REGULATION OF CERVICAL EPITHELIAL CELL PROLIFERATION BY VASCULAR ENDOTHELIAL GROWTH FACTOR

A Thesis
by
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Department of Biology

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ABSTRACT

REGULATION OF CERVICAL EPITHELIAL CELL PROLIFERATION BY VASCULAR ENDOTHELIAL GROWTH FACTOR. (May 2012)

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Cervical remodeling is a complex biological process, characterized in part by vascular leakage, an increase in edema, and epithelial growth. Here, we examined the effects of vascular endothelial growth factor (VEGF) primarily on cervical epithelial growth. Both timed-pregnant and non-pregnant mice were treated with either recombinant VEGF164 protein, vehicle only or VEGF blocker. The tissues were analyzed using a variety of techniques, including scanning electron microscopy, confocal immunofluorescence, BrdU, and quantitative real-time PCR. From these studies, we show that VEGF induces cervical epithelial cell, edema, and inter-epithelial immune cell migration into cervical lumen, possibly by altering expression of a tight junction molecule (claudin-5) and expression of itself (VEGF) and its receptors (KDR and Flt-1). These results demonstrate that VEGF is an important regulator in cervical remodeling, notably by altering various events in the cervical epithelial cells.

DEDICATION

To: Dad, Mom, Sarah, Thomas, Mackenzie, and Charlie, for all the love, support, and guidance all throughout my life. I would not be who I am today without you. Thank you for pushing me to pursue my dreams and aspirations.

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INTRODUCTION

One of the most important health problems in obstetrics currently is pre-term birth and its associated complications (Garfield, Maner, Shi, Shi & Saade, 2006). In humans, pre-term delivery is defined as birth prior to 37 weeks. Pre-term labor poses a significant challenge to health in that 5-25% of all deliveries are pre-term and up to 75% of all perinatal mortality (Mokuolu, Suleiman, Adesiyun & Adeniyi, 2010). Further, it (pre-term) is the second leading cause of infant mortality accounting for 16% of all infant deaths and is associated with significant morbidity (Fogleman et al., 2007). The underlying causes of premature birth have been and are still being studied extensively. However, there is no effective cure and the exact trigger of this anomaly is still not completely understood. This study aims to examine the effects of vascular endothelial growth factor (VEGF) on the proliferation of cervical epithelial cells. Cervical epithelial cell proliferation accounts for 50% of cervical tissue growth during pregnancy and is also believed to play a role in cervical tissue immune response (Burger & Sherwood, 1998).

Premature Birth

Pre-term birth can be caused by a number of factors, including infection, obesity, multiple births, assisted reproductive techniques, obstetric interventions, diet, genetics and (Hudic et al., 2009) endocrinologic factors, such as increasing androgen concentration, hyperprolactinameia, and defects in luteal phase (Furui, Imai & Ohno, 2007). Further, an

increasing number of women now have children in their mid-age and tend to delay marriage and child-bearing until later in life, when they have finished their education or when their professional careers are well-established (Tabcharoen, Pinjaroen, Suwanrath & Krisanapan, 2009). This trend may also account for the recent increase in pre-term labor rates (Tabcharoen et al., 2009). Of all these etiologies listed above, infection is one of the leading causes of pre-term birth, accounting for about 50% preterm and induces inflammation and vascular changes in the birth canal or cervix (Bosquiazzo et al., 2005).

Basic Anatomy of the Female Reproductive System

The basic structures of the female reproductive system include the **a**) ovaries, the source and site of egg maturity; **b**) fallopian tubes, site of fertilization and initial development of the embryo; and **c**) uterus, "house" for the fetus until birth. The most external reproductive tissue of the female reproductive system is the vagina. Between the vagina and the uterus is the cervix. My project focuses on the cervix.

The cervix is cylindrically-shaped and is located on the caudal part of the uterus and protrudes and opens through the upper anterior vaginal wall into the vagina. The surface of the ectocervix, the portion of the cervix extending in the vagina, is covered with squamous epithelium, while the endocervix is the portion that extends on the opposite end, into the uterus (Bauer et al., 2007). The endocervix forms the mucous membrane, with its glands and folds covered with columnar epithelium (Bauer et al., 2007). The cervix is composed mainly of fibrous connective tissue, with the main component being collagen. It is also composed of smooth muscle. The collagen is responsible for its rigidity and mechanical function (Bauer et al., 2007) and makes up the extracellular matrix (Garfield et al., 2006).

The Cervix and its Role during Pregnancy

One of the roles of the cervix during pregnancy and term is to provide mechanical resistance to the ever increasing gravitational force exerted by the growing fetus, and thus, ensure the normal development of the fetus (Garfield et al., 2006). This task by the cervix is possible because of its composition, i.e., its extracellular matrix, which enables it to function as a fiber-reinforced composite.

The cervix also serves as a mechanical and biochemical barrier that separates the vaginal bacterial flora from the sterile uterine-located fetal amniotic cavity. The changes that the cervix undergoes during and immediately after pregnancy are collectively termed cervical remodeling (CR) (Bauer et al., 2007). This complex process is regulated by various factors, which ultimately lead to catabolic processes such as degradation of collagen. However, the exact underlying mechanisms are not completely understood (Bauer et al., 2007). Here, we examine the role of VEGF in this process, particularly its role in cervical epithelial cellular growth.

Cervical Remodeling

CR is a progressive complex biological process that can be separated into four distinct but overlapping phases, namely softening, ripening, dilation and effacement, and post-partum repair (Challis, 2000). CR, ultimately, if accompanied by adequate uterine contraction, will lead to fetal passage. Failure of either or both of these events leads to birthing problems, such as preterm birth and protracted labor, which account for 75% of fetal mortality (Challis, 2000).

Studies in humans have shown that cervical softening starts as early as the first trimester, whereas in mice, softening usually begins by day 12 of the 19-day gestation period (Timmons & Mahendroo, 2007). During softening, there is a measurable decline in cervical tissue compliance or tensile strength (Leppert, 1995; Read, Word, Ruscheinsky, Timmons & Mahendroo, 2007; Timmons, Akins & Mahendroo, 2010). The second phase of CR, the ripening phase, is an accelerated phase during which there is a great loss of tissue compliance and integrity. This is the shortest of the four phases and occurs in the hours prior to birth in mice and up to a few weeks before birth in humans (Leppert, 1995; Read et al., 2007). The third phase of CR follows ripening and occurs as the cervix relaxes, dilates, and effaces and is initiated when the uterine contractions become more frequent. This ultimately leads to labor and the final passage of the fetus (Kelly, 2002). Immediately following birth, post-partum repair begins and the cervical tissue begins the restoration process to its non-pregnant, non-compliant state (Leppert, 1995). Dysfunction in any of the phases can lead to serious obstetrical complications. Some of the complications can include preterm birth and protracted labor (Leppert, 1995).

Although significant strides have been made in understanding mechanisms that underlie CR, the exact factors that regulate CR, and specifically events such as increase in cervical tissue and fluid content, are not fully understood. Other prominent features of CR, other than increase in tissue size and edema include vascular alteration such as increased permeability, angiogenesis and vasodilation (Collins, Wilson, Fischer-Colbrie & Papka, 2000; Mowa et al., 2004a). In particular, very little is known about the role of local microvascular alterations, the factors that regulate them and the relationships, if any, between

tissue edema and local cervical vascular changes. This project examines the relationship between vascular factors and changes in cervical size.

Cervical Size during Pregnancy

Increase in cervical tissue mass during pregnancy is believed to be influenced by factors such as relaxin, prostaglandin, and sex hormones (Burger & Sherwood, 1995). Based on scanning electron microscopy (SEM) data we have hypothesized that VEGF may play a role in this process, specifically by promoting cervical epithelial cellular growth, possibly by either directly stimulating endothelial cells to secrete growth factors that, in turn, stimulate proliferation of local cervical epithelial cells (Mowa et al., 2004a; Mowa & Papka, 2004b, Mowa et al., 2008). Secondly, VEGF may also induce vascular permeability leading to an increase in infiltration of local tissue and induction of epithelial growth by serum factors, which are a rich source of growth factors (Tomanek & Schatteman, 2000). Both of these mechanisms could be operational in the cervix, and in part, account for the phenomenal epithelial proliferation during cervical ripening, perhaps in collaboration with factors such as sex steroid hormones, prostaglandins, and relaxin. In our previous study, we used SEM to show pronounced effects of VEGF on cervical epithelial folds in ovariectomized rats treated with VEGF in comparison to the control group (Mowa et al., 2008). The present study builds on this earlier data and uses a more definitive test for evaluating VEGF-induced growth of epithelial cells, namely bromodeoxyuridine (BrdU).

