disease and preterm labor. Eventually, knowledge of the glycoforms found in both locations will be used to design

mechanistic tests and novel therapies for the prevention or treatment of preterm labor and periodontal disease.

B. BACKGROUND AND SIGNIFICANCE

B.1. The biological implication of carbohydrates at the cell surface.

Glycobiology, a branch of biology that studies the structure, biosynthesis, and function of the complex carbohydrates, is a rapidly growing field, and for good reason. The surface of all cells and many macromolecules is decorated with an array of monosaccharides joined together via specific linkages to form oligosaccharides, or glycans. These diversified structures are covalently attached to their protein carriers by various glycosyltransferases in a step-wise fashion co- or post-translationally, a process termed glycosylation. The modified proteins (or lipids) are referred to as glycoproteins (or glycolipids). The carbohydrate portions of these glycoconjugates are positioned distally at the cell surface, where they mediate a variety of cell-cell, cell-matrix, and cell-molecular interactions critical to the development and function of complex multicellular organisms (Marth and Grewal, 2008; Sperandio et al., 2009; Varki, 1999a). Because of this important role, alterations in glycosylation result in many diseases. A group of severe and multi-systemic disorders, collectively termed congenital disorders of glycosylation (CDG), represents a large and rapidly expanding category of diseases that illustrate the biological importance of carbohydrates (Haeuptle and Hennet, 2009). In addition, aberrant glycosylation is a hallmark of certain inflammatory

diseases, such as cystic fibrosis (Scanlin and Glick, 1999), inflammatory bowel disease (Campbell *et al*., 2001; Rhodes JM, 1996), rheumatoid arthritis (Parekh et al., 1988; Rademacher et al., 1988), and cancer (Ohyama, 2008; Reis et al., 2010; Szajda et al., 2008). Recent advances in the study of glycosyltransferases, particularly via the genetic modification of these enzymes in mice, have clearly demonstrated many unexpected *in vivo* biological functions for complex carbohydrate structures. These range from processes such as embryonic/neonatal development and spermatogenesis, through neurological and immunological functions, to pathologies such as oncogenesis (Furukawa et al., 2001).

B.2. Mucins and cellular recognition: Both the oral and uterine cavities in humans and mice are coated with glycoproteins, of which mucins are the major component. These molecules are characterized by a high level of *O*-linked oligosaccharides attached to serine or threonine residues along the peptide backbone. As oligosaccharide side chains generally carry negative charges, even simple motifs composed of only a few sugar residues exhibit a very large hydration volume that influences glycoprotein structure. For example, the densely *O*-glycosylated regions of mucins have a “bottle brush” conformation in which the protein stem is buried beneath a forest of carbohydrate residues (Jentoft, 1990).

Commonly, mucins are categorized as secretory or membrane-associated (Corfield, 1992; Kufe, 2009; Strous and Dekker, 1992; Van Klinken et al., 1995).

The secretory mucins are the principal protein components of the mucous layer that coats epithelial surfaces of the gastrointestinal, respiratory, and reproductive tracts. The mucous layer forms a selective physical barrier, protecting underlying tissue from mechanical, chemical, and microbial assaults while governing interactions between the epithelium and its milieu (Corfield, 1992; Kufe, 2009; Strous and Dekker, 1992; Van Klinken et al., 1995). The membrane-associated mucins have sufficient length to stick out well above the cell's glycocalyx. Therefore, in addition to serving as a selective, protective barrier, mucin-type oligosaccharides are ideally placed to mediate interactions with extracellular matrices and adjacent cells or pathogens. For example, attachment of microbial proteins to host cell-surface carbohydrates is considered an essential step for a successful infection (Karlsson, 1995). Thus, mucins play important roles in maintaining epithelial health and homeostasis. Due to their strategic locations and critical functions, aberrant mucin expression is observed in many pathological conditions such as inflammatory diseases and cancer (Beatty et al., 2007; Heazlewood et al., 2008; Kufe, 2009).

The human epithelial mucins and the genes that encode them have been studied extensively. Currently, 21 human epithelial mucin genes have been cloned and named in the order of their discovery as follows: MUC1-2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6-8, MUC11-13, MUC16, to MUC21 (Dekker *et al*., 2002; Kufe DW, 2009). Among these, MUC2, MUC5AC, MUC5B, and MUC6—the large, secretory, gel-forming mucins—are located at chromosomal

locus 11p15.5 (Dekker et al., 2002; Kufe, 2009). MUC7 is a small soluble secreted mucin whose expression is restricted to the oral cavity (Offner and Troxler, 2000). MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13 and MUC16 encode mucins whose C termini span the plasma membrane (Dekker et al., 2002; Kufe, 2009). Several other mucins (MUC8 and MUC11) are not easily assigned to the aforementioned groups (Dekker et al., 2002). Given the current rapid rate of advancements in mucin biology, this list will likely grow.

B.3. Endothelial mucins and sulfation: In terms of their cell type of origin, mucins exist in two classes: epithelial and endothelial/leukocyte. The structures and roles of epithelial mucins are described in Section B2. The endothelial and leukocyte mucins are adhesion molecules that are critical for leukocyte extravasation, the first step in inflammation and lymphocyte homing (Van Klinken et al., 1995). L-selectin, a protein that binds carbohydrate motifs presented by endothelial mucins, initiates leukocyte tethering and rolling adhesion, the first step in the extravasation process (Puri et al., 1998). Additionally, it is well documented that the bioactivity of endothelial ligands for L-selectin requires sulfation of the carbohydrate side chains (Hemmerich et al., 1994; Imai et al., 1993; Imai et al., 1991; Shailubhai et al., 1997). Although the exact nature of the oligosaccharide structures that comprise the high-affinity, sulfated L-selectin binding partners remains unclear, two specific modifications, Gal-6-SO_4 and GlcNAc-6-SO_4, which are carried by Lewis^x (Le^x) and sialyl Le^x (sLe^x) epitopes, are important aspects of the biological ligands (Hemmerich et al., 1995). Furthermore, two antibodies (G72 and G152) that are specific for the 6-sulfo-sLe^x

epitope inhibit L-selectin-mediated binding (Mitsuoka et al., 1998). In contrast, antibodies directed against 6′-sulfo-sLe^X or 6,6′-sulfo-sLe^X do not (Mitsuoka et al., 1998). This observation was further confirmed using MECA-79, an antibody that recognizes an extended core 1 glycan that contains GlcNAc-6-SO_4. MECA-79 immunostains high endothelial venules (HEV), specialized regions where lymphocytes exit the vasculature and enter lymph nodes. Addition of this antibody blocks L-selectin-dependent lymphocyte attachment to HEV (Streeter et al., 1988). Currently, MECA-79 reactivity is accepted as a predictor of L-selectin ligand activity (Clark et al., 1998; Fuhlbrigge et al., 1996; Uchimura and Rosen, 2006; Wagner et al., 1996). A recent study, which combined affinity chromatography and mass spectrometry, revealed that 6-sulfo-sLe^x on core-2 and extended core-1 *O*-glycans are the major epitopes for L-selectin recognition (Hernandez Mir et al., 2009).

Seven human carbohydrate sulfotransferases that generate the aforementioned sulfation modifications (Gal-6-SO_4 and GlcNAc-6-SO_4) have been characterized at the molecular level (Bowman and Bertozzi, 1999; Fukuda et al., 2001; Grunwell and Bertozzi, 2002; Hemmerich and Rosen, 2000; Uchimura and Rosen, 2006). Thus far, six murine carbohydrate sulfotransferases have also been identified and characterized. Previous studies showed that while chondroitin 6-sulfotransferase (Chst3), keratan sulfate Gal-6 sulfotransferase (Chst1), *N*-acetylglucosamine 6-*O*-sulfotransferase (GlcNAc6ST-1, also known as Chst2), and chondroitin 6-*O*-sulfotransferase 2 (GlcNAc6ST4, also known as

Chst7) have broad tissue expression patterns (Fukuta et al., 1997; Hiraoka et al., 1999; Uchimura et al., 1998c; Uchimura et al., 1998a; Uchimura et al., 1998b), high endothelial cell *N*-acetylglucosamine 6-*O*-sulfotransferase (HEC-GlcNAc6ST, GlcNAc6ST-2), also known as L-selectin ligand sulfotransferase (LSST, or Chst4) is restricted to HEV endothelial cells. Accordingly, this enzyme is likely to be the L-selectin ligand sulfotransferase (Bistrup et al., 1999; Hiraoka et al., 1999). In contrast, Intestinal GlcNAc6-sulfotransferase (I-GlcNAc6ST, GlcNAc6ST-3, also known as Chst5), is detected only in intestinal tissues (Lee et al., 1999). Whether or not this enzyme plays a role in lymphocyte homing in this region remains to be determined.

Accumulating evidence suggests that HEV-like vessels have been observed in several types of human inflammatory lesions (Kirveskari et al., 2000; Pablos et al., 2005; Salmi et al., 1997; Toppila et al., 2000; Toppila et al., 1999; Turunen et al., 1995), and in mouse models of collagen-induced arthritis (Yang et al., 2006). The HEC-GlcNAc6ST was detected in HEV-like vessels within ectopic lymphoid aggregates in mice (Bistrup et al., 2004). These data indicate that selectins and their specialized carbohydrate receptors are also involved in leukocyte recruitment to chronically inflamed non-lymphoid tissues.

B.4. Salivary mucins—important determinants of the oral ecology: Human salivary glands secrete two prototypical mucins: high-molecular-weight mucin MG1, encoded by MUC5B, and low-molecular-weight mucin MG2, encoded by

MUC7. As the major constituents of the mucous coating of the oral mucosa and tooth surface, MG1 and MG2 are essential for oral health, performing many diverse roles in the oral cavity (Amerongen et al., 1995; Offner and Troxler, 2000; Tabak, 1995; Tabak et al., 1985). Broadly, mucin oligosaccharides function in two ways—generally protecting epithelial surfaces by hydrating and lubricating, and specifically mediating adhesive interactions with leukocytes, bacteria, and/or viruses by binding particular receptors. With respect to the latter process, bacteria adhere to MG2 via T and sialyl-T carbohydrate antigens, as well as lactosamine sequences (Prakobphol et al., 1998). MG2 also carries Le^x and sLe^x carbohydrate structures that mediate interactions with neutrophils under conditions of shear stress (Karlsson and Thomsson, 2009; Prakobphol et al., 1999; Prakobphol et al., 1998). MG1, a product of the MUC5B gene with a molecular weight of over 1 million, carries a repertoire of unique oligosaccharides that is large and diverse (Thomsson et al., 2002). Interestingly, MG1 carbohydrate motifs (Lewis blood antigens) serve as microbial receptors for *Helicobacter pylori* (Bosch et al., 2000; Prakobphol et al., 2005). Additionally, MG1 carries MECA-79–reactive, high-affinity, sulfated L-selectin epitopes (Prakobphol et al., 2005), suggesting that this mucin may play important roles in leukocyte trafficking in the oral cavity. Furthermore, these highly specialized MECA-79 epitopes on MG1 appear to be under the regulation of the ovarian hormones estrogen and progesterone in humans (Prakobphol et al., 2005). MUC1 and MUC4 are expressed by the epithelial cells lining striated and excretory ducts and in some serous acinar cells of parotid and submandibular

glands, and MUC1 is also detected in ducts of minor salivary glands (Liu et al., 2002; Sengupta et al., 2001). Taken together, these data suggest that human salivary mucins play important roles in specifying the oral ecology through the carbohydrate structures they carry.

The glycosyltransferases that synthesize blood group antigens ABO(H) and Lewis antigens in oral tissue and saliva are well studied (Dabelsteen, 2002; Dabelsteen and Gao, 2005; Mandel et al., 1992; Ravn and Dabelsteen, 2000; Ravn et al., 1992). In oral epithelial cells, ABO(H) blood group antigen synthesis begins with fucosyltransferase 2 (FUT2, or secretor gene) that adds an α-1,2-fucose to the precursor glycans to form the H epitopes. Subsequently, A or B determinants are constructed on H epitopes by either an α-1,3-GalNAc transferase (A transferase) or an α-1,3-Gal transferase (B transferase), respectively. The Lewis blood group antigens are the products of the concerted actions of a structurally similar set of α-1,3/4-fucosyltransfereases (Lowe, 1999a). All of these enzymes are detected in human oral epithelial cells and their activities are correlated to the expression pattern of ABO(H) and Lewis blood group antigens in oral mucosa and saliva (Dabelsteen, 2002; Dabelsteen and Gao, 2005).

B.5. Mucins in the female reproductive tract: Many studies demonstrate that uterine mucins constitute a large portion of the mucous coating of the female reproductive tract (Carson et al., 1998; DeSouza et al., 1999; Gipson et al.,

1997). They play important barrier roles in reproductive processes and provide protection from bacterial infections (Carson et al., 1998; DeSouza et al., 1999). Interestingly, MUC5B, found in saliva, is a major gel-forming, oligomeric mucin of the human endocervix (Wickstrom et al., 1998). Other secretory mucins, such as MUC2, MUC5AC, and MUC6, are also detected in the reproductive tract at the mRNA and/or protein levels (Audie et al., 1995; Gipson et al., 1997; Gollub et al., 1993; Zhao et al., 2003). Additionally, the endocervical epithelium expresses other membrane-bound mucins, such as MUC1, MUC4, and MUC8 (Audie et al., 1995; Gipson et al., 1997; Gollub et al., 1993; Zhao et al., 2003). A study using semi-quantitative PCR determined that human endocervical mucins MUC5B and MUC4 mRNA level change during the menstrual cycle, with transcripts peaking right before or at midcycle (Gipson et al., 1999). Using an antibody to MUC5B and a quantitative ELISA technique, the highest level of MUC5B protein was detected also at midcycle in cervical mucus plugs of women (Gipson, 2001). Recently, the Fisher laboratory showed that uterine mucins carry high-affinity, sulfated L-selectin ligands (Genbacev et al., 2003). Furthermore, they found that expression of the specific carbohydrate structures that serve as selectin recognition determinants is strongly upregulated as the uterus becomes receptive. Interactions between these oligosaccharide motifs and L-selectin expressed on trophoblasts likely mediate the initial attachment step in human implantation (Genbacev et al., 2003). These data also suggest that sulfation is hormonally regulated, a hypothesis that will also be tested. MUC1 is the likely candidate scaffold for L-selectin ligands (Carson et al., 2006). To my knowledge,

human uterine mucins and salivary MG1 represent the only published examples of functional L-selectin ligands outside of the vascular system. Together, these data suggest that L-selectin ligands may be important candidates in our search for carbohydrate epitopes that govern infection in both the oral and uterine cavities.

The expression of ABO(H) and Lewis blood group antigens in the human reproductive tract has been well characterized (Ravn and Dabelsteen, 2000). The relevant glycosyltransferases have also been detected in these tissues (Ravn and Dabelsteen, 2000). The variable expression of these histo-blood group antigens in the endometrium during the menstrual cycle suggests that the corresponding glycosyltransferases are regulated by estrogen and progesterone.

B.6. Murine epithelial mucin biology: With genetic and physiological similarities to humans and facile genetic manipulation, the laboratory mouse has become the premier mammalian model system to study the pathogenesis of various human diseases, their therapy and prevention. However, in contrast to humans, murine epithelial mucins and the genes that encode them have not been extensively studied. Four mouse orthologs to human gel-forming mucins (Muc2, Muc5AC, Muc5B, and Muc6) have been cloned and sequenced (Aslam et al., 2001; Desseyn and Laine, 2003; Escande et al., 2002; Inatomi et al., 1997; Jonckheere et al., 2004; van Klinken et al., 1999). All are located on mouse chromosome 7 band F5, which is equivalent to human chromosome 11p15.5,

where the human orthologs are located. These data suggest that mucin genes may have been conserved among different species during evolution. Gene deletion studies in mouse models have revealed critical functions for these molecules in epithelial homeostasis. For example, $Muc2^{-/-}$ mice display aberrant intestinal crypt morphology and alterations in cell maturation and migration (Velcich et al., 2002). Notably, these transgenic mice frequently developed small intestine adenomas that progressed to invasive adenocarcinomas, as well as rectal tumors (Velcich et al., 2002). These results confirm that mucins play important roles in epithelial cells.

With the exception of Muc1, the mouse membrane-bound epithelial mucins are poorly studied. Muc1 expression by mouse uterine epithelia was confirmed both *in vitro* (Pimental et al., 1996) and *in vivo* (Braga and Gendler, 1993; Surveyor et al., 1995). Intensive research on this molecule shows important functions in reproductive processes (Carson et al., 1998). This mucin appears to be down-regulated before implantation occurs (Surveyor et al., 1995). The expression of Muc1 mRNA and protein is regulated by ovarian hormones during early pregnancy in mice (Surveyor et al., 1995). Furthermore, Muc1-null mice are highly susceptible to reproductive tract bacterial infection involving the cervix and vagina (Carson et al., 1998; DeSouza et al., 1999), an observation that correlates well with the known functions of mucins.

Likewise, the nature of mouse salivary mucins is less well understood. Although high-molecular-weight mucins were isolated from the mouse sublingual and submandibular glands (Amerongen et al., 1983; Denny and Denny, 1982; Denny et al., 1980; Roukema et al., 1976), little is known about these molecules. Recently, mouse salivary gland Muc6 mRNA expression was detected by RT-PCR (Desseyn and Laine, 2003). A few studies explored the oligosaccharide structures that mouse salivary mucins carry. Amerongen and co-workers showed that mouse submandibular mucins contain mannose, galactose and sialic acid residues (Nieuw Amerongen et al., 1987). Several high mannose-type oligosaccharide structures were also detected in the same study. Denny and colleagues described *N*-linked oligosaccharides on mucins isolated from mouse submandibular glands (Denny et al., 1995). In a recent mass spectrometry-based analysis of major sublingual mucins from the rat, *O*-glycans were dominated by sialylated core 3- and 4-type structures, while *N*-glycans featured non-bisected hybrid structures bearing sialylated type II lactosamine units (Yu et al., 2008).

During the last decade, a great deal of knowledge about glycosyltransferases has been generated from studies using laboratory mice (Lowe, 1999b). Novel functions of complex carbohydrates have been discovered from studies of mice with mutations of various glycosyltransferase genes (Furukawa et al., 2001). For example, Fut2 null mice display an altered glycan profile (Magalhaes et al., 2009). Moreover, these mice also have impaired *Helicobacter pylori* adhesion mediated by fucosylated carbohydrate receptors (Magalhaes et al., 2009) and

increased susceptibility to experimental vaginal candidiasis (Hurd and Domino, 2004). The Fut2-LacZ transgenic mice clearly demonstrate that their mRNA is expressed in uterine luminal and glandular epithelial cells and its abundance is regulated by estrogen (Domino and Hurd, 2004).

B.7. Preterm labor and delivery: Preterm births are defined as those that occur prior to 37 weeks gestational age. Approximately 50% of all preterm births result from the spontaneous onset of preterm labor, and 30% follow premature rupture of the membranes. Births attributable to these two events are collectively referred to as spontaneous preterm births. The remaining 20% follow a decision by the physician to deliver the pregnancy for specific maternal or fetal indications and, consequently, are referred to as elective or indicated preterm births. Due to advances in neonatal intensive care methods and surfactant therapy, the survival rate of preterm infants has dramatically improved over the last 20 years. However, the rate of long-term morbidity among survivors has not decreased (Goldenberg et al., 2008; Hack and Fanaroff, 1993; Philip, 1995; Rush et al., 1976; Shapiro et al., 1980). In the USA, the preterm delivery rate has been rising for the past two decades, increasing from 9.5% in 1981 to 12.7% in 2005 (Hamilton BE, 2006). Although growing numbers of indicated preterm births and preterm delivery of *in vitro* fertilized multiple pregnancies predominantly contribute to this rise (Goldenberg et al., 2008), the trend is alarming. Despite enormous financial investments and massive clinical prevention efforts, preterm delivery of PLBW infants still remains the number one challenge in obstetrics

today (Goldenberg et al., 2008; Hamilton BE, 2006; Iams et al., 2008; Rogowski, 1998; Saigal and Doyle, 2008).

B.8. Reproductive tract infection and spontaneous preterm birth: It is clear that preterm labor is a syndrome with a variety of different potential causes (Goldenberg et al., 2008; Romero et al., 1994). Known factors that are associated with preterm labor and delivery include maternal conditions (medical illnesses, anemia and uterine malformations), past medical events (prior obstetric complications, previous preterm labor, cervical surgery), intrinsic factors (reproductive tract infections, multiple fetuses, maternal age, short interpregnancy interval) and maternal behaviors (smoking and drug abuse) (Goldenberg et al., 2008; Robinson et al., 2001). Demographic variables such as race, employment and socioeconomic status can also associate with preterm labor (Goldenberg et al., 2008; Robinson et al., 2001).

In 1977, the first report was published describing the isolation of bacteria from the uterine cavities of 70% of women in preterm labor prior to membrane rupture (Bobitt and Ledger, 1977). Since that time, substantial data have accumulated indicating that reproductive tract infections, especially silent infections involving the upper tract, are the major initiating factor in spontaneous preterm birth, correlated with 30-40% of the cases (Andrews et al., 2000; Goldenberg et al., 2000; Goncalves et al., 2002; Terzidou and Bennett, 2002). The bacteria isolated from the upper reproductive tracts of women who have preterm births are

generally representative of the normal, low-virulence microbial flora of the cervix and vagina, such as *Ureaplasma urealyticum, Fusobacterium* species, *Mycoplasma hominis, Gardnerella vaginalis, Peptostreptococci* and *Bacteroides* species (Table 1.1) (Andrews et al., 1995a; Gibbs et al., 1992; Hauth and Andrews, 1998; Hillier et al., 1988; Krohn et al., 1995).

The ascending route from the lower reproductive tract is the most common pathway for intrauterine infections (Mazor et al., 1994). Only 12.5% of women with preterm labor, intact membranes and positive amniotic fluid cultures have clinical symptoms of chorioamnionitis (Romero et al., 1989). Consequently, clinical diagnosis of intra-uterine infections proves to be a very difficult task. Moreover, among women who have spontaneous preterm labor, the percentage of infections increases as the gestational age at delivery decreases (Andrews et al., 1995b; Watts et al., 1992). Although the subset of spontaneous preterm births that occur before 32 weeks of gestational age comprises only 1-2% of total affected pregnancies, this group accounts for the majority of adverse outcomes in terms of infant morbidity and mortality (Rogowski, 1998).

B.9. Periodontal disease and preterm labor: Periodontal disease, or periodontitis, the number one cause of tooth loss in adults, occurs in approximately 15% of the U.S. population (Williams, 1990). Periodontal disease is initiated by oral pathogens that induce host-mediated tissue destruction, which likely represents an excessive immune response to bacteria or bacterial products

that penetrate the periodontal tissues (Kornman and Van Dyke, 2008; Van Dyke, 2008; Williams, 2008). The periodontal pathogens are normal habitants of oral surfaces with low virulence, such as *Fusobacterium* species, *Peptostreptococci*, and *Bacteroides* species (Table 1.1) (Haffajee and Socransky, 2005; Socransky and Haffajee, 2005).

Periodontal disease takes various forms (Armitage, 2003). Chronic periodontitis, the most common type, is defined as an inflammatory disease of the supporting tissues around teeth, *i.e.,* periodontal ligament, cementum, and alveolar bone. It is most prevalent in adults in their late thirties and beyond. Chronic periodontitis is probably caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal supporting tissues, and leading to periodontal pocket formation, gingival recession, and eventually tooth loss. Aggressive periodontitis (AP) is characterized by rapid attachment loss and bone destruction. Patients with AP are generally healthy and the amount of microbial deposits does not correlate with disease severity. There appears to be a heritable component that confers susceptibility to this form of periodontitis, which typically develops in prepubescent or pubescent children and adults under 30 years of age.

The etiology of AP remains poorly understood. Certain cases of periodontitis appear to represent manifestation of systemic diseases (Armitage, 2003). The identified risk factors for periodontal diseases include smoking, diabetes mellitus

and other systemic conditions, the presence of specific pathogenic bacteria in the subgingival flora, and poor oral hygiene (Irfan et al., 2001; Nunn, 2003; Page, 2002; Page and Beck, 1997; Papapanou, 1999). Strikingly, African Americans are 15 times more likely to develop AP than their Caucasian peers (Brown et al., 1989; Saxby, 1987). Considering the relationship of infection to periodontitis, and the roles of carbohydrate receptors in bacterial adhesion, it is not surprising that a glycosyltransferase gene, GLT6D1, was identified as a susceptibility locus for aggressive periodontitis in a genome-wide association study in a German population (Schaefer et al., 2010). Although GLT6D1 appears to catalyze the formation of an α-1,3-Gal/GalNAc linkage, the function of this enzyme needs to be confirmed (Schaefer et al., 2010).

Recent studies in periodontal medicine strongly suggest a mild to moderate association between human periodontal disease and certain systemic disorders such as diabetic mellitus (Nishimura et al., 1998; Salvi et al., 1997), pneumonia (Scannapieco, 1999; Scannapieco and Ho, 2001), heart and vascular disease (Arbes et al., 1999; Chiu, 1999; Meyer and Fives-Taylor, 1998; Morrison et al., 1999), and preterm labor/delivery of PLBW babies (Agueda et al., 2008; Alves and Ribeiro, 2006; Boggess et al., 2006; Bosnjak et al., 2006; Goepfert et al., 2004; Jarjoura et al., 2005; Jeffcoat et al., 2001; Konopka et al., 2003; Lopez et al., 2002a; Marin et al., 2005; Mitchell-Lewis et al., 2001; Mokeem et al., 2004; Moreu et al., 2005; Offenbacher et al., 1996; Oittinen et al., 2005; Radnai et al., 2006; Romero et al., 2002; Sembene et al., 2000; Toygar et al., 2007). After

adjusting for all other risk factors, Offenbacher and colleagues determined that mothers with periodontal infections have a more than a seven-fold elevation in the risk of delivering a PLBW infant (Offenbacher et al., 1996). Furthermore, compared to the mothers with normal-birth-weight infants, the mothers with PLBW infants have much more severe periodontitis and a higher level of periodontal pathogens (Offenbacher et al., 1998).

Animal studies suggest that exposure to periodontal pathogens at a distant location can induce deleterious pregnancy outcomes. For example, in golden hamsters, a single intravenous challenge of endotoxin from *Porphyromonas gingivalis* (a potential periodontal pathogen) on day 8 of pregnancy reduces fetal weight and increases fetal resorption in a dose-dependent manner (Collins et al., 1994a). In a related study, live or heat-killed *Porphyromonas gingivalis* were introduced into a subcutaneous chamber before golden hamsters were mated. Then, the animals were challenged by inoculation with the same bacteria on day 8 of pregnancy. This treatment increased the inflammatory mediators prostaglandin E_2 (PGE_2) and tumor necrosis factor α (TNF-α) in the subcutaneous chamber, reduced fetal weight and increased fetal resorption and embryolethality (Collins et al., 1994b).

Two pilot intervention studies suggest that periodontal therapy may reduce preterm births in pregnant women with periodontal disease (Jeffcoat et al., 2003; Lopez et al., 2002b). These studies suggest that systemic host inflammatory

responses triggered by periodontitis may also stimulate the labor process, a theory supported by others (Madianos et al., 2001; Offenbacher et al., 1998). In addition, a hypothetical model in which periodontal pathogens that disseminate systemically in maternal blood gain access to the fetal compartment has been suggested (Champagne et al., 2000). In support of this scenario, an oral strain of *Bergeyella* was detected by PCR in amniotic fluids from a preterm labor patient (Han et al., 2006). Furthermore, in mice, diverse oral bacteria were able to transfer to the placenta through a hematogenous route (Fardini et al., 2010), indicating that periodontal pathogens might be able to translocate to and colonize the intrauterine cavity during pregnancy.

Despite the growing body of positive evidence, a number of other studies failed to find an association between periodontal disease and PLBW (Bassani et al., 2007; Davenport et al., 2002; Gomes et al., 2006; Holbrook et al., 2004; Lunardelli and Peres, 2005; Mitchell-Lewis et al., 2001; Moore et al., 2004; Moore et al., 2005; Rajapakse et al., 2005; Sanchez et al., 2007; Srinivas et al., 2009; Vettore et al., 2008; Wood et al., 2006). Although no mechanistic explanations have been offered to explain this discrepancy, it is noteworthy that the populations under study varied widely. For example, 58% of the subjects in the Offenbacher study that first identified the association were African American (Offenbacher et al., 1996). In contrast, in a study that did not detect any association between periodontal disease and preterm births, 53% of the subjects were of Bangladeshi origin (Davenport et al., 2002). In the context of our central

hypothesis, it is possible that these differences could be due, in part, to ethnic/racial differences in glycosyltransferase activities (Watkins et al., 1995).

In an attempt to clarify the conflicting data, several epidemiologists have conducted systematic reviews and meta-analyses to summarize the literature (Khader and Ta'ani, 2005; Scannapieco et al., 2003; Vergnes and Sixou, 2007; Vettore et al., 2006; Xiong et al., 2006; Xiong et al., 2007). A majority of these analyses identified maternal periodontal disease as a possible risk factor for adverse pregnancy outcomes, including preterm births. In the meta-analysis conducted by Vergnes and Sixou, which included 17 epidemiological surveys and enrolled 7151 women, the pooled odds ratio (OR) for pregnant women with periodontal disease to experience preterm births was 2.83 (Vergnes and Sixou, 2007). The authors recommended that further studies on this topic are needed, including ones to investigate the molecular mechanisms behind this association.

Because of the severe impact of periodontal disease and preterm births on society and the tremendous potential benefit if periodontal treatment reduces preterm birth rates, the National Institute of Dental and Craniofacial Research (NIDCR) sponsored three multi-center randomized clinical trials to study the effects of periodontal treatment on preterm births. All three studies recently published their results. Although periodontal treatment during pregnancy improves the periodontal health of pregnant women and is safe, the therapies failed to reduce the preterm birth rate (Macones et al., 2010; Michalowicz et al.,

2006; Offenbacher et al., 2009). As we do not understand the link between periodontal disease and adverse pregnancy outcomes at the molecular level, it is difficult for clinicians to design the appropriate strategies with regard to the timing and treatment methods for periodontal disease in pregnant women, a possible reason that the clinical trials failed to reduce preterm births.

B.10. Significance: The oral cavity and the reproductive tract are both coated with mucins presenting specialized oligosaccharide structures that function as receptors for leukocytes and bacteria. Provocatively, periodontal and uterine infections share similar bacterial ecologies with respect both to normal flora and pathogenic conditions. In both locations, pathogenic bacteria tend to be facultative or anaerobic, gram-negative species, that arise from normal flora with relatively low virulence (Table 1.1). Previous studies identified specific carbohydrate motifs carried by salivary mucins and other glycoproteins that mediate adhesion of both normal colonizers of the oral cavity and certain periodontal pathogens (Bosch et al., 2000; Edgerton et al., 1993; Gillece-Castro et al., 1991; Murray et al., 1992; Prakobphol et al., 1999; Prakobphol et al., 2000; Veerman et al., 1995). In parallel, a study using mice genetically deficient in Muc1 showed that this mucin plays an important role in reproductive tract infection (DeSouza et al., 1999). Furthermore, the highly specialized fucosylated and sulfated carbohydrate structures that mediate the initial attachment of leukocytes during inflammation and lymphocytes homing are present in both oral and uterine tissues (Genbacev et al., 2003; Prakobphol et al., 2005). Therefore, I hypothesize that carbohydrate receptors with similar structures govern adhesion

of bacterial pathogens in both the oral and uterine cavities, as well as recruitment of leukocytes into these locations, and that this correlation explains the enigmatic connection between periodontal disease and birth of PLBW infants. In the work described here, I explored this association using a mouse model as the experimental system. The resulting information describing the general feature of mouse oral and uterine glycomes will provide a valuable adjunct to studies in humans. In the future, with this basic knowledge in hand, I can use transgenic animals as a powerful method for continuing my studies designed to elucidate the connection between periodontal disease and preterm labor with the eventual goal of designing treatment and/or prevention strategies.

Previous work from Dr. Fisher's group showed that individual salivary components, unlike plasma/serum proteins, are glycosylated very differently. Although more research is needed to confirm whether uterine glycoproteins share this feature with their counterparts in the oral cavity, I determined that uterine and salivary glycoproteins of mice shared certain very specialized carbohydrate structures that can function as bacterial and leukocyte receptors. It is already well established that a subset of individuals who express the Le^b antigen on their gastric mucins are more susceptible to chronic *Helicobacter pylori* infection. As a consequence, these individuals are more prone to developing gastric ulcers and cancer (Ilver et al., 1998; Jones et al., 2001). Similarly, I propose that certain individuals express carbohydrates that make them susceptible to both periodontal disease and preterm labor. As glycan structure is dictated, in part, by heritable components, (*e.g.,* the ABH blood

groups), this concept could explain the discrepancy among the different epidemiological studies that have investigated this association. The results of these and future studies could serve as the basis of an important method for identifying pregnant women with a high risk of preterm labor, therefore enabling physicians to provide special preventative treatments, such as antibiotic therapies, to this subgroup.

Part II. MURINE GLYCOSYLATION-RELATED GENES ARE DIFFERENTIALLY REGULATED BY THE OVARIAN HORMONES ESTROGEN AND PROGESTERONE IN THE UTERUS BUT NOT THE MAJOR SALIVARY GLANDS

INTRODUCTION

Microarray-based gene expression profiling has been used to simultaneously monitor the transcription of thousands of genes, and to follow changes in expression correlated with development, pathology, or various treatment regimens (Lashkari et al., 1997; Schena et al., 1995). Unfortunately, genes relevant to the synthesis and function of various carbohydrate structures are not well represented and are poorly annotated on commonly available commercial array chips (Comelli et al., 2006). Recently, the Consortium for Functional Glycomics (CFG) has developed a focused and well-annotated glycogene-chip using highly-vetted Affymetrix technologies. Versions of this chip have been used to examine human and murine glycosylation-related gene expression through various developmental stages and under specific disease conditions (Comelli et al., 2006; Diskin et al., 2006; Saravanan et al., 2009, 2010). In collaboration with CFG, I used microarray CFG Glycov2 chips to examine the glycosylation-related gene expression patterns in the mouse uterus and major salivary glands. I also tested the effects of treatment with the ovarian hormones estrogen and progesterone on gene expression in terms of glycosylation-related pathways. As immune tissues present the most complete profile of glycogenes (Comelli et al.,

2006), submandibular lymph nodes were included as positive controls. I then confirmed the glycoarray results for a subset of genes at both the mRNA and protein levels using Quantitative PCR (Q-PCR) and protein-level techniques. The data demonstrated that the mouse uterus and major salivary glands contain a comprehensive set of glycosylation machinery, enabling the construction of diverse sets of carbohydrate structures. Interestingly, many of these glycosylation-related genes were differentially regulated by the ovarian hormones estrogen and/or progesterone in mouse uterine tissues.

