CERVICAL VIRAL INFECTION CAUSES PREMATURE CERVICAL RIPENING IN A MOUSE MODEL OF PREGNANCY

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ABSTRACT

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Preterm birth (PTB), or birth before 37 weeks of gestation, occurs in about 1 in every 10 pregnancies in the United States and is the leading cause of neonatal morbidity and mortality in the developed world. Furthermore, surviving preterm infants are at increased risk for brain, heart, breathing, metabolic, and immune function problems, with an estimated cost of $26 billion each year. Despite the health and financial implications of PTB, 50% of cases still have no attributed cause. Recently, cervical viral infection has been identified as a risk factor for PTB. The implication of viruses, specifically herpes simplex virus-2 (HSV-2), in pregnancy outcomes has not been well defined. Our approach was to utilize both in vivo and in vitro models to study cervical viral infection. Using a mouse model of pregnancy, we determined that intra-vaginal HSV-2 infection resulted in changes in both cervical structure and function. Viral infection caused stromal disorganization and increased risk for PTB associated with ascending bacterial infection. Using a cervical epithelial cell line, we discovered HSV-2 mediated changes in cell function by activating Src kinase, which affected hormone receptor stability and other down-stream effectors. We conclude that viral infection of the cervix leads to alterations in key pathways that are important to parturition and ultimately increase risk for PTB.

Dedicated to my Mom for instilling a sense of wonder and appreciation for how all things work and to my Dad for challenging me to be well-informed in what I discuss, and to think critically.

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KEY TO ABBREVIATIONS

PTB	Preterm Birth
Src	Src Kinase
4-MU	4-Methylumbelliferone
IVAG	intravaginal
sPTB	Spontaneous preterm birth
Src	Src kinase
ERα	Estrogen Receptor Alpha
i.p.	intraperitoneal
IVAG	intravaginal
SKI	Src Kinase inhibitor
PP1	Src Kinase inhibitor
C.I.	Cervical insufficiency
E.O.	External orifice
TFF1	Trefoil factor 1
SPINK5	Serine protease inhibitor Kazal type 5
GAG	Glycosaminoglycans
MMP	Matrix metalloproteinases
PG	Protaglandins
PGE2	Prostaglandin E2
HA	Hyaluronic acid or Hyaluronan
HAS	Hyaluronic acid synthase

MW	Molecular weight
KO	Knockout
17βHSD	17β-hydroxysteroid dehydrogenase type 2
20αHSD	20α-hydroxy-steroid dehydrogenase
5α-SRD	Steroid 5α-reductase
HPV	Human Papilloma Virus
HSV-2	Herpes Simplex Virus type 2
HSV-1	Herpes Simplex Virus type 1
MHV	Murid Herpes Virus
LPS	Lipopolysaccharide
IFN	Interferon
NK	Natural Killer Cells
pDC	plasmacytoid dendritic cells
PR	Pregesterone receptor
PgR	Progesterone receptor gene
P_4	Progesterone
ECM	Extracellular matrix
E_2	17 beta-estradiol
DME	Differentiated mucosal epithelial cells
NDE	Non-differentiated mucosal epithelial cells
SFK	Src Family Kinases
Gd	Gestational day
MHV	Murid Herpesvirus

CMV	Cytomegalovirus
HHV	Human Herpes virus
EBV	Epstein-Barr virus
Tyr	Tyrosine residue
DMSO	Dimethyl sulfoxide
ANOVA	Analysis of Variance
n.s.	Not significant

CHAPTER 1:

REVIEW OF THE LITERATURE

Significance of Preterm Birth

Preterm birth (PTB), or birth before 37 weeks of gestation, accounts for over 10% of all pregnancies worldwide and over 11% in the United States[1-4] and rates continue to rise, despite continued attempts at intervention [5] [6, 7]. Preterm delivery is the leading cause of neonatal morbidity and mortality resulting in nearly 1 million neonatal deaths per year [4, 8, 9]. These deaths stem from preterm infants' increased risk for neurologic and developmental disabilities [10], respiratory distress [11], dysregulated metabolism function [12], bone disease [13], heart defects [14], and immune function impairment [15]. Many of these problems arise because preterm infants have underdeveloped organs or organ systems [11]. Even when preterm infants survive past 5 years of age, they typically have poor long-term outcomes that include higher risk for vision and hearing impairment, chronic lung diseases, cardiovascular diseases, brain development and behavior problems such as increased hyperactivity, anxiety, and depression [16].

The cost associated with caring for a preterm infant is, understandably, quite high. The Institute of Medicine's Committee on Understanding Premature Birth and Assuring Healthy Outcomes found that the *minimum* cost burden of PTB on the US economy is predicted to be 26.2 billion dollars per year with 5.7 billion of that total coming from the disabilities associated with PTB. These estimates only consider four major debilitating conditions: cerebral palsy, mental retardation, vision impairment, and hearing loss; therefore, the estimates could be missing a significant amount of cost. Furthermore, the average cost per infant born preterm is about $51,600 [16], which can be an incredible financial burden to a new family, especially to an uninsured family.

Unfortunately, research to understand and prevent PTB is underfunded [17] and there is still much to learn about the causes and mechanism(s) of PTB [17].

Risk Factors for Preterm Birth

Bacterial infections and cervical insufficiency have been identified as important risk factors for PTB [18-20]. Up to 40% of preterm births are associated with intra-uterine infection [21] and, intra-vaginal, intra-uterine and/or systemic injection with bacteria or their conserved microbial motifs can induce PTB in animal models [22-28]. The majority of intra-uterine infections ascend from the vagina [29], and this is associated with cervical insufficiency [30]. Cervical insufficiency (CI) is characterized as mechanical weakness of the cervical tissue that affects the capacity of the cervix to support the fetus, and protect it from bacteria in the lower reproductive tract [31]. Unfortunately, diagnosing cervical insufficiency is challenging because of the limited criteria for use by clinicians [31]. While factors such as “short cervix” can be used as indicators of CI, by this time PTB is typically inevitable [32, 33].

The Function of the Cervix During Pregnancy

Throughout gestation the cervix functions as a structural and immune barrier for the developing fetus. During pregnancy, the cervical stroma is characterized by a dense extracellular matrix of proteins, including highly cross-linked fibrillar collagen, which provides mechanical strength to the tissue [34]. In humans and rodents, the cervical lumen is lined by epithelial cells that generate a cervical mucus plug, which

provides physical and immune protection against the vaginal flora [35-37]. At the end of gestation, the cervix must undergo a significant transformation into a pliable tissue to allow for the fetus to pass through the vaginal canal. The changes in the cervical structure, or "cervical remodeling", is a tightly regulated process that can be grouped into two different stages: cervical softening and cervical ripening [38].

Cervical Softening

In mid-gestation the cervix begins undergoing structural changes, appropriately named "cervical softening" by Dr. Word and colleagues, resulting in a progressively more compliant tissue but without loss of its mechanical competence [39]. In women softening begins at the end of the first trimester, and in mice is initiated by day 12 [39, 40]. Cervical softening is associated with decreased collagen cross-linking in the cervical stroma [41]. Collagen undergoes continuous turn-over, and during this time there is a down-regulation of genes, such as *Plod2* [41], that mediate the formation of the cross-linked collagen [42]. There are also changes in cervical epithelial cell function that could affect the characteristics of the cervical mucus barrier [38, 39]. For example, trefoil factor 1 (TFF1) and serine protease inhibitor Kazal type 5 (SPINK5) proteins incrementally increase in the epithelia during softening [39]. Trefoil factor 1 is an estrogen-regulated peptide, secreted by mucosal cells, that has a role in mucosal protection and epithelial repair [43]. The protease inhibitor encoded by SPINK5 also functions to maintain the epithelial barrier [44]. Increased expression of factors that protect the epithelial barrier is understandably important in preparation for vaginal

delivery. However, it is not known how changes in these factors or others affect the mucosal protection against bacteria.

Cervical Ripening

Over the last 2-3 days of gestation in the mouse, or in the last weeks of pregnancy in women, the cervix enters the phase of "cervical ripening" resulting in complete structural reorganization of the cervical stroma in preparation for labor. The stromal compartment becomes increasingly hydrated and the collagen fibers become larger in diameter and increasingly disorganized, a process initiated during cervical softening [41]. The changes in collagen structure are associated with increased collagen solubility, decreased cross-linking and down-regulation of collagen accessory proteins such as thrombospondin-2 and tenascin C [41]. Collagen degradation can also result from activation of matrix metalloproteinases in fibroblasts and activated immune cells [45].