Role of Cervical Epithelia

The functions of cervical epithelium during pregnancy are very specific for each stage of the pregnancy (Read et al., 2007). The major role of epithelia during the softening stage of pregnancy is in protection through increased expression of surveillance factors. Towards the end of pregnancy, the cervical epithelium expresses proteins that promote ripening and alter barrier properties of the cervix (Read et al., 2007). There is growing research interest aimed at understanding the roles and underlying mechanisms of the epithelia in CR (Garfield et al., 2006).

Vascular Endothelial Growth Factor

Vascular events in the remodeling cervix are prominent during and immediately after pregnancy. VEGF is the best studied vascular regulator and is known to be the key regulator of angiogenesis, which occurs during different physiological processes, such as embryogenesis, skeletal growth, and reproductive functions (Ferrara, Gerber & LeCouter, 2003). Angiogenesis is associated with endothelial cell proliferation, migration, and diminished apoptosis (Ferrara et al., 2003; Neufeld, Cohen, Gengrinovitch & Poltorak, 1999).

VEGF is a chemokine produced by cells such as fibroblasts and a member of a family of closely related growth factors that include VEGF-A, -B, -C, -D, -E, and placenta growth factor (PIGF) (Ferrara & Davis-Smyth, 1997). VEGF-A has well-established biological effects and exists as several splice variants (Ferrara & Davis-Smyth, 1997). The five different isoforms of VEGF are synthesized from alternative splicing from a single VEGF

gene. These isoforms contain 8 exons and are located at human chromosome 6p12-p21 (Ferrara & Davis-Smyth, 1997).

The different species of mRNA that encode for the VEGF protein have been identified and are found to be expressed in a tissue-specific manner (Ferrara & Davis-Smyth, 1997). These specific mRNA species increase from differential splicing, with the 165 amino acid form of VEGF lacking sequences encoded by exon 6, and the 121 amino acid form lacking exon 6 and 7 sequences (Ferrara & Davis-Smyth, 1997). The different VEGF isoforms differ in their molecular mass and biological properties. It is important to note here that there are three isoforms of VEGF-A that are known to be expressed in the cervix of rodents (164, 199, 205), the most predominant one being VEGF164 (Mowa et al., 2004a, b).

VEGF Receptors

Biological effects of VEGF are largely mediated by two receptors: VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), which have significantly different signaling properties and are almost exclusively expressed by endothelial cells (Ferrara et al., 2003). VEGF receptor 1 (VEGFR1 or Flt-1) is the high affinity receptor for VEGF and is the gene product of Flt-1, FMS-like tyrosine kinase. Kinase insert domain receptor, KDR, codes for VEGF receptor 2, VEGFR2, which is also known as Flk-1 (Ferrara & Davis-Smyth, 1997). Other cellular targets of VEGF that express VEGF receptors include osteoblasts, mononuclear phagocytes, hematopoietic cells, and some malignant cells (Chen, Ye & Xie, 2004). Our lab has localized the two primary VEGF receptors, KDR and Flt-1, in the endothelial, epithelial, and stromal cells in cervical tissues (Mowa et al., 2004a).

Purpose of Study

CR is a vital part of the birth process and maybe regulated by multiple cervical cell types. Recent data indicates that the cervical epithelial cells may be the key cell type that plays a primary role in regulating CR. The purpose of the present study is to determine if VEGF has a functional role in regulating cervical epithelial processes, such as proliferation.

MATERIALS AND METHODS

Animals and Treatments

Female C57BL6/129SvEv mice sourced from Charles Rivers were used in these studies (*n*=3) under different treatments (VEGF agents, i.e., recombinant protein or inhibitor), as described in further details below or physiological conditions (pregnant or ovariectomized non-pregnant). Prior to tissue harvest, animals were administered a lethal injection of Sodium Pentobarbital **(**Sleepaway®**,** Fort Dodge Laboratories Inc., Burlingame, CA) and perfused intra-cardially immediately thereafter with 0.9% normal saline. Cervical tissues were carefully harvested under a stereomicroscope to ensure only cervical tissue was harvested. The tissues were then processed and analyzed appropriately. Animals used were either pregnant or non-pregnant, as described in more details below. All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the local institution (Appalachian State University) and the NIH guidelines (NIH publication number 86-23), and efforts were made to minimize both animal suffering and numbers of animals used**.** Following treatments, tissues were analyzed using various techniques, including morphological techniques [basic immunohistochemistry (BrdU), SEM, immunofluorescence] and gene expression (Real time PCR) in order to elucidate the effects of VEGF on various cervical parameters and events, namely cervical epithelial structure and proliferation.

Surgery: Ovariectomization

Before treatments or harvesting of tissues in the non-pregnant mice, the mice are ovariectomized (ovariectomy = removal of ovaries). Prior to ovariectomy, animals were anaesthetized with ketamine and xylazine (43-129 mg ketamine and 8.6-26 mg xylazine per gram of body weight) and then allowed to rest for seven days post-surgery before performing the experiments, to allow complete removal of residual ovarian sex steroid hormones in plasma.

Treatments

Appropriate treatments with either vehicle only [negative control: 0.1 M PBS mixed with saturated (25%) pluronic gel mixed and kept in liquid form under ice] or VEGF agents [mouse recombinant VEGF 164 protein (Calbiochem, La Jolla, CA) or mouse recombinant VEGF Blocker]. Experiments were undertaken in this manner due to the fact that VEGF treatments (dose, route, frequency) had not been optimized in the cervix.

a) Dose-Dependent VEGF Study: Non-pregnant, ovariectomized mice were divided into two groups (n=1) and treated with 50µl of either: **1)** mouse recombinant VEGF 164 protein (50ng/mouse, 200ng/mouse, or 400ng/mouse), or **2)** vehicle (0.1M PBS). Both groups were injected in the intra-periotneal cavity (IP) in the morning and intra-vaginally (IV) in the evening over a period of four days. Following treatment, cervical tissues were harvested, split into ecto-cervices (end near vagina) and endo-cervices (end near uterus), and fixed in 2.5% glutaraldehyde (dissolved in 0.1M PBS) immediately after sampling to be analyzed using SEM (morphology).

b) Time-Dependent VEGF Study: Non-pregnant, ovariectomized mice were treated with a single injection (IP) of recombinant VEGF (200ng/mouse, IP) and divided into 3 different groups, based on the time that lapsed after VEGF treatment, i.e., 1hr 45min, 2hr 45min, and 4hr 45min. Animals were sacrificed on the second day, as described earlier, and tissues were then harvested, and fixed in 10% formalin and were analyzed using immunofluorescence (confocal microscopy).

c) Cell Proliferation Study: Ovariectomized mice were treated with a single injection (IP) of recombinant VEGF (400ng/mouse, IP) and divided into 3 different groups, based on the time that lapsed after VEGF treatment, i.e., 1hr 45min, 2hr 45min, and 4hr 45min. In addition, a day prior to and during VEGF treatment, both groups were also treated with 50µL (IP) BrdU, as described by the manufacturer (Invitrogen, Fredrick, MD). Animals were sacrificed on the second day, as described earlier, and tissues were then harvested, fixed in 10% formalin, and examined using immunohistochemistry (Inverted Olympus Light Microscope).

d) Optimal VEGF Treatment Study: Ovariectomized mice were divided into 2 treatment groups (n=3) as follows: **1)** Group 1 was given two consecutive injections of recombinant VEGF [200ng dissolved in 25µL of saturated pluronic gel and 0.1M PBS, 25µl/per injection, IV] administered at time = 0hr and 4hr. Animals were sacrificed at 8 hours post-initial injection, as described earlier, and tissues were then harvested and frozen at -80°C, and examined using quantitative real-time polymerase chain reaction (qRT-PCR).

e) Pregnant Baseline Gene Expression Study: Pregnant animals from days 11-17 of pregnancy (n=3) were used (untreated). Animals were sacrificed on every day of pregnancy prior to 12noon, tissues were then harvested and frozen at -80°C, and examined using qRT-PCR.

f) Pregnant VEGF, VEGF Blocker Gene Expression Study: Pregnant animals from days 11 and 17 of pregnancy were divided into three groups and treated with either VEGF (n=3), VEGF Blocker (n=3), or vehicle (n=3). **1)** Group 1 was given two consecutive injections of recombinant VEGF [200ng dissolved in 25µL of saturated pluronic gel and 0.1M PBS, 25µl/per injection, IV]. **2)** Group 2 was given two consecutive injections of VEGF Blocker [5mg VEGF Blocker/kg body weight, 50µL/per injection, IP]. **3)** Group 3 was given two consecutive injections of vehicle [25µL of saturated pluronic gel and 0.1 PBS, 25µl/per injection, IV]. All treatments were administered at time = 0hr and 4hr and tissues harvested at t = 8hr.