RESULTS

The mouse uterus and major salivary glands contain comprehensive and tissue-specific glycosylation machinery.

Using a global approach, I analyzed the glycosylation-related gene profiles of the mouse uterus, major salivary glands, and submandibular lymph nodes by the CFG GLYCOv2 array. In addition, to address the effects of the ovarian hormones estrogen and progesterone with respect to their impact on glycosylation in the reproductive tract, I ovariectomized the mice and supplemented them with estrogen and/or progesterone emulsified in sesame oil. The control group of mice received 0.1 ml oil subcutaneously. I chose dosages of estrogen (100 ng/mouse/day) and progesterone (2 mg/mouse/day) that are widely used in the studies of mammalian reproduction. These dosages are considered to be physiological because they induce a variety of reproductive events, such as implantation, decidualization, and uterine angiogenesis (Dey, 1996). Mice were

ovariectomized and treated with or without hormone(s) for 4 days. Then, uteri, major salivary glands (parotid, submandibular, and sublingual glands), and submandibular lymph nodes were collected. As additional controls for the hormonal treatments, major salivary glands and submandibular lymph nodes were also harvested from adult male mice (10 – 12 weeks old).

The mouse uterus becomes very thin after ovariectomy. Distinct morphological changes, such as uterine edema and tissue growth following ovariectomy and estrogen and/or progesterone supplementation were observed, which confirmed the effectiveness of the hormone treatments. No corresponding changes were observed in the major salivary glands and submandibular lymph nodes after hormone treatments (Ma and Fisher, data not shown).

For each treatment or control group, triplicate RNA samples were included for hybridization. A total of 42 array datasets were generated, and the expression signal values were calculated by using the RMA algorithm (Irizarry et al., 2003). The raw data can be accessed at the Consortium for Functional Glycomics website under CFG Data (http://www.functionalglycomics.org/publicdata/microarray.jsp; “Susan Fisher 1: Effects of estrogen and progesterone on glycosyltransferases expression in mice”).

Among the 925 mouse glycosylation-related genes included in this array, approximately 700 were detected in at least one tissue type (major salivary glands, submandibular lymph nodes, or uterus). These included glycosyltransferases, lectins, growth factors and receptors, chemokines, adhesion molecules, and molecules involved in nucleotide synthesis. For example, glycosyltransferases were well-represented in mouse uteri and major salivary glands. The enzymes involved in the synthesis of the core regions of *N*- and *O*-linked glycoproteins were ubiquitously expressed in all three tissue types and all treatment groups, with a few exceptions (Table 2.1). As for the enzymes that construct the highly versatile sub- and terminal carbohydrate structures (*e.g.,* sialyltransferases, fucosyltransferases, and sulfotransferases), expression patterns were more tissue-specific (Table 2.2). These data were in accord with prior reports documenting similar expression patterns in various tissues (Comelli et al., 2006; Fukui et al., 2002; Sutton-Smith et al., 2002; Wang et al., 2002).

As expected, lymph nodes yielded the most comprehensive set of terminal glycosyltransferases. The profile of these enzymes in the mouse uterus was reduced, but similar to that of lymph nodes, while major salivary glands had the simplest set of glycosyltransferases that add terminal sugar residues among the three tissue types. Interestingly, hormonal treatment differentially affected expression of the enzymes involved in creating terminal structures as compared to the glycosyltransferases involved in core assembly (Table 2.6), an observation that will be discussed in detail in later sections. Together, these data supported

the concept that differential expression of the enzymes that specify oligosaccharide termini, which confer their major functional activities, is a major factor in determining cell type- and tissue-specific glycosylation, as well as local responses to environmental cues such as hormones.

Lectins are proteins that specifically bind carbohydrates through recognition of particular structural motifs. Two major categories of animal lectins, the C-type (calcium dependent) and I-type (sialic acid immunoglobulin superfamily), play critical roles in protein-carbohydrate interactions involved in many immune functions, including inflammation, tumor surveillance and viral immunity (Varki, 1999). The expression data in Table 2.3 show that these proteins are expressed in a highly tissue-specific manner (Table 2.3). Most of the lectins included in the array were detected in the submandibular lymph nodes of all experimental and control groups. These expression patterns correlated well with the known immune-related functions of these carbohydrate-binding molecules (Cummings, 1999b; Varki, 1999c). In contrast, lectin expression patterns in the uterus and major salivary glands differed among the treatment groups, indicating that estrogen and/or progesterone differentially regulated their expression, a topic that will be discussed further in later sections.

Next, I examined the expression of glycoproteins including mucins, proteoglycans and other heavily glycosylated adhesion molecules (Tables 2.4 and 2.5). The expression patterns of these glycoproteins were highly tissue-

specific, with submandibular lymph nodes again expressing the highest number of the genes in question. A few glycoproteins displayed notable expression patterns. For instance, membrane-bound mucin, Muc1, was detected only in the mouse uterus, while two other membrane-bound sialomucins, Muc10 (Prol1, proline rich, lacrimal 1), and Muc14 (endomucin, Emcn), were observed in all three tissues. In contrast, the only two secretory mucins included in this array, Muc5AC and Muc5B, were not found in any of the tissues under study by this Glycov2 microarray system.

In summary, using a focused microarray chip, CGF Glycov2, I demonstrated that comprehensive tissue-specific glycosylation machinery is present in the mouse uterus, major salivary glands, and submandibular lymph nodes. The repertoire of expressed enzymes enables the construction of complex, functional glycans in mouse oral and uterine tissues.

Glycosylation-related genes are differentially regulated by the ovarian hormones estrogen and/or progesterone in the mouse uterus, but not in major salivary glands and submandibular lymph nodes.

To better understand the relationship among data sets, an unsupervised hierarchical clustering strategy using centered correlation and average linkage was used to compare the similarity or differences between expression profiles. As a result of this effort, a dendrogram was produced (Fig.2.1). Clusters that are merged low on the dendrogram are similar, whereas clusters merged high on the

dendrogram may be very heterogeneous. This analysis clearly showed distinct separation of the uterine data from different treatment groups (Fig.2.1). Neither the lymph node nor the major salivary gland datasets were separated based on hormonal treatment (Fig.2.1). These results indicate that there are true organ- and tissue-specific differences in the global gene expression profiles of the glycosylation machinery. We can confidently conclude that the ovarian hormone estrogens and/or progesterone differentially regulate the expression of glycosylation-related genes in the mouse uterus.

To identify specific genes differentially regulated by estrogen and/or progesterone, I performed a class comparison by using a two-tailed Student's *t*-test. A number of comparisons (E_2 vs. Oil, P_4 vs. Oil, E_2+ P_4 vs. Oil, E_2 vs. E_2+ P_4, P_4 vs. E_2+ P_4) were performed at the univariate significance level of 0.001. While the overall comparisons for major salivary glands and submandibular lymph nodes did not produce significant results even at the univariate significance level of 0.05, the comparison for uterine samples again generated a long list (~ 300) of genes that were significantly different according to distinct treatments (Fig. 2.2). Although both hormones differentially affected the expression levels of the genes that were interrogated, estrogen generally demonstrated a much greater impact. More than 260 genes were regulated by estrogen, whereas only about 50 genes were governed by progesterone (Fig.2.2). Co-administering both hormones reduced the number of regulated glycogenes to around 170 (Fig.2.2), indicating that progesterone can antagonize

the effects of estrogen on glycosylation-related gene expression. A heatmap was constructed of the 90 genes that were differentially regulated with a fold change ≥ 2 (Fig.2.3). A detailed list of more than 300 differentially regulated genes is presented in Supplemental Table 1.

The expression of mouse uterine genes involved in N- and O-glycosylation, glycosphingolipid biosynthesis, and many other glycosylation-related pathways are profoundly influenced by estrogen and/or progesterone.

To understand the effects of estrogen and/or progesterone on the expression of the glycogenes in a pathway context, I mapped the differentially regulated genes using Ingenuity Pathways Analysis software, which identifies biological pathways that are influenced by specific gene products (Ingenuity Systems, Palo Alto, CA, USA). The analysis revealed that a variety of canonical pathways, such as *O*-glycosylation and *N*-glycosylation, were affected by estrogen and/or progesterone treatment. The major pathways in the uterus that were governed by estrogen supplementation are shown in Figure 2.4. A detailed list of all affected pathways is provided in Supplemental Table 2. Furthermore, the glycosyltransferases that were differentially regulated by estrogen and/or progesterone, along with a description of their donor and acceptor specificities, are listed in Table 2.6. Information on glycosyltransferase function described in the table was obtained from the National Center for Biotechnology Information (NCBI) gene database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway analysis database.

Clearly, many enzymes involved in *N*-glycosylation were differentially regulated by estrogen and/or progesterone (Table 2.6). The glycosyltransferases that add the first two core *N*-acetyl-glucosamine (GlcNAc) monosaccharides (*Dpagt1* and *Alg13*) to the dolichol oligosaccharide precursor were upregulated 1.6- (*Dpagt1*) and 1.5-fold (*Alg13*) by estrogen. After synthesis of the precursor, the *N*-oligosaccharyl transferase complex (OST) on the ER membrane transfers the precursor *en bloc* to asparagine residues in nascent proteins inside the ER. The mRNA levels of three OST complex subunits, Rpn2, Dad1, and Ddost, increased (fold difference of 1.3- to 1.9-fold) with estrogen alone and estrogen + progesterone treatments. Finally, several enzymes involved in the processing of high mannose *N*-glycans to hybrid and complex types, including mannoside *N*-acetylglucosaminyltransferase IV c (*Mgat4c)*, UDP-Gal:β GlcNAc β 1,4-galactosyltransferase 1 (*β4galt1)* and UDP-Gal:β GlcNAc β 1,4-galactosyltransferase 3 (*β4galt3)* were also upregulated by estrogen and progesterone. An exception to this trend was mannoside *N*-acetylglucosaminyltransferase III (*Mgat3*) and mannoside *N*-acetylglucosaminyltransferase IV b (*Mgat4b)*. *Mgat3* was down-regulated by estrogen and estrogen + progesterone and *Mgat4b* was down-regulated by estrogen + progesterone. The possible implications of down-regulating *Mgat3*, a key glycosyltransferase that adds the important “bisecting” GlcNAc to the core *N*-glycan will be discussed below. Together, these data suggested that the

complexity of *N*-glycans in the mouse uterus increased as a consequence of estrogen and/or progesterone treatment.

As for *O*-glycosylation, the transfer of the monosaccharide β-D-N-acetylgalactosaminyl (GalNAc) from the UDP donor sugar to a serine or threonine residue in the protein backbone initiates this pathway. A group of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (Galnt), known as ppGalNAcTs, catalyzes this critical step. To date, nearly 20 ppGalNAcTs have been cloned and functionally expressed from various mammalian species (Ten Hagen et al., 2003). The isoforms of this family display tissue-specific patterns in adult mammals (Ten Hagen et al., 2003). In my studies, 7 uterine ppGalNAcTs were upregulated by estrogen, while one (*Galntl2*) was downregulated (Table 2.6). Strikingly, one member of this family, *Galnt3,* was upregulated 8.4-fold by estrogen. In general, progesterone had a negative influence on ppGalNAcT expression, however, transcripts of Galnt1 increased 1.5-fold after progesterone treatment. In a few cases (*e.g.,* Galnt3), the hormone combination down-regulated mRNA expression that had been upregulated by estrogen treatment alone. In aggregate, although ppGalNAcTs are differentially regulated by estrogen and/or progesterone stimulation, the synthesis of *O*-glycans will likely increase with hormonal treatments, a theory that is supported by the results of total carbohydrate staining by the Periodic Acid-Schiff's (PAS) method on uterine tissue sections, as described below.

Following the initiation of *O*-linked carbohydrate structures by ppGalNAcTs, the glycans can be elaborated into different core subtypes categorized according to the monosaccharides (and their linkages) appended to GalNAc-Ser/Thr. Core types 1-4 comprise the majority of *O*-glycan structures. Interestingly, two core-1 galactosyltransferases, *C1GalT1* and *C1GalT1c1,* were affected by hormone treatment—upregulated by estrogen 1.6- and estrogen + progesterone 1.8-fold, respectively (Table 2.6). In contrast, the core 2 enzyme *N*-acetyl-glucosaminyl transferase 3 (*Gcnt3*) was downregulated 1.6-fold at the mRNA level by estrogen + progesterone (Table 2.6). Several important oligosaccharide motifs are preferentially constructed on core 1 or 2 glycans. For example, the major L-selectin ligand, 6-sulfo-sLe^X, has been identified predominantely on core-2 structures and also on extended core-1 branchs of GlyCAM-1 in mouse HEV and CD34 in human tonsils (Hernandez Mir et al., 2009; Kawashima et al., 2005). Considering the functional importance of these glycoforms, the possible impact of the differential regulation of core 1 and 2 carbohydrate structures by estrogen and/or progesterone deserves further investigation.

After the core structures are in place, both *N*- and *O*-oligosaccharides are elaborated by the addition of specific monosaccharides to yield mature glycans bearing highly diversified sub-terminal and terminal structures. Various fucosyltransferases, sialyltransferases, and sulfotransferases perform these additions. The subterminal and terminal monosaccharides are positioned on the “outer” facets of glycoproteins and cell surfaces, and are thus critically important

to glycoconjugate function (Varki, 1999). For example, a recent study determined that ulcerative colitis, a chronic, relapsing inflammatory disorder of the digestive tract, is not regulated by the expression of MAdCAM-1, a mucin that carries carbohydrate-based ligands for lymphocyte adhesion. Rather, it is the expression of GlcNAc6ST-1, a carbohydrate sulfotransferase that installs a sulfate group onto select MAdCAM-1 glycans that controls the severity of the inflammatory disease. This effect is due to the fact that sulfation greatly increases the binding of particular carbohydrate structures to their selectin receptors, which mediates leukocyte extravasation and thus regulates inflammation (Kobayashi et al., 2009).

Fucose is a key element of the oligosaccharide motifs recognized by selectins. Therefore, structures containing this monosaccharide, such as the Le blood group antigens, have important biological functions. My study showed that four fucosyltransferases, including *Fut2, 8, and 9* were upregulated at the mRNA level by estrogen (Table 2.6). Fut9, an enzyme that likely adds terminal fucose to carbohydrates in non-immune tissues, was upregulated 6-fold by estrogen and 3.8-fold by estrogen + progesterone. Fut8, the enzyme that mediates core fucosylation on *N*-glycans, was also upregulated by estrogen + progesterone (Table 2.6).

In mammals, sialic acids usually cap carbohydrate side chains in terminal α–2,3- or α–2,6-linkages to β-D-galactose (Gal) residues, or α–2,6- linkages to a

GalNAc residue or a GlcNAc residue. Sialic acids are also found α–2,8-linked to other sialic acid residues in gangliosides and as part of the specialized oligosaccharide termed polysialic acid. A family of sialyltransferases catalyzes these additions (Harduin-Lepers et al., 2001). In my datasets, the α–2,3 sialyltransferases were differentially regulated by estrogen and/or progesterone in the mouse uterus (Table 2.6). For example, ST3 β-galactoside α-2,3-sialyltransferase 1 (*St3gal1*) was upregulated 1.4-fold by progesterone and 2-fold by estrogen + progesterone. In contrast, ST3 β-galactoside α-2,3-sialyltransferase 3 (*St3gal3*) was downregulated 1.4-fold by estrogen. Interestingly, ST3 β-galactoside α-2,3-sialyltransferase 6 (*St3gal6*), the sialyltransferase that likely contributes to the synthesis of sLe^X structures on core 2 glycans was downregulated 2.4-fold by estrogen and upregulated 1.4-fold by progesterone.

Sialyltransferases that synthesize α-2,6-linkages were also differentially regulated by estrogen and progesterone in the uterus (Table 2.6). In general, progesterone had an inhibitory effect on transcription of these enzymes, whereas estrogen alone tended to induce their expression. For instance, both β galactoside α-2,6-sialyltransferase 1 (*St6gal1*) and ST6 (α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3-*N*-acetylgalactosaminide α-2,6-sialyltransferase 2 (*St6galnac2*) were upregulated 2-fold by estrogen and downregulated 1.8- and 1.5-fold, respectively, by estrogen + progesterone. In contrast, ST6 α-*N*-acetyl-neuraminyl-2,3-β-galactosyl-1,3-*N*-acetylgalactosaminide α-2,6-sialyltransferase

5 (*St6galnac5*) was downregulated by all treatments, decreasing 1.9-, 1.5-, and 1.7-fold with estrogen, progesterone, and estrogen + progesterone, respectively. Finally, the polysialyltransferases responded differently to ovarian hormones. ST8 α-*N*-acetyl-neuraminide α-2,8-sialyltransferase 2 (*St8sia2*) was downregulated 1.7-fold by estrogen, while ST8 α-*N*-acetyl-neuraminide α-2,8-sialyltransferase 4 (*St8sia4*) was upregulated 1.8-fold by progesterone.

Several sulfotransferases were also differentially regulated by estrogen and/or progesterone (Table 2.6). Heparan sulfate 3-*O*-sulfotransferase 1 (*Hs3st1*) mRNA was upregulated 1.7- and 1.9-fold by estrogen and progesterone, respectively. Another broadly expressed carbohydrate sulfotransferase, *Chst7,* was also upregulated by estrogen 1.8-fold. However, heparan sulfate 6-*O*-sulfotransferase 1 (*Hs6st1*) was down-regulated 1.5-fold by estrogen + progesterone. My study showed that *Chst5*, *N*-acetylglucosamine 6-*O*-sulfotransferase (I-GlcNAc6ST), which may contribute to L-selectin ligand synthesis and whose expression is generally considered to be restricted to the intestine, was detected in the mouse uterus and was upregulated 1.7-fold by estrogen (Table 2.6).

Together, these data strongly indicated that the density and structure of fucosylated, sialylated, and/or sulfated terminal sugar species is likely under the influence of estrogen and/or progesterone. Compared to the various enzymes that synthesize the core structures of *N*- and *O*-glycans, hormonal regulation of

the enzymes that construct sub- and terminal carbohydrates structures was more complex. Considering the importance of these terminal glycan motifs in specifying glycoprotein functions, regulation by estrogen and/or progesterone could critically influence many biological and pathological events.

The glycosylation of lipids and sphingolipids requires ceramide-specific glucosyl- or galactosyltransferases to catalyze the addition of the first monosaccharide to ceramide. Further development of the oligosaccharide chains proceeds in a stepwise, untemplated fashion similar to that of glycoprotein synthesis. Many of the sub- and terminal glycolipid structures are believed to be catalyzed by shared glycosyltransferases that are also involved in glycoprotein synthesis (Varki, 1999). A number of galactosyltransferases and glucosaminyltransferases involved in glycolipid biosynthesis were differentially regulated by estrogen and/or progesterone (Table 2.6).

The differential expression of a subset of glycosylation-related genes in the mouse uterus is confirmed by Quantitative PCR (Q-PCR).

Next, for independent confirmation, I selected 8 genes whose uterine expression appeared to be strongly regulated by hormonal treatment. The selected genes encode key enzymes in the glycosylation process and/or their products generate biologically-important carbohydrate motifs. The list included: β-1,3-galactosyltransferase 5 (β3GalT5) and galactose-4-epimerase (Gale), which represented glycosyltransferases and related enzymes; mucin 1 (Muc1) and

surfactant associated protein D (Sftpd), which represented glycoproteins; insulin-like growth factor binding protein 3 (Igfbp3), a growth factor; chemokine (C-C motif) ligand 11 (Ccl11), a chemokine; and Notch homolog protein 4 (Notch4) and patched homolog 1 (Ptch1) from the hedgehog signaling pathway as developmental regulators. I performed gene-specific Q-PCR to confirm the changes in expression that were detected by microarray. Cyclophilin was chosen as an internal control on the basis of a preliminary experiment in which three common housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, β-glycuronidase, and cyclophilin) were tested for their variance among samples (Ma and Fisher, data not shown). A two-tailed Student's *t*-test was used to compare the relative fold changes between the estrogen and/or progesterone experimental groups to the oil control group. Overall, the Q-PCR data agreed with the microarray results, however different fold changes were detected by the two methods (Fig. 2.5). This discrepancy may possibly be explained by the fact that Q-PCR is a more sensitive technique than microarray analyses.

The total carbohydrate content of the mouse uterus, but not the major salivary glands, is upregulated by hormonal treatment.

Hematoxylin and Eosin (H&E) staining was performed on the tissue sections to examine the histological changes that resulted from estrogen and/or progesterone treatment (Fig. 2.6A). Both hormones induced cell proliferation in uterine tissues. Estrogen alone caused interstitial swelling of the stroma, and triggered the cuboidal to columnar transition of the luminal and glandular

epithelium. These changes were retarded in mice receiving both estrogen and progesterone. No visible histological changes associated with estrogen and/or progesterone supplementation were observed in the major salivary glands by H&E staining.

The total amount of carbohydrate macromolecules among different treatment groups was evaluated by Periodic Acid-Schiff's (PAS) staining of tissue sections from the uterus and major salivary glands (Fig. 2.6B). The staining intensity of the major salivary glands was similar in all the treatment groups. In the uterus, the oil control group stained very weakly, and PAS intensity was greatly increased by estrogen and/or progesterone supplements. The relative abundance of total carbohydrates in the major salivary glands and the uterus observed under different hormonal treatment conditions was consistent with the results of glycosylation-related gene expression as determined by microarray analyses.

Muc1 protein expression is upregulated by estrogen and/or progesterone in the mouse uterus.

Aberrant expression of Muc1, a cell surface-associated transmembrane glycoprotein with abundant *O*-glycans, is a feature associated with many types of cancer, such as ovarian, mammary gland, pancreatic, colon, and lung (Kufe, 2009). Translated as a single polypeptide, MUC1 protein undergoes autoproteolysis at the sea urchin sperm protein, enterokinase and agrin (SEA)

domain, located within its extracellular region, to form two products: the MUC1 N-terminal subunit (MUC1-N) and the MUC1 C-terminal transmembrane subunit (MUC1-C) (Julian et al., 2009; Kufe, 2009; Levitin et al., 2005; Macao et al., 2006). Altered glycosylation of the MUC1-N tandem repeats has been associated with human malignancies (Finn, 2008; Ichige et al., 1995). A monoclonal antibody DF3-P that recognizes underglycosylated MUC1 protein reacted with cell lines derived from ovarian carcinomas (Ichige et al., 1995). Moreover, the same antibody only stained ovarian tumor cells but not the surrounding tissues (Ichige et al., 1995). Further research on aberrant glycosylation in cancer demonstrated that MUC1 in cancer cells is incompletely glycosylated with only one sugar residue, GalNAc (known as Tn antigen), or the disaccharide, GalNAc-Gal (known as T antigen) (Finn, 2008). After MUC1-N is released from the cell surface, MUC1-C then acts as a receptor, which mediates diverse signaling pathways linked to transformation and tumor progression (Carson, 2008; Kufe, 2009).

Our glycoarray data showed that Muc1 mRNA in the mouse uterus was upregulated more than 14-fold by estrogen treatment (Fig. 2.3). This observation was confirmed by Q-PCR (Fig. 2.5 C). Next, I studied the effects of hormone treatment on Muc1 expression at the protein level, via immunolocalization and immunoblotting approaches (Fig. 2.7). A rabbit polyclonal antibody that recognizes the cytoplasmic domain of MUC1-C localized to both luminal and glandular epithelium in the mouse uterus (Fig. 2.7 A). This signal was greatly

increased by estrogen and/or progesterone treatment. The same polyclonal antibody was used for an immunoblotting experiment to probe electrophoretically-separated uterine lysates. A ~23 kDa band was detected, which corresponded to the cytoplasmic domain of Muc1. The intensity of this band was increased by hormone treatments (Fig. 2.7 B); the relative fold change of the Muc1 signal was calculated by ImageJ software (Fig. 2.7 C). The magnitude difference in Muc1 protein with estrogen and/or progesterone treatment was consistent with the changes observed at the mRNA level. Furthermore, our study is in agreement with previous observations that Muc1 expression is differentially regulated in the mouse uterine cavity during early pregnancy (Surveyor et al., 1995). The results from our study support these data and provide a mechanistic explanation for this regulation. Although MUC1 mRNA has been detected in human major salivary glands using PCR and Northern hybridization (Liu et al., 2002), here, neither MUC1 mRNA nor protein were detected in mouse major salivary glands using the microarray platform and the antibody against the cytoplasmic domain of MUC1-C (Ma and Fisher, data not shown).

Glycosyltransferases involved in the formation of Lewis blood group antigens are upregulated in the mouse uterus by estrogen and progesterone.

Le blood group antigens are functionally important structures that play particularly essential roles in immune cell trafficking. As ligands that govern the

first step in leukocyte extravasation, these carbohydrate motifs affect a number of homeostatic and pathologic processes, including immune surveillance and inflammation. Accordingly, I chose three glycosyltransferases, GalNt3, Fut9, and β1,3GalT5, involved in the synthesis of Le blood group antigens, and assessed their expression in uterine tissue sections by immunofluorescence. GalNt3, a member of the ppGalNAcT family, initiates *O*-linked glycosylation; β1,3GalT5, a galactosyltransferase, generates type 1 lactosamine units, which form the backbone of certain Le structures (Isshiki et al., 1999); and Fut9, a fucosyltransferase, installs α1,3 fucose residues. Expression of all three glycosyltransferases was detected in mouse uterine luminal and glandular epithelium, as well as in the secretory alveoli and tubular epithelium of major salivary glands (Figs. 2.8, 2.9, 2.10). The signal intensity for all three enzymes in the uterus was greatly increased by estrogen and/or progesterone supplementation, while signals in the major salivary glands were relatively unaffected (Fig. 2.8, 2.9, 2.10). The results demonstrated that the regulation of these glycosyltransferases at the protein level parallels that observed by microarray analyses at the mRNA level.

Although many fucosylated glycoconjugates have immunological roles, the fucosyltransferase Fut9 is expressed in certain non-immune tissues, such as kidney and brain (Comelli et al., 2006). Our study demonstrated that this enzyme is also present in the mouse uterus and major salivary glands. Changes in uterine Fut9 protein levels among different treatment groups were further

assessed by immunoblotting, using the same antibody that was employed for the immunofluoresence studies (Fig. 2.11). The changes observed in Fut9 at the protein level were in agreement with the relative mRNA levels detected by the microarray approach.

DISCUSSION

This study characterized glycosylation-related gene expression profiles at a global level in the mouse uterus and major salivary glands using the customized CFG Glycov2 microarray system. Then, I confirmed the array results for a subset of genes at both the mRNA and protein levels by real time PCR, immunostaining, and immunoblotting methods. As the mammalian female reproductive tract is highly responsive to the ovarian hormones estrogen and progesterone, which synchronize its functions (Dey, 1996), I designed these experiments to take into consideration of hormonal influences on glycosylation-related genes. Indeed, I found that glycosylation-related genes in the mouse uterus, but not the major salivary glands or submandibular lymph nodes, are highly regulated by these factors. This result is in accord with previous studies (Dutt et al., 1986; Kimber et al., 2001; Tulsiani et al., 1996), and the magnitude of the regulation observed was impressive. Among 700 murine glycosylation-related genes detected in this microarray analysis, about 300 exhibited highly statistically significant differential regulation by estrogen and/or progesterone in the mouse uterus ($p<0.001$). The relative fold changes observed ranged from +15 (Muc1, estrogen alone) to -8 (Patched 1, estrogen alone).

Using Ingenuity Pathway Analysis software, I indentified a long list of canonical pathways that were profoundly affected by estrogen and/or progesterone in the mouse uterus. My study detected glycosylation-related genes in the mouse uterus and major salivary glands that encoded proteins from important functional categories, *e.g.,* adhesion and signaling. Importantly, a comprehensive set of glycosyltransferases was present in the mouse uterus and major salivary glands, indicating that complex carbohydrate structures can be synthesized in these locations. In addition, their expression in the uterus was highly regulated by estrogen and progesterone, a phenomenon that likely greatly impacts reproduction. To the best of my knowledge, this is the first study that elucidates, in a comprehensive manner, the expression profiles of glycosylation machinery in the mouse uterus and major salivary glands, providing direct *in vivo* evidence of their regulation by estrogen and/or progesterone. These data serve as important evidence for my central hypothesis—that similar carbohydrate motifs govern bacterial ecology and leukocyte recruitment in both the uterine and oral cavities. Here, I have determined the machinery that is available in both tissues for the construction of particular oligosaccharide structures, which I directly characterized in Part III of this thesis.

Major glycosylation pathways, including the synthesis of *N*- and *O*-linked structures, glycosphingolipids, and heparan sulfate, are all profoundly impacted by estrogen and/or progesterone in the mouse uterus. *N*-linked carbohydrates,

one of the best studied glycan subsets, are characterized by a GlcNAc linkage to the amide group of an asparagine residue in the context of the *N*-linked consensus sequence NXT/S(C) (where X is any residue except proline). My study indicated that estrogen and/or progesterone upregulated *N*-glycosylation at every stage, starting from the formation of a lipid-linked precursor oligosaccharide, through the *en bloc* transfer of the oligosaccharide to the polypeptide, to later-stage processing of the oligosaccharide including trimming and elongation. A number of uterine genes involved in *N*-glycan biosynthesis were increased by estrogen and/or progesterone treatment. In particular, a group of mannoside *N*-acetylglucosaminyltransferases drew my attention. These enzymes regulate branching of *N*-linked glycans at the core mannose residues, resulting in tri- and tetrantennary structures. *Mgat3* was downregulated 1.5-fold with estrogen alone, and 1.3-fold with estrogen + progesterone. *Mgat4b* transcripts were reduced 1.5-fold after a combination estrogen + progesterone treatment, and *Mgat4c* mRNA was increased 1.6-fold following estrogen treatment as compared to the vehicle control.

Regarding the specificities of these enzymes, Mgat3 transfers a GlcNAc residue from UDP-GlcNAc to the β 1,4 mannose of the *N*-glycan core to form a so-called "bisecting" GlcNAc linkage, which is found in various hybrid and complex *N*-glycans (Ihara et al., 1993; Ohno et al., 1992). The addition of a bisecting GlcNAc inhibits the β 1,6 GlcNAc branch formation catalyzed by mannoside *N*-acetylglucosaminyltransferases V (Mgat5) because Mgat5 cannot use the

bisected oligosaccharide as an acceptor substrate (Gu et al., 2009; Ohyama, 2008; Schachter, 1986; Schachter et al., 1983; Taniguchi et al., 1999). When Mgat5 is free to act, *N*-glycans bearing β 1,6 GlcNAc-branches can be preferentially modified by β1,4 GalT and β1,3 GlcNAcT to form long poly-*N*-acetyllactosamine side chains, which are further processed to yield other carbohydrate motifs such as sLe^x. It has been reported that Mgat5 activity and β 1,6 GlcNAc-branched *N*-glycan levels are increased in highly metastatic tumor cell lines (Asada et al., 1997; Dennis et al., 1987; Pochec et al., 2003). Furthermore, cancer metastasis is reduced in Mgat5 null mice (Granovsky et al., 2000). As Mgat3 activity results in the loss of Mgat5 substrates, the former enzyme is considered an antagonist of the latter, and thereby contributes to the suppression of cancer metastasis (Gu et al., 2009; Taniguchi et al., 1999). Overexpression of Mgat3 in highly metastatic melanoma cells reduces β 1,6 GlcNAc branching in cell surface *N*-glycans and increases bisected *N*-glycans, resulting in reduced lung metastasis of B16 mouse melanoma (Yoshimura et al., 1995). The mechanism of this inhibition is partly explained by reduced synthesis of β-1,6-branched *N*-glycans on E-cadherin extra-cellular domains. It has been shown that these β-1,6-branching *N*-glycans could enhance cell-cell adhesion via prolonged residency of E-cadherin on cell surfaces because glycosylated E-cadherin exhibited delayed turnover and decreased release from cell surface (Guo et al., 2003; Yoshimura et al., 1996). As my study demonstrated that hormone treatments affect the expression of Mgat3, the biological implications of estrogen and progesterone on cancer progression and metastasis mediated by

these mannoside *N*-acetylglucosaminyltransferases definitely warrant further investigation.