Cervical ripening is also associated with changes in ECM components such as proteoglycans [46, 47]. Proteoglycan metabolism increases before the onset of labor in the term cervix and in preterm labor and elastin is low in women with cervical insufficiency [46, 47]. Interestingly, versican, dercorin, and heparan sulphate are proteoglycans that are highly expressed in the cervix, but don't change over the course of pregnancy [39, 48]. Perhaps what is more important than their regulation or expression is their interaction with other components in the cervix. These factors could still regulate cervical structure and function via their interactions with collagen or immune cell recruitment [38, 49]. For example, decorin can cause an increase in collagen fibrils and veriscan can also affect ECM disorganization in tissue remodeling

[50]. Heparan sulphate can interact with cytokines, such as IL-8 [49, 51], and can affect their half-life and functional potency within the tissue [52-54].

Hyaluronan, also known as Hyaluronic acid (HA), is a non-sulfated glycosaminoglycan that is a component of the ECM of most tissues [55]. It has been shown to affect tissue hydration [56], collagen interactions [57], and the immune response [58]. Hyaluronan is produced by epithelial, stromal, and immune cells in the cervix [55], then excreted into the ECM in multiple forms, ranging from high molecular weight (MW) HA, thought to influence tissue structure, and low molecular weight HA, potentially mediating tissue repair or the immune response [59]. It is present in the cervix in low amounts throughout pregnancy, but drastically increases during cervical ripening when the hyaluronan synthase enzyme gene, *Has2*, is upregulated [55, 60]. The HA that increases during ripening is high molecular weight, and has been implicated in weakening fibronectin-collagen interactions, thus destabilizing the ECM and making collagen vulnerable to collagenases [61]. Interestingly, when HA function was studied in the pregnant cervices of HA knockout mice, it was determined to have a role in epithelial and mucosal barrier function against pathogenic *E. coli* [62]. Indeed, loss of HA resulting in increased ascending bacterial infection and higher rates of infection-associated PTB[62].

Hormonal Regulation of Cervical Ripening

Cervical ripening is associated with the functional loss of progesterone (P_4) signaling within the cervix. Cervical ripening can be induced in women and rodents by

anti-progestin treatment, and vaginal P_4 can delay ripening and labor in women with anatomically short cervix [63]. Interestingly, the loss of cervical P_4 does not appear to be a result of declining P_4 in the circulation. In women, circulating P_4 does not significantly decrease before labor. In rodents, circulating P_4 does decline before labor, but cervical ripening is initiated prior to the decline [64]. Therefore, it is postulated that loss of P_4 function in the cervix is the result of tissue-specific changes in P_4 concentration and/or function [65]. The loss of P_4 function in the cervix preceding cervical ripening is not well defined but elegant work has demonstrated that there are changes in local P_4 metabolism.

Enzymes that metabolize progesterone and estradiol are expressed by cervical cells and are regulated over gestation. Enzymes that convert P_4 to an inactive form increase during ripening while enzymes that synthesize estradiol (E_2) increase at this time [66]. For example in early pregnancy, epithelial cells express 17β-hydroxysteroid dehydrogenase-2 (17β-HSD-2), which catalyzes the conversion of estradiol to inactive estrone. It also oxidizes its partner enzyme, 20α-HSD, which maintains progesterone concentrations. During cervical ripening, 17β-HSD is downregulated in epithelial cells, resulting in decreased local progesterone and increased estradiol [66-68]. The expression of the enzyme steroid 5α-reductase (5α-SRD) also converts progesterone to an inactive form and is upregulated in the cervical epithelia of mice during ripening. Indeed, 70% of mice lacking 5α-SRD had delayed parturition because local progesterone remained high, resulting in the inability to undergo cervical ripening [69]. Interestingly, there is little known about the role of estrogen in cervical ripening, although several studies suggest potential functions. For example, it was previously determined that estrogen administration induced cervical collagenase activity in women and rodents,

which could potentially affect collagen remodeling [70-73]. In vitro models have also demonstrated that estrogen induces collagen degradation activity, which can be blocked by progestin treatment [73, 74]. The key enzyme responsible for up-regulation of cervical HA during ripening, HAS2, is also regulated by estradiol [75], suggesting a role in HA-mediated immune protection.

Immune Regulation of Cervical Ripening

Immune cell recruitment, pro-inflammatory cytokines, prostaglandins and protease activation are also implicated in the regulation of cervical ripening. It is proposed that activated immune cells induce ripening by expressing proteases that degrade collagen and cytokines that affect tissue hydration [76]. These cells are also potential sources of prostaglandins, which regulate progesterone and induce labor [77-79]. There is some debate about the role of inflammation in the regulation of ripening. Some propose that infection induces cervical ripening via an inflammatory mechanism [80], and this is distinct from the mechanism associated with the functional loss of local progesterone.

Graham Liggins (1981) initially theorized cervical ripening was an inflammatory reaction mediated by immune cells [81]. It was postulated that neutrophils infiltrated during cervical ripening and mediated collagen degradation by secreting MMPs [82-85]. Recent studies, however, suggest neutrophils might have a more important role in cervical repair, postpartum [86-88]. These studies found that women and mice did not have increased neutrophils in the cervix at ripening, but they were increased post-partum [87, 88]. Also, depletion of neutrophils did not affect ripening or parturition in

mice, and there was no increase in myeloperoxidase, a marker of neutrophil function, until the period of post-partum repair [86, 88].

Monocyte recruitment and macrophage activation have also been characterized in the cervix during cervical ripening, although there is debate about the origin of these cells and how they function. Some have reported monocyte recruitment to the cervix during cervical ripening in mice, but without increased differentiated macrophages [89]. These data have led some to suggest that, like neutrophils, the role of macrophages is in post-partum cervical repair [38, 88]. Others have reported increased macrophage numbers and changes in functional markers at the time of cervical ripening, in direct conflict with the prior reports [90, 91]. In addition, macrophage depletion was able to prevent LPS-induced preterm birth in a mouse model [92]. These discrepancies are most likely associated with different methodologies, as evidenced by an elegant report from Steve Yellon's laboratory. They determined that if the cervices of mice were perfused prior to tissue collection and dispersion there was no increase in monocyte numbers. This observation suggested increased monocyte numbers previously reported were likely a result of the increased cervical vasculature, not a specific recruitment to the tissue. They also performed highly validated, 8-marker flow cytometric analysis of macrophage populations within the cervix, including markers for cell viability and activation. They discovered robust changes in functional populations of macrophages associated with ripening, supporting a potential role for these cells prior to post-partum repair.

Cervical Viral Infections and PTB

An increasing number of clinical studies suggest that cervical viral infection is a risk factor for PTB. Meta-analysis of Human Papilloma Virus (HPV) infections and preterm birth showed that HPV infections could more than double risk for preterm delivery [93]. This is important because, while HPV vaccines are used to combat cervical dysplasia and cancer, the HPV vaccine may provide an additional obstetrical benefit, and screens for HPV could be used as a predictor for PTB [93]. Several recent studies have also linked HSV-2 infections with increased risk for PTB, including a study with nearly 700,000 women that showed untreated genital HSV-2 infection nearly doubled the risk for PTD [94]. Another study in 2017 confirmed treatment of genital herpes with acyclovir decreased the chances of preterm birth in a cohort of with HSV-2 positive women in Uganda [95]. Early evidence of HSV-2's relationship to preterm birth was found in a 1996 study that associated asymptomatic genital shedding of the virus with increased incidence of preterm delivery [96]. Despite these associations there have been few studies into the mechanistic role that HSV-2 may have in incidences of preterm deliveries.

A mouse model of viral infection during pregnancy also suggests that cervical viral infection increases risk of PTB in the presence of bacteria. This mouse model was developed using a gammaherpes virus, MHV68, a latent DNA virus from the *herpesviridae* family, the same family of nearly all of the viruses that infect the woman's reproductive tract (HSV, CMV, EBV, HHV6, HHV7). When pregnant mice received an intraperitoneal (i.p.) injection of MHV68 on embryonic day (E)8.5 of pregnancy it

sensitized the response to a secondary i.p. injection of LPS on E15.5, resulting in PTB [97]. The same dose of LPS on that day did not induce PTB in mice without viral infection [97]. This suggested that while viral infection, alone, was not enough to induce labor, it acted as a "risk factor" to those with a secondary infection. **Importantly,** the pregnant cervix was the primary reproductive tract target for MHV68, and infection affected cervical function by decreasing cervical protection against ascending bacterial infections. This suggests the mouse can be a reasonable model for herpes viral-infection associated PTB, which is generally associated with cervical HSV-2 infection in women.