Techniques Used in the Study

I. Determination of VEGF's Effects on Cervical Epithelial Morphology Using SEM

Experiments were undertaken to visualize, in more depth, the effects of VEGF on the morphology of cervical epithelia in ovariectomized mice. After overnight fixation in 2.5% glutaraldehyde, the tissues were washed with the buffer, then dehydrated in a graded series of ethanol, and dried with a critical point drying apparatus (Polaron Instruments Inc., Doylestown, PA). All dried samples were mounted on aluminum stubs, sputter coated with gold, and imaged with a Quanta 200 SEM (FEI Company, Hilsboro, OR) at 20 kV.

II. Determination of VEGF's effects on expression of claudin-5 in cervical epithelial cells

Because our SEM data in VEGF-treated mice showed infiltration of WBC into the lumen of the cervix, we sought to examine the effects of VEGF on the expression of a key protein known to "glue" epithelial cells together, namely claudin-5, using confocal immunofluorescence. Cryostat sections were cut and processed for confocal immunostaining using Claudin-5 (primary dilution: 0.5μg/mL solution; secondary antibody (FITC) dilution: 1.0μg/mL solution, as described by the manufacturer, Santa Cruz Biotechnology). Following staining, sections were mounted using mounting medium and viewed using a confocal microscope (Zeiss LSM510, Thornwood, NY).

III. Determination of VEGF's effects on cervical epithelial proliferation using BrdU

This experiment was undertaken to test whether our earlier speculation that VEGF causes cervical epithelial cell proliferation, based on increased folds, as revealed by SEM data (Mowa et al., 2008), was valid. We utilized BrdU, the gold standard for marking cell proliferation. In order to determine the optimal time for VEGF's effects on cervical epithelial cell proliferation, mice were treated with VEGF and BrdU. Tissues were sectioned using a cryostat and processed for immunohistochemistry using anti-BrdU, as described by the manufacturer (Invitrogen, Fredrick, MD). Following staining, sections were counter-stained with hematoxylin, dehydrated in a series of ethanol and dipped in xylene before mounting. Mounted sections were then viewed and analyzed under a microscope (Inverted Olympus Light Microscope).

IV. Determination of VEGF, Flt-1, and KDR Gene Expression in the Cervix Studies

Gene expression analysis was performed using qRT-PCR to determine the extent to which different VEGF agents (recombinant VEGF164 protein and VEGF Blocker) influence mRNA expression of VEGF and its receptors, KDR and Flt-1, in the cervix of both pregnant and non-pregnant mice. Gene expression analysis was performed in three steps, as described below:

i) *Tissue processing, messenger RNA isolation, and quantification:* Following treatments, animals were euthanized and trans-cardially perfused with normal saline (0.9% sodium chloride). The cervices were harvested immediately, snap-frozen and either processed or stored at -80°C until processing. Total RNA was isolated from individual cervices using the RNeasy Mini Kit (Qiagen, Valencia, CA) and then the quality and quantity of each sample was estimated using Nanodrop Spectrophotometer (ND-1000 V3.1.2, 2001; Nanodrop Products,Wilmington, DE). Aliquots of total RNA were diluted in RNase-free deionized (DI) water and either stored at -80°C or processed for reverse transcriptase PCR, as described below.

ii) *Reverse transcriptase PCR (RT-PCR):* Total RNA from the cervical tissue was reverse-transcribed and amplified in an Eppendorf Master Cycler (Hamburg, Germany) using reagents from Applied Biosystems (Foster, CA). For generation of complementary DNA (cDNA), 1.0 µg of previously isolated total RNA was placed in a total volume of 9.5 µL per sample with RNase-free water, as was determined by Nanodrop Spectrophotometer, as described above. The RNA was incubated for 5 minutes at 65°C and cooled to room temperature for 10 minutes. During the cooling period, 9.5 µL of a reverse transcriptase master mix was added to each tube, which was comprised of the following: reverse

transcriptase buffer (2 µL per tube of RNA; Applied Biosystems, Foster, CA); $MgCl_2$ (2 µL per tube of RNA), dNTP (2 µL per tube of RNA); RNase inhibitor (0.5 µL per tube of RNA); RNAse-free water (2µL per tube of RNA); and random hexamers (1 µL per tube of RNA). Lastly, 1.0 µL of MuLV reverse transcriptase was added to each tube. One tube received no reverse transcriptase enzyme to serve as a non-template control for DNA contamination. The Thermocycler was programmed to run at 25°C for 10 minutes, 42°C for 2 hours, 95°C for 5 minutes, and stored at 4°C. The generated total cDNA was then used to evaluate mRNA levels of the genes of interest, as described below.

iii) *Real-time PCR (qRT-PCR):* Relative expressions of the genes of interest (VEGF, KDR, and Flt-1) were evaluated using qRT-PCR. Only fold changes equal to or greater than 2 were accepted as significant. TaqMan® Gene Expression Assays (Applied Biosystems, Foster, CA), which are pre-designed and pre-optimized gene-specific probe sets, were utilized and DNA amplification was performed using the Applied Biosystems qRT-PCR machine (ABI 7300 HT) with the GeneAmp 7300 HT sequence detection system software (Perkin-Elmer Corp.) The PCR reactions were set up in wells of 96-well plates in a volume of 25 µL per well. The reaction components included: 1000 ng (5.0 µL) of synthesized cDNA, 12.5 µL of 2X Taqman® Universal PCR Master Mix, 1.25 µL of 20X Assays-on-Demand™ Gene Mix (e.g. VEGF), and 6.25 µL of qRT-PCR-grade RNAse-free water. The program was set as follows: an initial step of 50°C for 2 min and 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 60 s. The relative amount was calculated from the threshold cycles with the instrument's software (SDS 2.0) according to the manufacturer's instructions. Relative expression levels of the target genes were normalized to the geometric mean of the endogenous control gene, GUSβ.

RESULTS

VEGF alters structure of cervical epithelial cells and induces WBC migration

SEM data analyzed from the ecto- and endo- cervices revealed specific VEGF-induced structural changes in the cervical epithelial cells compared to the control group (Figs.1-2). Specifically, in ecto-cervices, epithelial cells from VEGF-treated animals had more folds, appeared edematous, with less pronounced cellular borders (Fig. 1B, D, F), compared to the control group (Fig. A, C, E). The most obvious features in endo-cervices of mice treated with VEGF compared to the control group were dose-dependent migration of WBC into the cervical lumen cells, but also exhibited less prominent borders (Fig. 2B, D, F). When treated with 400ng of recombinant VEGF protein/mouse, there was induction of white blood cell (WBC) migration in the apical surface of the cervical lumen of endo-cervices and layers of the cervical epithelial cells peeled off in both ecto- and endo-cervices (data not shown).

VEGF alters gene expression of claudin-5, a cervical inter-epithelial adhesion molecule

SEM data revealed that VEGF treatment increases the cervical inter-epithelial cellular gaps. We sought, therefore, to understand the likely mechanisms underlying this phenomenon by examining alteration in the expression of an inter-epithelial adhesion molecule, claudin-5. Consistent with our SEM data, the intensity of the immunofluorescence of claudin-5 was found to be weak in the cervical epithelial of VEGF-treated mice compared to control, based on our subjective assessment (Fig. 3A) i.e., at 1hr 45min, claudin-5

expression was more intense and was confined to the inter-epithelial cellular space (data not shown); whereas, by 4hr 45min, the expression was more diffuse (Fig. 3B), and by 6hr, the localization pattern reverted to that seen earlier at 1hr 45min, i.e., it got confined to the inter-epithelial cellular space.