As compared to *N*-glycans, the analysis of *O*-glycosylation pathways remains more challenging and less understood (Jensen et al., 2010). Two groups of glycoproteins, mucins and proteoglycans, are heavily decorated by large numbers of *O*-linked saccharides. Like *N*-glycans, the carbohydrate portion of these molecules contributes greatly to their biological functions (Corfield, 1992; Strous and Dekker, 1992; Van Klinken et al., 1995). A family of nearly 20 glycosyltransferases, termed ppGalNAcTs, initiates *O*-glycan formation through the creation of the GalNAcα1-*O*-Ser/Thr linkage (Ten Hagen et al., 2003), a step considered to be the commitment to generating *O*-glycans. In this study, ppGalNAcTs in uterine tissues were generally upregulated by estrogen and downregulated by progesterone. Specifically, seven family members (*Galnt1, 3, 7, 10, 11, 12, 13*) were upregulated by estrogen at the mRNA level. The upregulation of Galnt3 was confirmed at the protein level by immunolocalization (Fig. 2.8). In contrast, another ppGalNAcT, Galnt-like 2 (*Galntl2*), was down-regulated by estrogen 3.5-fold at the mRNA level. My data suggested that despite catalyzing the same simple reaction, individual ppGalNAcTs may function in a tissue- and cell-specific manner, a theory that has been previously proposed (Ten Hagen et al., 2003). After ppGalNAcTs have catalyzed the addition of the first monosaccharide residue, *O*-glycan structures can be elaborated into various core structures (cores 1-8), defined by differential monosaccharide linkages to

the GalNAc-Ser/Thr. Many *O*-glycans contain the core 1 structure formed by the addition of Gal in a β1,3 linkage to the GalNAc-Ser/Thr by Core 1 synthases. Core 2 is then formed by the addition of a GlcNAc residue to the core 1 structure by core 2 synthases. In this study, the uterine expression of two core 1 synthase genes, *C1GalT1* and *C1GalT1c1*, was increased, and that of the core 2 synthase *Gcnt3,* was reduced by estrogen treatment. The data suggested that estrogen and progesterone promote the synthesis of core 1 *O*-glycans while inhibiting core 2. This observation may have important biological relevance because 6-Sulfo sLe^x is predominately located on core 2 and extended core 1 chains on human endothelial CD34 (Hernandez Mir et al., 2009).

While the core structures of various glycans can differ, certain outer structures, of which fucose, sialic acids, α-galactose, β-GalNAc, and β-GlcA are the major components, are often shared among different classes of glycans (Varki, 1999a). In mammals, fucosylated glycans linked to either proteins or lipids are involved in a variety of biological functions such as cell adhesion (Clarke and Watkins, 1996; Wiederschain et al., 1998), inflammation, leukocyte trafficking (Blander et al., 1999; Lowe, 1997), and fertilization (Zhu et al., 1995). Aberrant fucosylation is indicated in many pathological disorders, including cystic fibrosis (Glick et al., 2001; Scanlin and Glick, 2001) and cancer (Le Pendu et al., 2001; Miyoshi and Nakano, 2008). Human ABO and Le blood group antigens are a few examples of well-studied fucosylated oligosaccharides. These carbohydrate structures are widely expressed in many normal tissues, mainly by epithelial cells. Altered expression of these oligosaccharides occurs in many tumors and is often strongly

related to cancer prognosis (Le Pendu et al., 2001). Le^x and Le^y are considered to be tumor-associated markers (Gao et al., 2004; Le Pendu et al., 2001). Some of these antigens and their derivatives interact with selectins to mediate cell-cell adhesion and trafficking (Blander et al., 1999; Lowe, 1997). My results indicated that fucosylated oligosaccharides, including the ABO and Le blood group antigens, will likely be increased in the uterus after stimulation by estrogen and/or progesterone because several fucosyltransferases (Fut2, 8, 9) were upregulated at the mRNA level by these hormones. The functional impact of estrogen and progesterone on these fucosylated oligosaccharides deserves further investigation in terms of specific activities such as bacterial and leukocyte adhesion.

Sialylation is another important terminal sugar modification on glycoproteins and glycolipids with important functions in cell adhesion. Particularly interesting, in the context of my hypothesis, is the fact that sialic acid mediates many host-pathogen interactions (Schauer, 2009; Varki, 1999b). Sialic acid is added by a family of sialyltransferases with various specificities. A number of these enzymes were differentially regulated by estrogen and/or progesterone in the mouse uterus, indicating that the sialylation status of this tissue is also likely to be hormonally controlled. Considering the important roles played by sialylated oligosaccharides in cellular interactions, more research effort should be devoted to this topic.

Sulfation is another important post-translational modification with broad biological implications (Armstrong and Bertozzi, 2000; Bowman and Bertozzi, 1999; Falany, 1997; Glatt, 2000; Hooper et al., 1996). It is well established that the high-affinity endothelial ligands for L-selectin depend on the sulfation of certain carbohydrate-based determinants in high endothelial venules (HEVs) of lymph nodes (Crommie and Rosen, 1995; Hemmerich et al., 1994; Imai et al., 1993; Shailubhai et al., 1997). A study by Dr. Fisher and colleagues demonstrated that the same sulfated carbohydrates that serve as L-selectin ligands in HEVs also facilitate initial embryo attachment to the uterine lining during human implantation (Genbacev et al., 2003). Furthermore, the expression of these carbohydrate structures is strongly upregulated as the uterus becomes receptive (Genbacev et al., 2003). Similarly, during the window of receptivity for embryo implantation, mouse uterine epithelial cells also stain with the HECA-452 antibody, which reacts with high-affinity selectin ligands (Ma and Fisher, data not shown). My glycoarray data indicated that several glycosyltransferases and sulfotransferases that may play important roles in L-selectin ligand synthesis, such as *St3GalT III, St3GalT VI, fucosyltransferase IV, β4GalT, and sulfotransferases* (*Chst2, 5, 7*), are indeed differentially regulated by estrogen and/or progesterone (Table 2.6), suggesting that the final products of these enzymes are likely to be modulated during female reproductive processes. These data correlate very well with the aforementioned immunostaining observations in both mice and humans.

It has been suggested that the glycosyltransferases responsible for the synthesis of the *N*- and *O*-linked oligosaccharide cores are ubiquitously expressed, whereas the enzymes that elaborate terminal carbohydrate structures are expressed in a highly tissue-specific manner (Comelli et al., 2006). Interestingly, in our system, the hormonal regulation of the former group of enzymes proved to be more straightforward than that of the latter. Almost all of the differentially regulated enzymes involved in *N*-glycan core synthesis were upregulated by both estrogen and progesterone. *Mgat3 and 4b*, which were downregulated by treatment with both hormones, were the only exceptions (Table 2.6). As for the *O*-glycan cores, estrogen generally elevated mRNA expression, while the response to progesterone was mixed. PAS staining indicated that the total carbohydrate content at the uterine luminal and glandular epithelial surfaces was dramatically upregulated by all hormone treatments (Fig.2.6B), an observation that correlates with the regulation of core synthetic enzyme expression as noted above.

Compared to the enzymes that are involved in the synthesis of core structures, the differential regulation of terminal oligosaccharide glycosyltransferases by estrogen and/or progesterone was more complicated. Many members of the same gene family had distinct patterns of regulation by hormone treatments. The highly specific responses of the fucosyl-, sialyl-, and sulfotransferases to these stimuli may contribute to the cell- and tissue-specific nature of terminal

oligosaccharides under normal conditions and their aberrant expression in many diseases.

Consistent with previous findings, estrogen was generally a potent stimulator of glycosylation and progesterone had weaker activity. However, when given simultaneously, progesterone tended to antagonize the effects exerted by estrogen, which was reflected in the number of genes that were differentially regulated by estrogen and/or progesterone (Fig. 2.2) (Chilton et al., 1980; Coppola and Ball, 1966; Dutt et al., 1986; Isemura et al., 1981; Lambadarios et al., 1976; Nelson et al., 1975). However, the complexity of the effects of the combined estrogen and progesterone treatment on individual glycosylation-related genes as revealed by the microarray portion of this project belies this general conclusion. Several important glycoproteins in the mouse uterus, including Muc1, Emcn, and Prol1, were differently regulated by estrogen and/or progesterone (Supplemental Table 1). Specifically, MUC1 was tremendously upregulated by estrogen, both at the mRNA (15-fold) and the protein (3.5-fold) levels. Progesterone alone or in combination with estrogen also increased expression of this mucin (Figs. 2.5 and 2.7). In contrast, the ovarian hormones regulated Emcn and Prol1 very differently. *Emcn* was upregulated 1.7-fold by progesterone and downregulated 1.8-fold by estrogen; *Prol1* was upregulated 6.4-fold by estrogen + progesterone, but was minimally affected by treatment with either hormone alone. These results suggest that the glycoproteins Muc1, Emcn, and Prol1, and the carbohydrate structures that they carry may have

different functions *in vivo*. Because the combined actions of estrogen and progesterone are required for the mouse uterus to become receptive for embryo implantation, the role of Prol1 in receptivity certainly deserves further research.

As we enter into the postgenomic era, the importance of co- and post-translational modifications, such as glycosylation and sulfation, is becoming increasingly clear. Complex carbohydrate structures carried by glycoproteins and glycolipds, and synthesized by an array of glycosyltransferases, are major components of the cell membrane with crucial roles in host-pathogen interactions, cell differentiation, migration, tumor invasion/metastasis, and cell trafficking/signaling (Reis et al., 2010; Sperandio et al., 2009). Aberrant glycosylation patterns are related to many pathological conditions, including chronic inflammation and cancer (Ohyama, 2008; Reis et al., 2010). By elucidating the glycosylation machinery expressed in the mouse oral and uterine cavities, about which little is known, my work serves as a critical starting point for future studies using the laboratory mouse as a model system to explore local pathologies in the context of glycosylation, including periodontal disease, preterm birth, and the interrelationship between these two conditions.

Part III. DISTINCT GLYCAN PROFILES WERE PRESENT IN MOUSE UTERUS AND MAJOR SALIVARY GLANDS AND THE EXPRESSION OF THESE OLIGOSACCHARIDES WAS REGULATED BY ESTROGEN AND PROGESTERONE

Introduction

It is clear that glycosylation, the most complex posttranslational modification, mediates various biological events. The enormous structural diversity inherent in oligosaccharides, combined with their accessibility at the cell surface, places carbohydrates in a unique position to regulate cell-cell and cell-matrix interactions. The challenge in studying these molecules lies in their heterogeneous nature, a consequence of their non-template driven assembly by multiple, sequential, and partially competitive glycosylation reactions in the Golgi or ER (Varki, 1999a). This structural diversity also originates from the fact that monosaccharides can be assembled in a multiplicity of linkages, resulting in complex linear and branched polymers (Manzi, 1999). Therefore, a prerequisite to understanding oligosaccharide function is the ability to efficiently analyze glycan profiles at a global scale.

In order to characterize the glycan structures of the mouse uterus and major salivary glands, and to examine the effects of estrogen and progesterone on oligosaccharide structures in these locations, a newly developed high-throughput lectin array approach was used. Lectins are carbohydrate-binding proteins of

plants, bacteria, and animals (Cummings, 1999a). They can bind to multiple proteins that carry the same carbohydrate motifs. Lectins have been widely used in techniques such as blot overlays, flow cytometry, and histochemistry to characterize individual glycan structures. Recently, lectin arrays have been successfully used to rapidly analyze bacterial glycans (Hsu et al., 2006), and to profile carbohydrates on glycoproteins in cell lysates (Ebe et al., 2006; Kuno et al., 2005; Lee et al., 2006; Pilobello et al., 2007). This approach has also been used with intact cells to identify cell-specific and functionally significant mammalian cell surface glycans (Tao et al., 2008). Lectin arrays are particularly useful because they have the advantage of discriminating sugar isomers (Hirabayashi, 2004), a valuable alternative to the laborious process of direct structural analysis.

Here, I used this approach to profile uterine and salivary oligosaccharide structures. To confirm these profiling experiments, I performed immunoblotting and histochemistry with several well-characterized antibodies that recognize a group of very specialized oligosaccharides involved in leukocyte trafficking and inflammation.

Results

The distinct glycan profiles of the mouse uterus and major salivary glands were differentially regulated by hormone treatments.

To characterize the carbohydrate structures in the mouse uterus and major salivary glands, I used a lectin microarray system developed by Dr. Lara Mahal and her research group that contains a panel of 79 lectins with a variety of glycan specificities (Table 3.1) (Pilobello et al., 2007). Mouse uterus and major salivary glands from the various treatment groups were homogenized in lysis buffer. The lysates were sonicated, causing the formation of micelle-like structures from the membranes. The micellae were labeled with Cy3 or Cy5. Replicate samples from three female mice were included in each group for the lectin array assay. Major salivary glands from two male mice were also included as controls. Array hybridization and data analysis were completed in Dr. Mahal's laboratory at NYU. The results enabled construction of a heat map with dendrograms representing hierarchical clustering of 26 arrays (Fig. 3.1). Please note that a pooled reference standard containing aliquots from all tested samples was included as a comparator. Signals stronger or weaker than this reference were considered positive (red) or negative (green), respectively. Black indicates that the observed signal was equal to the pooled reference. The Pearson coefficient and average linkage analysis was used to produce this clustering.

The lectin array data clustered into two distinct groups according to sample origin—the uterus or the major salivary glands. This clustering clearly demonstrated that distinct glycan signatures existed in these two locations, proving the concept of tissue-specific glycosylation in my systems of interest. Within each of these major groupings, the samples clustered according to the

different hormonal treatments, with a few exceptions. This information strongly supported the conclusion that the ovarian hormones estrogen and progesterone regulated the expression of oligosaccharides in the mouse uterus and major salivary glands. Data from the male salivary glands (Male.MS) were grouped within the general cluster of major salivary glands samples, but were positioned slightly apart (closer to the uterine sample set). This distinction indicated that the glycan profile of the major salivary glands differed between male and female mice, additional information supporting hormonal regulation of glycan assembly in the major salivary glands.

Cell-surface oligosaccharides are assembled through the concerted actions of individual glycosyltransferases. The glycosylation-related gene expression profile of a cell or tissue will likely determine the profile of carbohydrate structures that it presents (Varki, 1999a). I demonstrated (Part II) that glycosylation-related genes in the mouse uterus, but not major salivary glands, were subject to differential regulation by estrogen and/or progesterone. In contrast, I observed that the products of these genes—the oligosaccharide structures themselves—were affected by hormones in both locations. Therefore, although the glycosyltransferase/glycan profiles in the uterus are internally consistent, the differential regulation of cell-surface oligosaccharides by estrogen and/or progesterone in the major salivary glands seems to contraindicate the lack of regulation on glycogenes at the mRNA level. However, because specific oligosaccharide structures are generally constructed by the collaborative action

of several glycosyltransferases, minor changes in the expression of individual transferases (that do not reach statistical significance) may indeed produce detectable differences in oligosaccharide profiles. I will discuss this discrepancy further below.

Examination of the lectin carbohydrate specificity clearly demonstrated that high mannose and terminal α/β Gal/GalNAc structures dominated the glycan profiles of mouse major salivary glands as reflected in the intense binding of a panel of mannose- and Gal/GalNAc-specific lectins [*i.e., Narcissus Pseudo-narcissus lectin* (NPA), *Vicia villosa lectin, mannose specific* (VVA man), Jacalin, *Helix pomatia agglutinin* (HPA), *Hippeastrum hybrid lectin* (HHL), *Vicia villosa lectin* (VVA), *Maulura pomifera lectin* (MPA), *Scytonema varium lectin* (SVN), *Griffithsia sp. Lectin* (GFRT), *Canavalia ensiformis lectin* (ConA), and *Allium sativum lectin* (ASA)] that clustered together on the heat map. In contrast, the binding activity of these lectins to the uterine samples was low (Fig. 3.1). Instead, lectins that recognize complex *N*-glycans with heavily sulfated and sialylated terminal sugars hybridized strongly to uterine lysates. These lectins, which clustered in the center of the heat mapncluded *Trichosanthes japonica lectin I* (TJA-I; sialylated and sulfated LacNAc), *Psophocarpus tetragonolobus lectin I* (PTL-I; fucosylated LacNAc) and the sialic acid-binding lectins *Sambucus nigra lectin* (SNA), *Maackia amurensis lectin II* (MAL-II), *Vibrio cholerae lectin* (cholera), and *Polyporus squamosus lectin* (PSL).

Hormone treatments induced uterine synthesis of more highly branched structures with heavily fucosylated, sulfated, and sialylated terminal oligosaccharides.

Binding of the lectins *Ulex europaeus lectin I* (UEA-I), *Aspergilus oryzae lectin* (AOL), and *Aurentia lectin* (AAL) to mouse uterine micellae was tremendously upregulated by estrogen treatment (Fig. 3.1). These proteins recognize α-fucose; AOL specifically distinguishes α-1,6-fucose modifying an *N*-linked core GlcNAc. As compared to the oil control, hybridization of these lectins did not change with progesterone treatment, but greatly increased in mice supplemented with estrogen. Estrogen + progesterone only slightly elevated the binding of these lectins, indicating that progesterone antagonized the effects of estrogen on the synthesis of fucosylated oligosaccharides. These results were in agreement with the differential regulation of several fucosyltransferases that catalyze these modifications (Table 2.5).

Regarding sialylated structures, the binding of SNA and TJA-I to uterine micellae was upregulated by estrogen alone and estrogen + progesterone. SNA prefers sialic acids in α-2,6-linkages and TJA-I recognizes sialylated, sulfated LacNAc units, which are often located on branched *N*- and *O*-glycans.

The hormonal regulation of *Phaseolus vulgaris lectin E* (PHA-E) and PTL-I binding to uterine oligosaccharides is very interesting. The binding activity of these two lectins was upregulated by a combination of estrogen + progesterone,

but was reduced by estrogen alone. PTL-I recognizes fucosylated LacNAc units and PHA-E (sugar specificity: Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-6)Man) binds to the β-1,6 arm of branched complex *N*-glycans, which is preferentially extended by polylactosamine units. Polylactosamine units are frequently modified by terminal fucose, sialic acids, and sulfate molecules.

As compared to the uterus, estrogen and progesterone had a lesser impact on glycan assembly in the mouse major salivary glands. The overall trend appeared to be downregulation of high mannose and terminal Gal/GalNAc structures with stimulation by the individual hormones. The combination of estrogen + progesterone yielded results similar to the oil controls (Fig. 3.1). The reactivity of a group of lectins, which included *Datura stramonium lectin* (DSA), *Sambucus nigra lectin II* (SNA-II), *Psophocarpus tetragonolobus lectin II* (PTL-II), *Tulipa sp. Lectin* (TL), *Arachis hypogaea lectin* (PNA), *Wisteria floribunda lectin* (WFA), and *Trichsanthes japonica lectin II* (TJA-II), distinguished the glycan profiles of major salivary glands from male and female mice. These lectins all hybridized more strongly to micellae from male mice (Fig. 3.1). Regarding the specificities of these lectins, PNA and WFA bind Gal/GalNAc-containing structures. SNA-II distinguishes α-2,6 sialic acid. DSA recognizes lactosamine units, while PTL-II and TJA-II prefer fucosylated lactosamine units. Finally, TL recognizes complex biantennary *N*-glycans. Expression of these ligand structures was reduced in the major salivary glands by estrogen and/or progesterone treatment. The biological significance of this observation awaits further investigation.

The blood group antigens H, Le^x, and Le^y were differentially regulated by estrogen and/or progesterone in the mouse uterus and major salivary glands

To further expand our knowledge of the cell-surface glycans presented by the mouse uterus and major salivary glands, I took advantage of several well-characterized monoclonal antibodies that recognize the blood group antigens H, Le^x, and Le^y. Although the sugar determinants for all three antibodies include terminal fucose [H: Fucα1-2Galβ1-3/4GlcNAc; Le^y: (Fucα1-2)Galβ1-4(Fucα1-3)GlcNAc; Le^x: Galβ1-4(Fucα1-3)-GlcNAc], the linkages between fucose and the underlying monosaccharides differ. Please note that Le^y epitopes share the common Fucα1-2Galβ1 linkage with H and Fucα1-3-GlcNAcβ linkage with Le^x.

First, I performed immunoblotting of lysates prepared from uterine tissues, as well as sublingual and parotid glands. The results are presented in Fig.3.2 (uterus) and Figs.3.3 and 3.4 (salivary tissues). Multiple immunoreactive bands were detected by all antibodies in all tissue types, indicating that several proteins [molecular weight (MW) ~30 KDa to >191 kDa] carry these sugar modifications. For uterine lysates, blood group H and Le^y determinants were dramatically upregulated by estrogen (Fig.3.2, A and C), while the combination of estrogen and progesterone increased the expression of Le^x determinants (Fig.3.2, B). Three major immunoreactive bands of similar MW were detected by the anti-H

and -Le^y antibodies (Fig.3.2, A and C), and four major bands ranging from 60 to >191 kDa were observed with the anti-Le^x antibody (Fig.3.2B). These data suggested that assembly of the specific blood group H, Le^x, and Le^y sugar determinants is differentially regulated by estrogen and/or progesterone in a protein-specific manner in the mouse uterus.

Blood group H, Le^x, and Le^y epitopes were also detected by immunoblotting of mouse salivary glands (Figs. 3.3 and 3.4). With respect to parotid lysates, a prominent reactive band of ~ 130 kDa was detected with the anti-H antibody; the intensity of this signal was similar for all treatment groups. With respect to sublingual tissues, multiple reactive bands, including one of ~ 180 kDa, were detected, and the signal was reduced by estrogen. These data showed that estrogen inhibited the expression of H carbohydrate determinants in sublingual, but not in parotid glands. Le^x oligosaccharides were also detected by immunoblot in parotid and sublingual gland lysates (Figs. 3.3B and 3.4B). Interestingly, this epitope was downregulated by estrogen and upregulated by estrogen + progesterone in sublingual glands (Fig. 3.4B). Among the three major salivary glands, the Le^y epitope was only detected by immunoblotting in sublingual tissues (Fig. 3.4C). The epitope was observed in association with two bands of ~ 70 and ~ 130 kDa. Progesterone treatment differentially downregulated immunoreactivity of the ~ 130 kDa band. Blood group H, Le^x, and Le^y were not detected in submandibular gland lysates by immunoblotting (data not shown).

To examine the subcellular and cellular locations of the carbohydrates detected by immunobloting, I used the same anti-H, -Le^x, and -Le^y antibodies to perform immunofluorescent staining of tissue sections from the mouse uterus (Fig.3.5), and salivary glands (Fig. 3.6). In the uterus, both luminal (arrows) and glandular epithelium (arrowheads) stained positively with these antibodies in a plasma membrane associated pattern. Estrogen treatment dramatically increased the intensity of the blood group H, Le^x, and Le^y immunoreactivity. Estrogen + progesterone had a similar effect.

As for the salivary glands, anti-blood group H and Le^y antibodies did not react with tissue sections from the three major salivary glands. The anti-Le^x antibody did not stain sublingual sections (data not shown). In parotid and submandibular salivary glands, this antibody strongly stained both the plasma membrane and cytoplasm of tubular epithelial cells (Fig. 3.6). No obvious differences in signal were observed between different hormonal treatment groups. A possible explanation for the discrepancy between the immunoblotting and immunofluoresence results is that the scaffolds that present the relevant oligosaccharide motifs may interfere with antibody detection in different contexts.

Uterine expression of L-selectin ligands was differentially regulated by estrogen and/or progesterone.

L-selectins and its carbohydrate-based ligands mediate the initial tethering and rolling adhesion between leukocytes and endothelial cells that line the vasculature. These interactions are critical for the recruitment of leukocytes into sites of acute or chronic inflammation and for lymphocyte homing to secondary lymphoid organs. As described above, very specialized oligosaccharide with restricted expression patterns comprise these ligands that are expressed on sialomucin family members. A recent study completed in Dr. Fisher's laboratory demonstrated that interactions between L-selectin and its carbohydrate ligands also mediate the initial attachment between the human embryo and uterine epithelial cells during implantation (Genbacev et al., 2003). To examine this important oligosaccharide class and its regulation by estrogen and/or progesterone, I used HECA-452, an antibody that recognizes sialyl Le^x and can functionally block L selectin-mediated interactions in multiple experimental systems (Tu et al., 1999b). Specifically, I used HECA-452 to perform immunoblotting of uterine, sublingual, and parotid gland lysates. Two major reactive bands (MW >97 kDa) carried reactive saccharides in the mouse uterus (Fig. 3.2D). Importantly, expression of these oligosaccharides was upregulated by progesterone and estrogen + progesterone, but downregulated by estrogen alone. Immunofluorescent staining with HECA-452 on uterine tissue sections confirmed the immunoblotting results (Fig. 3.5, M-P). Both membrane-bound and cytoplasmic staining was observed.

With regard to salivary glands, HECA-452 detected a reactive band of ~191 kDa in sublingual glands; the signal was not changed by hormone treatments (Fig. 3.3, D). Multiple bands <191 kDa were noted in parotid gland lysates (Fig. 3.4, C). No obvious differences in signal intensity were observed among treatment groups. HECA-452 reacted with both the plasma membrane and cytoplasm of the secretory alveolus cells in tissue sections of parotid, submandibular, and sublingual glands (Fig. 3.7). This signal was reduced in the parotid glands after progesterone, or estrogen + progesterone supplementation, an observation that suggested expression of these oligosaccharides was regulated in a tissue-specific manner by estrogen and progesterone.

Discussion

After profiling the expression of glycosylation-related genes in the mouse uterus and major salivary glands and elucidating their differential regulation by the ovarian hormones estrogen and progesterone, I took on the challenge of characterizing the final products of the glycosylation machinery in both locations—cell-surface glycan structures. The lectin array, a high-throughput approach, was used in this study to provide a global and systemic analysis of the cell-surface glycan profiles, and to examine the effects of ovarian hormones on glycan expression in these tissues. In addition, several well-characterized antibodies that recognize highly specified oligosaccharide structures were included in the analyses. The finding that mouse uterine cells assembled a

complex glycan signature that was subject to differential regulation by estrogen and progesterone agreed with the parallel gene expression profiling studies (Part II). Moreover, with estrogen and/or progesterone stimulation, the cell-surface glycans shifted from a simpler to a more complex profile with increased branching and heavily sialylated, fucosylated, and sulfated terminal sugars. Considering the important roles of these highly specialized terminal oligosaccharides play in a variety biological functions, insight into how these steroids regulate glycan assembly significantly advances our understanding of glycosylation, the most common but poorly studied post-translational modification.

An unexpected finding was that the glycan structures produced by the major salivary glands appeared to be under the influence of ovarian hormones. This disagrees with the fact that the expression of mRNA encoding glycosylation-related genes was not significantly affected in these tissues. Several possibilities may explain this discrepancy. First, due to the relatively low sensitivity of microarray techniques (reflected by the fact that the relative fold changes detected by Q-PCR for a subset of differentially regulated genes were generally greater than the relative fold changes observed in the microarray datasets, see Fig. 2.5), this approach might not be able to detect the subtle differences in expression among the different control and experimental groups. Second, the sample size of each group for the microarray analysis (n=3) might have been too small to reach statistical significance. Third, and most likely, as the assembly of

oligosaccharides requires the action of a group of glycosyltransferases, insignificant changes in the expression of individual molecules may produce significant differences in the expression of certain oligosaccharide structures.

In any case, the differential regulation of glycan profiles in mouse major salivary glands by estrogen and/or progesterone might have important biological implications. For example, the mouse model developed in this study may be used to explore why women are more prone to Sjogren's Syndrome, a chronic autoimmune disease in which a person's own lymphocytes attack her salivary glands. Carbohydrate-based homing mechanisms could play a role. Further investigation of this and related theories may provide new information on women's health, an interesting topic that will be further discussed in the final part of this thesis (Part V).

A few previous studies investigated the effects of the steroid hormones estrogen and progesterone on glycan assembly in mouse uterine cells (Dutt et al., 1988; Dutt et al., 1986; Kuo et al., 2009). An *in vitro* experiment determined that estrogen stimulates *N*-glycan synthesis in cultured uterine tissue (Dutt et al., 1986). The same research group demonstrated that estrogen specifically upregulates polylactosamine units with highly branched structures in the mouse uterus (Dutt et al., 1988). A recent mass spectrometry-based structural analysis showed that Le^x and Le^y epitopes on glycoprotein carrier 24p3 (lipocalin 2) and

lactotransferrin predominated in mouse uterine luminal fluid after stimulation with diethylstilbestrol, a synthetic non-steroidal estrogen agent (Kuo et al., 2009).

There is strong evidence indicating that Le^x and Le^y epitopes are typically located on polylactosamine units (Lowe, 1999a). The oligosaccharide structures elucidated by the lectin array and immunolocalization methods used in the current study included the aforementioned structures. The high-throughput approach I chose for these studies tremendously expanded the knowledge of cell-surface glycans expressed in the mouse major salivary glands and uterus. Based on the sugar specificity of each lectin (Table 3.1), a detailed oligosaccharide profile of these tissues was obtained. Similar to the glycan structures detected in human saliva, mouse major salivary glands included, but were not limited to, two distinct groups of oligosaccharides. The first group contained T (Galβ1-3GalNAc), sialyl T (NeuAcα2-3Galβ1-3GalNAc), and Le^x (Galβ1-4(Fucα1-3)-GlcNAcβ1) antigens, as well as lactosamine units (Galβ1-4GlcNAc). The structures could serve as receptors mediating the adhesion of several commensal and pathogenic bacteria, such as *Actinomyces naeslundii, streptocci, Helicobacter pylori,* and *Fusobacterium nucleatum* (Bosch et al., 2000; Edgerton et al., 1993; Prakobphol et al., 1999; Prakobphol et al., 1998; Veerman et al., 1995). The second group included sLe^x related structures as defined by HECA-452 antibody reactivity, which could include ligands for L-selectin (Rosen and Bertozzi, 1994). The glycans of the mouse uterus consisted of these and more complicated structures. Whether the oligosaccharides function as receptors

for bacteria and leukocytes in the uterine cavity, and the potential influence of ovarian hormones on these adhesion events remains to be tested.

The ability to form branched structures with highly diversified terminal sugar moieties gives glycans enormous structural complexity, diversity and specificity, conferring the ability to mediate a variety of cell-cell and cell-matrix interactions (Varki, 1999a). A group of GlcNAc transferases (Gnt or Mgat) generate the branched hybrid and complex *N*-glycans found in higher organisms, such as mammals. The microarray analysis showed that several members of this GlcNAc transferase family were differentially regulated by estrogen and/or progesterone (Table 2.5). Mgat3 deserves particular attention because of its inhibitory effect on synthesis of β-1,6-branch of *N*-glycans (Gu et al., 2009; Ohyama, 2008; Schachter, 1986; Schachter et al., 1983; Taniguchi et al., 1999). The β-1,6 branch initiated by Mgat5 is the preferred precursor for polylactosamine units. The latter motif again serves as a candidate structure for subsequent modifications, *e.g.,* fucosylation and sulfation, to build functional determinants such as Le^x and Le^y (Lowe, 1999a). The microarray data showed that mouse uterine Mgat3 was downregulated by estrogen and estrogen + progesterone and Mgat4c was upregulated by estrogen (Table 2.5), suggesting that estrogen probably promotes branching of *N*-glycans in the uterus. Indeed, the results of lectin array analysis and immunoblotting experiments confirmed this prediction. The binding activity of PTL-I, which recognizes complex branched *N*-glycans was upregulated by estrogen and/or progesterone (Fig. 3.1). Similar changes were

also observed for TJA-I, which binds to polylactosamine units (Fig. 3.1). Reactivity of the fucose-binding lectins UEA-I, AOL, and AAL was upregulated by estrogen. Furthermore, immunoreactive bands detected by Le^x and Le^y antibodies were greatly upregulated by estrogen and/or progesterone in mouse uterine lysates (Figs. 3.2 and 3.5). These results also correlate well with the estrogen-mediated increase in uterine fucosyltransferase 2 and 9 expression, as these enzymes probably contribute to the synthesis of Le^x and Le^y (Table 2.5). Fut8, which transfers a fucose residue onto the *N*-glycan core, was also upregulated by estrogen and progesterone (Table 2.5); expression of the latter fucosyltransferase likely accounts for the increased binding activity of AOL to uterine micellae after hormone treatment.

Hormonal regulation of the HECA-452 epitope in mouse uterine tissue differed from that observed for blood group antigen H, Le^x, and Le^y. As shown by both immunoblotting and immunofluoresence approaches, estrogen predominantly upregulated the expression of the H and Le^y epitopes and estrogen + progesterone promoted assembly of the Le^x epitope (Figs. 3.2, 3.3). In contrast, estrogen slightly reduced, while progesterone strongly upregulated, HECA-452 immunoreactivity, observed by immunoblot analyses of mouse uterine lysates. The latter treatment induced the expression of two HECA 452-reactive bands, one ~ 130 kDa and the other > 191 kDa (Fig. 3.2). Estrogen + progesterone also upregulated HECA 452-reactivity. Interestingly, the combination of these steroids differentially upregulated the larger, but not the smaller band. The

immunofluoresence pattern agreed with the immunoblotting results (Fig. 3.5, M-P).

Although L-selectin-mediated adhesion requires a sialylated, fucosylated, and sulfated ligand (Hemmerich et al., 1994; Tu et al., 1999a), the HECA-452 antibody recognizes a sialylated and fucosylated epitope (Rosen and Bertozzi, 1994). Candidate glycosyltransferases with the specificities required for creating the HECA-452 epitope were observed in the microarray data. The Fut9 which adds α-1,3-fucose, was upregulated 6- and 3.8-fold by estrogen and estrogen + progesterone, respectively (Table 2.5). The sialyltransferase St3gal6, which transfers sialic acid α-2,3-linked to Gal, was downregulated 2.4-fold by estrogen and upregulated 1.4-fold by progesterone at the mRNA level (Table 2.5). The regulation of the latter gene matched the expression pattern of the HECA-452 epitope. This information suggests that while both fucosylation and sialylation are required for HECA-452 antibody recognition, sialylation may play a more critical role in this protein-carbohydrate interaction. Neither the mouse uterus, nor the major salivary glands presented detectable amounts of the epitope that react with MECA-79, an antibody that recognize high-affinity L-selectin ligands in a sulfate-dependent manner (data not shown). Whether carbohydrate sulfation is required for leukocyte/endothelial cell interactions in the mouse uterus and major salivary glands needs further investigation.