Herpes Simplex-2 Virus

The *Herpesviridae* family is a large group of double-stranded DNA viruses that are ubiquitous in the human population [98-101]. Herpes Simplex Virus (HSV) -1 is predicted to be present in 67% of the world's population [98], and HSV-2 in over 11% [99]. HSV-2 generally manifests as a genital infection, and has received special attention during pregnancy for the its potential for vertical transmission from mother to neonates [99, 100]. HSV-1 remains important since there has been a rising number of genital herpes cases documented as being caused by HSV-1 [102].

HSV-2 poses a unique problem for its host because, after an infection episode, it has the ability to establish latency within neurons and later reactivate in a lytic cycle [103]. HSV-2 primarily and efficiently infects mucosal epithelial cells, which is the classical site of primary infection and HSV is also present in these cells during its lytic cycle which is essential for transmission [104]. HSV-2 targets sensory neurons for long term residency, important for its latent life [104]. There are a few well documented

stimuli that induce reactivation of viruses including UV light exposure, emotional stress, fever, tissue damage, and immune suppression [103]. HSV-2 also has documented cases of lytic cycles, which result in viral shedding, occurring without the presence of clinically noticeable symptoms, and this is postulated to be why HSV-2 is so effective in its virulence [105-108].

Herpes simplex virus -2 is an enveloped virus that utilizes host cell surface receptors for attachment and subsequent breach of cells [109]. Herpes simplex virus has been shown to bind to glycosaminoglycans and interacts with plasma membrane integrin αvβ3 for entry into genital tract epithelial cells [109, 110]. Interestingly, HSV-2 causes phosphorylation of the integrin's downstream focal adhesion kinase even in the absence of viral entry [109]. Specifically, herpes viruses have been shown to regulate Src family kinase activity as a means of producing efficient viral yields [111, 112]. It is not known how these cellular changes might affect the cervical epithelial cells in the context of pregnancy.

CHAPTER 2:

CERVICAL VIRAL INFECTION CAUSES PREMATURE CERVICAL RIPENING IN A MOUSE MODEL OF PREGNANCY

Abstract

Preterm birth occurs in about 10% of all pregnancies worldwide, and about 12% of pregnancies in the United States. Preterm infants have an increased risk for brain, heart, breathing, metabolism, and immune function problems, which is, in-part, why it is the leading cause of neonatal morbidity and mortality in the developed world. 50% of all spontaneous PTB cases have no cause attributed to them. Viruses, specifically HSV-2, have been implicated in potentially contributing to preterm delivery (PTD) and require a deeper examination. In order to do this, we utilized both an in vivo mouse model and in vitro cell line model of virus infections to study both outcomes and mechanisms by which the virus acts on cervix tissue and cells. We observed that intravaginal infections with HSV-2 of our pregnant E15.5 mice show cervices with characteristics of cervical insufficiency compared to controls. Viral infection of both mice and ECT1 cells with HSV-2 results in a stabilization of the transcription factor estrogen receptor alpha (ERa) which is mediated by the focal adhesion kinase, Src. We also found an upregulation of progesterone receptor (PR) in our virally treated mice cervices. ELISA analysis of hyaluronic acid (HA) showed that it is upregulated in response to viral infection in mice cervical flushes and human cervical epithelial cells. HA was linked as an important factor for high viral copy numbers, and to the focal adhesion kinase, Src, by its downregulation when pSrc is inhibited. Our results show that intravaginally infected mice by E15.5 show characteristics of cervical insufficiency (i.e. dilute collagen, tissue spacing) and HSV-2 infected cervical cells show increases in factors important for parturition suggesting that viral infections of the cervix could contribute to compromised cervix integrity, and subsequent preterm labor.

Introduction

Preterm birth (PTB), or birth before 37 weeks' gestation, affects approximately 12% of pregnancies in the United States [9, 113-118] and is the leading cause of neonatal mortality worldwide. Unfortunately, there has been little progress in predicting, preventing and treating PTB due to the complexity of the condition. Indeed, PTB is more accurately defined as a syndrome, resulting from an accumulation of risk factors that, in isolation, may not precipitate labor [114]. While some risk factors have been identified, including infection/inflammation, low progesterone (P_4), cervical insufficiency, and race/ethnicity, we have had limited success using them to predict PTB. Furthermore, nearly half of PTB have no known risk factors and are unexplained. Therefore, the best strategy for predicting or preventing PTB will be to identify the common biological pathways that can be influenced by multiple risk factors.

Appropriate regulation of cervical structure and function is essential to pregnancy maintenance. Throughout most of gestation, the cervical stroma contains a dense extracellular matrix (ECM) rich in highly organized fibrillar collagen. This provides structural support for the developing fetus [119]. The cervical epithelial cells also synthesize a highly effective mucus barrier that protects the uterus from ascending bacteria. This barrier consists of mucins, proteoglycans, anti-microbial peptides, cytokines, proteases and associated protease inhibitors [120]. At the end of gestation there is profound remodeling of the stromal ECM resulting in a structurally compliant tissue in preparation for vaginal birth. At this time the collagen fibers become larger in diameter and increasingly disorganized as a result of increased collagen solubility, decreased cross-linking, increased stromal hydration and/or enzymatic degradation [39].

There are also significant changes in the mucus barrier and epithelial cell function. Collectively, this process is termed “cervical ripening”, and premature cervical ripening precedes preterm labor.

The initiation and regulation of ripening is not well-understood, but there is evidence it is influenced by a tissue-specific decline in progesterone (P_4) concentration and/or function[65]. Cervical ripening can be induced in women and rodents by anti-progestin treatment, and vaginal P_4 can delay ripening and labor in women. Changes in P_4 concentration could be mediated by an increase in local P_4 metabolism or changes in the expression of steroid hormone receptors genes for P_4 and E_2 (*PgR*/*ESR1*). Interestingly, very little is known about the role of E_2 in cervical ripening.

Ripening is also associated with increased synthesis of hyaluronan (HA). The function of HA in the pregnant cervix is not fully understood, but it has been identified as an important mediator of epithelial cell differentiation, epithelial barrier permeability and ascending bacterial infections [121]. The concentration and size of HA may also affect stromal remodeling. High molecular weight (HMW) HA increases in the stroma during ripening, and may contribute to ECM disorganization and loosening because of its large size (>500 kDa) and high level of glycosylation [60].

One of the most common causes of PTB is ascending bacterial infection, which typically indicates cervical function is compromised. Normally, cervical epithelial cells synthesize a highly effective mucus barrier that protects the uterus from ascending bacteria. This barrier consists of mucins, proteoglycans, anti-microbial peptides, cytokines, proteases and associated protease inhibitors. During cervical ripening there is increased epithelial cell proliferation [122], increased synthesis of hyaluronan (HA) [55],

activation of proteases [123], and immune cell infiltration resulting in the enzymatic degradation of the mucus barrier[121]. When ripening is premature, these changes can lead to ascending infection, which can trigger in utero inflammation and PTB. Therefore, anything that can induce cervical ripening, or in any way compromise the mucus barrier, can increase risk for PTB in response to secondary bacterial infection.

Very little is known about how viral infection affects cervical function during pregnancy. Since HSV-2 was shown to increase risk of sPTB, we hypothesized that intra-vaginal herpesvirus infection would affect the function and structure of the cervix, resulting in reduced cervical competency. We discovered that herpesvirus infection increased ER in cervical epithelia and upregulated cervical HA synthesis, in vitro and in vivo. Furthermore, these changes were associated with dramatic remodeling of collagen within the stromal ECM, in vivo, suggesting that viral infection affected the structural integrity of the cervix.

Previously, a study including nearly 700,000 women reported that untreated genital herpes simplex virus-2 (HSV-2) infection nearly doubled the risk of sPTB[124]. Furthermore, in a mouse model of pregnancy, herpesvirus infection significantly increased the risk for PTB in the presence of low levels of bacterial endotoxin, another risk factor. In this model, the cervix was the primary viral target in the reproductive tract [125]. Infection decreased expression of numerous innate immune genes in the cervix and reduced cervical protection from artificial ascending bacterial infection. Based on these data, we hypothesize herpesviruses can increase risk for sPTB by affecting cervical function.

Materials and methods

Pregnant mice and infections

Animals were maintained at Michigan State University's Animal Facility and all procedures were approved by Michigan State University's Institutional Animal Care and Use Committee (IACUC #11-14-198-00). C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME); adult female mice (8–12 wks of age) were mated and confirmed pregnant when vaginal plug was detected. Mice received HSV-2 (10^6), MHV68 (10^6), or vehicle, intra-vaginally, on E10.5 after sedation with isoflurane. Redness and swelling was observed with mice treated with HSV-2, indicating infection. Mice were humanely sacrificed on E15.5 using CO_2 followed by cervical dislocation. The cervix was flushed with sterile 1XPBS (3x with 30µl) and fixed in 4% PFA overnight. Animal numbers: HSV-2, n=4; MHV68, n=4; control, n=8.