VEGF induces proliferation of cervical epithelial cells

Treatment of ovariectomized mice with VEGF induced pronounced proliferation of cervical epithelial cells in a time-dependent manner, with the peak expression observed at 4hr 45min (Fig. 4B, E) and the least amount of expression observed at both 1hr and 6hr, respectively (Fig. 4A, C, D, E). Intestines from either group (VEGF or vehicle only treated groups) were used as positive control (Fig. 4G).

VEGF alters gene expression of VEGF and its Receptors

To understand or elucidate the underlying mechanism of VEGF's effects reported above, we sought to examine the effects of VEGF protein on its self and receptors using gene expression. Data generated from real-time PCR analysis showed VEGF down-regulated expression of Flt-1 (Fig. 5A), KDR (Fig. 5B), and itself (Fig. 5C) compared to control (n=3). These results revealed that VEGF is having an effect on the cervical epithelium based on the fact that animals treated with VEGF have a lower expression of the receptors for VEGF and VEGF itself (Fig. 5).

VEGF alters gene expression of VEGF and its Receptors in cervices of pregnant mice

Levels of VEGF mRNA and the mRNAs of both of its receptors were found to remain constant from gestational age days 11 through 16, but increased sharply by day 17 of

gestational age ($p<0.05$) (Fig. 6). In VEGF-treated animals compared to control (n=3), at day 11 and 17 of pregnancy; VEGF, KDR, and Flt-1 are being up-regulated. In VEGF-blocker treated animals compared to control (n=3), at day 11 and 17 of pregnancy; VEGF, KDR, and Flt-1 are being down-regulated (Fig. 7).

DISCUSSION

We have previously shown, using SEM, that exogenous VEGF administered to ovariectomized mice induces folding of the cervical epithelia layer, and based on these findings, suggested that VEGF induces proliferation of cervical epithelial cells. The present study builds on these earlier observations and was aimed at investigating the effect of VEGF on cervical epithelial cell proliferation using a more definitive technique, namely, BrdU. The key findings of this study are: VEGF 1) induced pronounced proliferation of cervical epithelial cells in a temporal- and dose-dependent manner; 2) promotes WBC migration into the cervical lumen, by diminishing the expression of cervical inter-epithelial cell adhesion proteins, such as claudin-5; 3) induces folding and edema in cervical epithelial cells; and 4) VEGF Blocker alter mRNA expression of VEGF and its receptors (Flt-1 and KDR), in a physiologically-dependent manner. Collectively, these findings suggest that VEGF plays a possible role in CR by influencing the overall growth of the cervix, specifically, events in the cervical epithelial cells, which (epithelial cells) are now thought to play the central role in cervical remodeling.

The induction of edema in cervical epithelial cells by VEGF shown in this study is consistent with previous studies that have reported that cervical edema is a prominent feature of CR in experimental animals and humans (Burger & Sherwood, 1998; Myers, Socrate, Tzeranis & House, 2009). However, these earlier studies did not identify the factors that induce this condition. Here, we show that VEGF is a likely candidate for inducing this condition (edema) in the cervix. This conclusion is not unusual in that VEGF's ability to

induce edema in tissues is a well-established phenomenon, especially in tissue types such as uterine tissue (Van Bruggen et al., 1999). In fact, VEGF was first described as a potent vascular permeability factor (Senger et al., 1983). For instance, when compared to one of the most potent endogenous vascular permeability factors, namely histamine, which is released during a bee sting, VEGF is capable of inducing edema about 50,000 times more than histamine (Van Bruggen et al., 1999). It is, therefore, likely that VEGF may account for the cervical edema that is prominently present in the cervix of humans and experimental animals during pregnancy. Future studies in our lab will attempt to address this knowledge gap.

In edematous tissues, infiltration of WBCs is not uncommon. However, migration or presence of immune cells into the cervical lumen as observed in the present study is an interesting phenomenon that has not been reported before, and its functional significance in CR, for now, is unclear. It is also not clear at this point whether this observation is physiological, i.e., whether it occurs during pregnancy. If indeed it does, it could perhaps signify the formation or presence of the first line of defense during pregnancy. More specifically, these cervical luminal migrant immune cells may prevent or inhibit the possible entry of vaginal microbes into the uterus. If vaginal microbes break through the epithelial barrier and into the uterus, the consequences would be contamination of the sterile fetal amniotic fluid, ultimately leading to placental infection, which is one of the most potent and major inducers of preterm birth (Bosquiazzo et al., 2005). Thus, these immune cells may create a buffer zone between the sterile fetal amniotic fluid housed in the uterus and the microbe-rich vaginal environment. This line of defense may complement the well-known mechanical barrier to infection provided by epithelial cells which are generally observed at the body's entry points, which, in this particular case, are cervical epithelial cells. To date the

types of immune cells infiltrating the cervical lumen remains unknown. However, our preliminary data in other projects have demonstrated the presence of macrophages in the cervical epithelial during pregnancy and postpartum using the macrophage marker primary antibody from Santa Cruz Biotechnolgy (unpublished data). Taken together, if indeed our current interpretation is true, VEGF may play an important role in preventing infection-induced CR and preterm labor in general.

The exact process mediating VEGF-induced infiltration of immune cells through the tightly-regulated cervical inter-epithelial cell border, as revealed by SEM data, is currently unclear. However, this phenomenon is not unusual in vascular endothelial cells (Antonetti, Barber, Hollinger, Wolpert & Gardner, 1999; Timmons & Mahendroo, 2007). VEGF readily induces infiltration of local tissues by microvascular-derived immune cells by vaso-permeability through alterations in the expression of cell adhesion molecules (Antonetti et al., 1999; Timmons & Mahendroo, 2007). Indeed, we have previously shown that VEGF alters expression of vascular cell adhesion molecule (VCAM-1) in the cervix of non-pregnant and pregnant rodents using DNA microarray studies and real time PCR analysis (Mowa et al., 2004a). However, the present study is the first to demonstrate VEGF's ability to increase intercellular gaps between cervical epithelial cells, which, like all epithelial cells, belong to the same general family as vascular endothelial cells. It is feasible that VEGF altered the expression of cervical inter-epithelial tight junction (TJ) proteins, such as claudin-5 shown in this study.

Tight junctions have two main components or group of molecules, namely occludin and claudin. More specifically, claudin is a member of a family of proteins consisting of over 20 members (Gonzalez-Mariscal, Betanzos, Nava & Jaramillo, 2003). It has been

shown that over-expression of mutant forms of occludin in epithelial cells leads to changes in the "gate" functions of the TJ (McCarthy et al., 1996). Of relevance to the present study, claudins are also regulated in cervical epithelial cells during pregnancy and have a comparable temporal and spatial relationship with VEGF (Mowa et al., 2004a). Tight junctions are also found between endothelial cells. Claudin-5 is a transmembrane protein and is primarily present in TJs of endothelia and has recently been localized to an epithelial cell line derived from human colon (HT-29/B6). Three types of transmembrane proteins located in epithelial and endothelial TJs have been identified (Amasheh et al., 2005). Although functional studies of claudin-5 have just begun recently, it has been localized to the tight junctions of both endothelial and epithelial cells in the intestine (Amasheh et al., 2005). Therefore, it is possible that cluadin-5 may mediate VEGF induction of immune cell migration into the cervical lumen.

The cervical epithelium serves multiple roles in the female, depending on the physiological conditions. The cervical epithelia, along with mucus, play an important protective role during pregnancy and the birth process (Timmons et al., 2010). These roles also include prevention of intra-uterine infection, and protection against mechanical assault during delivery. Epithelia accomplish these roles by undergoing marked proliferation during pregnancy (Timmons et al., 2010). Other non-physical or mechanical roles include innate and adaptive immunity (Timmons et al., 2010). The cervical epithelium has recently been shown to secrete cytokines and chemokines that recruit and activate inflammatory cells and antimicrobial factors that eliminate invading pathogens (Timmons et al., 2010).

VEGF causes epithelial cell structural changes in the cervix. The present study shows that VEGF treatment affects cervical epithelial cell structure and the amount of ciliated cells

present in the epithelium. Based on observations, cervical epithelial cells in VEGF-treated animals appeared swollen with less defined boundaries in comparison to the control group; in addition, ciliated cells were also less abundant. This suggests that VEGF may not only influence the growth of cervical epithelial cells, but also their overall viability, structure, as well as the presence of ciliated cells. In addition, VEGF likely plays an important role in CR, in part, by influencing growth of cervical epithelial cells, a cell type believed to play a central role in CR, according to recent studies.