High mannose and sialylated non-bisected triantennary hybrid oligosaccharides have been detected on mouse submandibular mucins by a combination of exoglycosidase treatments and electrophoresis with oligosaccharide standards (Denny et al., 1995). The same study also indicates that the sialic acids found in mouse submandibular mucin exist mainly in α-2,3 linkages. The lectin array data in the current study also revealed that terminal/internal mannose and Gal/GalNAc residues dominate the glycan profiles of mouse major salivary glands, which was reflected in the binding activity of lectins that clustered together on the heat map (Fig. 3.1). Several lectins that bind sialic acid, *e.g., Triticum vulgaris lectin* (WGA), and specifically to those with α-2,3 linkages, *e.g., Maackia amurensis lectin* (MAA), hybridized strongly to micellae from the major salivary glands (Fig. 3.1). These results were consistent with the microarray data described in Part II of this thesis showing that St6galnac II and IV mRNA were expressed in mouse major salivary glands (Table 2.2). One interesting observation that Denny and colleagues reported is that these *N*-linked oligosaccharides lack fucose, a common terminal sugar molecule (Denny et al., 1995). However, my data disagreed with this finding. As shown in Figs. 3.6 and 3.7, fucosylated oligosaccharides were detected in tissue sections of mouse submandibular glands by immunofluorescence localization using anti-Le^x and HECA-452 antibodies. Similar fucosylated oligosaccharides were also detected in parotid and sublingual glands by immunostaining and immunoblotting with antibodies that recognize H, -Le^x, and -Le^y blood group antigens, as well as HECA-452 (Figs. 3.3, 3.4, 3.6, and 3.7). Fucosyltransferases that transfer fucose in α-1,3,

and α-1,6 linkages were detected in mouse major salivary glands (Table 2.2). Although neither Fut1 nor Fut2, α-1,2 fucosyltransferases, were detected in mouse major salivary glands according to the method that was used to process RMA raw data, Fut2 gave marginal hybridization signals in major salivary glands (Ma and Fisher, data not shown). These data support the immunolocalization studies described above. A recent report describing the glycan structures of rat sublingual gland mucins as determined by mass spectrometry-based approaches detects minor amounts of fucose, accounting for about 1.2% (w/w) of the total carbohydrates content of the samples (Yu et al., 2008). In the future, a similar strategy could be used to confirm whether mouse major salivary glands synthesize fucosylated carbohydrates.

Although high mannose oligosaccharides dominated the glycan profiles of mouse major salivary glands, these structures appeared to be reduced in abundance by estrogen and progesterone treatment (Fig. 3.1). Complex branched structures were also detected by DSA, SNA-II, PTL-II, TL, and TJA-II in the lectin array, although with overall weaker hybridization signals as compared to the pooled reference. DSA, PTL-II, and TJA-II recognize LacNAc units, which form the backbone for the addition of fucose, sialic acid, and sulfate modifications to yield more complex motifs such as sLe^x and sLe^y. The lectin TL, which binds to complex *N*-glycan structures, hybridized weakly to micellae from the major salivary glands (Fig. 3.1). Please note that the pooled standard included uterine micellae that were rich in hybrid and complex *N*- and *O*-linked structures.

Therefore, the signals from major salivary gland micellae, although weak as compared to the pooled reference, represented an important class of more complex oligosaccharides present in these tissues. These data are in support of a previous study that detected hybrid *N*-glycans on mouse salivary mucins (Denny et al., 1995).

Expression of the very specialized tetrasaccharide recognized by the HECA-452 antibody was also detected in association with major salivary glands by immunoblotting and immunolocalization methods (Figs. 3.3, 3.4, and 3.7). This information suggested that although high mannose and simple *O*-linked structures dominated the glycan profiles of mouse major salivary glands, more complex structures were also present in these tissues.

The current study demonstrated that the lectin array, a new global approach, is a powerful tool for characterizing glycan profiles from tissue lysates. It is also a sensitive technique as demonstrated by the ability to detect subtle changes in the mouse uterine and major salivary gland glycome caused by estrogen and/or progesterone treatment. In combination with antibodies, the lectin arrays identified oligosaccharide structures in the uterus and major salivary glands with the potential to serve as functional receptors for the adhesion of bacteria and leukocytes. Furthermore, the glycans were differentially regulated by estrogen and progesterone, a finding that has important implications for many specific biological functions and women's health issues in general. With this fundamental

information in hand, the next phase of my research will involve elucidating the pathogenesis of certain oral and uterine disorders, including periodontal and intra-uterine infections in the context of carbohydrate mediated adhesive interactions.

Part IV. MATERIALS AND METHODS

Animals and experimental design

Eighty 8-week old BALB/c female mice (Charles River Laboratories, Wilmington, MA, USA) were ovariectomized and rested for 14 days to exhaust the endogenous supply of estrogen (E_2) and progesterone (P_4). Then, the mice were divided into 4 groups for 4 days of treatment as follows. E_2 (17β-Estrodial, Sigma, St. Louis, MO, USA) and P_4 (Sigma, St. Louis, MO, USA) were emulsified in sesame oil (Sigma, St. Louis, MO, USA). Mice received subcutaneous injections of 100 ng/day E_2, 2 mg/day P_4, 100 ng/day E_2 and 2 mg/day P_4, or vehicle alone as a negative control. Mouse uteri, major salivary glands including parotid, submandibular, and sublingual glands, as well as submandibular lymph nodes were collected and snap frozen in liquid nitrogen 24 h after the last injection. Uteri and major salivary glands were collected and fixed in 10% Formalin/PBS on ice, then embedded in Optimal Cutting Temperature (OCT compound) mounting medium (Sakura Finetek USA inc., Torrance, CA, USA) prior to sectioning. All samples were kept at -80°C until use. The major salivary glands and submandibular lymph nodes were also collected from 10 BALB/c adult male mice for inclusion in the microarray experiments. The experimental design was approved by the Institutional Animal Care and Use Committee at University of California San Francisco (UCSF). Mice were housed in the animal facility at UCSF in compliance with all applicable federal and state laws.

RNA sample preparation and cDNA synthesis

Total RNA samples from mouse uteri, major salivary glands, and submandibular lymph nodes from individual mice were isolated by Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality was assessed by using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA samples were sent to the Microarray Core facility at the Consortium for Functional Glycomics (CFG). RNA samples from three individual animals were prepared independently and used to synthesize cDNA according to the standard protocol used in the Microarray core facility. Chip hybridization was performed by the Microarray Core at the CFG according to their standard protocols as described previously (Comelli et al., 2006).

Glycov2 array analysis

The Glycov2 microarray is a custom-designed Glyco-gene chip array that contains probe sets to monitor the expression of approximately 2000 human and 925 mouse glycosylation-related genes, including glycosyltransferases, glycan-binding proteins (GBPs), glycan degradation proteins and other genes that are relevant to the CFG. It was developed by the Consortium and manufactured by Affymetrix (Santa Clara, CA, USA). All samples were hybridized to the Glycov2 array and Robust Multichip Average (RMA) (Irizarry et al., 2003) was used to convert the intensity values to expression values. RMA consists of a three step approach that performs background correction and quantile normalization, then summarizes the probe set information using Tukey's median polish algorithm.

Replicate probeset copies were averaged and gene expression patterns were analyzed using hierarchical clustering (Simon et al., 2007) and class comparison methods. The resulting class comparisons used a univariate cut-off of 0.001 and a multivariate permutation-based false discovery rate calculation.

Quantitative PCR analysis

Eight genes from different functional categories were chosen for quantitative PCR to confirm the microarray results. The experiments were performed by the Genomic Core at the University of California San Francisco Cancer Center. Taqman probes for each target gene were purchased from Applied Biosystems (Foster City, CA, USA). The probe catalog numbers were as follows: UDP-Gal: βGlcNAc β 1,3-galactosyltransferase 5 (*β13GalT5*), #Mm00473621; UDP-galactose-4-epimerase (*Gale*), #Mm00617772; Mucin 1(*Muc1*), #Mm00449604; Surfactant associated protein D (*Sftpd*), #Mm00486060; Insulin-like growth factor binding protein 3 (*Igfbp3*), #Mm00515156; Chemokine C-C motif ligand 11(*Ccl11*), #Mm00441238; Notch homolog 4 (*Notch4*), #Mm00440525; Patched homolog 1 (*Ptch1*), #Mm00436026. A two-tailed Student's *t*-test was used to analyze the data.

Ingenuity Pathway analysis

The microarray data were analyzed by Ingenuity Pathways Analysis (Ingenuity Systems, Palo Alto, CA, USA). The changes in gene expression among different treatments were analyzed in the context of canonical pathways, as well as

individual genes. The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used as a reference.

Muc1, Fut9, anti-blood group H, -Le^x, -Le^y, and HECA452 immunoblotting analysis

The following antibodies were used in immunoblotting experiments: a rabbit polyclonal antibody Muc1 (Cat. #RB-9222, Thermo Scientific, Fremont, CA) that recognizes the cytoplasmic domain of Muc1; a goat polyclonal antibody against Fut9 (Cat. #sc-14889, Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal antibodies against blood group H (Cat. #ab24222, Abcam Inc., Cambridge, MA, USA), Le^x (Cat. #555400, BD Pharmingen, San Jose, CA, USA), Le^y (Cat. #3359-500, Abcam Inc., Cambridge, MA, USA), and the rat monoclonal antibody HECA452 (Cat. #550407, BD Pharmingen). Tissue lysates were prepared as follows. Briefly, frozen tissues were thawed on ice and washed three times with cold PBS to remove residual red blood cells. Tissues were then homogenized in Sigma CelLytic MT Mammalian tissue Lysis/Extraction Regent (Sigma, St. Louis, MO, USA) with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Then, samples were centrifuged at 4°C for 2 min at 4000 rpm to remove insoluble debris. The protein concentration of the samples was estimated using the Bio-Rad Protein Assay based on the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots were frozen at -80°C until needed. Lysates (20 μg) were separated on NuPAGE Novex Bis-Tris 4-12% mini gels (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose

membranes (Bio-Rad Laboratories). The membranes were blocked with 5% non-fat milk (Bio-Rad Laboratories) in 1x PBST (1X PBS in 0.1% Tween-20) for 1 h at room temperature (RT), followed by overnight incubation with anti-Muc1, anti-blood groups H, -Le^x, -Le^y, and HECA-452 (1:1000 dilution in blocking buffer) and anti-Fut9 (1:250 dilution in blocking buffer) at 4°C. The membranes were washed with Tris-Buffered saline containing Tween-20, then incubated for 2 h at RT with a 1:5000 dilution of horseradish peroxidase-conjugated antibodies—donkey anti-rabbit for Muc1 and donkey anti-goat for Fut9 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Horseradish peroxidase-conjugated goat-anti-mouse light chain specific secondary antibodies (for blood group H, Le^x, and Le^y antibodies) and goat-anti-rat light chain specific secondary antibodies (for HECA452) (Jackson ImmunoResearch Laboratories) were incubated for 2 h at RT. Immunoreactive bands were visualized by using Amersham ECL Plus Western Blotting Detection Reagents using High Performance Chemiluminescence Film (GE Healthcare, Buckinghamshire, UK). As a negative control, identical blots were processed without the primary antibody alone. To evaluate sample loading, the same nitrocellulose membranes were stripped with Restore^{TM} plus Western Blot Stripping buffer (Thermo Scientific, Fremont, CA, USA) and reprobed with a rabbit anti-α tubulin antibody (Thermo Scientific) at 1:5,000 for 2 h at RT or overnight at 4°C followed by the appropriate secondary antibody for 2 h at RT. Bands were visualized as described above. The experiment was repeated three times using different tissue lysates with the same results. Densitometry analysis was performed using ImageJ software.

Immunofluorecence analysis Muc1, Galnt3, Fut9, and β1,3-GalT5, anti-blood group H, -Le^x, -Le^y, and HECA-452

Serial sections (5 mm) of formalin-fixed mouse uterus and major salivary glands were individually stained with the following antibodies: rabbit polyclonal antibodies against Muc1 (Cat. #RB-9222, Thermo Scientific); Galnt3 (Cat. #HPA007613, Sigma-Aldrich); goat polyclonal antibodies against Fut9 (Cat. #sc-14889, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β1, 3-GalT5 (Cat. #sc-22060, Santa Cruz Biotechnology); antibodies against blood group H, Le^x, Le^y, and HECA452 as described above. A previously published protocol (Drake et al., 2001) was followed. Briefly, tissue sections were washed three times in PBS, then blocked with 3% BSA (Sigma) in PBS for 30 min at RT for Muc1, Galnt3, Fut9, and β1, 3-GalT5. To reduce background staining, the product of reactions between the secondary antibody and endogenerous mouse immunoglobins, a blocking reagent from Vector Laboratory Mouse on Mouse (M.O.M.) kit (Vector Laboratory, Burlingame, CA, USA) was used to block and to dilute the primary antibodies for blood group H, Le^x, Le^y, and HECA-452, as well as the appropriate secondary antibodies. Then, the sections were incubated with primary antibody (1:100 in 0.5% BSA in PBS or Vector M.O.M. agent) for 2 h at RT or overnight at 4°C. As a negative control, sections were incubated without primary antibody. Next, the tissue sections were washed 3 times in PBS and incubated with fluorescene-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 2 h at RT. Then they were washed in PBS

and mounted with Vectashield containing DAPI (Vector Laboratories), which allowed visualization of the nuclei. This experiment was repeated at least 3 times using tissues from different animals with similar results. Images were captured using a Leica DFC 350FX digital camera (Leica Microsystems, Bannockburn, Germany).

Hematoxylin and Eosin (H&E) staining

Sections (3–5 mm) of frozen and formalin-fixed tissue biopsies of mouse uterus and major salivary glands were washed in PBS briefly. Sections were stained with Gill's #4 hematoxylin (Fisher Scientific) for 1-2 min, then washed under running tap water for 5 min. Slides were cleared in acidic alcohol for a few seconds and the nuclei were blued in Scott's water (Fisher Scientific) for 1 min. After dehydration in 95% alcohol, sections were stained with eosin (Fisher Scientific) to the desired intensity. Then they were further dehydrated with 100% ethanol, cleared with xylenes, and mounted with Permount (Fisher Scientific). Images were captured using a Leica DFC 450FX digital camera (Leica Microsystems).

Periodic Acid-Schiff's (PAS) staining

Formalin-fixed tissue sections (3-5 mm) were washed briefly in tap water. After rinsing briefly with distilled water, the sections were incubated with 0.5% periodic acid (Sigma) for 7 min. Then, they were rinsed quickly with distilled water and stained in Schiff's solution (American MasterTech Scientific, Lodi, CA, USA) for

15 min. Next, slides were rinsed in running tap water for 5 min and stained with 1% Light Green (Sigma) until the desired intensity was achieved. Mounting and image capture was performed as described above for H&E staining.

Cellular micellae sample preparation and Cy3/Cy5 labeling

Flash frozen uteri and salivary glands from each experimental treatment group were defrosted on ice, washed three times with cold PBS to remove residual red blood cells, and macerated with a pre-chilled glass Potter-Elvehjem homogenizer. Then the samples were sonicated with three pulses at 70% power and centrifuged at 5 °C for 1 hour at 100,000 x g. The resulting pellets were resuspended in 0.1 M $NaCO_3$, pH 9.3. The protein concentration was estimated using Bio-Rad Protein Assay reagents based on Bradford methods (Bio-Rad Laboratories). Aliquots were frozen at -80°C until needed. Cy3/Cy5-NHS (GE Life Sciences) labeling was performed according to the manufacturer's instructions. Briefly, 10 μg of Cy3- or Cy5-NHS was added to 1 mg micellae, and incubated at RT for 30 min. The labeled samples were kept in -80°C until the lectin array analyses.

Lectin microarray and data analysis

All lectins used in this study were printed at concentrations optimized to give a minimal signal of 1,000 arbitrary fluorescent units (A.U.) under fixed scanning conditions with a range of labeled glycoproteins. Two replicate arrays on each slide were used for quality control hybridizations with glycoprotein standards.

Routinely, the specificity of lectin binding was confirmed by inhibition with the relevant monosaccharides. The fluorescence signals were scanned and data were extracted using the standard image analysis software GenePix Pro 5.1. To control for any discrepancies due to dye labeling, the signals from the Cy3 and Cy5 arrays were averaged, a method described by Yang (Yang et al., 2002) to calculate the dye bias-corrected hybridization signals. A pooled reference sample was included in this analysis as a comparator for the hybridization signals of individual samples. Hierarchical clustering of the resultant data sets using the Pearson correlation coefficient with average linkage analysis produced a heat map illustrating glycan profiles observed in the mouse uterus and major salivary glands from mice in various experimental groups.

Part V. SUMMARY AND FUTURE DIRECTIONS

A. SUMMARY

Using multiple approaches, I profiled glycosylation-related genes in the mouse uterus and major salivary glands, and characterized the complex carbohydrate structures assembled at both sites. These analyses were designed to take into consideration the effects exerted by the ovarian hormones estrogen and/or progesterone. The results demonstrated that a comprehensive set of glycosylation-related genes was expressed by the mouse uterus and major salivary glands enabling the assembly of complex glycans. Indeed, complex glycans with distinct tissue and hormonally-regulated profiles were detected, demonstrating that the glycosylation machinery in these locations was functional. Moreover, estrogen and progesterone differentially affected the expression of both glycosyltransferases and oligosaccharide structures in the mouse uterus and major salivary glands. Specifically, under the influence of these hormones, the uterine glycan profile shifted from a simpler pattern to more complex branched structures, with heavily fucosylated, sialylated, and sulfated termini. Considering that these complex oligosaccharides play critical roles in cell-cell and cell-matrix interactions, and that the uterus can quickly increase or decrease production of these structures in a hormone-regulated manner, these results strongly suggested that glycosylation may play very critical roles in reproductive

events such as implantation, a theory for which strong evidence has already been demonstrated in humans (Genbacev et al., 2003).

The effects of estrogen and progesterone on glycosylation in the reproductive tract have been previously investigated in many model systems (Chilton et al., 1980; Coppola and Ball, 1966; Dutt et al., 1986; Isemura et al., 1981; Lambadarios et al., 1976; Nelson et al., 1975). However, the current study is the first to use a global approach to systematically examine the effects of these steroids on uterine glycosylation. As a result, the influence of estrogen and progesterone on glycosylation was dissected in a detailed and comprehensive manner. Estrogen and progesterone affected almost every aspect of glycosylation in the mouse uterus (Table 2.6). In accord with previous studies, my work also demonstrated that, in general, estrogen promotes glycosylation, while progesterone alone has a mildly stimulatory effect (Fig.2.2). When administered together, progesterone tended to antagonize the effects of estrogen (Fig.2.2). However, the complexity of hormonal regulation on individual genes as revealed by the microarray data did not allow for a general conclusion. Indeed, the number of genes affected and magnitude of changes observed in this study were truly unparalleled. Therefore, the information obtained from this study will serve as a valuable source for future research on glycosylation.

An important finding from this thesis project is that terminal glycosylation, *i.e.,* fucosylation, sialylation, and sulfation, was differentially regulated by estrogen

and/or progesterone in the mouse uterus. In agreement with the microarray data showing that the glycosyltransferases that transfer these modifications are subject to hormonal control, the abundance of the fucosylated, sialylated, and sulfated oligosaccharides was also influenced by estrogen and/or progesterone (Results; Part III). Although the formation of glycans is controlled at multiple levels (Marth and Grewal, 2008) ranging from the transcriptional regulation of glycosyltransferases to the accessibility of substrates, my results illustrated that control of glycosyltransferase expression at the mRNA level plays a critical role in this process.

The story of glycosylation in the mouse major salivary glands proved to be equally interesting. For the most part, estrogen and progesterone did not affect the expression of their glycosylation-related genes. Nevertheless, their glycan repertoire appeared to be under the influence of these hormones. This conclusion was supported by evidence that major salivary gland samples clustered according to hormonal treatment groups in the lectin array analyses. Furthermore, the glycan profile of male mouse major salivary glands also differed from that of female mice, additional evidence that estrogen and progesterone regulated glycan synthesis in these tissues. A possible explanation for the apparent discrepancy between hormonal regulation of glycosylation-related genes and oligosaccharide expression is the combinatorial nature of carbohydrate synthesis. Minor changes in the expression of a number of individual glycosyltransferases may be amplified into detectable changes at the

level of their carbohydrate products. In any case, my results suggested that the major salivary glands are ovarian responsive, a conclusion that may have important biological implications for women's oral health.

In agreement with previous studies, the current work also identified high mannose, terminal Gal/GalNAc oligosaccharides, and sialylated species as the dominant sugar motifs associated with mouse major salivary glands. An important group of fucosylated and/or sialylated structures, including H, Le^x, Le^y, and HECA-452 determinants, was also detected by lectin array and antibody-based methods. Interestingly, fucosylated oligosaccharides, particularly the Le^x epitope, were upregulated by estrogen + progesterone. Again, these results suggested that the major salivary glands are an ovarian responsive tissue. As a result, the expression of functionally important oligosaccharides was under the influence of estrogen and progesterone.

In summary, using powerful global approaches, I profiled the glycosylation-related genes and glycans in the mouse uterus and major salivary glands. In addition, I illustrated the differential regulation of glycosylation-related genes and glycan structures by estrogen and progesterone. Highly specialized oligosaccharides that can function as bacterial receptors and selectin ligands were detected in these tissues. Moreover, these specialized oligosaccharides were differentially regulated by estrogen and progesterone, an observation indicating that they may play critical roles in females. With the fundamental

information obtained from this project, I can extend my research using laboratory mice as a model to identify the molecular link between periodontal disease and preterm birth in the context of carbohydrate-mediated cell-cell adhesive interactions. Future research will show whether similar or different oligosaccharides govern bacterial and leukocyte adhesion in the murine oral cavity and reproductive tract. In either case, this important information could be used to design novel therapies for the prevention or treatment of preterm labor and periodontal disease.

B. FUTURE DIRECTIONS

As one of the four fundamental macromolecular components of all cells, glycans are essential for cell viability and normal function (Haltiwanger and Lowe, 2004; Ohtsubo and Marth, 2006; Paulson et al., 2006; Raman et al., 2005). Due to their strategic location and structural diversity, cell surface glycans mediate cell-cell and cell-matrix interactions that are crucial for the development, growth, function, and survival of the organism (Varki, 1999a). Aberrant glycosylation remains a key molecular change associated with many disorders, including various cancers and inflammatory diseases (Marth and Grewal, 2008; Miyoshi and Nakano, 2008; Ohyama, 2008). A growing body of research strongly demonstrates that bacterial invasion and host-mediated inflammation are key components in the pathogenesis of both periodontal disease and preterm labor (Part I; Section B9). Therefore, I propose to study the mechanism of carbohydrate-mediated bacterial

adhesion in both oral and uterine cavities using laboratory mice as the model system. With completion of this thesis project, I have learned a great deal about the expression of glycosylation-related genes and oligosaccharides in the mouse uterus and major salivary glands, as well as their regulation by estrogen and progesterone. In support of my central hypothesis, the oligosaccharides that could potentially serve as functional receptors for bacteria and leukocytes were detected in both tissues. I plan to continue my future research in two separate but complementary directions. First, I will continue use laboratory mice as a model system to uncover the molecular links between periodontal disease and preterm labor, focusing on carbohydrate-mediated bacterial and leukocyte adhesion. Second, I will expand my research to humans by using a similar approach as in the mouse study. My ultimate goal is to develop a new strategy to prevent and treat periodontal disease and preterm labor.

My short-term goals involve a continuation of my thesis project, using the mouse model that I have developed. I will study the adhesive functions of specific protein scaffolds and their carbohydrate modifications in promoting bacterial and leukocyte adhesion in the mouse uterus and major salivary glands. Pathogens that promote periodontal disease and intrauterine infections (Table 1.1) will be used in whole cell ligand binding assays to detect the specific oligosaccharide ligands carried by uterine and salivary glycoproteins that interact with bacterial receptors. A suite of exoglycosidases and carbohydrate-specific antibodies will be used to prove structural specificity. Many strains of mice that are deficient in a

specific glycosyltransferase or other glycosylation-related gene are available. I will use these mice to test whether specific carbohydrate linkages play critical roles in mediating bacterial adhesion in the oral and uterine cavities. The frozen tissue overlay assay will be used to identify receptors that mediate leukocyte adhesion (Prakobphol et al., 1995). Freshly isolated peripheral blood lymphocytes and certain cell lines that serve as positive and negative controls will be used. Once adhesion is detected, the specificity of the interaction will be determined by the addition of mono/oligosaccharide and antibody inhibitors.

Whenever possible, specific glycosyltransferase-deficient mice will be used to determine the critical oligosaccharides that mediate such interactions. For example, Fut1-/- and Fut2-/- mice have already been generated (Domino et al., 2001; Magalhaes et al., 2009). Fut1/Fut2 double knock-out mice lack α-1,2-fucosylated glycans in their reproductive tract (Domino et al., 2001). Moreover, Fut2-/-mice have impaired BabA-mediated *H. pylori* adhesion to gastric mucosa (Magalhaes et al., 2009). These mice will be a valuable tool for me to examine the roles of the α-1,2-fucosylated oligosaccharides in periodontal disease and intrauterine infections. In addition, I will control estrogen and progesterone levels in these mice, using approaches similar to those described in this thesis. After specific carbohydrate structures that mediate bacterial and leukocyte adhesion are identified, further experiments will be designed to address, at a mechanistic level, the interesting and unexplained association between periodontal disease and preterm labor. My research efforts will be directed at how to prevent or

promote such interactions. As previously described, I will use monosaccharides and carbohydrate-specific antibodies to block glycan-mediated interactions, thereby determining the binding specificity of bacterial and/or leukocyte adhesion.

Strong evidence also suggests that periodontal disease is associated with many systemic disorders besides preterm labor, for example heart and vascular diseases. A similar mouse model could be used to address the association between periodontal disease and other systemic disorders.

Once animal studies are underway, I will extend my research to humans. Similar approaches as used for the mouse experiments in this thesis will be applied to studies in humans. First, I will profile the glycosylation-related genes and the uterine as well as salivary glycomes to expand what is known about the glycosylation machinery and oligosaccharide repertoire in these regions. My focus will be to discover aberrant glycosylation that is related to both periodontal disease and preterm labor. To my knowledge, this type of project has yet to be attempted in humans. Salivary samples will be collected from healthy individuals and from patients with periodontal disease. Uterine tissue from women who undergo endometrial biopsies for various reasons will also be used. Both glycoarrays and lectin arrays will be used in these experiments. The results will provide valuable insights into the pathogenesis of periodontal and preterm labor. This fundamental knowledge will enable the design of experiments to identify

specific carbohydrate structures that mediate bacterial and leukocyte adhesion in the human oral and uterine cavities. The effects of estrogen and progesterone on these interactions will be considered. Novel strategies to prevent or promote the glycan-mediated bacterial or leukocyte adhesion will be explored.

It has always been an enigma that women are more prone to certain autoimmune diseases than men. For example, middle-aged women are the primary population that develops Sjogren's Syndrome, an autoimmune disease in which a person's leukocytes attack their salivary glands. The results of my thesis project indicate that major salivary glands are very likely ovarian responsive tissues with oligosaccharide profiles that are under the influence of estrogen and/or progesterone. The cyclic changes in the expression of the glycans that serve as ligands for leukocyte adhesion may eventually result in the trafficking of autoreactive leukocytes, which attack salivary gland cells, a hypothesis that my work suggests and should be investigated.

As we enter the post-genomic era, glycosylation has attracted more attention. After completion of my Ph.D. training, as a young periodontist and glycobiologist, I am on the path to conduct independent research aimed at providing new insights into the pathogenesis of periodontal disease and preterm labor. My ultimate goals are to develop novel preventive and therapeutic strategies for women who experience either or both disorders.

Table 1.1. Representitives of pathogenic microorganisms that are associated with periodontal disease and preterm births

Genus	Species	Found in vaginal flora	Found in oral flora	Association with periodontitis	Association with PTB[a]
Bacteroides	*B. forsythia (T. forsythia)*	x	x	x	x
Fusobacterium	*F. nucleatum sub nucleatum*	x	x	x	x
Gardnerella	*G. vaginalis*	x			x
Lactobacillus	*L. fermentum*	x	x		x
Peptostreptococcus	*P. micros*	x	x	x	x
Porphyromonas	*P. gingivalis*	x	x	x	x
Prevotella	*P. intermedia*	x	x	x	x
Actinomyces	*A. actinomycetemcomitans*		x	x	x
Campylobacter	*C. gracilis*		x	x	x
Capnocytophaga	*C. sputigena*		x	x	x
Mobiluncus	*M.curtisii*	x			x

References: Hill GB. 1998: Socranskv SS et al.. 1998: Socranskv SS and Haffaiee AD. 2005

Table 2.1. Tissue gene expression profiles of transferases involved in the synthesis of the core regions of N- and O-linked glycoproteins and of glycolipids.

Category	Common name	Abbreviation	GenBank	Ut				MS					LN				
				oil	E	P	EP	oil	E	P	EP	M	oil	E	P	EP	M
N-Glycan																	
Lipid-linked glycan	Dolichol phosphate-mannose synthase 1	*Dpm1*	AB004789	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Dolichol phosphate-mannose synthase 2	*Dpm2*	NM_010073	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Mannosyltransferase ALG3	*Alg3*	AA215144	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-glycans transferases	Defender against cell death 1 protein	*Dad1*	U83628	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Oligosaccharyltransferase 48	*Ddost*	D89063	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Ribophorin I	*Rpn1*	BC016080	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Ribophorin II	*Rpn2*	NM_019642	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Processing GlcNAc-T	N-acetylglucosaminyltransferase I	*Mgat1*	L07037	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	N-acetylglucosaminyltransferase II	*Mgat2*	BC010583	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannosidases	Mannosidase, beta a	*Manba*	NM_027288	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Mannosidase II, a 1	*Man2a1*	X61172	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Mannosidase II, a b1	*Man2b1*	U29947	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O-Glycan																	
Polypeptide transferase	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 1	*Galnt1*	U73820	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 2	*Galnt2*	AF348968	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 3	*Galnt3*	U70538	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 4	*Galnt4*	NM_015737	—	+	—	—	+	—	—	+	+	—	—	—	—	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 5	*Galnt5*	NM_172855	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 6	*Galnt6*	AJ133523	—	+	—	—	—	+	—	—	—	+	+	+	+	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 7	*Galnt7*	BC007484	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 9	*Galnt9*	NM_198306	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 10	*Galnt10*	NM_134189	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 11	*Galnt11*	NM_144908	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 12	*Galnt12*	NM_172693	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 13	*Galnt13*	NM_173030	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 14	*Galnt14*	AK078292	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 15	*Galnt15*	NM_026449	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 16	*Galnt16*	AB045325	—	—	—	—	—	—	—	—	—	—	—	—	—	—

	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 17	*Galnt17*	AK006215	+	+	+	+	+	+	+	+	+	+	+	+	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 18	*Galnt18*	NM_173739	+	+	+	+	+	+	—	—	+	+	+	+	+	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase-like 2	*Galntl2*	AK019470	+	+	+	+	—	—	—	—	—	+	+	+	+	+
	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 20	*Galnt20*	NM_145218	+	+	+	+	—	—	—	—	—	—	—	—	—	—
Gal T	glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1	*C1galt1*	NM_052993	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	core 1 beta1,3-galactosyltransferase 2	*C1galt1c1*	AY159320	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GlcNAc-T	core2-GlcNAc-transferase 1	*C2GnT1*	NM_010265	—	—	+	—	—	—	—	—	—	+	+	+	+	+
	core2-GlcNAc-transferase 2 (mouse)	*C2GnT2 (M)*	AA762195	—	+	+	—	—	—	—	—	—	—	—	—	—	—
	core 2 beta-1,6-*N*-acetylglucosaminyltransferase 3	*C2GnT3*	CeleraC2GnT	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Glycolipids																	
Ceramide transferase	UDP-glucose ceramide glucosyl transferase	*Ugcg*	D89866	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ceramide 1--galactosyltransferase (CGT) EST		BG060736	—	—	—	—	+	+	+	+	—	—	—	—	—	—

Ut, uterine tissue; MS, major salivary glands; LN, submandibular lymph nodes; oil, oil vehicle control group; E, estrogen supplemental group; P, progesterone supplemental group; EP, estrogen + progesterone supplemental group; M, male mice. Data were obtained by microarray analysis using CFG Glycov2 chips. The RMA algorithm was used to obtain the expression signal values. Present (+) or absent (-) absolute calls were determined with the MicroArray Suite (MAS) 5.0 Affymetrix algorithm. Glycogenes were considered present in one tissue if they had been assigned a present call in at least two of three replicate samples. All marginal calls were considered as absent.

Table 2.2. Tissue expression profiles of terminal glycosyltransferases: sialyl-, fucosyl-, and sulfotransferases.