Histology

Cervices were harvested from mice and immediately placed into 4% paraformaldehyde rocking at room temperature overnight. Tissues were then washed in 70% ethanol, placed into Thermo Scientific Excelsior ES tissue processor for routine processing, and embedded in paraffin wax. For immunohistochemical (IHC) analysis of ER protein, 6µm sections underwent antigen retrieval by boiling in sodium citrate buffer and treatment with endogenous biotin inhibitor (X059030-2, Dako, Santa Clara, CA). Tissue sections were incubated with primary antibody (1:100, ER-α, MC20, Santa Cruz Biotechnology, CA) in

antibody diluent (S080981-2, Dako, Santa Clara, CA) overnight at 4C. The following day, tissue was incubated with secondary antibody (ab6720, 1:200, goat anti-rabbit IgG-biotinylated, Abcam, Cambridge, ME) for 1h, treated with an inhibitor of endogenous peroxidase (S200380-2, Dako, Santa Clara, CA), and developed with diaminobenzidine (DAB) substrate-chromogen (K346711-2, Dako, Santa Clara, CA). Negative controls underwent the same protocol but without primary antibody. For collagen staining, 10μm tissue sections were stained with picrosirius red (Polysciences Inc, Warrington, PA). Using a 40x objective and polarized light, a photomicrograph was taken of 10 sections, at least 20μm apart, per animal. The optical density (OD) of three non-overlapping sections of each photo was analyzed. Specifically, after photomicrographs were converted to gray scale, they were inverted and OD was calculated using a calibrated threshold and the Rodbard standard curve (NIH Image J software). In this analysis, areas of dark collagen staining have low OD values, and areas with light staining have high OD values. To account for cell density, the OD was divided by cell nuclei/area. Animal numbers per group: HSV-2, n=4; MHV68, n=4; control, n=8.

Cell culture and treatments

Immortalized human ectocervical cells (ECT1, CRL-2614, ATCC, Grand Island, NY) were cultured in keratinocyte serum free medium (17005-042, Gibco, Grand Island, NY) with bovine pituitary extract and hEGF supplementation as recommended by ATCC under 5% CO2 at 37°C. Cells were confirmed to be mycoplasma free (13100-01, Southern Biotechnology, Birmingham, Al). Inhibitors included Src inhibitor cocktail, SKI-1/PP1 (ab120839, ab120859, Abcam, Cambridge, ME) resuspended in DMSO (5μM) and an

inhibitor of HA synthesis, 4-methylumbelliferone (4-MU, M1381, Sigma Aldrich, St Louis, MO) resuspended in methanol (600nM). Cells were treated with inhibitors for 1h prior to HSV-2 infection (10^6). Supernatants and cells were collected 24 or 48h after HSV-2 infection, as indicated. To infect ECT-1 with HSV-2, cells were inoculated with HSV-2 (10^6) in 500 µl for 1h prior to addition of 2.5 mL media (protocol for 35mm plate). Results from cell culture experiments are representative of 4 independent experiments.

Virus production and quantification

MHV68 was passaged on M2-10B4 cells (CRL-1972, ATCC, Grand Island, NY) in DMEM plus 10% FBS. HSV-2 was passaged in Vero Cells (CL-81, ATCC, Grand Island, NY) in DMEM plus 10% FBS. After lysis supernatants were harvested, filtered (0.45 µm pore) and titered by 10-fold serial dilutions on confluent monolayers. To detect viral titers in mice, DNA was extracted from the cervix using DNeasy blood and tissue kit (69504, Qiagen, Valencia, CA). 100ng total DNA was then assayed using primers specific for MHV68 (forward primer: 5'-CCA-CTG-AGG-GAC-GTA-TGT-GT-3', reverse primer: 5'-TGG-GAT-CAC-CCA-AGA-AAC-CAC-3') or HSV-2 (forward primer: 5'-GCT-CGA-GTG-CGA-AAA-AAC-GTT-3', reverse primer: 5'-TGC-GGT-TGA-TAA-ACG-CGC-AGT-3'), and compared to a standard curve. Results reported as copies/100ng DNA.

RNA, cDNA synthesis and qPCR

RNA was extracted from ECT-1 cells using RNeasy RNA extraction kit (74104, Qiagen, Valencia, CA). RNA concentration and purity was analyzed using spectrophotometric analyses of 260/280 ratios with exclusions for samples that were below 1.7. For real-time

quantitative analysis of mRNA, 1 µg of RNA was reverse transcribed for each sample using iScript cDNA synthesis kit (170-8891, Bio-Rad, Hercules, CA). The cDNA was diluted 1:20 in nuclease-free water and 5µl was mixed with SsoAdvanced Universal SYBR green superscript (172-5270, Bio-Rad, Hercules, CA) and gene specific primers for ER-α (forward primer: 5'-GGC-CCC-AGC-TCC-TCC-TCA-T-3', reverse primer: 5'-ACG-TTC-TTG-CAC-TTC-ATG-CTG-TA-3') and GAPDH (forward primer: 5'-AGG-GCT-GCT-TTT-AAC-TCT-GGT-3', reverse primer: 5'-CCC-CAC-TTG-ATT-TTG-GAG-GGA-3'). Samples were evaluated with the Applied Biosystems Q-RT PCR machine. Values were normalized to GAPDH and calculated using delta delta Ct method; delta delta Ct= delta ct treated- delta Ct control; results expressed as fold differences are 2ˆ-(average delta delta Ct) for negative delta delta Ct values or – (2ˆ[average delta delta Ct]) for positive delta delta Ct values.

Hyaluronic Acid ELISA

Hyaluronic acid concentration was assessed using ELISA (DHYAL0, R&D systems, Minneapolis, MN). ECT1-conditioned medium was diluted 1:40, cervical flushes were diluted 1:160, and samples were assayed according to manufacturer's protocol. Wavelength correction was used by subtracting 540 nm readings from all readings at 450 nm. The subtraction was used to correct for optical imperfections in the plate as recommended by manufacturer.

Western blot analysis

Cells were lysed in M-PER Mammalian Protein Extraction Reagent buffer (78503, Pierce, Rockford, IL), with HALT protease inhibitor. Total protein concentrations were quantified using BCA assay (23227, Pierce, Rockford, IL). Twenty-five micrograms of total proteins were dissolved in 1X sample buffer, boiled for 5 minutes and separated on a 5-20% SDS-PAGE gel in 1X Tris-Glycine SDS running buffer (Novex, Carlsbad, CA) at a constant voltage of 125V for 2h. The proteins were transferred to PVDF membranes (0.45 µm, Novex, Carlsbad, CA) in an XCell II Blot module apparatus (Novex, Carlsbad, CA) at a constant 25 V for 2hrs. Non-fat milk (NFM) (5%) was used to block non-specific signals and immunoblotting was performed with a 1:1000 dilution of primary antibodies against total Src and phospho-Src-Tyr527 (2105, 32G6, Cell Signaling, Danvers, MA), ER-α (MC20, Santa Cruz Biotechnology, CA), or 1:10,000 dilution of beta-actin (ab16039, Abcam, Cambridge, ME) in 2% NFM at 4°C overnight. Membranes were washed in 1X PBST and a 1:10,000 dilution of goat anti-rabbit or goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Cell Signaling, Danvers, Mass) was used as appropriate. Membranes were developed using HRP substrate (Amersham ECL Prime Detection Reagent; General Electric, Buckinghamshire, UK) and immunoreactive proteins were visualized using the Bio-Rad ChemiDoc XRS+.

Statistical analysis

Differences were determined using analysis of variance (ANOVA) (multiple comparisons, Tukey's test), or independent t-test functions of Graph pad inSTAT statistical software (La

Jolla, CA). A p-value of ≤0.05 was considered significant, different letters denote significant differences. Data is presented as mean ± standard error of the mean (SEM).

Results and figures

Cervical viral infection affects stromal tissue organization

We first determined if viral infection affected the cellular and/or extra-cellular structure of the cervical stroma during pregnancy. Mice were infected with MHV68, HSV2 or vehicle, intra-vaginally, at gestational day (gd)10.5, and cervices were collected at gd15.5. Tissue was fixed, stained for collagen, and examined using bright light, and polarized light, microscopy. Under bright light, it was readily apparent that infected cervices had a looser arrangement of collagen fibers compared to controls, as demonstrated by representative sections from cervices of mice treated with HSV2, MHV68 or vehicle (Figure 1a). To quantify collagen objectively, the sections were first viewed under polarized light and converted to gray scale (Figure 1b). Images were then inverted and OD was calculated using a calibrated threshold and the Rodbard standard curve (NIH Image J). In this analysis, areas of dark collagen staining have low OD values, and areas with light staining have high OD values, and the OD is normalized to cell number by dividing by number of nuclei per μm^2. The infected cervix tissue had higher OD compared to the controls, indicating the collagen was less dense in infected tissue (Figure 1c), (t-test, p=.0007). The cervical stroma was also less cellular in infected animals, which was quantified by analyzing the number of nuclei per μm^2 (Figure 1d), (t-test, p=.02).