It is important to note that in the data presented in this paper, there were some significant differences between the endo-cervix and the ecto-cervix. This could be due to the fact that the endo-cervix is comprised of simple columnar epithelial cells (Timmons et al., 2010). For instance, in some tissue types, including the cervix, simple columnar epithelium secrete mucus, which may act as a lubricant (Timmons & Mahendroo, 2007). In contrast, ecto-cervical epithelium consists of stratified squamous, non-keratinized cells. This type of epithelium is typically multilayered and found in tissues that with-stand mechanical or chemical insult, such as our skin and the vagina (Timmons et al., 2010).

The functional significance of VEGF's effects on cervical epithelia growth, as demonstrated here, is for now unclear. During CR, the cervix undergoes pronounced growth over the course of pregnancy (Burger & Sherwood, 1998). In a previous study it was found that in control rats, nearly 75% of the epithelial cells and 55% of the stromal cells in the cervix at term had proliferated during the second half of pregnancy (Burger & Sherwood, 1998). This dramatic increase in cervical epithelial growth may reflect its overall role in CR. Indeed, recent reports have suggested that cervical epithelial may play the central role during CR (Timmons et al., 2010). Since the epithelia accounts for a good portion of cervical

growth, it may be the major contributing tissue that provides the biomechanical resistance to the ever-increasing gravitational force exerted by the growing fetus on the cervix. Our current results are consistent with previous studies using fetal lung, showing VEGF-induced increase in epithelia growth (Brown, England, Goss, Snyder & Acarregui, 2001).

To date, cervical growth during pregnancy has been largely attributed to relaxin, a protein hormone produced by the ovarian corpus luteum and the breast during pregnancy (Burger & Sherwood, 1995). Like VEGF, relaxin also enhances angiogenesis and is known to induce vasodilation in some tissues, such as the kidneys (Mookerjee et al., 2006). Relaxin also increases proliferation of epithelial and stromal cells in the rat cervix during the second half of pregnancy (Burger & Sherwood, 1995), and is known to stimulate expression of VEGF in cultured human endometrial cells (Burger & Sherwood, 1995). Taken together, we suggest that VEGF and relaxin in the cervix could either have a synergistic relationship or that VEGF maybe downstream of relaxin or vice versa. Future studies should decipher the effects of VEGF on cervical epithelium growth from those of relaxin.

This study has shown that mRNA levels of VEGF and its receptors, KDR, and Flt-1, are lower earlier in pregnancy (day 11), but peak sharply in late pregnancy (day 17). This trend of mRNA expression of VEGF and its receptors resembles those reported in our previous studies which used pregnant rats using Western blot, ELISA and confocal immunofluorescence (Mowa et al., 2004a). In our earlier pregnant rat study, protein expression of VEGF and its receptors were overall lower in early pregnancy (day 8) compare to late pregnancy (day 22) (Mowa et al., 2004a).

The treatment data of pregnant mice when treated with VEGF protein agents shows up-regulation of mRNA levels of VEGF and its receptors. When treated with VEGF blocker

protein there was a down-regulation of mRNA levels of VEGF and its receptors, which implies that rising levels of VEGF over the course of pregnancy may stimulate a further increase in VEGF. It is interesting that the effects of exogenous VEGF on the expression of endogenous VEGF mRNA in ovariectomized non-pregnant mice are just the opposite, i.e., instead of inducing VEGF expression, it down-regulates it. It is not clear at this point why there seem to be an apparent discrepancy between these data. It is possible that presence of sex steroid hormones in pregnant mice may account for this difference.

In conclusion, the present study has shown that VEGF induces multiple alterations in the cervical tissues of mice, notably increased epithelia growth, folding, edema, down regulation of tight junction molecules, such as claudin 5, which possibly results in increase in epithelial cell paracellular spaces leading to migration of WBC into cervical lumen. Collectively, these findings suggest that VEGF plays a role in CR by inducing physiological inflammation (WBC infiltration, tight junction molecules and paracellular spaces, and edema) and cervical growth (epithelial growth) during CR.

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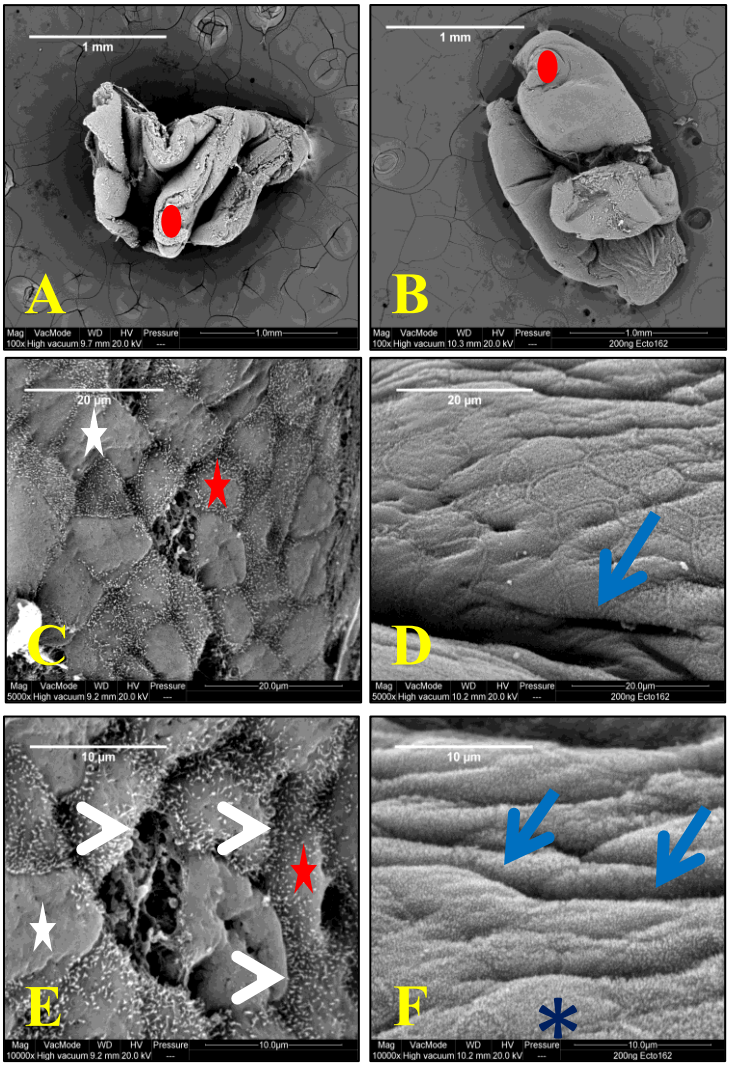

Figure 1. *Effect of VEGF on the ultra-structure of the ecto-cervical epithelial cells in non-pregnant ovariectomized mice as revealed by scanning electron microscopy:* mice treated with VEGF recombinant protein 164 (B, D, F) (200ng/mouse, single treatment of either IP or intra-vaginal) induces folds (blue arrows) and a general swelling in cervical epithelial sheets compared to the control group (A, C, E), where cell types, ciliated (red stars) and non-ciliated (white stars), borders (short white arrows), are more distinct than in VEGF-treated mice (*). Red circle in A and B are sites on the *ecto*-side of the cervix from which images C-F were taken (n=1). Magnification: A,B=x100; C,D = x 5,000; E,F=x10,000.