Category	Common name	Abbreviatic	GenBank	Ut				MS					LN				
				oil	E	P	EP	oil	E	P	EP	M	oil	E	P	EP	M
Sialyltransferases																	
	ST3 beta-galactoside alpha-2,3-sialyltransferase I	*St3gal I*	X73523	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ST3 beta-galactoside alpha-2,3-sialyltransferase II	*St3gal II*	X76989	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ST3 beta-galactoside alpha-2,3-sialyltransferase III	*St3gal III*	BC006710	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ST3 beta-galactoside alpha-2,3-sialyltransferase IV	*St3gal IV*	BC011121	—	—	+?	—	+	+	+	+	+	—	—	—	—	—
	ST3 beta-galactoside alpha-2,3-sialyltransferase V	*St3gal V*	AF119416	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ST3 beta-galactoside alpha-2,3-sialyltransferase VI	*St3gal VI*	NM_01878	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Beta-galactoside alpha-2,6-sialyltransferase I	*St6gal I*	BB768706	+	+	+	+	—	—	—	—	—	+	+	+	+	+
	Beta-galactoside alpha-2,6-sialyltransferase II	*St6gal II*	NM_17282	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase I	*St6galnac I*	NM_01137	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase II	*St6galnac II*	X93999	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase III	*St6galnac II*	Y11342	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase IV	*St6galnac IV*	Y15779	+	+	+	+	+	+	—	—	+	+	+	+	+	+
	alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase V	*St6galnac V*	AB028840	+	+	+	+	—	—	—	—	—	+	+	+	+	+
	alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase VI	*St6galnac V.*	BB772604	+	+	+	+	—	—	—	—	—	+	+	+	+	+
	ST8 alpha-*N*-acetyl-neuraminide alpha-2,8-sialyltransferase I	*St8sia I*	NM_01137	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	ST8 alpha-*N*-acetyl-neuraminide alpha-2,8-sialyltransferase II	*St8sia II*	X83562	+	—	+	+	—	—	—	—	—	—	—	—	—	—
	ST8 alpha-*N*-acetyl-neuraminide alpha-2,8-sialyltransferase III	*St8sia III*	X80502	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	ST8 alpha-*N*-acetyl-neuraminide alpha-2,8-sialyltransferase IV	*St8sia IV*	X86000	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ST8 alpha-*N*-acetyl-neuraminide alpha-2,8-sialyltransferase V	*St8sia V*	NM_01366	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	ST8 alpha-*N*-acetyl-neuraminide alpha-2,8-sialyltransferase VI	*St8sia VI*	NM_14583	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fucosyltransferases																	
alpha 1,2 fut	fucosyltransferase 1	*Fut1*	NM_00805	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	fucosyltransferase 2	*Fut2*	NM_01887	—	+	—	—	—	—	—	—	—	—	—	—	—	—
alpha 1,3/4	fucosyltransferase 4	*Fut4*	NM_01024	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	fucosyltransferase 7	*Fut7*	NM_01352	—	—	—	—	—	—	—	—	—	+	+	+	+	+
alpha 1,6	fucosyltransferase 8	*Fut8*	BC010666	+	+	+	+	+	+	+	+	+	+	+	+	+	+
alpha 1,3/4	fucosyltransferase 9	*Fut9*	AB015426	+	+	—	+	—	—	—	—	—	—	—	—	—	—
alpha 1,3	fucosyltransferase 10	*Fut10*	BF181631	+	+	+	+	—	—	—	—	—	+	+	+	+	+
	fucosyltransferase 11	*Fut11*	AK014029	+	+	+	+	+	+	+	+	+	+	+	+	+	+
protein fut	protein O-fucosyltransferase 1 (from EST)		BB024471	+	+	+	+	—	—	—	—	—	+	+	+	+	+
	protein O-fucosyltransferase 3		BC018194	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sulfotransferases																	
	chondroitin 4-O-sulfotransferase 1 (CS4ST1)	*Chst11*	NM_02143	+	+	+	+	—	—	—	—	—	+	+	+	+	+
	chondroitin 4-O-sulfotransferase 2 (CS4ST2)	*Chst12*	NM_02152	+	+	+	+	—	—	—	—	—	+	+	+	+	+
	chondroitin 6-O-sulfotransferase 1 (C6ST1)	*Chst3*	NM_01680	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	galactose-3-O-sulfotransferase 1	*Galst1*	NM_01692	+	+	+	+	—	—	—	—	—	+	+	+	+	—

galactose-3-O-sulfotransferase 2	*Galst2*	BB617404	—	—	—	—	—	—	+	—	—	—	—	—	—	—
galactose-3-O-sulfotransferase 4	*Galst4*	XM_28415[illegible]	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N-acetylglucosamine 6-O-sulfotransferase 1 (GlcNAc6ST-1)	*Chst2*	AB011451	+	+	+	+	—	—	—	—	—	+	+	+	+	+
N-acetylglucosamine 6-O-sulfotransferase 2 (GlcNAc6ST-2)	*Chst4*	AF109155	—	+	+	+	—	—	—	—	—	+	+	+	+	+
N-acetylglucosamine 6-O-sulfotransferase 3 (GlcNAc6ST-3)	*Chst5*	NM_01995(	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N-acetylglucosamine 6-O-sulfotransferase 4 (GlcNAc6ST-4)	*Chst7*	NM_021715	—	+	—	—	—	—	—	—	—	—	—	—	—	—
heparan sulfate 2-O-sulfotransferase 1	*Hs2st1*	AF060178	+	+	+	+	+	+	+	+	+	+	+	+	+	+
heparan sulfate (glucosamine) 3-O-sulfotransferase 1	*Hs3st1*	NM_01047[illegible]	+	+	+	+	+	+	+	+	+	+	+	+	+	+
heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	*Hs3st3b1*	NM_01880[illegible]	+	+	+	+	—	—	—	—	—	+	+	+	+	+
heparan sulfate 6-O-sulfotransferase 1	*Hs6st1*	NM_01581[illegible]	+	+	+	+	+	+	+	+	+	+	+	+	+	+
heparan sulfate 6-O-sulfotransferase 2	*Hs6st2*	NM_01581[illegible]	—	—	—	—	—	—	—	—	—	—	—	—	—	—
heparan sulfate 6-O-sulfotransferase 3	*Hs6st3*	NM_01582(	—	—	—	—	—	—	—	—	—	—	—	—	—	—
carbohydrate (keratan sulfate Gal-6) sulfotransferase 1 (KS6ST-1)	*Chst1*	NM_02385(	+	—	—	—	+	—	+	—	—	+	+	+	+	+
N-deacetylase/*N*-sulfotransferase (heparan glucosaminyl) 1	*Ndst1*	AF074926	+	+	+	+	—	—	—	—	—	+	+	+	+	—
N-deacetylase/*N*-sulfotransferase (heparan glucosaminyl) 2	*Ndst2*	NM_010811	+	+	+	+	—	—	—	—	—	+	+	+	+	+
N-deacetylase/*N*-sulfotransferase (heparan glucosaminyl) 3	*Ndst3*	AF221095	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N-deacetylase/*N*-sulfotransferase (heparan glucosaminyl) 4	*Ndst4*	AB036838	—	—	—	—	—	—	—	—	—	—	—	—	—	—

The abbreviation and methods to obtained data are described in Table 2.1

Table 2.3. Tissue expression profiles of C-type and I-type lectins.

Category	Common name	Abbreviatio	GenBank	Ut				MS					LN				
				oil	E	P	EP	oil	E	P	EP	M	oil	E	P	EP	M
C-type																	
1-Proteoglycan	Aggrecan	Acan	L07049	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Brevican	Bcan	X87096	—	—	—	—	—	—	—	—	—	—	+	—	—	—
	Neurocan	Ncan	X84727	—	—	—	—	—	—	—	—	—	—	+	—	—	—
	Versican (CSPG2, PG-M)	Vcan	NM_019389	+	+	+	+	—	—	—	—	—	—	—	—	—	—
2-Type 2 Recepto	Asialoglycoprotein receptor MF1		S36676	—	—	—	+	—	—	—	—	—	—	—	—	—	+
	Asialoglycoprotein receptor MF2		AY103461	+	—	+	+	—	—	—	—	—	+	+	+	+	+
	Asialoglycoprotein receptor R1		D13517	—	—	—	—	+	—	+	—	+	—	+	—	—	—
	Asialoglycoprotein receptor R2		X53042	—	—	—	—	—	—	—	—	—	—	+	—	—	—
	C-type lectin domain family 4, member n	Clec4n	AF240357	+	+	+	+	—	—	—	—	—	+	+	+	+	+
	C-type lectin domain family 4, member a2	Clec4a2	AJ133533	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	C-type lectin domain family 4, member b1	Clec4b1	NM_027218	+	+	+	+	+	+	+	+	—	+	+	+	+	+
	dendritic cell-specific ICAM-3-grabbing non-integrin	DC-SIGN	AF373408	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	DC-SIGN-related protein 1	DC-SIGN-R	AF373409	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	DC-SIGN-related protein 2	DC-SIGN-R	AF373410	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	DC-SIGN-related protein 3	DC-SIGN-R	AF440280	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	DC-SIGN-related protein 4	DC-SIGN-R	AF373412	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	DC-SIGN-X5	DC-SIGN-X	BC025069.1	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	DC-SIGN-X6	DC-SIGN-X	ENSMUSG00000047357	—	—	+	—	—	—	—	—	—	—	+	—	—	—
	DC-SIGN-X7	DC-SIGN-X	ENSMUSG00000040141	—	—	—	—	+	+	+	+	—	+	+	+	+	—
	Fc receptor, IgE, low affinity II, alpha polypeptide	Fcer2a	NM_013517	—	—	+	—	—	—	—	—	—	+	+	+	+	+
	Langerin	Langerin	AJ302711	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	Mincle	Mincle	BC003218	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	Scavenger receptor with CTLD		AB038519	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Collectin L1		NM_173422	—	—	—	—	—	—	—	—	—	—	+	—	—	—
3-Collectin	Mannose-binding protein A	Mbl1	NM_010775	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Mannose-binding protein C	Mbl2	S42294	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Sftpa: Surfactant associated protein A- Long Trans	Sftpa	NM_023134.3	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Surfactant associated protein SP-D	Sftpd	NM_009160	+	+	—	+	+	—	+	—	—	—	—	—	—	—
4-Selectin	E-Selectin	E-Selectin	NM_011345	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	L-selectin	L-selectin	NM_011346	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	P-selectin	P-selectin	M87861	+	—	+	—	—	—	—	—	—	+	+	—	—	—
5-NK Receptors	CD69	CD69	L23638	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	CD72	CD72	NM_007654	+	+	+	+	+	+	—	+	—	+	+	+	+	+
	CD94	CD94	NM_010654	+	—	+	+	—	—	—	—	—	+	+	+	+	+
	CLEC-2	CLEC-2	AF201457	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	CLLEC-1	CLLEC-1	NM_175526	+	+	+	+	+	+	+	+	—	+	+	+	+	+
	Dectin-1	Dectin-1	AF262985	+	+	+	+	—	+	—	+	—	+	+	+	+	+
	lymphocyte antigen 49b	Ly49b	U10304	—	—	—	—	—	—	—	—	—	—	—	—	+	—
	lymphocyte antigen 49c	Ly49C	U10305	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	killer cell lectin-like receptor subfamily G, member 1	Klrg1	NM_016970	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	myeloid DAP12-associating lectin 1	MDL-1	AF139769	—	—	—	—	—	—	—	—	—	+	+	+	+	+
6-MMR	macrophage mannose receptor 1	Mrc1	Z11974	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8-Simple Type 1 r	Layilin homolog		AF311699	+	+	+	+	—	—	—	—	—	—	—	—	—	—
9-Tetranectins	Tetranectin		X79199	+	+	+	+	—	—	—	—	—	+	+	+	+	+
10-Polycystin	Polycystin		U70209	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11-Attractin	Attractin homolog		NM_181415	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12-CTLD + acidi	Eosinophil major basic protein		AK010901	+	+	+	+	+	+	—	—	+	+	+	+	+	+
13-IDD	IDD		D78641	+	+	+	+	+	+	—	—	+	+	+	+	+	+
14-Endosialin	14-Endosialin		AF081789	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Endosialin		AF388572	+	+	+	+	—	—	—	—	—	—	—	—	—	—
	Thrombomodulin (Thbd)		X14432	+	+	+	+	+	+	+	+	+	+	+	+	+	+
I-type																	
Non-Siglec	CD83	CD83	NM_009856	+	—	+	—	—	—	—	—	—	+	+	+	+	+

Siglec	sialic acid binding Ig-like lectin 1	Siglec-1	NM_011426	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	sialic acid binding Ig-like lectin 10	Siglec-10	PROPIGLEC2	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	sialic acid binding Ig-like lectin 2	Siglec-2	NM_009845	—	+	+	+	—	—	—	—	—	+	+	+	+	+
	sialic acid binding Ig-like lectin 3	Siglec-3	NM_021293	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	sialic acid binding Ig-like lectin 4	Siglec-4	M31811	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	sialic acid binding Ig-like lectin E	Siglec-E	NM_031181	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	sialic acid binding Ig-like lectin F	Siglec-F	AF293371	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	sialic acid binding Ig-like lectin H	Siglec-H	PROPIGLEC3	—	—	—	—	—	—	—	—	—	+	+	+	+	+

The procedure of obtaining data and abbreviations are described in Table 2.1. Lectin classification is according to Taylor and Drickamer (2003).

Table 2.4. Tissue expression profiles of glycoproteins.

Category	Common name	Abbreviation	Genbank	Ut				MS					LN				
				oil	E	P	EP	oil	E	P	EP	M	oil	E	P	EP	M
mucins	mucin 1	Muc1	NM_013605	+	+	+	+	—	—	—	—	—	—	—	—	—	—
	mucin 10	Muc10	NM_008644	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	mucin 3	Muc3	AF027131	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	mucin 5AC	Muc5AC	L42292	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	mucin 5B	Muc5B	AF369933	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Adhesion Molecule	Endoglycan	Endoglycan	NM_176973	—	—	+	—	—	—	—	—	—	—	—	—	—	—
	Endomucin (Muc14)	Emcn (Muc14)	NM_016885	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BCM1 (CD48)	Bcm1(Cd48)	X53526	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	CD2 (LFA-2)	Cd2	NM_013486	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	Neurothein (CD147)	Neurothein (CD147)	BC010270	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	CD34	Cd34	BC006607	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	glycosylation dependent cell adhesion molecule 1	Glycam1	M93428	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	mucosal vascular addressin cell adhesion molecule 1	Madcam1	NM_013591	—	—	—	—	—	—	—	—	—	+	+	+	+	+
	platelet (p-selectin) ligand	Psgl-1	NM_009151	+	+	+	+	+	+	—	—	—	+	+	+	+	+

The procedure of obtaining data and abbreviations are described in Table 2.1.

Table 2.5. Tissue expression profiles of proteoglycans.

Category	Common name	GenBank	Ut				MS					LN				
			oil	E	P	EP	oil	E	P	EP	M	oil	E	P	EP	M
BMPG	Agrin	AF190858	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Bamacan short (CSPG6)	NM_007790	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Perlecan (HSPG2)	M77174	+	+	+	+	—	—	—	—	—	+	+	+	+	+
Glypican	Glypican-1 (Gpc-1)	AF185613	+	+	+	+	—	—	—	—	—	+	+	+	+	+
	Glypican-2 (Gpc-2, cerebroglycan)	AK010515	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Glypican-3 (Gpc-3, OCI-5)	AF185614	+	—	+	—	—	—	—	—	—	—	—	—	—	—
	Glypican-4 (Gpc-4)	BC006622	—	—	—	+	—	—	+	+	—	—	—	—	—	—
	Glypican-5 (Gpc-5)	AF001463	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Glypican-6 (Gpc-6)	AF105268	+	+	+	+	—	—	—	—	—	+	+	+	+	+
Miscellaneou	CD44 (Epican)	BC005676	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Collagen IX a2	NM_007741	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Collagen Type XIV	AJ131395	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Invariant Chain CD74	AK002232	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Serglycin	X16133	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SLRP	Biglycan (BGN, PGI)	BC005452	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Decorin (DCN, PGII)	X53929	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Fibromodulin	X94998	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Lumican	BC005550	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Syndecan	Syndecan-1	NM_011519	—	+	—	—	—	—	—	—	—	—	—	—	—	—
	Syndecan-2	U00674	+	+	+	+	—	—	—	—	—	+	+	—	—	—
	Syndecan-3	U52826	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Syndecan-4 long	NM_011521	—	—	—	—	+	+	+	+	+	—	—	—	—	—

The procedure of obtaining data and abbreviations are described in Table 2.1.

Table 2.6. Glycosyltransferases that were differentially regulated by ovarian hormones estrogen and/or progesterone in mouse uterus.

Category	Abbreviation	GenBank	Common name	E	P	EP
N-glycosylation						
N-glycans-transferase	*Rpn2*	NM_019642	ribophorin II (dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2)	1.8		1.9
N-glycans-transferase	*Dad1*	U83628	defender against cell death 1(dolichyl-diphosphooligosaccaride-protein glycosyltransferase subunit DAD1)	1.3		1.5
N-glycans-transferase	*Ddost*	D89063	dolichyl-diphosphooligosaccharide-protein glycosyltransferase	1.5		1.6
GlcNAc-T	*Dpagt1*	X65603	dolichyl-phosphate (UDP-N-acetylglucosamine) acetylglucosaminephosphotransferase 1 (GlcNAc-1-P transferase)	1.6		1.4
GlcNAc-T	*Alg13*	AA215144_at	asparagine-linked glycosylation 13 homolog (S. cerevisiae)	1.5		
Mannosidase	*Manba*	NM_027288	Mannosidase, beta a	1.4		
Mannosidase	*Man2a1*	X61172	Mannosidase II a	1.3		
Mannosidase	*Man2b1*	U29947	Mannosidase II b	2.1		1.7
GlcNAc-T	*Mgat2*	BC010583	mannoside acetylglucosaminyltransferase 2	2		2
GlcNAc-T	*Mgat3*	NM_010795	mannoside acetylglucosaminyltransferase 3	-1.5		-1.3
GlcNAc-T	*Mgat4a*	AI155765	mannoside acetylglucosaminyltransferase 4, isoenzyme A	1.3		
GlcNAc-T	*Mgat4b*	AB053218	mannoside acetylglucosaminyltransferase 4, isoenzyme B			-1.5
GlcNAc-T	*Mgat4c*	AK033482	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme C (putative)	1.6		
Gal-T	*B4galt1*	J03880	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	1.5	1.7	
Gal-T	*B4galt3*	BC013619	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 3	1.6		
O-glycosylation						
GalNAc-T	*Galnt1*	U73820	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1		1.5	
GalNAc-T	*Galnt3*	U70538	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3	8.4		2
GalNAc-T	*Galnt7*	BC007484	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7	2.8	-1.4	
GalNAc-T	*Galnt10*	NM_134189	N-acetylgalactosaminyltransferase 10	2		
GalNAc-T	*Galnt11*	NM_144908	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 11			2.2
GalNAc-T	*Galnt12*	NM_172693	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12	4.5	-1.7	-1.4
GalNAc-T	*Galnt13*	NM_173030	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 13	1.7		
GalNAc-T	*Galntl2*	AK019470	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	-3.5		-1.8
Gal-T	*C1galt1*	NM_052993	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1	1.6		
Gal-T	*C1galt1c1*	AY159320	C1GALT1-specific chaperone 1			1.8
GlcNAc-T	*Gcnt3*	AA762195_s_a	glucosaminyl (N-acetyl) transferase 3, mucin type			-1.6
Gal-T	*B3galt5*	1428397_at	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	2.5		
Fucosyltransferase						
Fucosyl-T	*Fut2*	NM_018876	fucosyltransferase 2 (alpha-1,2)	1.7		
Fucosyl-T	*Fut8*	BC010666	fucosyltransferase 8 (alpha-1,6)			1.5

Fucosyl-T	*Fut9*	AB015426	fucosyltransferase 9 (alpha-1,3)	6		3.8
Fucosyl-T	*Pofut2*	BC018194	protein O-fucosyltransferase 2			1.4
Sialyltransferase						
Sia-T	*St3gal1*	X73523	ST3 beta-galactoside alpha-2,3-sialyltransferase 1		1.4	2
Sia-T	*St3gal3*	BC006710	ST3 beta-galactoside alpha-2,3-sialyltransferase 3	-1.4		
Sia-T	*St3gal6*	NM_018784	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	-2.4	1.4	
Sia-T	*St6gal1*	BB768706	b galactoside alpha-2,6-sialyltransferase 1	2		-1.8
Sia-T	*St6galnac2*	X93999	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	2		-1.5
Sia-T	*St6Galnac5*	AB028840	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5	-1.9	-1.5	-1.7
Sia-T	*St8sia2*	X83562	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2	-1.7		
Sia-T	*St8sia4*	X86000	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4		1.8	
Sulfotransferase						
Sulfo-T	*Chst5*	NM_019950	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 5	1.7		
Sulfo-T	*Chst7*	NM_021715	carbohydrate (N-acetylglucosamino) sulfotransferase 7	1.8		
Sulfo-T	*Hs3st1*	NM_010474	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	1.7	1.9	
Sulfo-T	*Hs6st1*	NM_015818	heparan sulfate 6-O-sulfotransferase 1			-1.5
Glycosphingolipid biosynthesis						
Gal-T	*B3galnt1*	BC003835	UDP-GalNAc:betaGlcNAc beta 1,3-galactosaminyltransferase, polypeptide 1	-1.5		
GlcNAc-T	*B3gnt5*	NM_054052	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	1.3	1.9	
GlcNAc-T	*B3gnt8*	NM_146184	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8	1.7		1.6
GalNAc-T	*B4Galnt1*	NM_008080	beta-1,4-N-acetyl-galactosaminyl transferase 1	2.3		2
GlcNAc-T	*Gcnt2*	AB037597	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	-2		-1.9
Gal-T	*Ggta1*	AF297615	glycoprotein galactosyltransferase alpha 1, 3	-2.1	2.3	

The procedure of obtaining signal density and abbreviations are described in Table 2.1. Processing of the data was performed within the Bioconductor project using R software. The fold changes and standard errors were estimated by fitting a linear model for each gene and empirical Bayes smoothing was applied to the standard errors for all the samples at the same time. The linear modeling approach and the empirical Bayes statistics as implemented in the Limma package in the R software were employed for differential expression analysis. Statistics were obtained for transcripts with the multiple testing adjusted (Benjamini-Hochberg) p-values level of .05. Filtering was performed so that probe-sets with a fold change of <1.4 were excluded from the results. Positive numbers indicate the expression level was up-regulated and negative numbers indicating down-regulation.

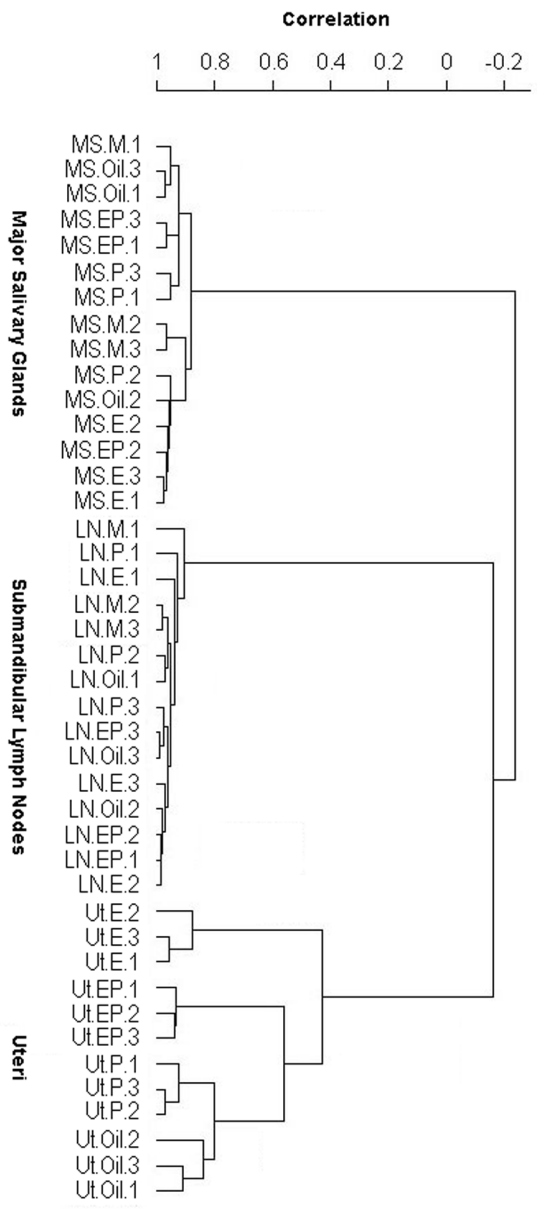


Fig.2.1. Dendrogram analysis shows distinct differences in gene expression among treatment groups in uterine samples, but not major salivary glands and submandibular lymph nodes. Forty-two expression arrays were hierarchically clustered with the algorithm BRB Array Tools 3.0.2 and displayed with Tree View. The centered correlation distance and average linkage dendrogram construction methods were used to produce this cluster. The individual samples were clustered in branches of the dendrogram based on overall similarity in patterns of gene expression. Samples are labeled as follows: the first abbreviation denotes tissue type (Ut: uterus, LN: lymph nodes, MS: major salivary glands), the second indicates treatment groups (E: estrogen treatment, P: progesterone treatment, EP: estrogen + progesterone treatment, Oil: sesame oil control treatment, M: male control group), and the number identifies individual mice used in the study. Note that only uterine samples regrouped together based on treatment regimen. This result suggests that glycosylation-related genes are differentially regulated by estrogen and/or progesterone in the mouse uterus, but not major salivary glands or submandibular lymph nodes.

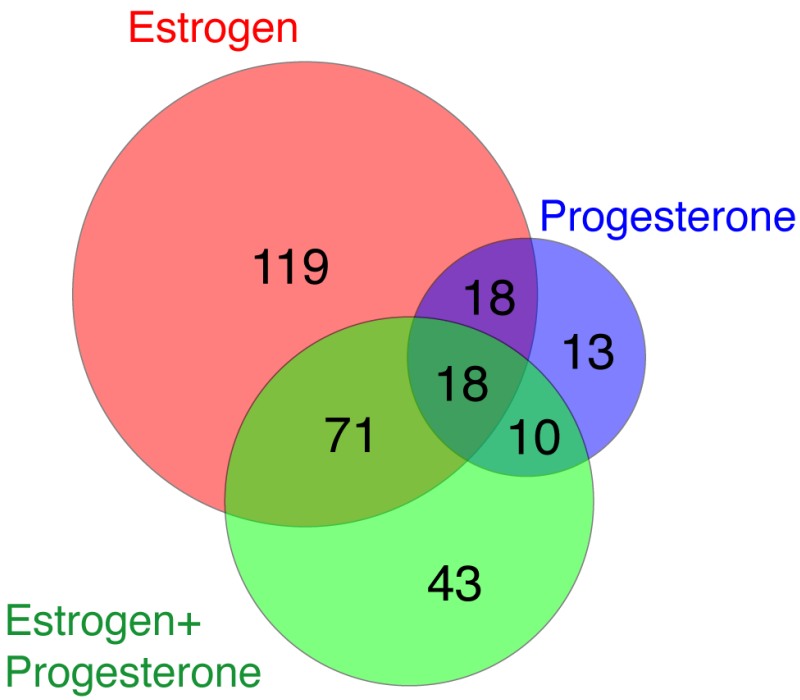
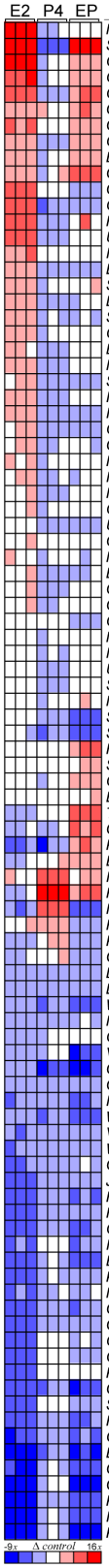


Fig.2.2. Glycosylation-related genes are regulated in the mouse uterus by estrogen and/or progesterone treatments. A comparison of gene expression levels in the mouse uterus among treatment groups (estrogen, progesterone, estrogen plus progesterone, and oil) was performed for the glycoarray data. A total of 292 glycosylation-related genes were identified as differentially regulated at the univariate significance level of 0.001. This included 226 and 59 glycogenes that were affected by estrogen or progesterone alone, respectively. Treatment with both hormones modulated mRNA levels of 143 genes. These results indicate that both estrogen and progesterone govern glycogene expression in the mouse uterus, and often act in opposition to each other. However, in aggregate, estrogen exhibits a more profound influence than progesterone.



Symbol	Title	E2	P4	EP
Muc1	mucin 1, transmembrane	14.8		1.4
Sftpd	surfactant associated protein D	12.9	-1.9	15.5
Galnt3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3	7.1		2
Golm1	golgi membrane protein 1	9.6	1.7	3.3
Gmds	GDP-mannose 4, 6-dehydratase	7.9		5.4
Ccl8	chemokine (C-C motif) ligand 8	2.5		3
Clec4a3	C-type lectin domain family 4, member a3	3.1		2.5
Gale	galactose-4-epimerase, UDP	3.2		2.2
B4galnt1	beta-1,4-N-acetyl-galactosaminyl transferase 1	2.3		2
Clec11a	C-type lectin domain family 11, member a	2.8		5.8
Ccl11	chemokine (C-C motif) ligand 11	6.6		-1.6
Galnt12	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12	4.5	-1.7	-1.4
Fut9	fucosyltransferase 9	6		3.8
Ugt1a6a	UDP glucuronosyltransferase 1 family, polypeptide A6A	4		2
Ifngr2	interferon gamma receptor 2	3.7	1.5	1.4
B3galt5	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	2.5		
Sulf2	sulfatase 2	3.7	1.5	2.2
Lgals9	lectin, galactose binding, soluble 9	3.8		1.4
Smpd1	sphingomyelin phosphodiesterase 1, acid lysosomal	2.9		
Gusb	glucuronidase, beta	3.1	1.4	1.7
Bmp8a	bone morphogenetic protein 8a	2.4		1.7
Ncstn	Nicastrin	2.7		1.9
St6galnac2	ST6-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	2		-1.5
Il4ra	interleukin 4 receptor, alpha	2.8		1.6
Igfbp2	insulin-like growth factor binding protein 2	2.2		
Galnt7	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 7	2.8	-1.4	
Ccl7	chemokine (C-C motif) ligand 7	2		
Nans	N-acetylneuraminic acid synthase (sialic acid synthase)	2.1		1.9
Il1a	interleukin 1 alpha	8.3		2.1
Neu1	neuraminidase 1	2.2		2.1
Gba	glucosidase, beta, acid	2.3		2.1
Ugdh	UDP-glucose dehydrogenase	2.3		
Ccr2	chemokine (C-C motif) receptor 2	2.1		
Nubp1	nucleotide binding protein 1	2		
Btg2	B-cell translocation gene 2, anti-proliferative	2.4		1.3
Ctsa	cathepsin A	2.5		1.8
Ccl9	chemokine (C-C motif) ligand 9	2.2		1.6
Galnt10	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10	2		
Man2b1	mannosidase 2, alpha B1	2.1		1.7
Naga	N-acetyl galactosaminidase, alpha	2		1.4
Uap1	UDP-N-acetylglucosamine pyrophosphorylase 1	2		1.8
Slc35a2	solute carrier family 35 (UDP-galactose transporter), member A2	2.1		1.7
Mgat2	mannoside acetylglucosaminyltransferase 2	2		2
St6gal1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	2		-1.8
Sc4mol	sterol-C4-methyl oxidase-like			-2.2
Figf	c-fos induced growth factor	1.7	1.5	6.1
St3gal1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1		1.4	2
Glb1	galactosidase, beta 1	2		2.5
Bmp1	bone morphogenetic protein 1		1.5	2
Tgfb2	transforming growth factor, beta 2		2	8.5
Csf1	colony stimulating factor 1 (macrophage)			4.8
Prol1	proline rich, lacrimal 1			6.4
Bgn	biglycan		1.8	2.1
Igf1	insulin-like growth factor 1	1.8	4	1.9
Fst	follistatin		12.7	4.4
Npl	N-acetylneuraminate pyruvate lyase	-2.1	6.3	-2.8
Fbln5	fibulin 5	1.9	3.4	-1.5
Pdgfd	platelet-derived growth factor, D polypeptide	-2.4		
Col14a1	collagen, type XIV, alpha 1		2.5	
Lgals12	lectin, galactose binding, soluble 12			-2.7
Ly75	lymphocyte antigen 75			-2.2
Wnt4	wingless-related MMTV integration site 4		-1.8	-2.5
Madcam1	mucosal vascular addressin cell adhesion molecule 1	-2.2		
Galnt11	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 11			2.2
Vcan	versican			-2.1
Cxcl14	chemokine (C-X-C motif) ligand 14		-2.4	-2.6
Cd209a	CD209a antigen	-2.2		
Il1r1	interleukin 1 receptor, type I	-2.1		
Wnt7a	wingless-related MMTV integration site 7A	-1.3		-2
Vegfb	vascular endothelial growth factor B	-2.1		-1.5
Wnt6	wingless-related MMTV integration site 6	-2		-1.7
Ccl5	chemokine (C-C motif) ligand 5	-2.2		
Jag1	Jagged1 - Long trans	-2.4		-1.4
Fzd7	frizzled homolog 7 (Drosophila)	-2		-1.8
Fzd2	frizzled homolog 2 (Drosophila)	-2.1		-1.6
Gcnt2	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	-2		-1.9
Ihh	Indian hedgehog	-2.4	1.5	-2.2
Bmp7	bone morphogenetic protein 7	-2		-2.1
Khk	ketohexokinase	-2.1		
Mgl2	macrophage galactose N-acetyl-galactosamine specific lectin 2	-2.8		
Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex)	-2.9		
Cxcl12	chemokine (C-X-C motif) ligand 12	-2.3		-1.6
Ggta1	glycoprotein galactosyltransferase alpha 1, 3	-2.1	2.3	
Gpc6	glypican 6	-2.9	2.3	
Pdgfra	platelet derived growth factor receptor, alpha polypeptide	-2.2	2.4	1.6
Cxcr4	chemokine (C-X-C motif) receptor 4	-2.5	-1.7	-2.5
St3gal6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	-2.4	1.4	
Fgfr2	fibroblast growth factor receptor 2	-2.5		
Galntl2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	-3.5		-1.8
Bmp2	bone morphogenetic protein 2	-3.7		-3.3
Cxcl15	chemokine (C-X-C motif) ligand 15	-2.7		-2.2
Cd83	CD83 antigen	-3.9		-4.3
Prdm1	PR domain containing 1, with ZNF domain	-4.6		-2.3
Igfbp3	insulin-like growth factor binding protein 3	-6.9		-3.2
Ptch1	patched homolog 1	-8.3		-5.8

Fig. 2.3. Hormone treatments cause differential expression of 97 glycosylation-related genes in the mouse uterus. Genes whose expression levels changed >2 fold, with a statistical significance of $p \leq 0.001$, as determined by microarray data are depicted. Signals are defined as up- or downregulated (red or blue, respectively) as compared to the oil control group. Fold changes are represented both visually, by the intensity of red or blue (see scale at the bottom of the figure), and numerically, as listed at the right. Each column presents data from individual mice (n = 3 per treatment group); rows correspond to a single probe set.