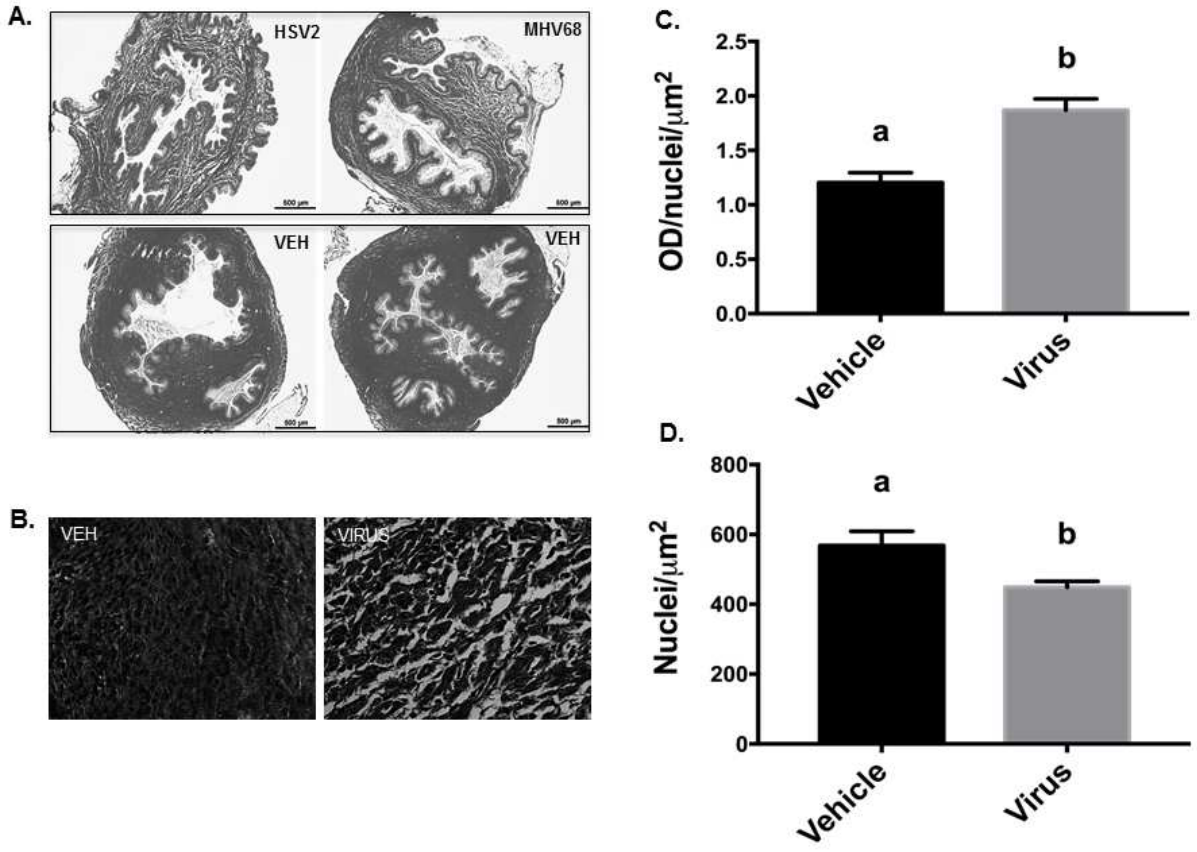

Figure 1: Cervical viral infection affects stromal tissue organization

Histological sections from cervices of animals infected with MHV68, HSV-2 or vehicle were stained with picrosirius red to characterize collagen organization. (A) A representative section from pregnant mice infected with HSV2, MHV68 and representative sections from two control mice (VEH) were imaged under bright light microscopy (4x) and shown in gray scale. (B) Representative images from control (VEH) and infected (VIRUS) animals under polarized light and gray scale conversion (40x). (C) Collagen content and structure was quantified by calculating the optical density (OD) using 3 non-overlapping photos from 10 sections per animal, normalized to cell nuclei/μm^2. Specifically, after photomicrographs were converted to gray scale (as shown

Figure 1 (cont'd): in (B)), they were inverted and OD was calculated using a calibrated threshold and the Rodbard standard curve. In this analysis, areas of dark collagen staining have low OD values, and areas with light staining have high OD values (t-test, p=.0007). (D) Cell density analysis, represented as the number of nuclei per μm^2 (t-test, p=.02). Animal treatments: HSV-2, n=4; MHV68, n=3; vehicle, n=8.

Viral infection increases ER-α protein in cervical epithelial cells

Normally, at term, cervical remodeling is mediated by changes in steroid hormone concentrations within the tissue, characterized by a shift from progesterone to estrogen dominance [126]. Since viral infection induced similar changes in the cervical stroma, we examined how infection affected the known mediators of the local hormonal shift. Initially, the human cervical epithelial cell line, ECT-1, was used to screen the effect of HSV-2 infection on known regulators of cervical endocrinology. While HSV-2 did not affect progesterone receptor (PR), or 17β -hydroxysteroid dehydrogenase (17 β -HSD)(data not shown), the infection did increase ER-α (Figure 2a). Furthermore, there was no change in *ESR1* mRNA (Figure 2b), (analysis of variance (ANOVA), n.s.), suggesting HSV-2 stabilized the protein, as opposed to increasing transcription.

We also examined ER-α expression in cervices from infected and control mice at gd15.5 using immunohistochemistry. In infected mice, there were irregular pockets of cervical epithelium expressing ER-α, which can be identified by the brown positive staining (Figure 2c). The cellular staining pattern suggested the receptor was localized in the cytoplasm, as well as in or around the nucleus of the non-differentiated epithelial cells (NDE), which were small, lacked mucus and resided beneath the differentiated mucosal epithelial cells (DME) (Figure 2c). The DME contained large mucus vacuoles and had little to no positive ER-α staining (Figure 2c). This was in stark contrast to control tissues that had very little, if any, epithelial cells positive for ER-α (Figure 2c).