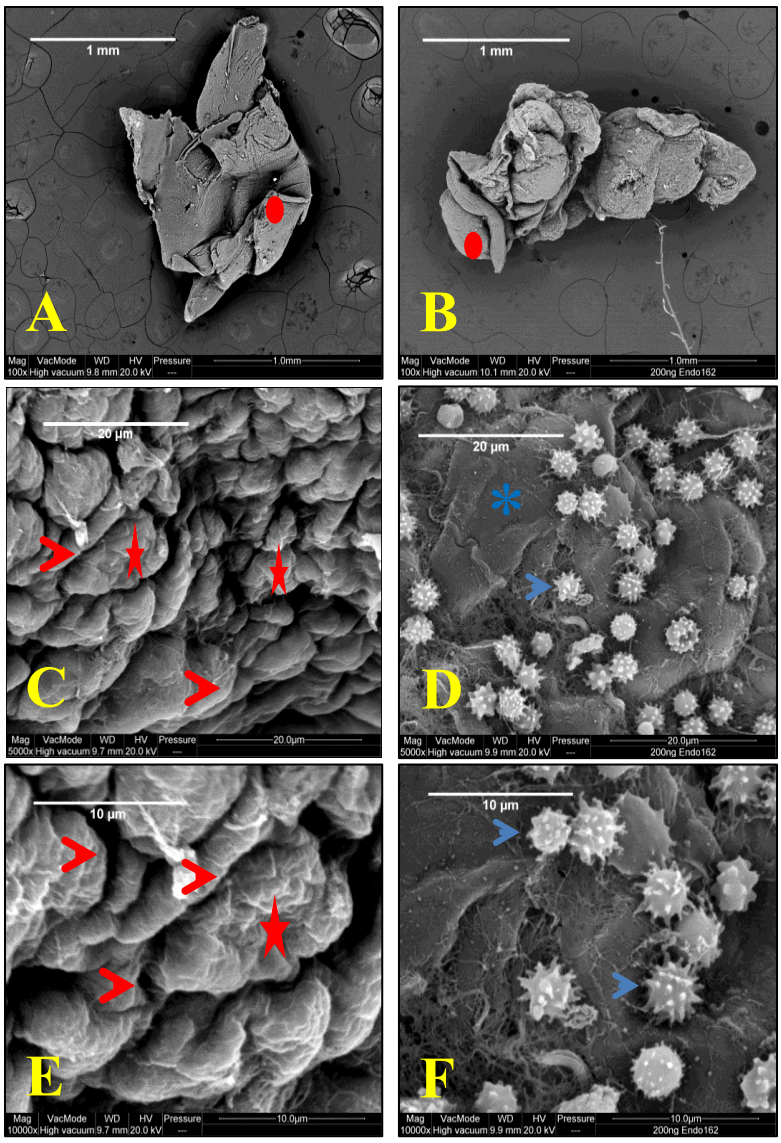

Figure 2. *Effect of VEGF on the ultra-structure of the endo-cervical epithelial cells in non-pregnant ovariectomized mice as revealed by scanning electron microscopy:* mice treated with VEGF recombinant protein 164 (B, D, F) (200ng/mouse, single treatment of either IP or intra-vaginal) causes WBC (blue arrows) migration from cervical tissue into the cervical lumen and a change in cellular shape compared to the control group (A, C, E). Cells in control group (red stars) have distinct borders (red arrows) compared to those in VEGF-treated mice (*). Red circle in A and B are sites on the *endo-* side of the cervix from which images C-F were taken (n=3). Magnification: A, B=x100; C, D = x 5,000; E, F=x10,000.

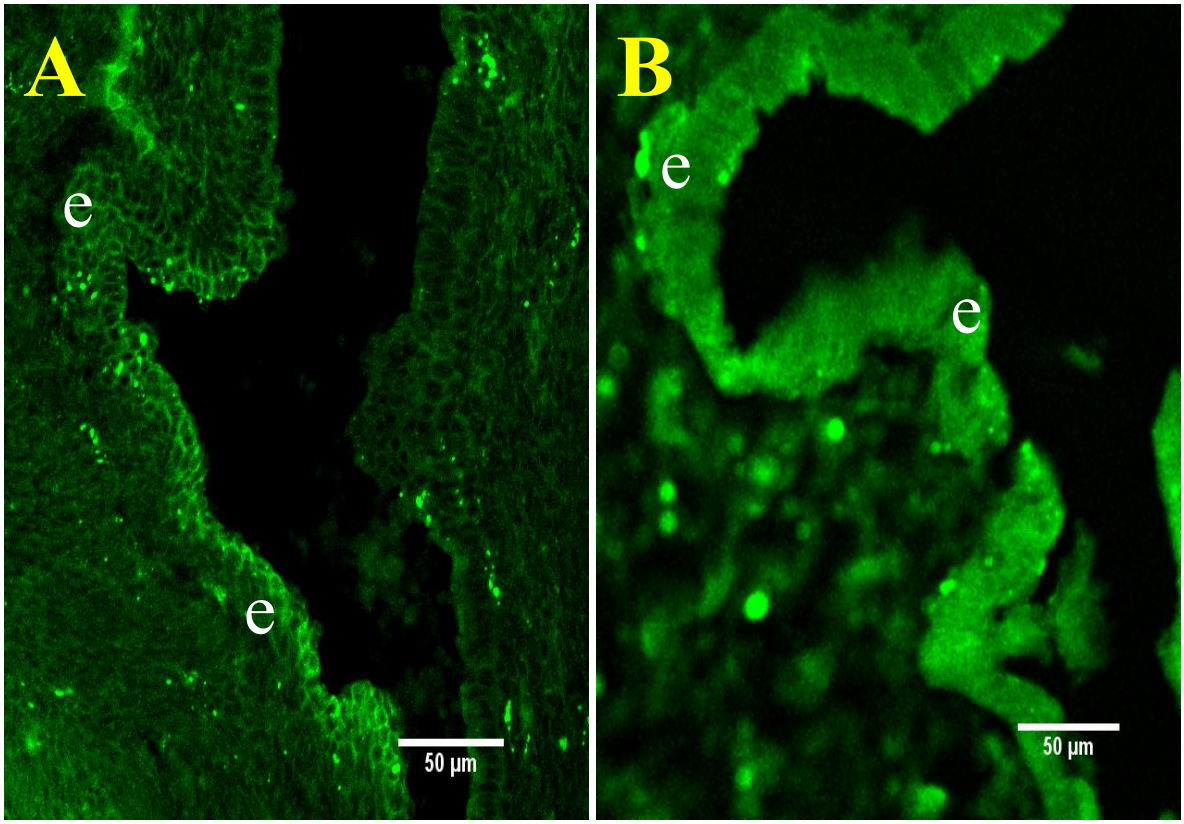

Figure 3. *Effect of VEGF on the expression of claudin-5 protein in cervical epithelial cells of non-pregnant, ovariectomized mice as revealed by immunofluorescence:* VEGF recombinant protein 164 (200ng/mouse, single treatment of either IP or intra-vaginal) diminished expression of claudin-5 protein (B) in a time-dependent fashion between intercellular gaps of cervical epithelial cells (**e**) in ovariectomized mice, shown here using confocal immunostaining. The maximum effects of VEGF occurred at about 4hrs (B). Claudin-5 expression in control group (A) were more pronounced and localized between the inter-epithelial space, the normal locale of claudin-5 (n=3).