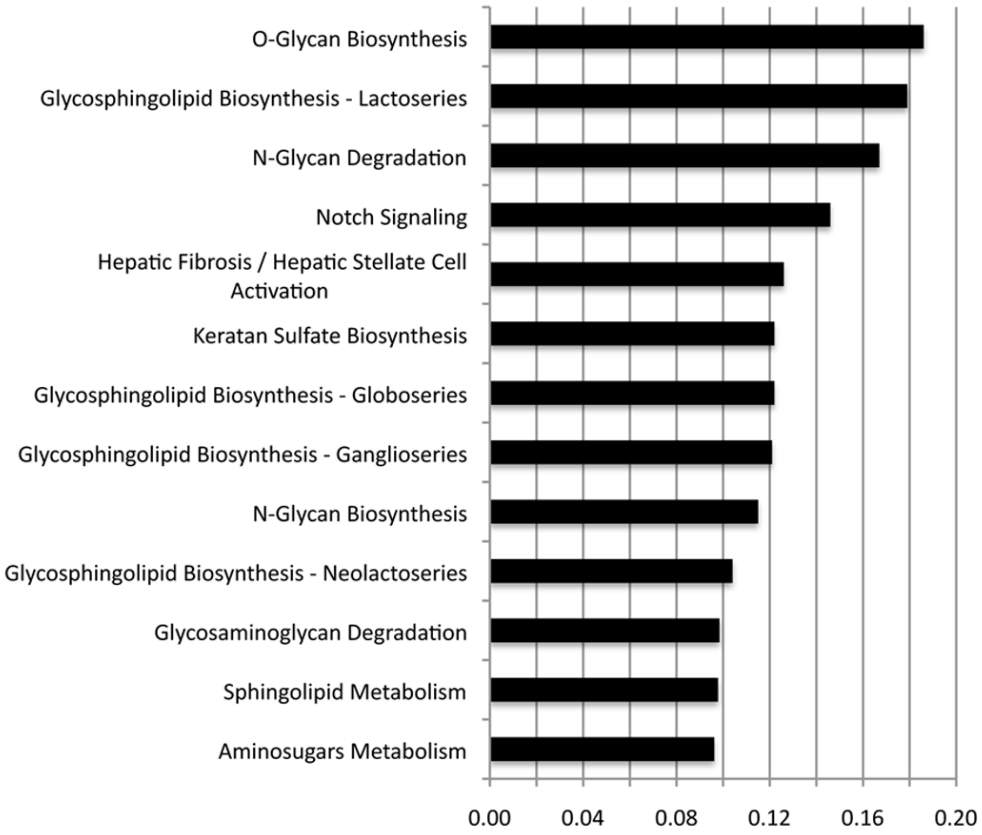


Fig. 2.4. Estrogen treatment affects the expression of genes in a number of carbohydrate-related signaling and biosynthetic pathways in the mouse uterus. Ingenuity Pathways software was used to analyze microarray data to determine canonical pathways that were most affected by treatment with estrogen and/or progesterone. Bars represent the percentage of genes in each pathway whose expression changed as a result of hormonal treatments.

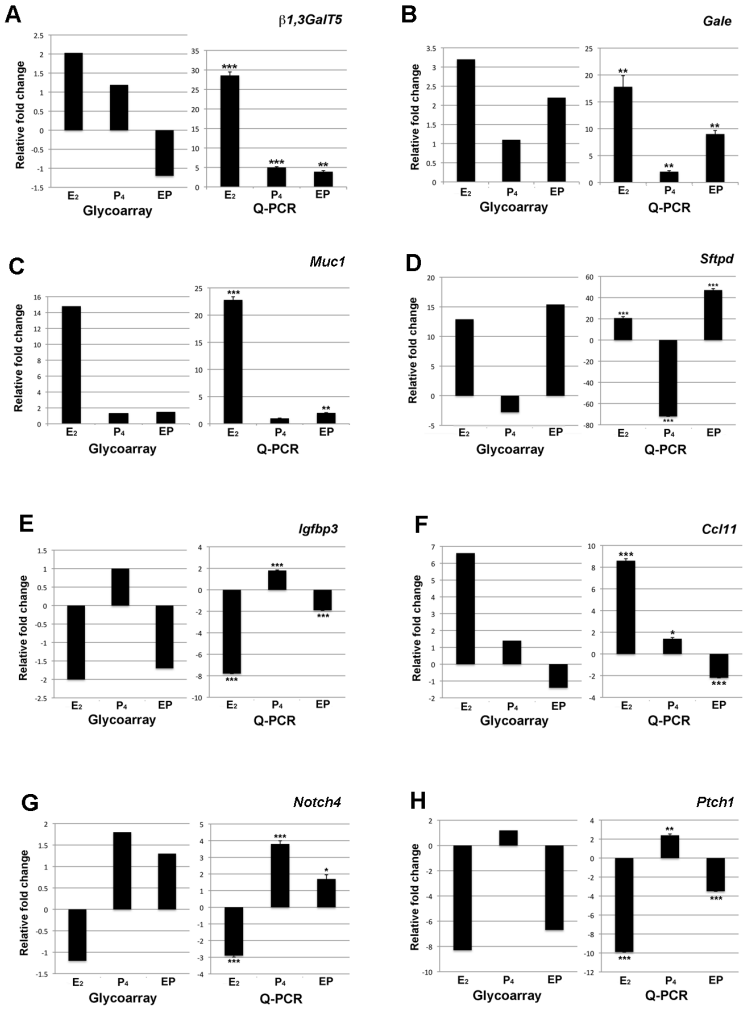


Fig.2.5. Q-PCR analyses confirmed the microarray expression patterns of a subset of genes that were differentially regulated in the mouse uterus by hormone treatment. Total uterine RNA was analyzed with TagMan probe sets for 8 selected genes: UDP-Gal: bGlcNAc b 1,3-galactosyltransferase 5 (*b13GalT5*), Panel A; UDP-galactose-4-epimerase (*Gale*), Panel B; Mucin 1(*Muc1*), Panel C; Surfactant associated protein D (*Sftpd*), Panel D; Insulin-like growth factor binding protein 3 (*Igfbp3*), Panel E; Chemokine C-C motif ligand 11(*Ccl11*), Panel F; Notch homolog 4 (*Notch4*), Panel G; Patched homolog 1 (*Ptch1*), Panel H. Relative RNA levels were normalized to the housekeeping gene cyclophilin, then divided by the oil control sample, which served as a calibrator. Microarray data for the genes in question are presented for comparison with the Q-PCR results (left and right sides of each panel, respectively). Each bar represents the mean ± SD of mRNA levels observed in individual mice (n = 3). Different cohorts of mice were used for each type of analysis. Significance was determined using two-tailed Student's *t*-test; asterisks denote the following p values: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

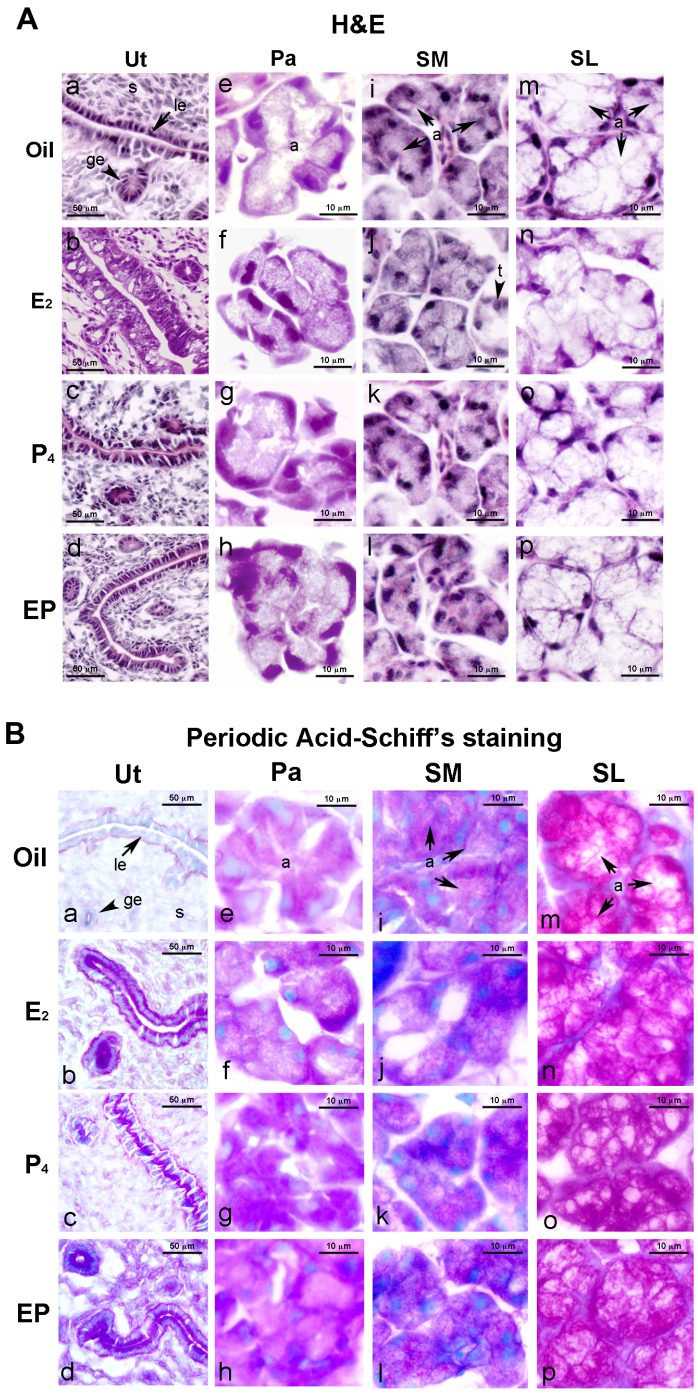


Fig.2.6. Hormonal treatments induced histological changes in the uterus, and upregulated the carbohydrate content of uterine epithelia. Hematoxylin and eosin (H&E; Panel A) and Periodic Acid-Schiff's (PAS; Panel B) staining was performed on serial sections from formalin-fixed biopsy specimens of uterus, parotid, submandibular, and sublingual glands from different treatment groups. For uterine tissues, areas including both the lumen and tubular glands are shown (Panels A and B, subpanels a-d). Scale bar for subpanels a-d and e-p: 50 and 10 μm, respectively. Abbreviations: le: luminal epithelium, ge: glandular epithelium, s: stroma, a: alveolus, t: tubular epithelium.

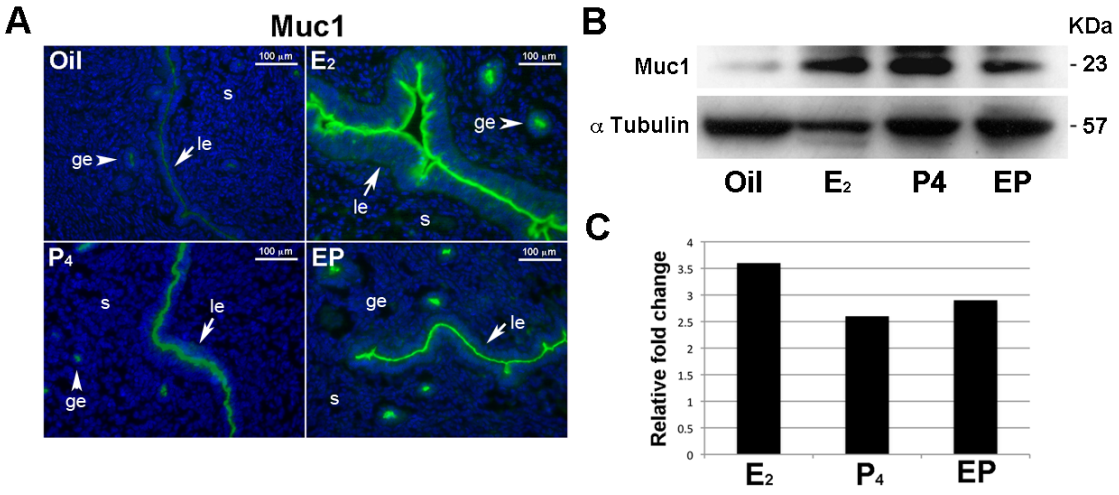


Fig. 2.7. Ovarian hormones upregulate Muc1 protein on uterine epithelia.
A. Muc1 protein in uterine tissue sections was detected with an anti-Muc1 antibody and visualized by a fluorescien-conjugated secondary antibody. Both luminal (arrows) and glandular epithelium (arrowheads) were minimally Muc1+ under control conditions (Oil). However, staining intensity dramatically increased after estrogen and/or progesterone treatment (E_2, P_4 and EP). Nuclei were stained with DAPI. Abbreviations: le: luminal epithelium, ge: glandular epithelium, s: stroma. Arrows designate the uterine lumen, and arrowheads, glands. Scale bar: 100 mm.
B. Uterine lysates from mice treated with estrogen, progesterone, both hormones or vehicle control were electrophoretically separated, transferred to nitrocellulose and immunoblotted with a polyclonal antibody against the cytoplasmic tail domain of Muc1. A single band of the appropriate molecular weight (23 KDa) was observed. The blot was stripped and reprobed with an anti-a tubulin antibody to demonstrate total protein loading.
C. The density of each band in Panel B was measured using ImageJ software. The a tubulin signal was used to normalize the total protein content in all lanes. Then, the fold change of Muc1 protein levels in experimental as compared to control groups was calculated.

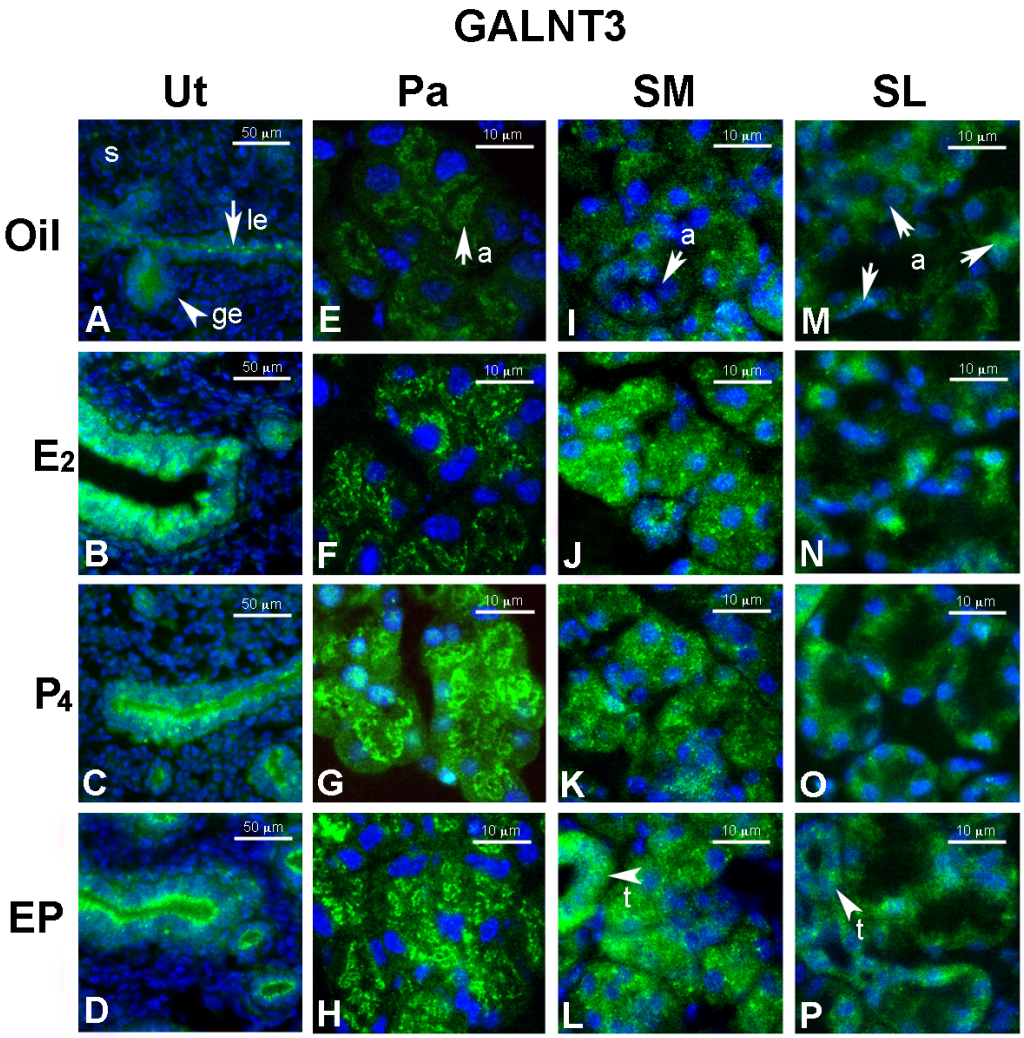


Fig. 2.8. Galnt3 protein is upregulated in uterine epithelia following exposure to ovarian hormones. Tissue sections of mouse uterus and major salivary glands from animals treated with estrogen (E_2), progesterone (P_4), both hormones (EP), or vehicle only (Oil) were probed with a polyclonal antibody against Galnt3 and visualized with a fluorescently-conjugated secondary antibody. Nuclei were stained with DAPI. Abbreviations: le: luminal epithelium, ge: glandular epithelium, s: stroma, a: secretory alveolus, t: tubular epithelium. Scale bar for panels A-D and E-P: 50 and 10 mm, respectively.

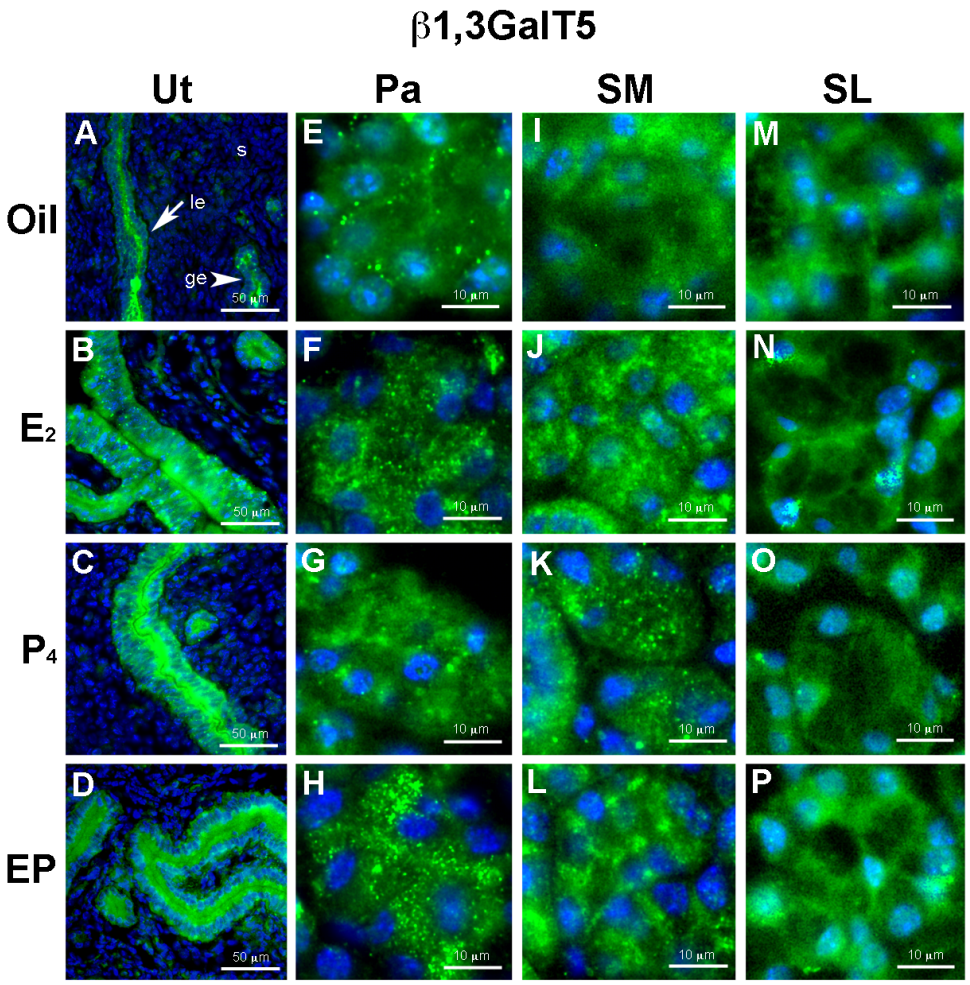


Fig. 2.9. Ovarian hormones induce the epithelial expression of β1,3GalT5 protein in the mouse uterus. Tissue sections of mouse uterus and major salivary glands from animals treated with estrogen (E_2), progesterone (P_4), both hormones (EP), or vehicle only (Oil) were probed with a polyclonal antibody against β1,3GalT5 and visualized with a fluorescently-conjugated secondary antibody. Nuclei were stained with DAPI. Abbreviations: le: luminal epithelium, ge: glandular epithelium, s: stroma, a: secretory alveolus, t: tubular epithelium. Scale bar for panels A-D and E-P: 50 and 10 μm, respectively.

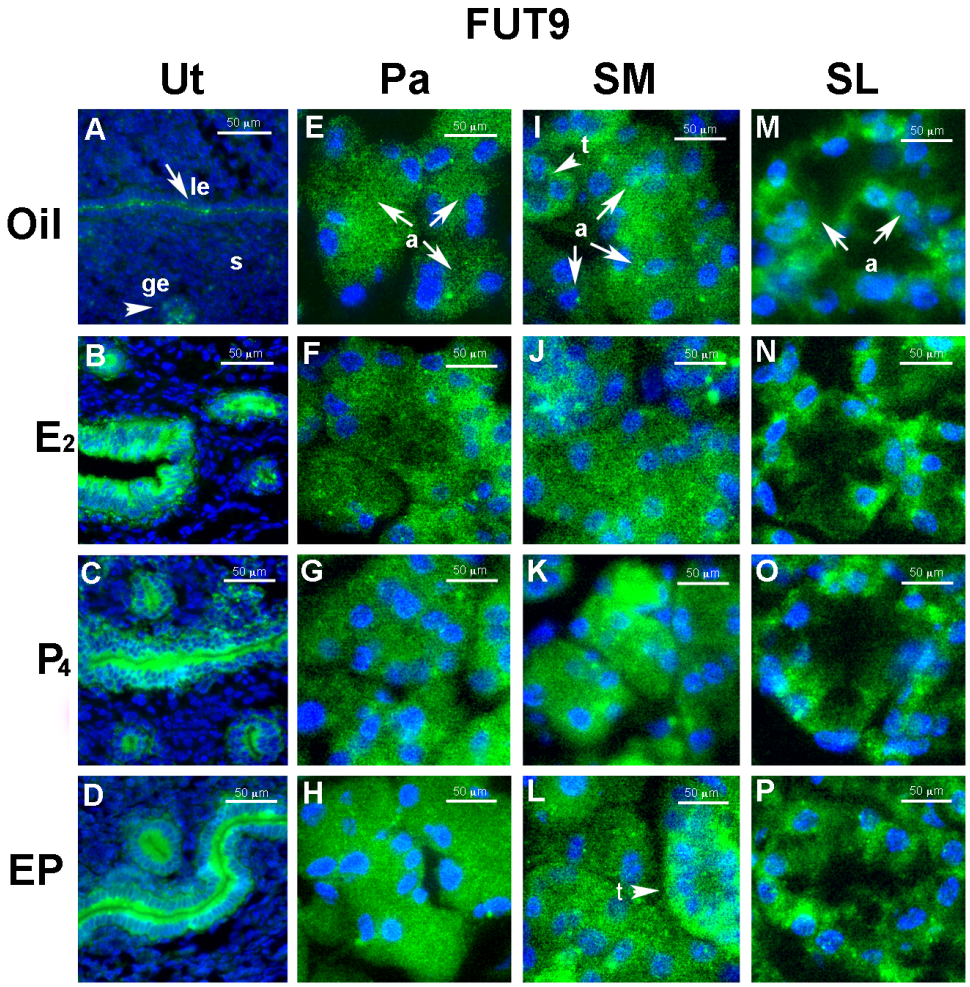


Fig. 2.10. Estrogen and progesterone upregulate Fut9 protein in uterine epithelial cells. Tissue sections of mouse uterus and major salivary glands from animals treated with estrogen (E_2), progesterone (P_4), both hormones (EP), or vehicle only (Oil) were probed with a polyclonal antibody against Fut9 and visualized with a fluorescently-conjugated secondary antibody. Nuclei were stained with DAPI. Abbreviations: le: luminal epithelium, ge: glandular epithelium, s: stroma, a: secretory alveolus, t: tubular epithelium. Scale bar for panels A-D and E-P: 50 and 100 µm, respectively.

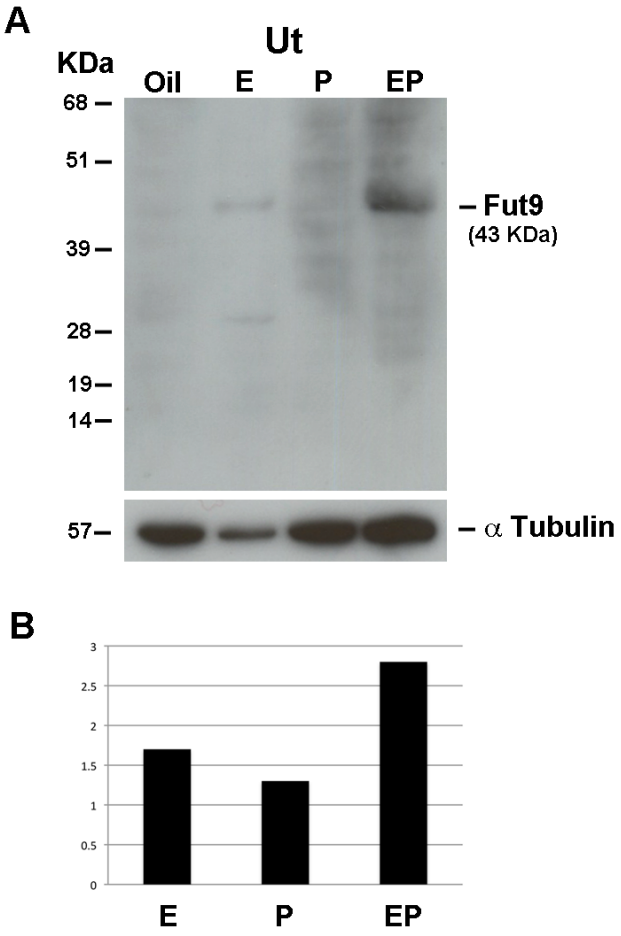


Fig. 2.11. Ovarian hormones upregulate Fut9 protein in uterine tissue lysates.
A. Uterine lysates from mice treated with estrogen (E), progesterone (P), both hormones (EP) or vehicle control (Oil) were electrophoretically separated, transferred to nitrocellulose and immunoblotted with a polyclonal antibody against Fut9. A single band of the appropriate molecular weight (43 KDa) was observed. The blot was stripped and reprobed with an anti-a tubulin antibody to demonstrate total protein loading.
B. The density of each band in Panel A was measured using ImageJ software. The a tubulin signal was used to normalize the total protein content in all lanes. Then, the fold change of Fut9 protein levels in experimental as compared to control groups was calculated.

Table 3.1. Lectins used in the lectin array analysis

Abbreviation	Full name/source species	Specificity	Ref.
AAA	Anguilla anguilla lectin (Fresh water eel)	α-Fuc	1
AAL	Aurentia lectin	α-Fuc	1
ACA	Amaranthus caudatus lectin (Amaranthin)	GalNAc or clusters of Galβ1,3GlcNAc	2
AOL	Aspergillus Oryzae	α-1,6 Fucose (core fucosylation)	Matsumura K et al., 2007
APA	Abrus precatorius lectin (Jequirity bean)	Galβ-1,3 GalNAc (T antigen)	1
ASA	Allium sativum lectin (Garlic)	High mannose chains (Man9-GlcNAc2..)	1
Blackbean	Blackbean lectin (Black bean)	GalNAc	1
BPA	Bauhinia purpurea lectin	Galβ-1,3/1,4	3
CA	Colchicum autumnale lectin (Meadow saffron)	Terminal Galβ	2
Cholera	Vibrio Cholerae	Pentasaccharide (Gal, GalNAc, Glc, Lactose, and sialic acid) on GM1	Merritt EA et al., 1992
ConA	Canavalia ensiformis (Jack bean)	Branched and terminla mannose, terminal GlcNAc	1
CPA	Cicer arietinum lectin (Chick pea)	Complex	2
CVN	Nostoc ellipsosporum lectin (Blue algae)	Oligosaccharides Man8 (Man8GlcNAc2) and Man9 (Man9GlcNAc2)	Botos et al., 2002
DBA	Dolichos biflorus lectin (Horse gram)	GalNAcα	2
DSA	Datura stramonium lectin	GlcNAcβ-1,4GlcNAc oligomers (CFG annotation, actually LacNAc binder)	1
ECA	Erythrina cristagalli lectin (Coral tree)	Terminal Galβ1-4GlcNAc	2
Gal3	Galectin 3	Galβ1-3GlcNAc, lactosamine (LacNAc), polylactosamine	1
Gal9	Galectin 9	Galβ1-3GalNAc, LacNAc, $(LacNAc)_2$	Nagae et al., 2006
GRFT	Griffithsia sp. Lectin (Red algae)	Oligosaccharides Man9 (Man9GlcNAc2)	Ziolkowska et al., 2006
GNA	Galanthus nivalis agglutinin (Snowdrop)	Terminal α-1,3 Man	1
GS-I	Griffonia simplicifolia lectin	α-Gal	Wearne et al., 2006
GS-II	Griffonia simplicifolia lectin	Terminal GlcNAc	Wearne et al., 2006
HHL	Hippeastrum Hybrid	α-1,3/1,6 Man	Wearne et al., 2006
HPA	Helix pomatia agglutinin (Roman snail, edible snail)	Terminal α-GalNAc	2
IAA	Iberis amara lectin	GalNAc	3
Jacalin	Jackfruit lectin (Artocarpus heterophyllus)	α-GalNAc (O-linkage)	1
LBA	Phaseolus lunatus lectin (Lima bean)	GalNAcα-1,3 (Fuca-1,2) Gal	2
LCH	Lens culinaris lectin (Lentil)	Complex (Man/GlcNAc core with Fucα1-6)	Wearne et al., 2006
LEA	Lycopersicon esculentum (Tomato) lectin	β-1,4 GlcNAc oligomers (prefer trimer and tetramer)	3
LPA	Limulus polyphemus lectin (Horseshoe crab)	α-Sia	1
MAA	Maackia amurensis lectin	α-2,3 Sia	2
MAL-I	Maackia amurensis lectin I	Galβ1-4GlcNAc oligomers	3
MAL-II	Maackia amurensis lectin II	α-2,3 Sia-Galβ1-4GlcNAc	3
MOA	Marasmium oreades agglutinin (Mushroom)	Ga1α1,3 Gal	1
MPA	Maulura pomifera lectin (Osage orange)	α-Gal/GalNAc	1
NPA	Narcissus pseudo-narcissus lectin (Daffodil)	Terminal and internal Man	2
PAA	Perseau americana lectin (Avocado)	unknown	N/A

PHA-E	Phaseolus vulgaris lectin (Red kidney bean)	Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ	1
PHA-L	Phaseolus vulgaris lectin (Red kidney bean)	Galβ1-4GlcNAcβ1-2Man	2
PNA	Arachis hypogaea lectin (Peanut)	Terminal Galβ	1
PSA	Pisum sativum	Man	1
PSL	Polyporus squamosus lectin (Mushroom)	α-2,6 Sia	1
PTA	Psophocarpus tetragonolobus lectin (Winged bean)	Gal	3
PTL-I	Psophocarpus Tetragonolobus Lectin I	α-GalNAc, GalNAcα-1,3 (Fucα-1,2) Galβ-1,3/1,4 GlcNAcβ	1
PTL-II	Psophocarpus Tetragonolobus Lectin II	Fucα-1,2 Galβ-1,4 GlcNAcβ1,3 oligomer	1
Ricin B	Ricin B	Gal/GalNAc	Wearne et al., 2006
RPA	Robinia pseudoacacia lectin (Black locust)	Complex	N/A
SJA	Sophora japonica lectin (Pagoda tree)	GalNAc	2
SNA	Sambucus nigra lectin (Elcerberry bark)	α-2,6 Sia	2
SNA-II	Sambucus nigra lectin II (Elderberry bark)	GalNAcα 1,2/1,3/1,6 Gal	1
SVN	Scytonema varium lectin (Cyanobacterium)	high mannose oligosaccharides with terminal Manα1-2Man	Adams et al., 2004
TJA-I	Trichosanthes japonica I	Sialylated and sulfated LacNAc	Yamashita K et al., 1992
TJA-II	Trichosanthes japonica II	Fuc α−1,2-Galβ-1,3/4-GlcNAc, GalNAcβ-1,4-Galβ-1	Yamashita K et al., 1992
TKA	Trichosanthes kirilowii lectin (China gourd)	β-Gal, LacNAc	2
TL	Tulipa sp. Lectin (Tulip)	Biantennary complex N-glycan	2
UDA	Urtica dioica lectin (Stinging nettle)	GlcNAcβ-1,4-GlcNAc oligomers	1
UEA-I	Ulex europaeus lectin I (Gorse, Furze)	α-Fuc	Wearne et al., 2006
UEA-II	Ulex europaeus lectin II (Gorse, Furze)	GlcNAcβ, Fucα1-2Galβ1-4GlcNAc	Wearne et al., 2006
VFA	Vicia fava lectin (Fava bean)	Man	1
VGA	Vicia graminea lectin	Galβ-1,3 GalNAc clusters (O-linkage)	2
VRA	Vigna radiata lectin (Mung bean)	α-Gal	2
VVA	Vicia villosa lectin (Hairy vetch)	GalNAc	2
VVA (Man)	Vicia villosa lectin (Hairy vetch, mannose specific)	Man	3
WFA	Wisteria floribunda lectin (Japanese wisteria)	GalNAc	2
WGA	Triticum vulgaris lectin (Wheat germ)	β-GlcNAc, sialic acid, GalNAc	1

1. the Consortium for Functional Glycomics
2. EY laboratories, Inc., Lectin Specificility chart
3. Product information from EY or Vector laboratories
N/A: not available