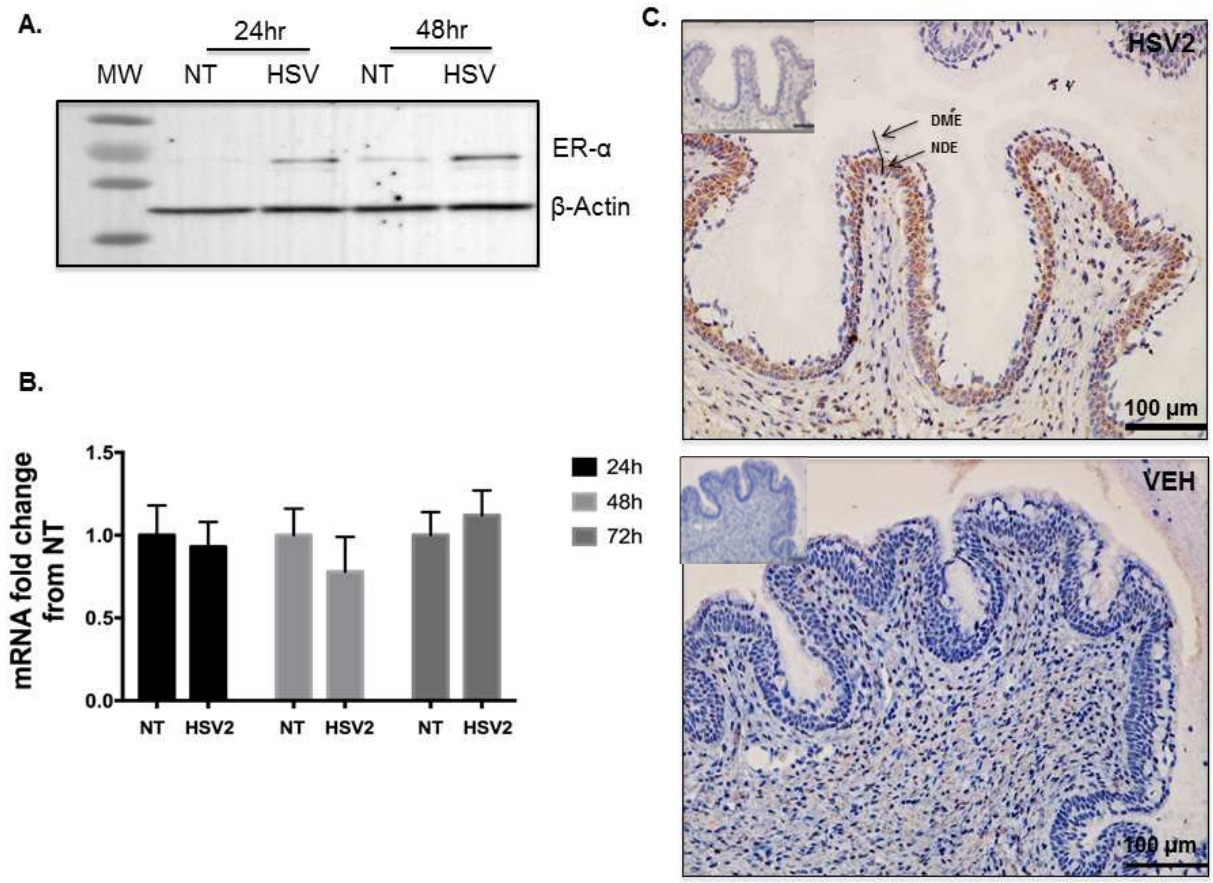

Figure 2: Viral infection increases ER-α protein in cervical epithelial cells

ECT-1 cells were treated with vehicle (VEH) or HSV-2 for 24, 48h and 72h. (A) Representative Western blot analysis of ER-α, and actin loading control. (B) Quantitative PCR was used to analyze ERA mRNA expression in ECT-1 with and without HSV-2 (ANOVA, n.s.). (C) Representative images from control (VEH) and infected (HSV-2) animals analyzed for ER-α protein using immunohistochemistry. NDE, non-differentiated epithelial cells; DME, differentiated mucosal epithelial cells. Figures represent results of 4 independent in vitro experiments. Animal treatments: HSV-2, n=4; MHV68, n=3; vehicle, n=8.

Hyaluronic acid is increased by HSV-2 in the pregnant cervix and ECT1 cells

We then considered how the virus, and the associated increase in ER-α protein in the epithelia, might be related to the changes in the tissue stroma. Hyaluronic acid (HA) is an important component of the cervical mucus barrier and the stromal ECM. It is also regulated by estradiol and increases at the end of gestation. Therefore, we analyzed cervical flushes from pregnant mice with and without viral infection and discovered HA was significantly increased in the infected mice (Figure 3a, t-test, p=.012). Similarly, HSV-2 increased HA in ECT-1 conditioned medium at 48h post-infection (Figure 3b), (24h NT vs HSV2: t-test, n.s.; 48h NT vs HSV2: t-test, p=.017).

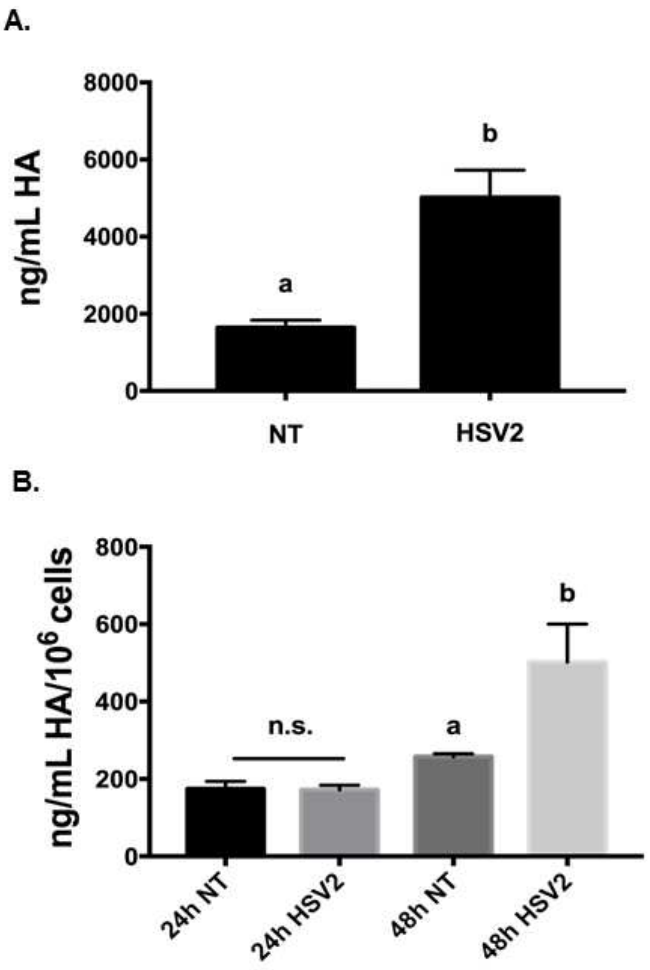

Figure 3: Hyaluronic acid is increased by HSV-2 in the pregnant cervix and ECT1

(A) Hyaluronic acid was quantified at gd15.5 in cervical flushes from infected and control animals using ELISA (t-test, p=.012). (B) Hyaluronic acid was quantified in ECT-1 conditioned medium 24 or 48h after treatment with vehicle or HSV-2 using ELISA (24h Veh vs Virus: t-test, n.s.; 48h Veh vs Virus: t-test, p=.017). Animal treatments: HSV-2, n=4; MHV68, n=3; vehicle, n=8. HSV-2 and MHV68 treatments analyzed together compared to vehicle. In vitro results represent 3-4 independent experiments.

Herpes simplex virus-2 activation of Src kinase stabilizes ER-α and regulates HA

We next determined how HSV-2 might stabilize ER-α protein and/or induce changes in HA expression in cervical epithelial cells. The tyrosine kinase Src was previously shown to be activated by toll-like receptor signaling following viral infection [125], and Chu *et al.* showed it to interact with ER-α, therefore we first tested its role in HSV-associated ER-α stabilization. First we treated ECT-1 with HSV-2 and measured phosphorylation of Src-Tyr527 at multiple time points from 30m up to 8h post-infection. This phosphorylation site is an inhibitor of function and acts as the primary regulator of Src, therefore loss of phosphorylation at Tyr527 indicates Src is in its active form. We found that Src Tyr527 phosphorylation was not changed at early timepoints following infection (30m-2h, data not shown), but viral infection reduced phosphorylation, or activated Src, at 6h and 8h post-infection (Figure 4a). To determine if Src could also affect ER-α, ECT-1 cells were treated with Src inhibitor, SKI/PP1, which resulted in decreased ER-α protein (Figure 4b). To test if HSV-associated changes in ER-α were mediated by Src kinase, we inhibited Src (SKI-PP1) prior to HSV-2 infection and again measured ER-α protein. When ECT-1 cells were treated with the Src inhibitor, HSV-2 infection no longer caused an increase in ER-α protein (Figure 4c) suggesting HSV-2 mediates ER-α protein stability by activating Src kinase in cervical epithelial cells. Finally, we tested how activation of Src affected HSV-associated changes in HA. When we infected ECT-1 with HSV-2 in the presence of Src inhibitors (SKI-PP1), the virus-associated increase in HA was diminished (Figure 4d), (ANOVA; Tukey's multiple comparison; a<b, p=.012; a>c, p=.026; b>c, p=.0003). Therefore, Src kinase could be an important mediator of the cervical response to HSV-2.