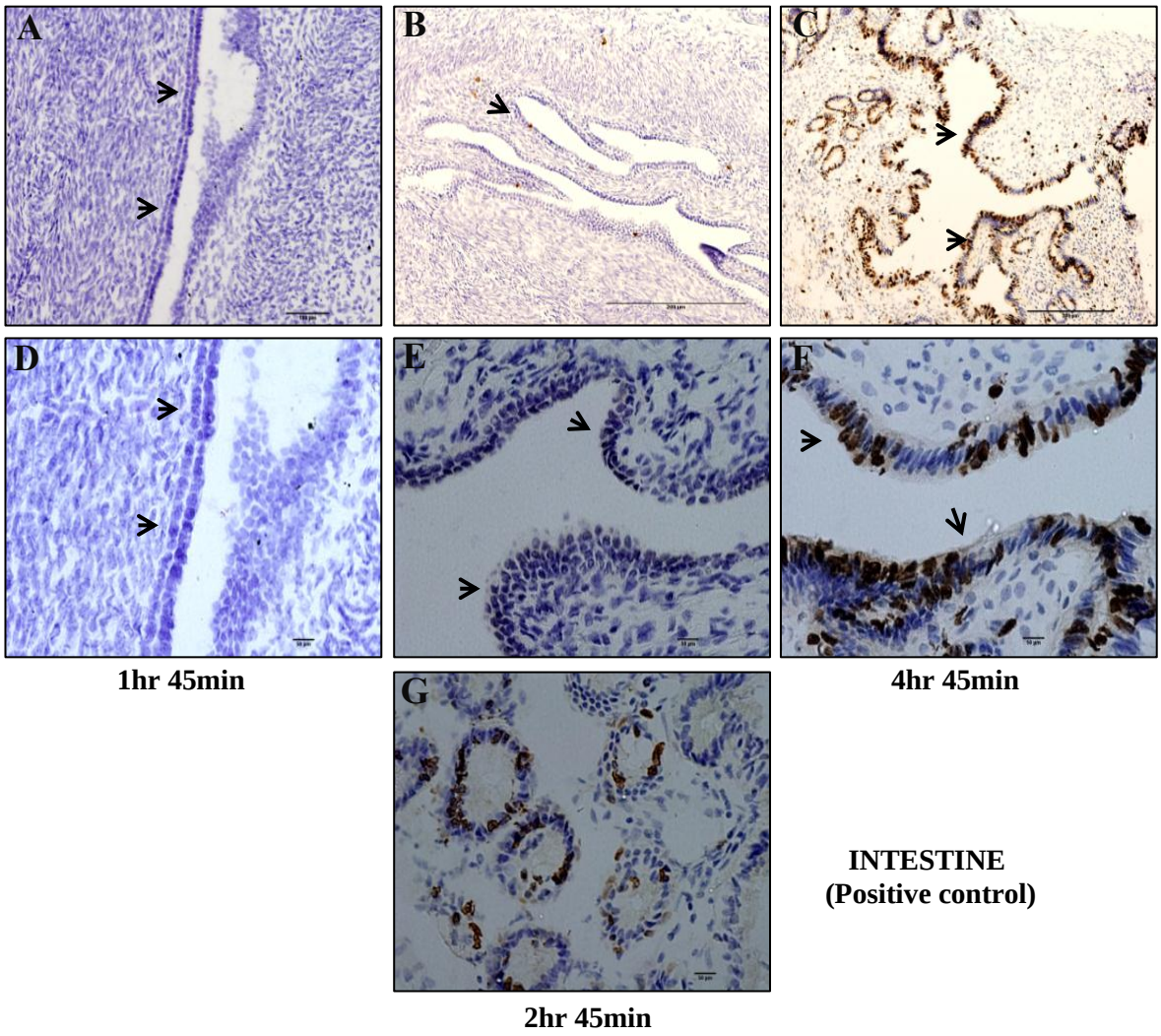

Figure 4. *Effect of VEGF on cervical epithelial cell proliferation in non-pregnant ovariectomized mice as revealed by immunohistochemistry:* VEGF recombinant protein 164 (200ng/mouse, single treatment of either IP or intra-vaginal) induces proliferation of cervical epithelial cells in ovariectomized mice, shown here using bromodeoxyuridine (BrdU) immunostaining, a marker for proliferation (brown staining). The maximum proliferative effects of VEGF was observed at about 5hrs (C, F) and by the 6th hr post VEGF treatment, BrdU immunostaining significantly diminished. Small intestines, which have a robust proliferation, were used as positive control tissue (G)(n=3).

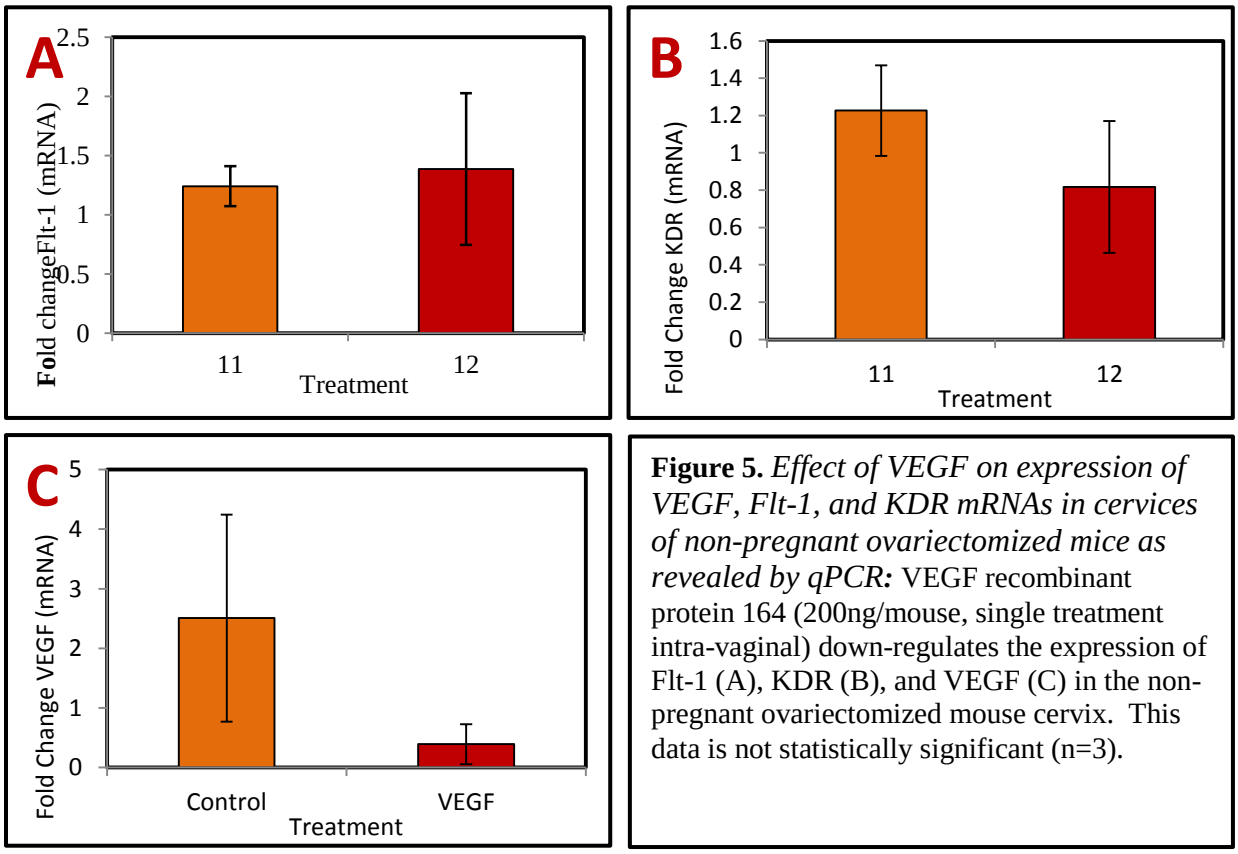

Figure 5. *Effect of VEGF on expression of VEGF, Flt-1, and KDR mRNAs in cervices of non-pregnant ovariectomized mice as revealed by qPCR:* VEGF recombinant protein 164 (200ng/mouse, single treatment intra-vaginal) down-regulates the expression of Flt-1 (A), KDR (B), and VEGF (C) in the non-pregnant ovariectomized mouse cervix. This data is not statistically significant (n=3).