Table 3.2. Antibodies and their carbohydrate epitopes used in the study

Antibody	Carbohydrate epitope
Blood group H	Fucα1-2Galβ
Le^x	Galβ1-4(Fucα1-3)-GlcNAcβ
Le^y	(Fucα1-2)Galβ1-4(Fucα1-3)GlcNAcβ
HECA452	NeuAcα2-3Galβ1-4(Fucα1-3)-GlcNAcβ

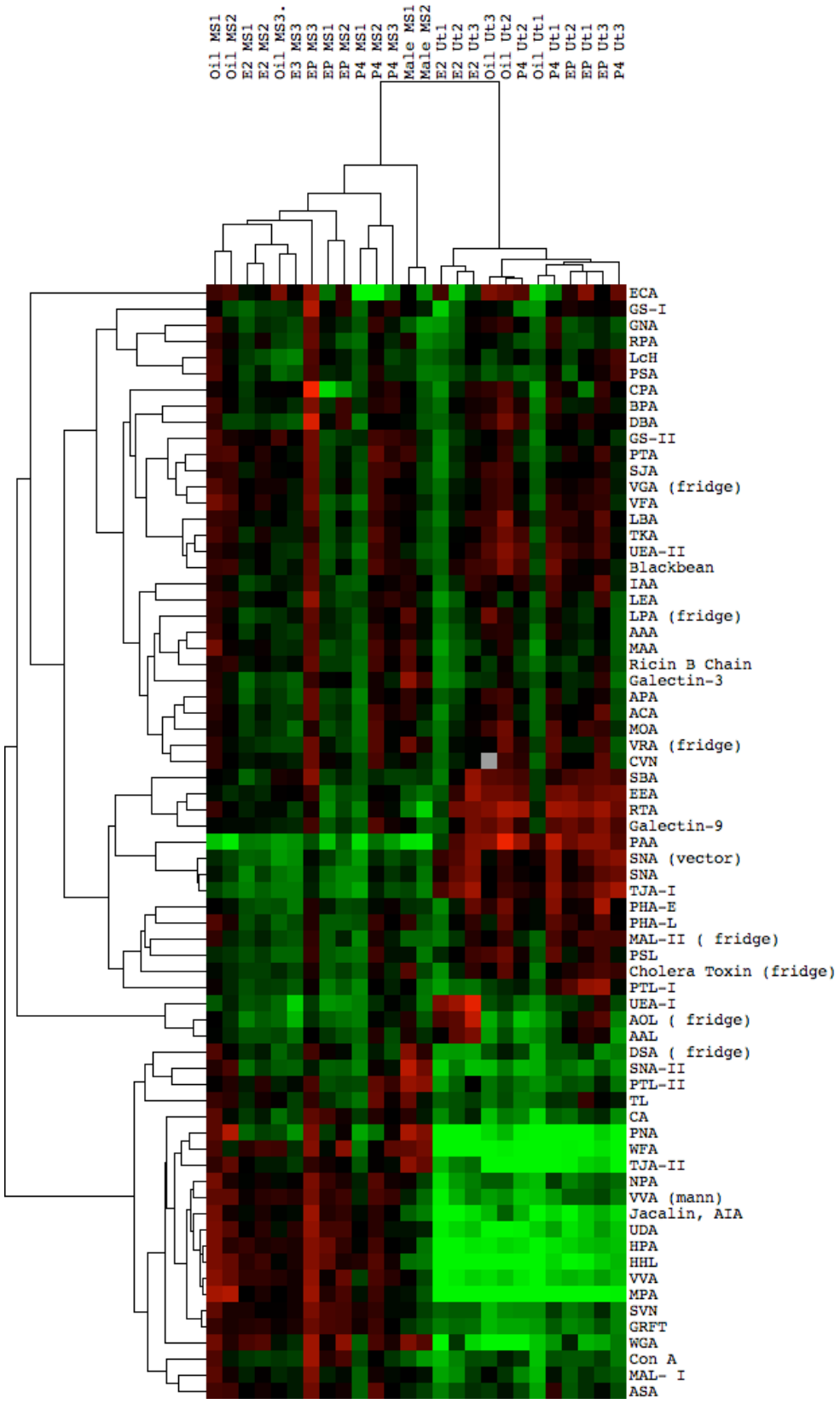

Oil MS1
Oil MS2
E2 MS1
E2 MS2
Oil MS3.
E3 MS3
EP MS3
EP MS1
EP MS2
P4 MS1
P4 MS2
P4 MS3
Male MS1
Male MS2
E2 Ut1
E2 Ut2
E2 Ut3
Oil Ut3
Oil Ut2
P4 Ut2
Oil Ut1
P4 Ut1
EP Ut2
EP Ut1
EP Ut3
P4 Ut3
ECA
GS-I
GNA
RPA
LcH
PSA
CPA
BPA
DBA
GS-II
PTA
SJA
VGA (fridge)
VFA
LBA
TKA
UEA-II
Blackbean
IAA
LEA
LPA (fridge)
AAA
MAA
Ricin B Chain
Galectin-3
APA
ACA
MOA
VRA (fridge)
CVN
SBA
EEA
RTA
Galectin-9
PAA
SNA (vector)
SNA
TJA-I
PHA-E
PHA-L
MAL-II (fridge)
PSL
Cholera Toxin (fridge)
PTL-I
UEA-I
AOL (fridge)
AAL
DSA (fridge)
SNA-II
PTL-II
TL
CA
PNA
WFA
TJA-II
NPA
VVA (mann)
Jacalin, AIA
UDA
HPA
HHL
VVA
MPA
SVN
GRFT
WGA
Con A
MAL- I
ASA

Fig. 3.1. The mouse uterus and major salivary glands present distinct cell-surface glycan profiles. Heat map with dendrograms of hierarchical clustering for mouse uterine and major salivary glands in different treatment groups. The heat map was generated by Cluster 3.0 and Java TreeView with the Pearson correlation as the distance metric for the arrays with average linkage analysis. The lectins were grouped by an uncentered Pearson correlation. Signals from individual samples were compared to a pooled reference comprised of all samples. Black, red, and green indicate that the sample signal was equal to, greater than, or less than the reference signal, respectively. A brighter red designates a stronger hybridization signal. Duplicate arrays using Cy3- and Cy5-labeled samples were averaged before the analysis. Abbreviations: Oil, vehicle only; E2, estrogen treatment; P4, progesterone treatment; EP, estrogen + progesterone treatment; MS: major salivary glands; UT: uterus. Numbers 1,2,3 indicate individual mice used in the experiment.

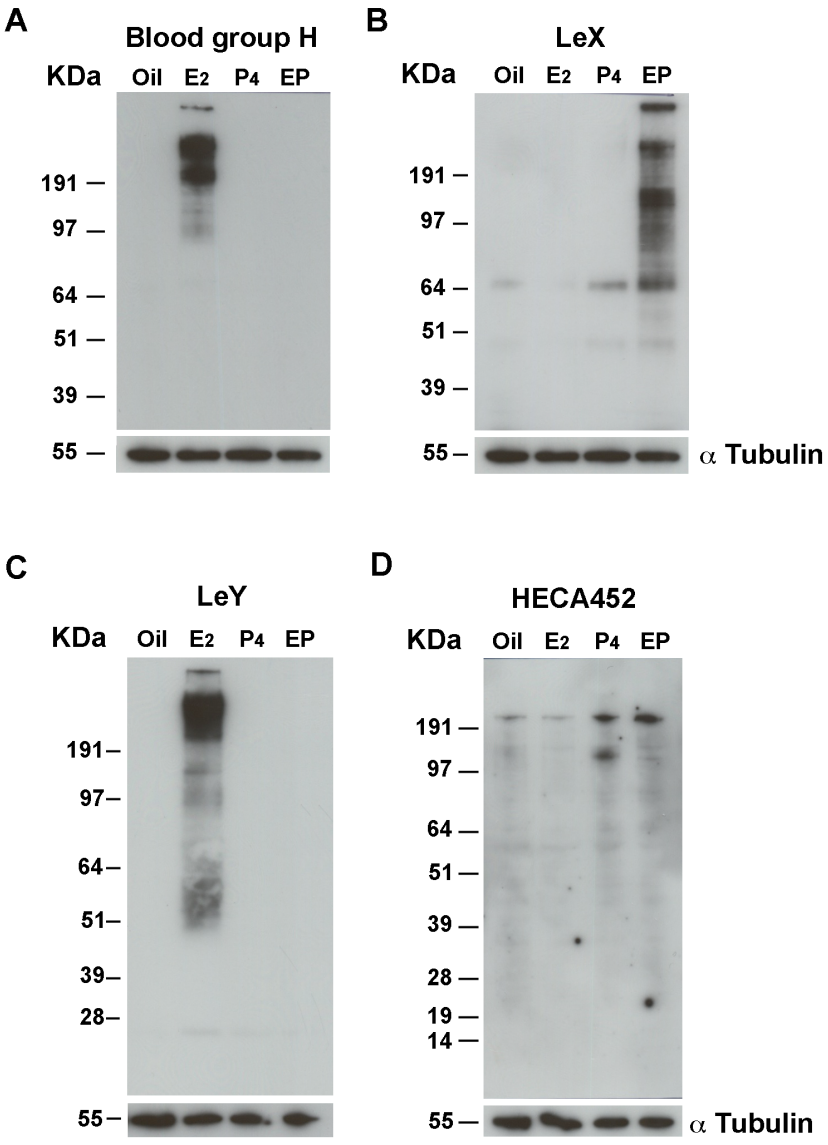


Fig. 3.2. The highly-specialized oligosaccharide epitopes blood group antigen H, Le^x, Le^y, and HECA-452 were differentially regulated in the mouse uterus by estrogen and progesterone. Uterine lysates from mice treated with estrogen (E_2), progesterone (P_4), both hormones (EP) or vehicle control (Oil) were electrophoretically separated, transferred to nitrocellulose and immunoblotted with monoclonal antibodies against the blood group antigen H (Panel A), Le^x (Panel B), Le^y (Panel C), or with the HECA-452 antibody (Panel D). The blots were stripped and reprobed with an anti-α tubulin antibody to demonstrate total protein loading.

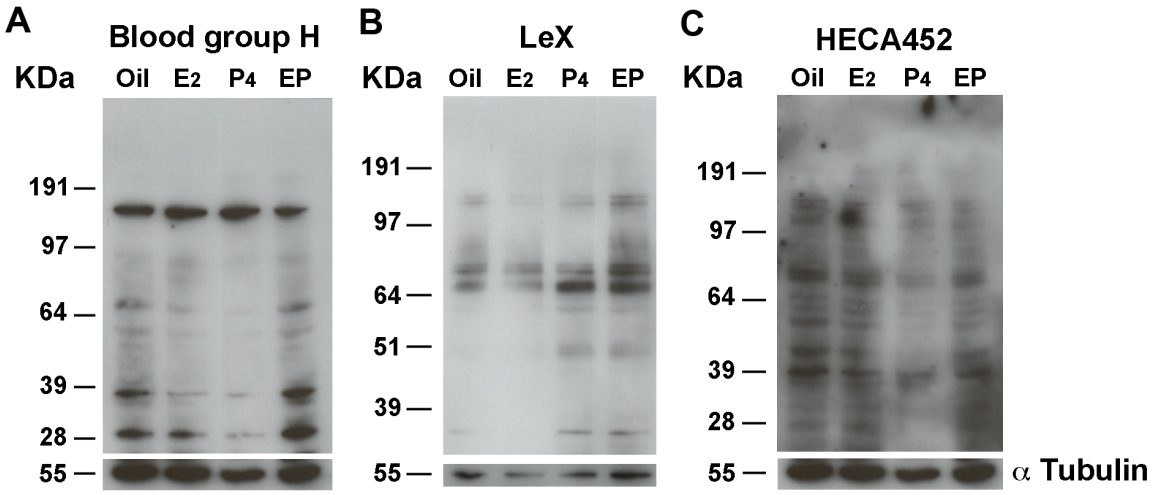


Fig. 3.3. Mouse parotid glands expressed the fucosylated oligosaccharide structures blood group antigen H, Le^x, and HECA-452.

Parotid gland lysates from mice treated with estrogen (E_2), progesterone (P_4), both hormones (EP) or vehicle control (Oil) were electrophoretically separated, transferred to nitrocellulose and immunoblotted with monoclonal antibodies against the blood group antigen H (Panel A), Le^x (Panel B), or with the HECA-452 antibody (Panel C). The blots were stripped and reprobed with an anti-α tubulin antibody to demonstrate total protein loading.

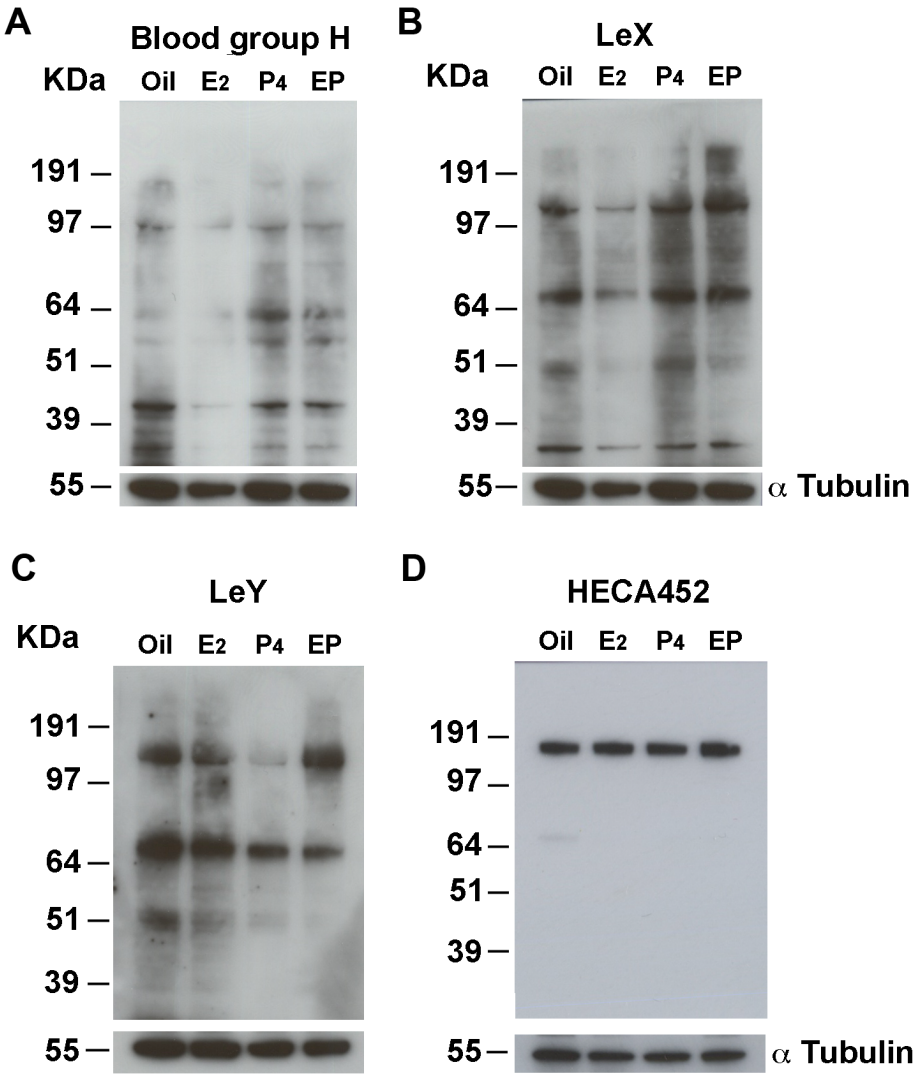


Fig. 3.4. The functionally-interesting terminal carbohydrate structures blood group antigen H, Le^x, Le^y, and HECA-452 are presented by mouse sublingual salivary glands. Sublingual gland lysates from mice treated with estrogen (E_2), progesterone (P_4), both hormones (EP) or vehicle control (Oil) were electrophoretically separated, transferred to nitrocellulose and immunoblotted with monoclonal antibodies against the blood group antigen H (Panel A), Le^x (Panel B), Le^y (Panel C), or with the HECA-452 antibody (Panel D). The blots were stripped and reprobed with an anti-α tubulin antibody to demonstrate total protein loading.

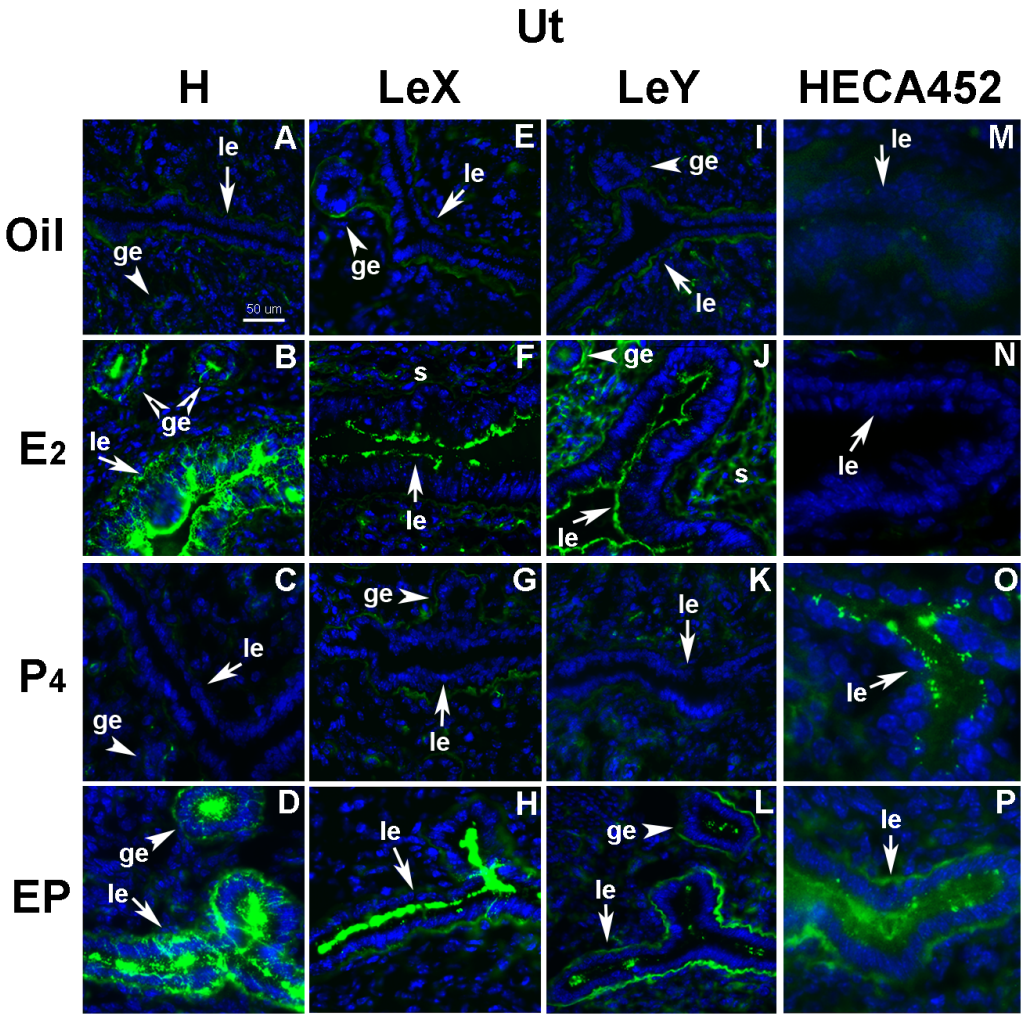


Fig. 3.5. The carbohydrate motifs blood group antigen H, Le^x, Le^y, and HECA-452 demonstrate distinct expression patterns and are differentially regulated by estrogen and progesterone in the mouse uterus.
Tissue sections of mouse uterus from animals treated with estrogen (E_2), progesterone (P_4), both hormones (EP), or vehicle only (Oil) were probed with monoclonal antibodies against blood group H, Le^x, and Le^y, and with the HECA-452 antibody, and visualized with a fluorescently-conjugated secondary antibody. Nuclei were stained with DAPI. Abbreviations: le: luminal epithelium, ge: glandular epithelium, s: stroma. Scale bar: 100 μm.

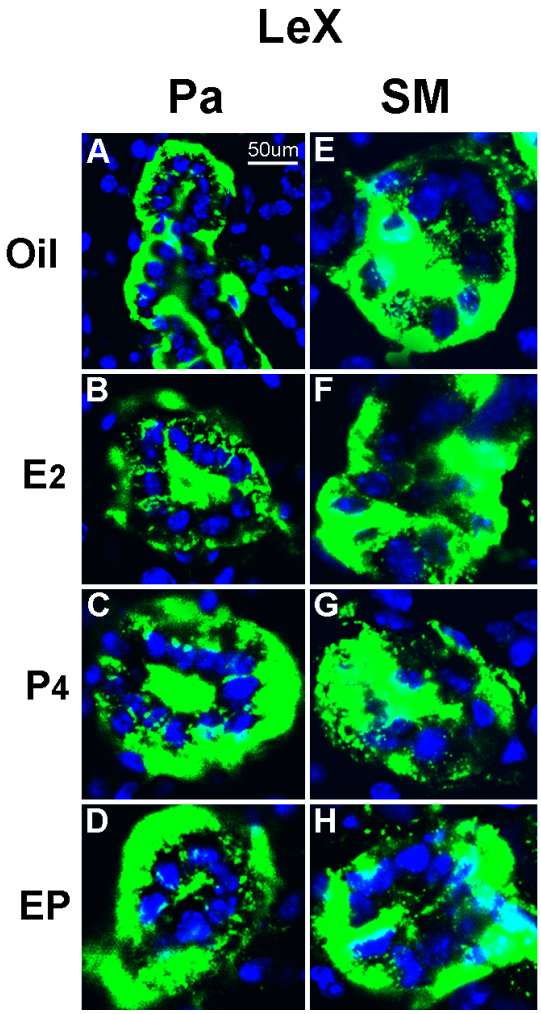


Fig. 3.6. The mouse parotid and submandibular glands express the Le^x epitope. Tissue sections of parotid (Pa) and submandibular (SM) glands from animals treated with estrogen (E_2), progesterone (P_4), both hormones (EP), or vehicle only (Oil) were probed with the monoclonal antibody HECA-452, and visualized with a fluorescently-conjugated secondary antibody. Nuclei were stained with DAPI. Scale bar: 100 μm.

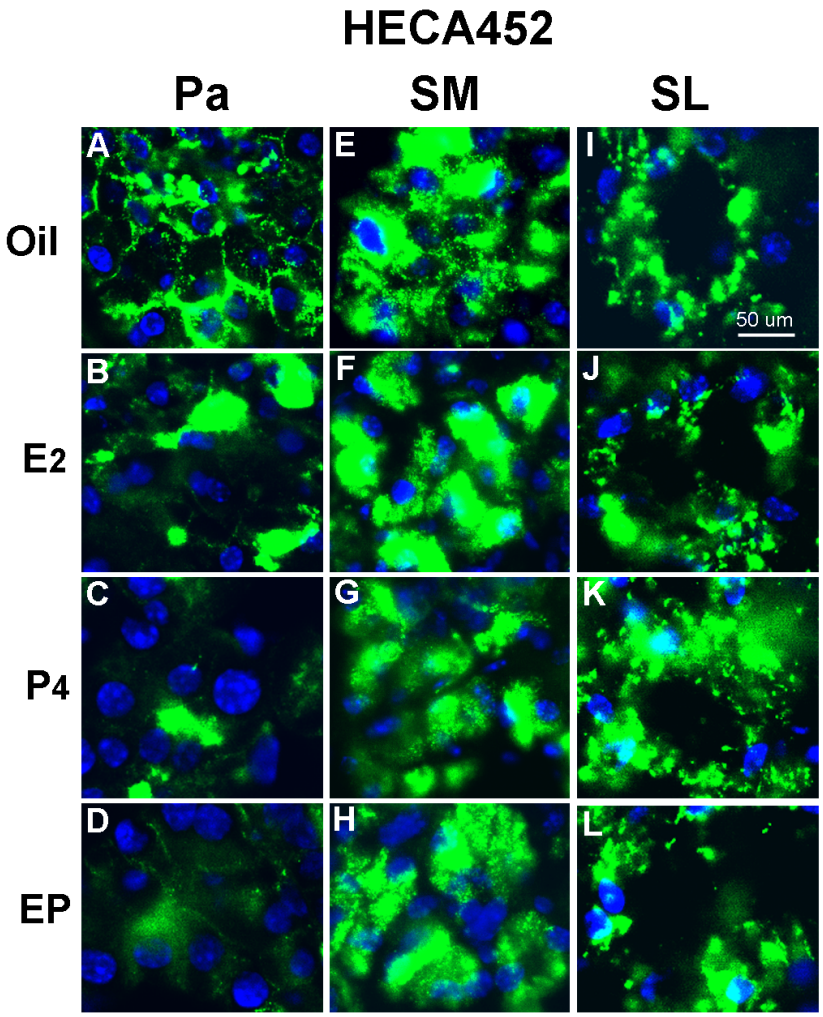


Fig. 3.7. The L-selectin ligands that are recognized by the HECA-452 antibody are present in mouse major salivary glands.
Tissue sections of parotid (Pa), submandibular (SM), and sublingual (SL) glands from animals treated with estrogen (E_2), progesterone (P_4), both hormones (EP), or vehicle only (Oil) were probed with the monoclonal antibody HECA-452, and visualized with a fluorescently-conjugated secondary antibody. Nuclei were stained with DAPI. Scale bar: 100 μm.

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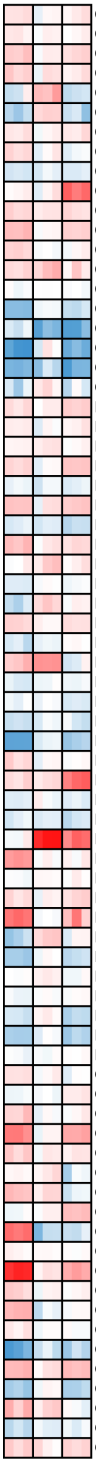
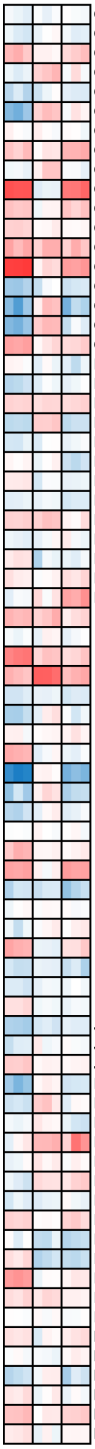
Supplemental table 1. The list of uterine glycosylation-related genes that are differentially regulated by estrogen and progesterone.

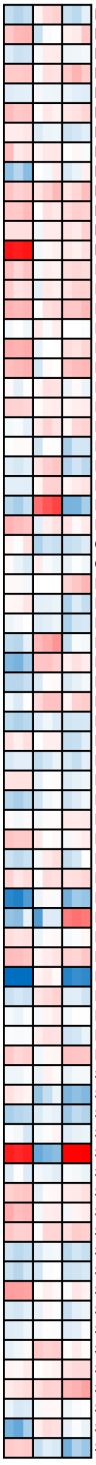
E2	P4	EP	Symbol	Title	E	P	EP	Probe	Accession
			1110067D22Rik	Mus musculus RIKEN cDNA 1110067D22 gene	1.5		-1.4	1424318_at	BY740213
			Acvr1	activin A receptor, type 1	1.4			1448460_at	NM_007394
			Acvr2b	activin receptor IIB	-1.8	-1.5		1419140_at	NM_007397
			Acvrl1	activin A receptor, type II-like 1			1.4	NM_009612_at	NM_009612
			Aldoa	aldolase A, fructose-bisphosphate	1.8			NM_007438_s_at	NM_007438
			Alg13	asparagine-linked glycosylation 13 homolog (S. cerevisiae)	1.5			AA215144_at	AA215144
			Angpt1	angiopoietin 1	-1.6		-1.3	NM_009640_at	NM_009640
			Areg	amphiregulin		1.8		NM_009704_at	NM_009704
			Arfgef1	ADP-ribosylation factor guanine nucleotide-exchange factor 1(brefeldin A-inhibited)	1.4			1415711_at	BC025221
			Arsa	arylsulfatase A	1.5		1.6	AK004540_x_at	AK004540
			Asah1	N-acylsphingosine amidohydrolase 1	1.7			NM_019734_at	NM_019734
			Atox1	ATX1 (antioxidant protein 1) homolog 1 (yeast)	1.4			1415760_s_at	BC027632
			Atp6v0d1	ATPase, H+ transporting, lysosomal V0 subunit D1	1.6	1.4	1.5	1415671_at	NM_013477
			Atrnl1	attractin like 1	-1.4		-1.5	1419922_s_at	NM_181415
			Aup1	ancient ubiquitous protein 1	1.4		1.4	1415742_at	BC016485
			B3galnt1	UDP-GalNAc:betaGlcNAc beta 1,3-galactosaminyltransferase, polypeptide 1	-1.5			BC003835_at	BC003835
			B3galt5	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	2.5			1428397_at	NM_033149
			B3gnt5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	1.3	1.9		NM_054052_at	NM_054052
			B3gnt8	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8	1.7		1.6	1425128_at	NM_146184
			B4galnt1	beta-1,4-N-acetyl-galactosaminyl transferase 1	2.3		2.0	NM_008080_s_at	NM_008080
			B4galt1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	1.5	1.7		J03880_at	J03880
			B4galt3	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 3	1.6			BC013619_at	BC013619
			B4galt6	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6	-1.4			NM_019737_at	NM_019737
			BC031181	cDNA sequence BC031181			-1.5	1415752_at	BC016084
			Bgn	biglycan		1.8	2.1	BC005452_s_at	BC005452
			Bmp1	bone morphogenetic protein 1		1.5	2.0	AK004995_s_at	AK004995
			Bmp2	bone morphogenetic protein 2	-3.7		-3.3	1423635_at	NM_007553
			Bmp4	bone morphogenetic protein 4	-1.4		-1.4	NM_007554_at	NM_007554
			Bmp7	bone morphogenetic protein 7	-2.0		-2.1	NM_007557_at	NM_007557
			Bmp8a	bone morphogenetic protein 8a	2.4		1.7	NM_007558_at	NM_007558
			Bsg	basigin	1.5			BC010270_s_at	BC010270
			Btg2	B-cell translocation gene 2, anti-proliferative	2.4		1.3	M64292_s_at	M64292
			C1galt1	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1	1.6			NM_052993_at	NM_052993
			C1galt1c1	C1GALT1-specific chaperone 1			1.8	1416655_at	AY159320
			Canx	calnexin		1.3	1.3	1422845_at	NM_007597
			Ccl11	chemokine (C-C motif) ligand 11	6.6		-1.6	NM_011330_at	NM_011330
			Ccl12	chemokine (C-C motif) ligand 12	1.8			NM_011331_s_at	NM_011331
			Ccl17	chemokine (C-C motif) ligand 17	-1.5		-1.4	1419413_at	NM_011332
			Ccl21b	chemokine (C-C motif) ligand 21B	-1.4	-1.5		NM_011335_s_at	NM_011335
			Ccl5	chemokine (C-C motif) ligand 5	-2.2			NM_013653_s_at	NM_013653
			Ccl7	chemokine (C-C motif) ligand 7	2.0			NM_013654_at	NM_013654
			Ccl8	chemokine (C-C motif) ligand 8	2.5		3.0	NM_021443_s_at	NM_021443
			Ccl9	chemokine (C-C motif) ligand 9	2.2		1.6	NM_011338_at	NM_011338
			Ccr1l1	chemokine (C-C motif) receptor 1-like 1	-1.6			NM_007718_at	NM_007718
			Ccr2	chemokine (C-C motif) receptor 2	2.1			NM_009915_s_at	NM_009915
			Ccr4	chemokine (C-C motif) receptor 4	-1.4			NM_009916_s_at	NM_009916
			Ccr5	chemokine (C-C motif) receptor 5	1.5		1.5	D83648_s_at	D83648
			Cd209a	CD209a antigen	-2.2			AF373408_at	AF373408
			Cd209c	CD209c antigen	-1.4			1421562_at	AF373410
			Cd33	CD33 antigen	-1.4			NM_021293_at	NM_021293
			Cd34	CD34 antigen		1.8	1.4	BC006607_s_at	BC006607
			Cd44	CD44 antigen	-1.4			BC005676_at	BC005676
			Cd69	CD69 antigen	-1.4			L23638_at	L23638
			Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	-2.9			AK002232_at	AK002232
			Cd83	CD83 antigen	-3.9		-4.3	NM_009856_at	NM_009856
			Cd93	CD93 antigen	-1.7	1.9		1419589_at	AF081789
			Chodl	chondrolectin			1.7	AF311699_at	AF311699
			Chrd	chordin	-1.3			NM_009893_at	NM_009893
			Chst12	carbohydrate sulfotransferase 12			1.3	NM_021528_s_at	NM_021528
			Chst2	carbohydrate sulfotransferase 2	-1.4			AB011451_s_at	AB011451
			Chst5	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 5	1.7			NM_019950_at	NM_019950
			Chst7	carbohydrate (N-acetylglucosamino) sulfotransferase 7	1.8			NM_021715_at	NM_021715
			Cic	capicua homolog (Drosophila)	-1.3		-1.3	1415746_at	AF363690
			Clec10a	C-type lectin domain family 10, member A			1.8	S36676_at	S36676
			Clec11a	C-type lectin domain family 11, member a	2.8		5.8	AB009245_at	AB009245
			Clec14a	C-type lectin domain family 14, member a		1.8		1419467_at	NM_025809
			Clec1a	C-type lectin domain family 1, member a	-1.7			1456318_at	NM_175526
			Clec2d	C-type lectin domain family 2, member d	1.5			1419477_at	AF350409
			Clec4a2	C-type lectin domain family 4, member a2	1.4		1.5	AJ133533_s_at	AJ133533
			Clec4a3	C-type lectin domain family 4, member a3	3.1		2.5	1429954_at	NM_153197
			Clec4b1	C-type lectin domain family 4, member b1			1.4	1425407_s_at	NM_027218
			Clec4e	C-type lectin domain family 4, member e	-1.6			BC003218_s_at	BC003218
			Clec7a	C-type lectin domain family 7, member a	1.6			AF262985_at	AF262985