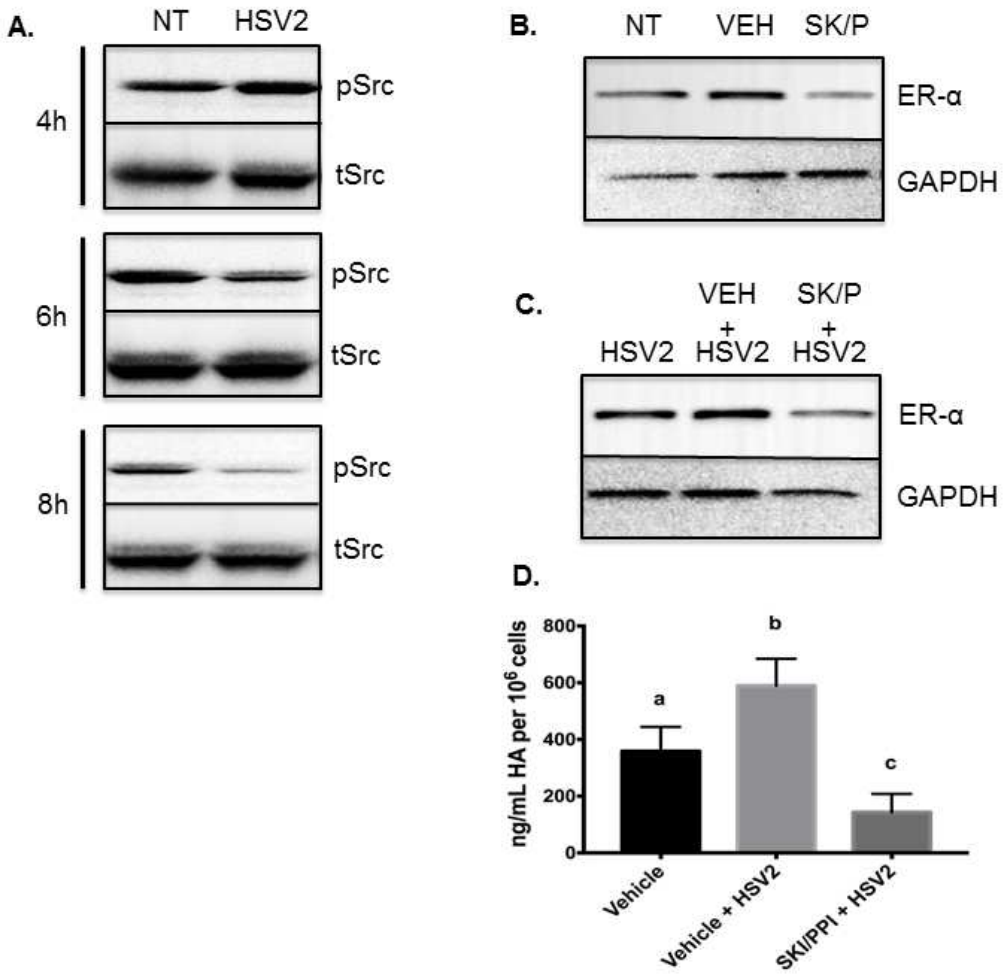

Figure 4: Herpes simplex virus-2 regulates ER-α and HA via Src kinase activation

(A) Src kinase activation was characterized in ECT-1 cells 4, 6, and 8h post-infection with HSV-2 using Western blot analysis with an antibody specific for Src Tyr527 phosphorylation or total Src. (B) ECT-1 cells were treated with vehicle (DMSO) or Src inhibitor (SKI/PP1) and ER-α protein was quantified using Western blot analysis. (C) ECT-1 cells were treated with HSV-2, HSV-2 and DMSO, or HSV-2 and Src kinase inhibitor, and ER-α protein was quantified using Western blot analysis. (D) ECT-1 were treated with vehicle (DMSO), vehicle and HSV-2, or Src inhibitor (SKI-PP1) and HSV-2

Figure 4 (cont'd): for 24h and HA was quantified in the conditioned medium using ELISA (ANOVA with Tukey's multiple comparison; a<b, p=.012; a>c, p=.026; b>c, p=.0003). Figures represent results of 4 independent in vitro experiments.

Hyaluronic acid mediates HSV-2 infection

Interestingly, HA has also been shown to facilitate viral infection of host cells [127]. Therefore, since HSV-2 upregulated HA, we tested its role in mediating HSV-2 infection of cervical epithelial cells. ECT-1 cells were treated with 4-methylumbelliferone (4-MU), an inhibitor of HA synthesis, which resulted in down-regulation of HA (Figure 5a), (t-test, p=.003). Cells were then infected with HSV-2, and viral titers were analyzed 24h post-infection by measuring gene copy number. Inhibition of HA resulted in a drastic decrease in HSV-2 infection (Figure 5b), (t-test, p=.019). Inhibition of Src kinase (SKI-PP1) also resulted in a similar decrease in HSV-2 infection (Figure 5c), (t-test, p=.006). Therefore, the HSV-associated increases in HA could result in increased susceptibility to epithelial cell infection, in addition to the potential changes in cervix tissue structure.

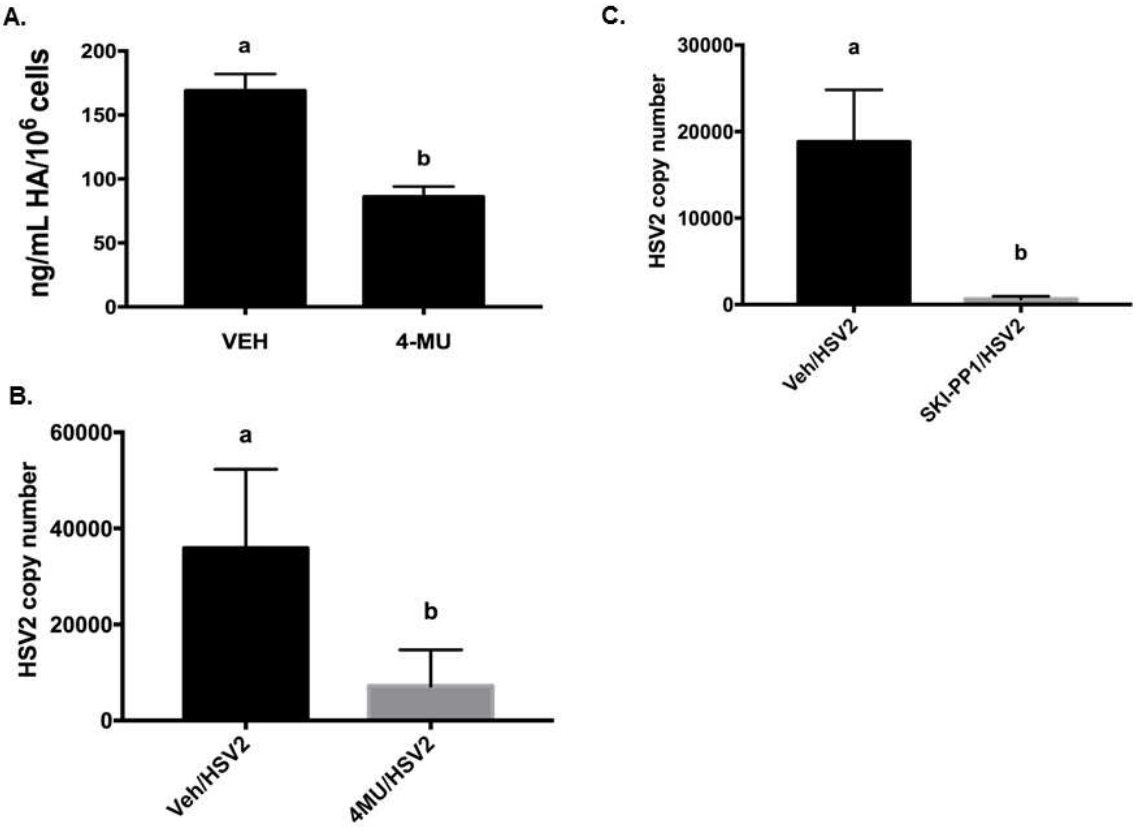

Figure 5: Hyaluronic acid mediates HSV-2 infection

(A) ECT-1 cells were treated with vehicle (VEH, methanol) or 4-MU, an inhibitor of HA synthesis, and HA was quantified in conditioned medium (t-test, p=.003). (B) ECT-1 cells were treated with vehicle (VEH) or 4-MU preceding HSV-2 infection, and viral copy number was analyzed using qPCR (t-test, p=.019). ECT-1 were treated with vehicle (VEH) or Src inhibitor (SKI-PP1) preceding HSV-2 infection and HSV-2 copy number was analyzed using qPCR (t-test; p=.006). Figures represent results of 4 independent in vitro experiments.

CHAPTER 3:

CONCLUSIONS AND DISCUSSION

For the first time, to our knowledge, we report that intra-vaginal herpesvirus infection causes cervical remodeling in pregnant mice. Herpesvirus infection of the cervical epithelium was associated with increased expression of ER-α, increased cell proliferation, and upregulation of HA synthesis and/or secretion. These changes were associated with significant disorganization and/or loss of collagen within the cervical stroma. Using ECT-1 cells, we discovered that HSV-2 activated Src kinase, which mediated the increase in ER and HA. Infection with HSV-2, activation of Src, and increased HA also increased the susceptibility of cervical epithelial cells to HSV-2 infection. These results demonstrate viral infection can significantly change the cervix during pregnancy. We propose viral infection can induce cervical insufficiency, which increases risk of ascending infection, intra-amniotic inflammation and preterm labor[128].