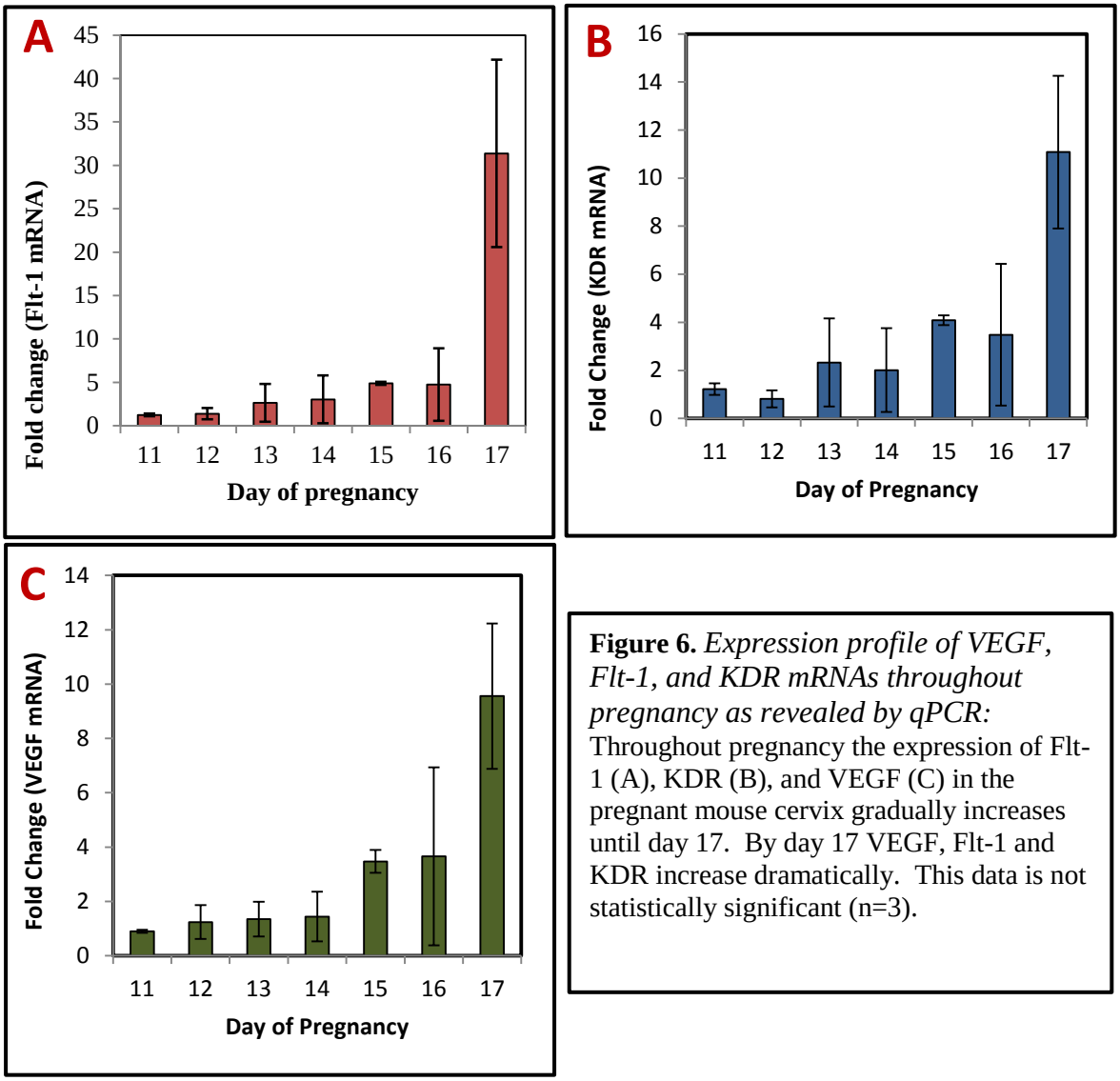

Figure 6. *Expression profile of VEGF, Flt-1, and KDR mRNAs throughout pregnancy as revealed by qPCR:* Throughout pregnancy the expression of Flt-1 (A), KDR (B), and VEGF (C) in the pregnant mouse cervix gradually increases until day 17. By day 17 VEGF, Flt-1 and KDR increase dramatically. This data is not statistically significant (n=3).

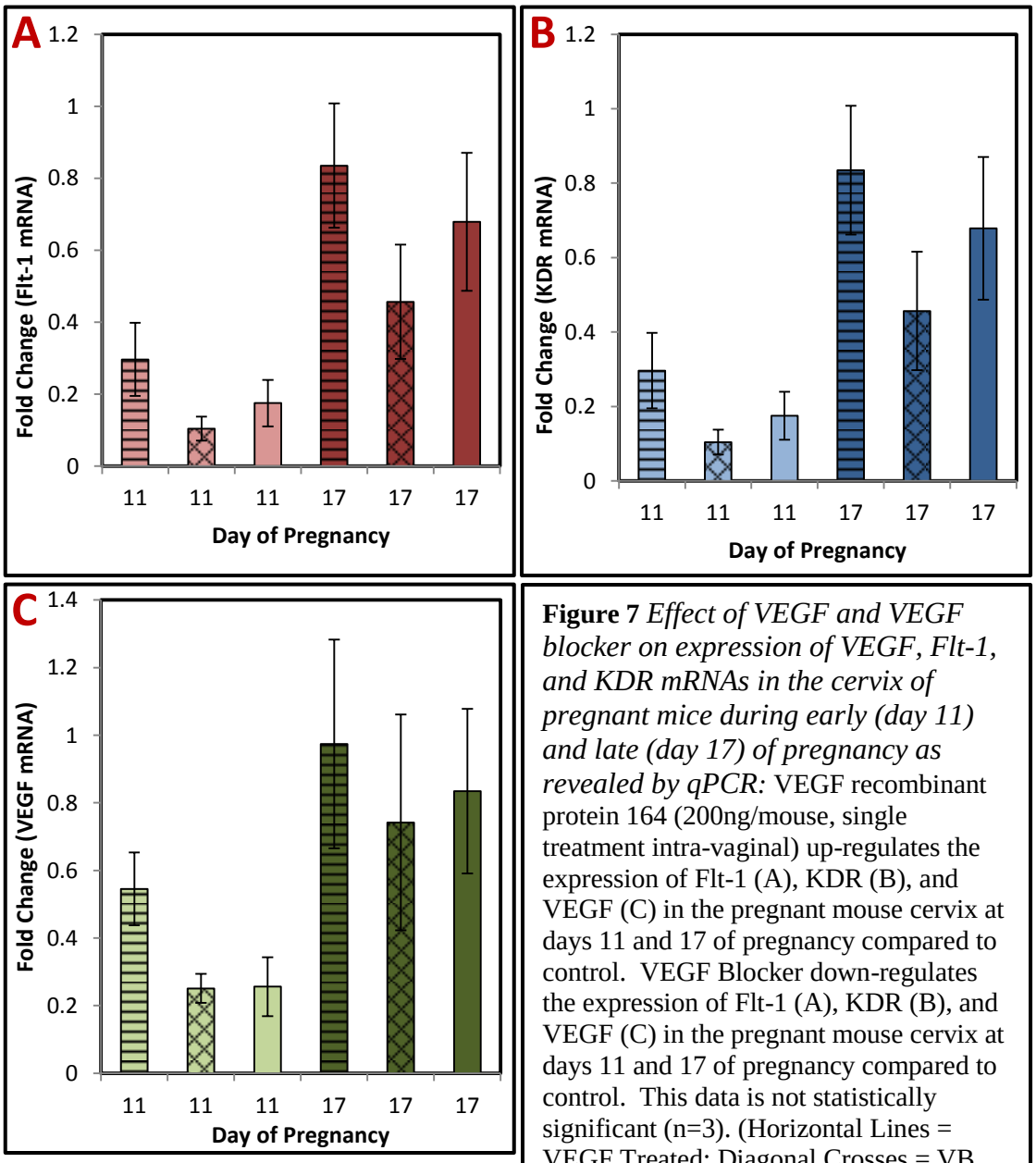

Figure 7 *Effect of VEGF and VEGF blocker on expression of VEGF, Flt-1, and KDR mRNAs in the cervix of pregnant mice during early (day 11) and late (day 17) of pregnancy as revealed by qPCR:* VEGF recombinant protein 164 (200ng/mouse, single treatment intra-vaginal) up-regulates the expression of Flt-1 (A), KDR (B), and VEGF (C) in the pregnant mouse cervix at days 11 and 17 of pregnancy compared to control. VEGF Blocker down-regulates the expression of Flt-1 (A), KDR (B), and VEGF (C) in the pregnant mouse cervix at days 11 and 17 of pregnancy compared to control. This data is not statistically significant (n=3). (Horizontal Lines = VEGF Treated; Diagonal Crosses = VB Treated

Biographical Sketch

Siobhan Maureen Donnelly was born in Helena, Montana to parents Peter and Lynn on July 4, 1986. She became a younger sister to Sarah, who is 13 months older and was born with Downs Syndrome. Three years later, Sarah and Siobhan were blessed with the addition of their brother, Thomas, and five years after Tom, Mackenzie was born. The four of the Donnelly children grew up in the beautiful mountains of Montana and were introduced to a variety of sports at a very young age. Siobhan and her siblings became US and world travelers due to the fact that their father was originally from Australia. They would make frequent trips down under to visit family and friends.

A 2004 Helena High graduate, Siobhan was a two-sport athlete in basketball and volleyball. Following high school, she traveled to eastern Montana to attend Miles Community College on a basketball scholarship. After one year at MCC, Siobhan transferred to a 4-year university to pursue her Bachelor's degree. University of Great Falls became the new campus and basketball team to Siobhan over the next 4 years. She graduated from UGF in May 2010 with a Bachelors of Arts degree in pre-professional Biology with a minor in chemistry.

Upon college graduation, she decided to complete a Master of Science degree at Appalachian State University. She has been studying female reproduction, specifically cervical remodeling, under the guidance of Dr. Nathan Mowa for the last two years. After graduation, Siobhan will pursue a career in the cell and molecular field of research.