Cmas	cytidine monophospho-N-acetylneuraminic acid synthetase	1.6		1.4	AJ006215_at	AJ006215
Cog4	component of oligomeric golgi complex 4	1.4			1416993_at	NM_133973
Cog6	component of oligomeric golgi complex 6	1.7		1.5	1426216_at	NM_026225
Cog8	component of oligomeric golgi complex 8	1.4			1426821_at	NM_139229
Col14a1	collagen, type XIV, alpha 1		2.5		AJ131395_at	AJ131395
Colec12	collectin sub-family member 12	-1.9		-1.4	1419693_at	AB038519
Copg	coatomer protein complex, subunit gamma	1.5		1.4	1415670_at	BC024686
Cox18	COX18 cytochrome c oxidase assembly homolog (S. cerevisiae)	1.8			1415710_at	BM123013
Cpsf7	cleavage and polyadenylation specific factor 7	-1.5			1415730_at	BG972112
Csf1	colony stimulating factor 1 (macrophage)			4.8	NM_007778_s_at	NM_007778
Csf1r	colony stimulating factor 1 receptor	1.5		1.5	NM_007779_at	NM_007779
Ctsa	cathepsin A	2.5		1.8	NM_008906_at	NM_008906
Cx3cl1	chemokine (C-X3-C motif) ligand 1	1.3			NM_009142_s_at	NM_009142
Cx3cr1	chemokine (C-X3-C) receptor 1	-1.4			BC012653_at	BC012653
Cxcl11	chemokine (C-X-C motif) ligand 11	-1.5			NM_019494_at	NM_019494
Cxcl12	chemokine (C-X-C motif) ligand 12	-2.3		-1.6	NM_021704_s_at	NM_021704
Cxcl14	chemokine (C-X-C motif) ligand 14		-2.4	-2.6	1418457_at	NM_019568
Cxcl15	chemokine (C-X-C motif) ligand 15	-2.7		-2.2	NM_011339_at	NM_011339
Cxcr4	chemokine (C-X-C motif) receptor 4	-2.5	-1.7	-2.5	D87747_at	D87747
Cxcr6	chemokine (C-X-C motif) receptor 6	-1.7			NM_030712_at	NM_030712
Dad1	defender against cell death 1	1.3		1.5	U83628_at	U83628
Dcdc2b	doublecortin domain containing 2b	1.4		1.4	1415763_a_at	BE853401
Dctn5	dynactin 5		1.4		1415748_a_at	NM_021608
Ddost	dolichyl-di-phosphooligosaccharide-protein glycotransferase	1.5		1.6	D89063_at	D89063
Dgcr2	DiGeorge syndrome critical region gene 2			-1.3	1421810_at	D78641
Dhrs1	dehydrogenase/reductase (SDR family) member 1	1.5		1.6	1415677_at	NM_026819
Dlg1	discs, large homolog 1 (Drosophila)	-1.3		-1.7	1415691_at	BQ176806
Dpagt1	dolichyl-phosphate (UDP-N-acetylglucosamine) acetylglucosaminephosphotransferase 1 (GlcNAc-1-P transferase)	1.6		1.4	1448549_a_at	X65603
Egfr	epidermal growth factor receptor		1.4		NM_007912_at	NM_007912
Eif5	eukaryotic translation initiation factor 5	-1.5		-1.4	1415723_at	BQ176989
Emcn	endomucin	-1.8	1.7		NM_016885_at	NM_016885
Erbb3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	1.4			L47240_at	L47240
Extl3	exostoses (multiple)-like 3	-1.4			NM_018788_at	NM_018788
Fbln5	fibulin 5	1.9	3.4	-1.5	NM_011812_at	NM_011812
Fgf10	fibroblast growth factor 10	-1.6			NM_008002_at	NM_008002
Fgf12	fibroblast growth factor 12			-1.6	AK011712_at	AK011712
Fgf18	fibroblast growth factor 18	-1.4			NM_008005_at	NM_008005
Fgfr2	fibroblast growth factor receptor 2	-2.5			1420847_a_at	NM_010207
Fibp	fibroblast growth factor (acidic) intracellular binding protein	1.4			NM_021438_at	NM_021438
Figf	c-fos induced growth factor	1.7	1.5	6.1	NM_010216_s_at	NM_010216
Fktn	fukutin	-1.4			1451853_at	NM_139309
Fryl	furry homolog-like (Drosophila)			-1.4	1415758_at	BM118442
Fst	follistatin		12.7	4.4	NM_008046_at	NM_008046
Fut2	fucosyltransferase 2	1.7			NM_018876_s_at	NM_018876
Fut4	fucosyltransferase 4	-1.3			1450834_at	NM_010242
Fut8	fucosyltransferase 8			1.5	BC010666_at	BC010666
Fut9	fucosyltransferase 9	6.0		3.8	AB015426_at	AB015426
Fzd1	frizzled homolog 1 (Drosophila)	-1.8	1.8		NM_021457_at	NM_021457
Fzd2	frizzled homolog 2 (Drosophila)	-2.1		-1.6	NM_020510_at	NM_020510
Fzd4	frizzled homolog 4 (Drosophila)	-1.5			1449416_at	#N/A
Fzd5	frizzled homolog 5 (Drosophila)	-1.3			1422937_at	NM_022721
Fzd6	frizzled homolog 6 (Drosophila)	-1.4		-1.7	1417301_at	NM_008056
Fzd7	frizzled homolog 7 (Drosophila)	-2.0		-1.8	1450044_at	NM_008057
Fzd9	frizzled homolog 9 (Drosophila)	-1.6			AF088850_s_at	AF088850
G3bp2	GTPase activating protein (SH3 domain) binding protein 2	1.6			1415697_at	BG069656
Gaa	glucosidase, alpha, acid			1.4	U49351_at	U49351
Galc	galactosylceramidase	1.4			NM_008079_at	NM_008079
Gale	galactose-4-epimerase, UDP	3.2		2.2	AK009083_at	AK009083
Galns	galactosamine (N-acetyl)-6-sulfate sulfatase	1.5			NM_016722_at	NM_016722
Galnt1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1		1.5		U73820_at	U73820
Galnt10	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10	2.0			1418195_at	NM_134189
Galnt11	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 11			2.2	1424748_at	NM_144908
Galnt12	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12	4.5	-1.7	-1.4	1437760_at	NM_172693
Galnt13	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 13	1.7			1457045_at	NM_173030
Galnt3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3	7.1		2.0	1417588_at	NM_015736
Galnt4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 4	1.3			1423637_at	NM_015737
Galnt7	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 7	2.8	-1.4		BC007484_at	BC007484
Galntl1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 1	1.3			1416760_at	AB045325
Galntl2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	-3.5		-1.8	1429236_at	AK019470
Gba	glucosidase, beta, acid	2.3		2.1	NM_008094_at	NM_008094
Gcnt2	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	-2.0		-1.9	AB037597_s_at	AB037597
Gcnt3	glucosaminyl (N-acetyl) transferase 3, mucin type			-1.6	AA762195_s_at	AA762195
Gdf1	growth differentiation factor 1	-1.6			NM_008107_s_at	NM_008107
Gdf15	growth differentiation factor 15	1.4		1.4	NM_011819_s_at	NM_011819



Gdf2	growth differentiation factor 2	-1.4			AF156890_at	AF156890
Gdnf	glial cell line derived neurotrophic factor	-1.5			1419080_at	NM_010275
Gfpt1	glutamine fructose-6-phosphate transaminase 1	1.8		1.4	BC010516_at	BC010516
Gfpt2	glutamine fructose-6-phosphate transaminase 2	-1.4			NM_013529_at	NM_013529
Gfra2	glial cell line derived neurotrophic factor family receptor alpha 2	-1.5			1423007_a_at	NM_008115
Ggta1	glycoprotein galactosyltransferase alpha 1, 3	-2.1	2.3		AF297615_at	AF297615
Gla	galactosidase, alpha	-1.4			NM_013463_at	NM_013463
Glb1	galactosidase, beta 1	2.0		2.5	NM_009752_at	NM_009752
Glce	glucuronyl C5-epimerase	-1.3			AF325532_at	AF325532
Gmds	GDP-mannose 4, 6-dehydratase	7.9		5.4	AI852418_at	AI852418
Gmppa	GDP-mannose pyrophosphorylase A	1.8		1.7	BC008116_s_at	BC008116
Gnb1	guanine nucleotide binding protein (G protein), beta 1	1.6			NM_008142_s_at	NM_008142
Gnpda1	glucosamine-6-phosphate deaminase 1	1.5	1.5	1.4	NM_011937_s_at	NM_011937
Golm1	golgi membrane protein 1	9.6	1.7	3.3	1415698_at	BC011152
Gpc1	glypican 1	-1.8			AF185613_s_at	AF185613
Gpc3	glypican 3	-1.9			AF185614_at	AF185614
Gpc6	glypican 6	-2.9	2.3		AF105268_at	AF105268
Gusb	glucuronidase, beta	3.1	1.4	1.7	NM_010368_s_at	NM_010368
Hbegf	heparin-binding EGF-like growth factor			-1.3	NM_010415_at	NM_010415
Hdac5	histone deacetylase 5	-1.6			1415743_at	NM_010412
Hexa	hexosaminidase A	1.4			NM_010421_at	NM_010421
Hgf	hepatocyte growth factor	-1.8	1.8	-1.6	1425379_at	D10213
Hk1	hexokinase 1	-1.5		-1.3	1437974_a_at	NM_010438
Hmgn2	high mobility group nucleosomal binding domain 2			-1.6	NM_016957_s_at	NM_016957
Hnrnpul2	heterogeneous nuclear ribonucleoprotein U-like 2	1.4			1415765_at	BI080136
Hp1bp3	heterochromatin protein 1, binding protein 3			-1.4	1415751_at	BC020024
Hs3st1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	1.7	1.9		NM_010474_s_at	NM_010474
Hs3st3b1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	-1.3			NM_018805_at	NM_018805
Hs6st1	heparan sulfate 6-O-sulfotransferase 1			-1.5	NM_015818_s_at	NM_015818
Hspg2	perlecan (heparan sulfate proteoglycan 2)			1.5	M77174_s_at	M77174
Hyal1	hyaluronoglucosaminidase 1	-1.7			AF422176_at	AF422176
Icam2	intercellular adhesion molecule 2	1.8	1.7		NM_010494_at	NM_010494
Ifnar1	interferon (alpha and beta) receptor 1	-1.4			NM_010508_s_at	NM_010508
Ifngr2	interferon gamma receptor 2	3.7	1.5	1.4	NM_008338_s_at	NM_008338
Igf1	insulin-like growth factor 1	1.8	4.0	1.9	NM_010512_s_at	NM_010512
Igf1r	insulin-like growth factor I receptor	-1.4			1426565_at	AF056187
Igf2	insulin-like growth factor 2	-1.7			NM_010514_s_at	NM_010514
Igfbp1	insulin-like growth factor binding protein 1			1.4	1418918_at	NM_008341
Igfbp2	insulin-like growth factor binding protein 2	2.2			NM_008342_s_at	NM_008342
Igfbp3	insulin-like growth factor binding protein 3	-6.9		-3.2	1423062_at	NM_008343
Ihh	Indian hedgehog	-2.4	1.5	-2.2	NM_010544_s_at	NM_010544
Il11ra1	interleukin 11 receptor, alpha chain 1	-1.5			NM_010549_at	NM_010549
Il16	interleukin 16	1.7			NM_010551_at	NM_010551
Il1a	interleukin 1 alpha	8.3		2.1	X01450_at	X01450
Il1f6	interleukin 1 family, member 6	1.7		1.8	AF206697_at	AF206697
Il1r1	interleukin 1 receptor, type I	-2.1			1448950_at	NM_008362
Il25	interleukin 25	-1.3			1420740_at	NM_080729
Il2rb	interleukin 2 receptor, beta chain	-1.4			NM_008368_at	NM_008368
Il4ra	interleukin 4 receptor, alpha	2.8		1.6	NM_010557_at	NM_010557
Il5ra	interleukin 5 receptor, alpha	-1.7		-1.9	NM_008370_at	NM_008370
Il6ra	interleukin 6 receptor, alpha	-1.4			NM_010559_at	NM_010559
Itch	Mus musculus itchy, E3 ubiquitin protein ligase	1.5			1415769_at	NM_008395
Jag1	Jagged1 - Long trans	-2.4		-1.4	1434070_at	NM_013822
Jag2	jagged 2	-1.4			1426431_at	NM_010588
Junb	Jun-B oncogene	1.4			U20735_at	U20735
Khk	ketohexokinase	-2.1			BC013464_at	BC013464
Kit	kit oncogene	-1.6	1.5		NM_021099_at	NM_021099
Klra18	killer cell lectin-like receptor, subfamily A, member 18			-1.7	1426127_x_at	AF288377
Klra3	killer cell lectin-like receptor, subfamily A, member 3			1.7	1425436_x_at	U10305
Klrc2	killer cell lectin-like receptor subfamily C, member 2	-1.5			AF106010_at	AF106010
Klrg1	killer cell lectin-like receptor subfamily G, member 1			1.3	1420788_at	NM_016970
L1cam	L1 cell adhesion molecule	-1.3			NM_008478_at	NM_008478
Lamp2	lysosomal-associated membrane protein 2	1.5		1.3	NM_010685_at	NM_010685
Lgals12	lectin, galactose binding, soluble 12			-2.7	AF223223_at	AF223223
Lgals3	lectin, galactoside-binding, soluble, 3		-1.6	-1.7	1426808_at	NM_010705.1
Lgals9	lectin, galactose binding, soluble 9	3.8		1.4	1455002_at	U55061
Lipa	lysosomal acid lipase A	1.4	1.4		NM_021460_s_at	NM_021460
Ly75	lymphocyte antigen 75			-2.2	U19271_at	U19271
Mad2l1bp	MAD2L1 binding protein	1.3		1.4	1415720_s_at	AK011037
Madcam1	mucosal vascular addressin cell adhesion molecule 1	-2.2			1425253_a_at	NM_013591
Magi2	membrane associated guanylate kinase, WW and PDZ domain containing 2	-1.3			1420532_at	NM_015823
Man2a1	mannosidase 2, alpha 1	1.3			X61172_at	X61172
Man2b1	mannosidase 2, alpha B1	2.1		1.7	U29947_at	U29947
Manba	mannosidase, beta A, lysosomal	1.4			NM_027288_s_at	NM_027288



Mdk	midkine	-1.7	1.8		NM_010784_s_at	NM_010784
Met	met proto-oncogene	1.9			NM_008591_s_at	NM_008591
Mfng	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	-1.5			1416992_at	NM_008595
Mgat2	mannoside acetylglucosaminyltransferase 2	2.0		2.0	BC010583_at	BC010583
Mgat3	mannoside acetylglucosaminyltransferase 3	-1.5		-1.3	NM_010795_x_at	NM_010795
Mgat4a	mannoside acetylglucosaminyltransferase 4, isoenzyme A	1.3			AI155765_at	AI155765
Mgat4b	mannoside acetylglucosaminyltransferase 4, isoenzyme B			-1.5	1424720_at	AB053218
Mgat4c	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme C (putative)	1.6			1421178_at	AK033482
Mgl2	macrophage galactose N-acetyl-galactosamine specific lectin 2	-2.8			1438467_at	AY103461
Mpi	mannose phosphate isomerase	1.4		1.5	AF244360_at	AF244360
Mrc1	mannose receptor, C type 1	1.9		1.6	Z11974_at	Z11974
Mrpl43	mitochondrial ribosomal protein L43	1.5		1.6	1415681_at	NM_053164
Muc1	mucin 1, transmembrane	14.8		1.4	NM_013605_s_at	NM_013605
Naga	N-acetyl galactosaminidase, alpha	2.0		1.4	AF079458_at	AF079458
Naglu	alpha-N-acetylglucosaminidase (Sanfilippo disease IIIB)	1.5		1.5	1417706_at	NM_013792
Nans	N-acetylneuraminic acid synthase (sialic acid synthase)	2.1		1.9	AB041263_at	AB041263
Ncan	neurocan	-1.5			1423604_at	X84727
Ncstn	Nicastrin	2.7		1.9	1418570_at	NM021607
Neu1	neuraminidase 1	2.2		2.1	BC004666_at	BC004666
Neu2	neuraminidase 2	-1.4			NM_015750_at	NM_015750
Nmt1	N-myristoyltransferase 1	1.4			1415683_at	BC016526
Nog	noggin			1.4	NM_008711_at	NM_008711
Notch1	Notch gene homolog 1 (Drosophila)			-1.7	1418634_at	NM_008714
Notch3	Notch gene homolog 3 (Drosophila)	-1.4	1.6	-1.6	1421965_s_at	NM_008716
Notch4	Notch gene homolog 4 (Drosophila)	-1.3			1449146_at	NM_010929
Npl	N-acetylneuraminate pyruvate lyase	-2.1	6.3	-2.8	AK002734_at	AK002734
Nubp1	nucleotide binding protein 1	2.0			NM_011955_at	NM_011955
Odz4	odd Oz/ten-m homolog 4 (Drosophila)		-1.6	-1.4	1451888_a_at	NM_011858
Opn4	opsin 4 (melanopsin)	-1.4			NM_013887_at	NM_013887
Papss2	3'-phosphoadenosine 5'-phosphosulfate synthase 2			1.5	NM_011864_at	NM_011864
Pdgfa	platelet derived growth factor, alpha	1.3		-1.6	1449187_at	NM_008808
Pdgfc	platelet-derived growth factor, C polypeptide			-1.6	NM_019971_at	NM_019971
Pdgfd	platelet-derived growth factor, D polypeptide	-2.4			1426319_at	AF335583
Pdgfra	platelet derived growth factor receptor, alpha polypeptide	-2.2	2.4	1.6	NM_011058_at	NM_011058
Pdgfrb	platelet derived growth factor receptor, beta polypeptide	-1.5			NM_008809_s_at	NM_008809
Pecam1	platelet/endothelial cell adhesion molecule 1		1.6		BC008519_at	BC008519
Pfdn5	prefoldin 5			-1.7	1415736_at	NM_020031
Pgm2	phosphoglucomutase 2			-1.3	BC008527_s_at	BC008527
Phactr1	phosphatase and actin regulator 1	-1.3		-1.4	AI848515_at	AI848515
Pigq	phosphatidylinositol glycan anchor biosynthesis, class Q	1.4			NM_011822_s_at	NM_011822
Pkd1	polycystic kidney disease 1 homolog	-1.7			1460210_at	U70209
Pmm1	phosphomannomutase 1			1.4	AF007267_at	AF007267
Pmm2	phosphomannomutase 2	1.7			NM_016881_at	NM_016881
Podxl2	podocalyxin-like 2	-1.4			1455622_at	NM_176973
Pofut2	protein O-fucosyltransferase 2			1.4	BC018194_at	BC018194
Prdm1	PR domain containing 1, with ZNF domain	-4.6		-2.3	NM_007548_s_at	NM_007548
Prol1	proline rich, lacrimal 1			6.4	NM_008644_s_at	NM_008644
Psen1	presenilin 1	1.6			1421853_at	NM_008943
Psmb5	proteasome (prosome, macropain) subunit, beta type 5	1.8		1.4	1415676_a_at	NM_011186
Ptch1	patched homolog 1	-8.3		-5.8	1428853_at	NM_008957
Ptch2	patched homolog 2	-1.4			NM_008958_at	NM_008958
Rbpj	recombination signal binding protein for immunoglobulin kappa J region	-1.4			1448957_at	NM_009035
Rfk	riboflavin kinase		1.5	-1.4	1415737_at	NM_019437
Rpn2	ribophorin II	1.8		1.9	NM_019642_at	NM_019642
Sap30l	SAP30-like	-1.3			1415718_at	BI152383
Sc4mol	sterol-C4-methyl oxidase-like			-2.2	NM_025436_at	NM_025436
Sdc2	syndecan 2	-1.9		-1.9	U00674_at	U00674
Sdc4	syndecan 4	-1.4		-1.4	NM_011521_s_at	NM_011521
Sftpd	surfactant associated protein D	12.9	-1.9	15.5	NM_009160_s_at	NM_009160
Siglece	sialic acid binding Ig-like lectin E	-1.4			NM_031181_at	NM_031181
Slc35a2	solute carrier family 35 (UDP-galactose transporter), member A2	2.1		1.7	AB027147_s_at	AB027147
Slc35a3	solute carrier family 35 (UDP-N-acetylglucosamine (UDP-GlcNAc) transporter), member 3	1.9			BC024110_at	BC024110
Slc35b1	solute carrier family 35, member B1	1.5		1.4	D87990_s_at	D87990
Smc3	structural maintenace of chromosomes 3	-1.5		-1.4	NM_007790_x_at	NM_007790
Smo	smoothened homolog (Drosophila)	-1.4		-1.3	AF089721_at	AF089721
Smpd1	sphingomyelin phosphodiesterase 1, acid lysosomal	2.9			1448621_a_at	NM_011421
Snapin	SNAP-associated protein	-1.3			1415756_a_at	BB667523
Snrnp27	small nuclear ribonucleoprotein 27 (U4/U6.U5)	-1.3		-1.3	1415714_a_at	BC027564
Srgn	serglycin		1.6		X16133_at	X16133
Ssr3	signal sequence receptor, gamma			1.4	1415700_a_at	BC011111
St3gal1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1		1.4	2.0	X73523_at	X73523
St3gal3	ST3 beta-galactoside alpha-2,3-sialyltransferase 3	-1.4			BC006710_at	BC006710
St3gal6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	-2.4	1.4		NM_018784_at	NM_018784
St6gal1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	2.0		-1.8	BB768706_s_at	BB768706

St6galnac2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	2.0		-1.5	X93999_at	X93999
St6galnac5	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5	-1.9	-1.5	-1.7	AB028840_at	AB028840
St8sia2	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2	-1.7			X83562_s_at	X83562
St8sia4	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4		1.8		X86000_s_at	X86000
Sulf1	sulfatase 1	-1.6	1.8		1438200_at	NM_172294
Sulf2	sulfatase 2	3.7	1.5	2.2	1430388_a_at	NM_028072
Tcea1	transcription elongation factor A (SII) 1			-1.5	NM_011541_at	NM_011541
Tgfb1	transforming growth factor, beta 1			1.4	NM_011577_at	NM_011577
Tgfb2	transforming growth factor, beta 2		2.0	8.5	NM_009367_s_at	NM_009367
Tgfbr1	transforming growth factor, beta receptor I			-1.5	1420895_at	NM_009370
Tgfbr2	transforming growth factor, beta receptor II			1.5	1425444_a_at	NM_009371
Tgfbr3	transforming growth factor, beta receptor III	-1.6		1.7	AF039601_at	AF039601
Tmem165	transmembrane protein 165		1.4		1415741_at	NM_011626
Tmsb10	thymosin, beta 10	1.8			NM_025284_at	NM_025284
Tpst1	protein-tyrosine sulfotransferase 1	-1.6		-1.4	1421733_a_at	NM_013837
Tsnax	translin-associated factor X			-1.9	NM_016909_at	NM_016909
Txndc12	thioredoxin domain containing 12 (endoplasmic reticulum)	1.6			1415738_at	NM_025334
Uap1	UDP-N-acetylglucosamine pyrophosphorylase 1	2.0		1.8	BC016406_at	BC016406
Ube2r2	ubiquitin-conjugating enzyme E2R 2	1.6			1415768_a_at	AV054417
Ugdh	UDP-glucose dehydrogenase	2.3			AF061017_s_at	AF061017
Ugp2	UDP-glucose pyrophosphorylase 2			-1.6	AF424698_at	AF424698
Ugt1a6a	UDP glucuronosyltransferase 1 family, polypeptide A6A	4.0		2.0	U16818_s_at	U16818
Vcan	versican			-2.1	1421694_a_at	NM_019389
Vegfb	vascular endothelial growth factor B	-2.1		-1.5	NM_011697_s_at	NM_011697
Vta1	Vps20-associated 1 homolog (S. cerevisiae)	1.3			1415722_a_at	AK003213
Wnt1	wingless-related MMTV integration site 1	-1.4			BC005449_at	BC005449
Wnt10b	wingless related MMTV integration site 10b	-1.3			1426091_a_at	NM_011718
Wnt11	wingless-related MMTV integration site 11	-1.7			NM_009519_at	NM_009519
Wnt16	wingless-related MMTV integration site 16	-1.3			1422941_at	NM_053116
Wnt4	wingless-related MMTV integration site 4		-1.8	-2.5	NM_009523_at	NM_009523
Wnt5a	wingless-related MMTV integration site 5A	-1.4			NM_009524_at	NM_009524
Wnt5b	wingless-related MMTV integration site 5B	-1.4			1439373_x_at	NM_009525
Wnt6	wingless-related MMTV integration site 6	-2.0		-1.7	NM_009526_s_at	NM_009526
Wnt7a	wingless-related MMTV integration site 7A	-1.3		-2.0	NM_009527_at	NM_009527
Xylt2	xylosyltransferase II	-1.3			AJ291751_at	AJ291751
Ybx1	Y box protein 1	1.5			NM_011732_s_at	NM_011732
Ywhab	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	1.5			NM_018753_at	NM_018753
Zc3h11a	zinc finger CCCH type containing 11A	-1.5		-1.4	1415764_at	AK003350
Zranb1	zinc finger, RAN-binding domain containing 1	-1.6			1415712_at	AJ250693

Supplemental tabel 2. Ingenuity canonical pathways that were affected by estrogen in mouse uterus.

Ingenuity Canonical Pathways	-log(p-value)	Ratio	Molecules
Bile Acid Biosynthesis	0.31	0.01	LIPA
Stilbene, Coumarine and Lignin Biosynthesis	0.54	0.01	GBA (includes EG:2629)
Selenoamino Acid Metabolism	0.42	0.01	PAPSS1 (includes EG:9061)
Glutamate Receptor Signaling	0.23	0.01	GNB1
Cyanoamino Acid Metabolism	0.55	0.02	GBA (includes EG:2629)
Retinol Metabolism	0.28	0.02	UGT1A6
Phototransduction Pathway	0.24	0.02	GNB1
Sulfur Metabolism	0.57	0.02	PAPSS1 (includes EG:9061)
Hepatic Cholestasis	0.44	0.02	IL1A, IL1F6, IL1R1
FXR/RXR Activation	0.39	0.02	IL1A, IL1F6
Pentose and Glucuronate Interconversions	1.37	0.02	UGDH, UGT1A6, GUSB
Glycerolipid Metabolism	0.77	0.02	NAGA, LIPA, GLB1
PTEN Signaling	0.38	0.02	PDGFRA, PDGFRB
VEGF Signaling	0.42	0.02	VEGFB (includes EG:7423), FIGF
IL-8 Signaling	0.54	0.02	GNB1, VEGFB (includes EG:7423), ANGPT1, FIGF
Androgen and Estrogen Metabolism	0.86	0.02	UGT1A6, ARSA, SULF2
Nitric Oxide Signaling in the Cardiovascular System	0.48	0.02	VEGFB (includes EG:7423), FIGF
Acute Phase Response Signaling	0.55	0.02	IL1A, IL6R, IL1F6, IL1R1
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	0.33	0.02	YWHAB
Xenobiotic Metabolism Signaling	0.69	0.02	LIPA, HS6ST1, IL1A, UGT1A6, CHST7, HS3ST1
LPS/IL-1 Mediated Inhibition of RXR Function	0.71	0.03	HS6ST1, CHST7, SLC35A2, HS3ST1, IL1R1
Antigen Presentation Pathway	0.35	0.03	CD74
PDGF Signaling	0.52	0.03	PDGFRA, PDGFRB
Amyotrophic Lateral Sclerosis Signaling	0.75	0.03	VEGFB (includes EG:7423), IGF1, FIGF
Nucleotide Sugars Metabolism	2.06	0.03	UGDH, GALE
p38 MAPK Signaling	0.71	0.03	IL1A, IL1F6, IL1R1
Ephrin Receptor Signaling	1.26	0.03	GNB1, VEGFB (includes EG:7423), ANGPT1, SDC2, CXCL12, FIGF
Neuregulin Signaling	0.79	0.03	DCN, ERBB3, PSEN1
Pentose Phosphate Pathway	1.63	0.03	GPI, PGM1, ALDOA
Interferon Signaling	0.43	0.03	IFNGR2
Cysteine Metabolism	1.39	0.04	HS6ST1, CHST7, HS3ST1
Glycolysis/Gluconeogenesis	1.73	0.04	HK1, GPI, GALK1, PGM1, ALDOA
Glutamate Metabolism	1.66	0.04	GFPT1, NAGK, GFPT2
Amyloid Processing	0.70	0.04	NCSTN, PSEN1
Inositol Metabolism	0.69	0.04	ALDOA
Starch and Sucrose Metabolism	3.98	0.04	HK1, GPI, UGDH, UGT1A6, GBA (includes EG:2629), GALK1, PGM1, GUSB
Clatrin-mediated Endocytosis	1.90	0.04	MET, FGF16, VEGFB (includes EG:7423), FGF10, IGF1, FGF18, FIGF
IL-6 Signaling	1.26	0.04	IL1A, IL6R, IL1F6, IL1R1
Macropinocytosis	1.06	0.04	MET, MRC1, HGF
TREM1 Signaling	1.18	0.04	CCL2, CCL7, CD83
TGF-β Signaling	1.43	0.05	BMP4, BMP2, BMP7, ACVR2B
NF-κB Signaling	2.10	0.05	IL1A, BMP4, BMP2, PDGFRA, IL1F6, IL1R1, PDGFRB
Chondroitin Sulfate Biosynthesis	1.44	0.05	HS6ST1, CHST7, HS3ST1
BMP signaling pathway	1.49	0.05	BMP4, BMP2, BMP7, BMP8A
PPAR Signaling	1.86	0.05	IL1A, PDGFRA, IL1F6, IL1R1, PDGFRB VEGFB (includes EG:7423), BMP4, BMP2,
			PTCH1, WNT6, FZD1, PTCH2, GNB1, WNT7A,
			IGF1, SDC2, CXCL12, FZD6, FIGF, BMP7, FZD2
Axonal Guidance Signaling	5.86	0.05	BMP8A, WNT11, WNT5A, FZD7
Galactose Metabolism	3.92	0.05	NAGA, HK1, GLB1, GALE, GALK1, PGM1

IGF-1 Signaling	1.88	0.05	IGF1, YWHAB, IGF1R, IGFBP3, IGFBP2
IL-10 Signaling	1.76	0.06	IL1A, CCR5, IL1F6, IL1R1
Fructose and Mannose Metabolism	5.83	0.06	HK1, GMPPA, GMDS, KHK, GALK1, ALDOA, PMM2, MPI
LXR/RXR Activation	2.31	0.06	IL1A, CCL2, CCL7, IL1F6, IL1R1
Wnt/β-catenin Signaling	3.59	0.06	WNT7A, CD44, FZD6, WNT6, FZD1, ACVR2B, FZD2, WNT11, FZD7, WNT5A
Sonic Hedgehog Signaling	1.09	0.06	PTCH1, PTCH2
Chemokine Signaling	2.23	0.07	CCR5, CCL2, CCL7, CXCL12, CCL11
FGF Signaling	3.53	0.08	MET, FGF16, FGF10, FGF18, FGFR1, HGF, FGFR2
Aminosugars Metabolism	8.13	0.10	HK1, GNPDA1 (includes EG:10007), GFPT1, NAGK, CMAS, GALK1, HEXA, UAP1, NANS, GFPT2
Sphingolipid Metabolism	6.38	0.10	NEU1, NAGA, GALC, GBA (includes EG:2629), GLB1, SMPD1, ARSA, SULF2, ASAH1
Glycosaminoglycan Degradation	5.32	0.10	GLB1, HEXA, NAGLU, GALNS, SULF2, GUSB
Glycosphingolipid Biosynthesis - Neolactoseries	6.99	0.10	FUT2, GCNT2, ST3GAL6, FUT9, B3GNT5, ST3GAL5, GGTA1
N-Glycan Biosynthesis	8.31	0.12	MGAT3, ST6GAL1, ALG3, RPN2, MGAT4B, DPAGT1, DDOST, RPN1, MGAT2, B4GALT3
Glycosphingolipid Biosynthesis - Ganglioseries	7.27	0.12	B3GALT4, B4GALNT1, GLB1, ST3GAL1, ST6GALNAC2, HEXA, ST3GAL5
Glycosphingolipid Biosynthesis - Globoseries	4.54	0.12	NAGA, ST3GAL1, FUT2, FUT9, HEXA
Keratan Sulfate Biosynthesis	3.97	0.12	HS6ST1, CHST7, ST3GAL1, HS3ST1, ST3GAL3, B4GALT3
Hepatic Fibrosis / Hepatic Stellate Cell Activation	10.80	0.13	CCR5, IL1A, VEGFB (includes EG:7423), FGFR1, IL6R, IFNGR2, FGFR2, IL1R1, MET, IGF1, CCL2, HGF, IGFBP3, PDGFRA, IGF1R, FIGF, PDGFRB
Notch Signaling	4.51	0.15	DLL1, JAG2, MFNG, NCSTN, JAG1, PSEN1
N-Glycan Degradation	4.27	0.17	NEU1, GLB1, HEXA, MAN2B1, MANBA
Glycosphingolipid Biosynthesis - Lactoseries	5.71	0.18	ST3GAL1, FUT2, B3GNT5, ST3GAL3, GGTA1
O-Glycan Biosynthesis	8.00	0.19	GALNT7, ST3GAL1, GALNT3, GALNT4, GALNT12, GALNTL2, C1GALT1, GALNT10

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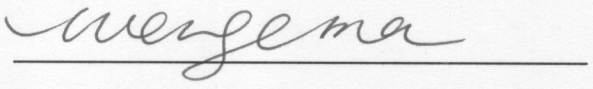

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