Cervical ripening in response to hormones has been best characterized in rodent models. Typically, and in the absence of bacterial infection, cervical ripening is initiated by the switch from high P_4 to high E_2 at the end of pregnancy. Previously this raised concerns about the model, since rodents experience a sharp decline in circulating P_4 at the end of pregnancy, while P_4 is maintained in women. These concerns were in some way overcome by the discovery that, in both species, cervical ripening was regulated by changes in local hormone metabolism, which resulted in the loss of P_4 within the tissue. In rodents, upregulation of SRD5α1 decreases P_4 concentration, while in humans downregulation of 17β-HSD reduces P_4 and increased E_2 synthesis in the cervix. Therefore, since we considered the rodent model reasonable, we used a mouse model to study herpesvirus infection of the cervix during pregnancy.

We were intrigued to discover viral infection increased ER-α in cervical epithelial cells. Availability of ER has been shown to be directly associated with E_2 function in multiple cell types, therefore viral infection likely increases E_2 sensitivity in the epithelium. We also observed dramatic reorganization of the cervical stroma, including reduced cell density and loosening of the collagen fiber network. These changes can be triggered by the hormone environment, or by an inflammatory cascade mediated by immune cells [129]. We speculate that viral infection could affect the cervix both ways, by increasing local E_2-sensitivity and activating immune cells. Future investigation into the mechanism of virus-induced cervical changes will be important for development of clinical interventions.

Hyaluronic acid synthesis is also associated with hormone changes [130], and infection [62], induced cervical ripening and is a result of increased expression of HA synthase-2 or -3 (*HAS2/3*). Interestingly, *HAS2* is positively regulated by estradiol in the cervix[131], which is relevant to our study because HA was increased in infected cervices, which was associated with the increased ER-α in cervical epithelia. We could not use the ECT-1 to determine the role of ER in HSV-induced HA synthesis because cervical epithelial cells do not properly respond to E_2 without the stroma, but we did determine HA synthesis was dependent on Src kinase activation following viral infection. Therefore, Src kinase could be an important mediator of the virus-associated changes in cervical function. Although the role of ER in our model is still unknown, we hypothesize it mediates some of the cervical changes downstream of viral infection and Src activation. This is being tested in ongoing in vivo studies of infection with an ER antagonist.

Finally, we tried to identify factors that might affect the susceptibility of the cervix to HSV-2 infection. Herpes simplex virus-2 can readily infect primary human cervical epithelial cells, and infection can be reduced using the antiviral drug, Acyclovir, or the ligand for toll-like receptor-3, Poly (I:C) [132]. Infection can also be affected by sex-hormone treatment. In primary human epithelial and stromal cell co-cultures, treatment with E_2 resulted in increased infection while P_4 decreased HSV-2 infectivity [132]. In a mouse model, there was no susceptibility to genital infection during estrus (high E_2), while animals in diestrus or treated with P_4 had high susceptibility to HSV-2 [133, 134]. It is still unclear how genital tract infection is affected by sex hormones and susceptibility to HSV-2 during pregnancy has not been established. Interestingly, HA and other glycosaminoglycans, such as heparin sulfate, are established mediators of herpesvirus viral entry into host cells. We determined that HA did affect HSV-2 infection of cervical epithelial cells. Indeed, loss of HA by direct inhibition with 4-MU or inhibition of Src kinase drastically reduced HSV-2 infection of ECT-1. Collectively, the data suggests that HSV-2 infection upregulates HA, which in turn, further increases viral susceptibility.

In conclusion, we propose that virus-associated activation of Src kinase, and the associated stabilization of ER, and HA synthesis in the epithelium, affects the structure of the cervical stroma and further increases the susceptibility to infection (Figure 6). Ongoing studies are investigating the causal link between the virus-associated changes in the epithelia and the stromal remodeling. Also, while not addressed in this study, local immune cells are also affected by cervical viral infection, and it is likely that these cells also contribute to the remodeling of the ECM. Studies like this are necessary to understand how viral infection could increase the risk for sPTB in women. Based on these

results, we could predict that HSV-2 infection might weaken the cervix or even induce premature cervical ripening in some women. It is our hope that understanding the mechanism of virus-induced cervical remodeling will help us provide targets for clinical intervention.

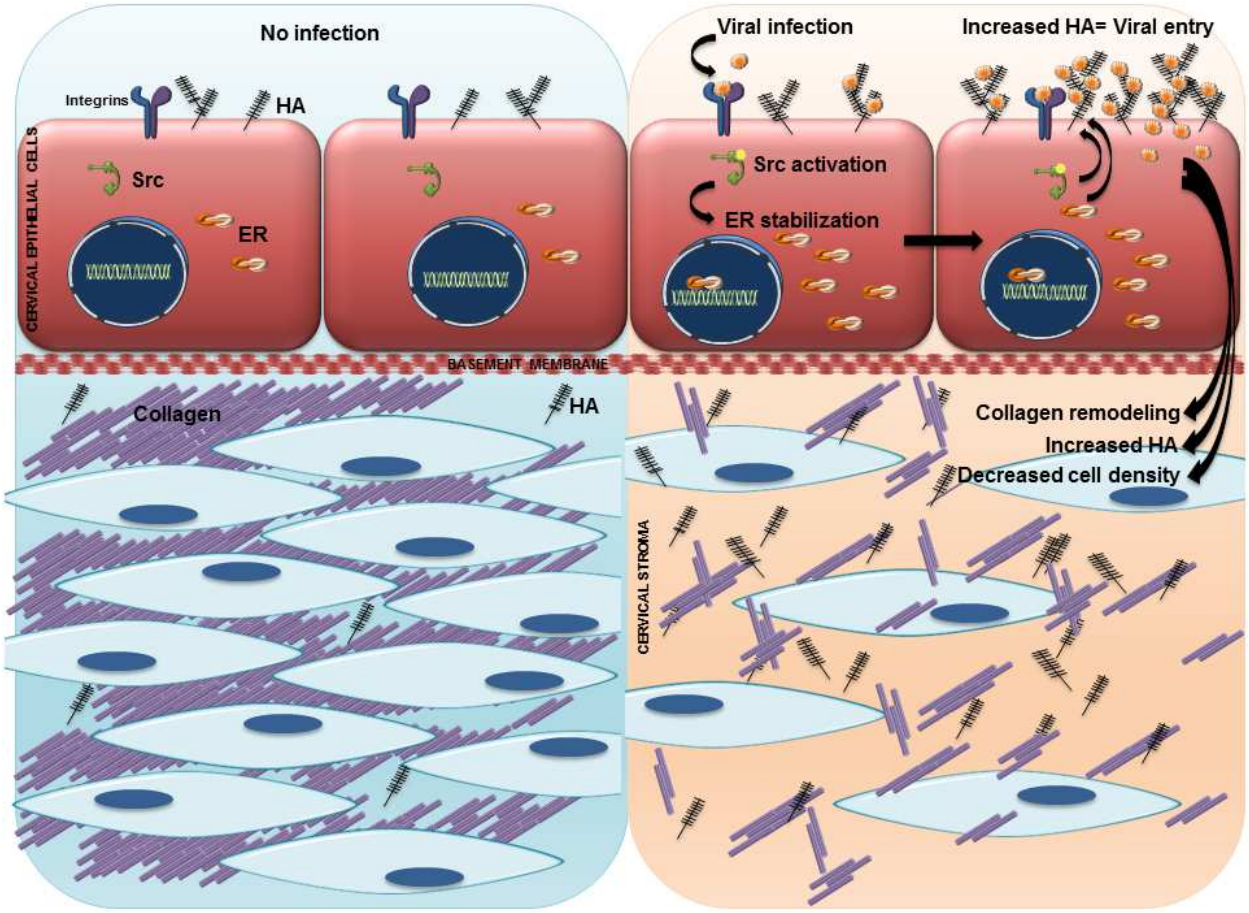

Figure 6: Proposed model of cervical viral infection during pregnancy

Herpesvirus infection of the pregnant cervix bind surface receptors, such as integrins and HA, resulting in viral entry. Infection causes activation of Src kinase, which stabilizes ER, increasing ER concentration and potentially estrogen sensitivity. These changes result in increased HA expression on the surface of epithelial cells, which in turn, increases viral entry into the cells. Increased soluble HA affects the structure of the cervical stroma. These changes are associated with decreased collagen and cellular density of the stroma.

As we continue this investigation we hope to address several remaining questions. One of the primary questions is the potential role that immune cells are playing in remodeling after the initial infection. If immune cells are recruited to the cervix, what is their function there? We want to study MMPs and their potential role in the changing collagen. Understanding if MMPs are being created immune cells, fibroblasts, or both could create potential for development of therapeutic treatments for at-risk women. Better understanding the role of estrogen and its receptors can be achieved with utilizing an estrogen receptor antagonist, and would allow us to both identify critical factors controlled by estrogen and turn to the potential of rescuing a phenotype of term-birth. Finally, to address the idea of PTB as a syndrome, we could attempt to test viral infections with other causes of preterm birth in mice.

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