Determining the Role of the Perivascular Microenvironment on Reproductive Function in an Organ-on-Chip Model of the Human Endometrium

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This work is dedicated to my supportive family and friends and the women who kindly donated their uterus for reproductive research.

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Chapter 1

Introduction

> "*Molecular studies of the endometrium have been impeded by the lack of a cell culture system that retains the hormonally induced proliferation and differentiation occurring in vivo. [Furthermore], it is probable that for full functional differentiation, epithelium and stroma requires correct spatial relationship within a well-defined extracellular matrix*".
>
> – Dr. John Aplin, 1989[1]

The human female reproductive tract is a complex system of interactive organs that not only maintains local hormonal control but also significantly influences the metabolic state of other organ systems[2,3]. Defining the biological homeostasis of uterine function is fundamental to our overall comprehension of reproductive tract function. In particular, the current understanding of the menstrual cycle has vastly grown in the last century due to key technological advancements such as immunohistochemistry, radioimmunoassay (RIA), high-resolution imaging techniques and genetic analysis[1,4,5]. However, advancements in reproductive medicine and the development of treatments for uterine disorders have been hindered by the lack of appropriate experimental models that physiologically and functionally mimic the human condition. As described in the quote above, reproductive research has been urgently missing human models robust enough to capture the numerous

morphological and biochemical changes associated with the microenvironment of endometrial physiology. Although some of the anatomical and molecular events of the endometrium have been highly documented during the menstrual cycle, this information is constrained by the end-point nature of these assays, which only offer an oversimplified static snapshot of the events that occur in a cycling endometrium. This suppresses the ability to examine the spatio-temporal framework of changes in real-time that are critical to comprehend endometrial receptivity during implantation and endometrial-related disorders. This issue has limited our ability to understand the mechanistic biology behind the menstrual cycle, and thus the pathophysiology of disease and the development of novel therapeutics. Specifically, the mechanisms that drive dysregulation of tightly regulated inflammatory processes within the reproductive tract due to environmental or pathological stimuli remain elusive. To address these limitations, in this thesis we introduce a platform to recapitulate the cellular components of the human endometrial microenvironment to provide analysis of cell-cell communication and specifically model the immune-endocrine pathways of the cyclic endometrium in a controllable microfluidic system. Although the enthusiasm for an *in vitro* model of the endometrium was initially voiced decades ago, we now have technological advances to accomplish this goal. Novel organ-on-a-chip (OoC) tissue-engineered microfluidic devices can provide an innovative solution for overcoming these technical boundaries.[6–10] Altogether, integration of engineering microfabrication technologies and cell-culture techniques now opens the opportunity to recreate an *in vitro* endometrial menstrual cycle by developing an Endometrium-on-a-Chip (EndoChip) (Supplementary Figure 1-4). In this thesis, we develop the first OoC of the endometrial perivascular microenvironment.

1.1 The endometrium as a paradigm for reproductive function

The female reproductive tract represents the collection of organs and specialized tissues that evolved in order to orchestrate and fulfil the primal necessity of live birth, allowing mammalian species to exist. This organ system is comprised by the vagina, cervix, uterus, fallopian tubes and ovaries in ascending order and utilizes hormones to maintain communication and dictate timely and orchestrated functions.[2] Regulation of such a delicate system is maintained by multifactorial events which include, but are not limited to, endocrine, paracrine and immunological processes that occur in a spatio-temporal manner. Additionally, reproductive organs are necessary to maintain systemic hormonal fluxes that subsequently influences many organs at distant sites[2,3]. In women of childbearing age, the first phase of cyclic reproductive function is influenced by follicular estrogen production during the growth and release of an egg from the mature ovarian follicle in process referred to as ovulation. This event marks the transition between the ovarian follicular phase dominated by estrogen and the luteal phase, which includes progesterone production. The vagina plays a critical role during fertilization of the female gamete by providing an open tract for insemination while conferring a protective function for the female reproductive tract.[2] Ovulation and the subsequent production of progesterone primes the fallopian tubes and the uterus for fertilization and implantation of an embryo, respectively. During a fertile cycle in which the establishment of pregnancy occurs, the endometrial lining of the uterus undergoes a series of physiological and hormone dependent changes to accommodate and maintain the developing embryo. Specifically, the gravid, or pregnant uterus, undergoes a maternal differentiation process that both allows implantation and contributes to the development of a new interim organ

known as the placenta. Other tissues within the reproductive tract, such as the fallopian tube and cervix, also adapt novel roles during establishment of pregnancy within the uterine corpus, for example, determining the timing of fertilization and producing a mucus barrier that helps protect the environment of the uterine compartment. Altogether, these sex organs individually and collectively provide the necessary and orchestrated series of processes that are required for successful fertilization, embryo implantation and maintenance of pregnancy.

Reproductive health is a complex, and delicate system that is vulnerable to perturbations from environmental and pathological stimuli. Unfortunately, our understanding of the role of individual cells and their functional interactions within individual sex organs is limited. Therefore, the pathophysiology of diseases associated with these tissues that threatens reproductive success, such as infertility, pre-term birth, endometriosis, and infections, remains elusive. As discussed above, the uterus is the major hormone-responsive organ during pregnancy and uterine function during pregnancy is essential to reproductive success because it is responsible for the establishment and maintenance of pregnancy[11] Although the uterus is directly associated with several reproductive disorders, unfortunately, our understanding of the mechanisms that drive the endocrine-mediated physiology of this organ is limited.

Table 1-1. Specialized functions of the endometrium.

Table 1. Specialized functions of the human endometrium:
• Produce glandular secretions that permit efficient sperm migration through the uterine cavity
• Produce glandular secretions which nourish sperm and blastocyst
• Permits and limits implantation of the blastocysts
• Allows placental development and enhances fetal nourishment
• Precipitates in the process leading to the onset of labor
• Undertakes several immunological surveillance roles to defend against microbes, but permit blastocyst acceptance and maintenance
• Undergoes decidual shedding and remodeling post-partum
• Undergoes complete surface repair cycles and prevents scarring and adhesions from developing
• Remains inactive during childhood, lactation and post-menopause
• Performs various complex endocrine and paracrine roles.

The uterus undergoes numerous cyclical changes that make the function of this organ an interesting biological paradigm that represents the intricate spatial and temporal molecular regulation of the female reproductive function. Anatomically, the average uterus is a pear-shaped organ typically ranges in size from eight to ten by six centimeters (cm) and weighs approximately 6 ounces.[12] It is a hollow organ primarily composed of an outer layer of muscular tissue called the myometrium. The inner lining of the uterus, the endometrium, is a unique tissue that undergoes monthly cyclical changes in response to increasing levels of the ovarian-derived sex hormones noted above relative to follicular development and ovulation, namely estradiol (E2) and progesterone (P4).[5,13] The specialized endometrium is a histologically distinct tissue within the inner cavity of the uterus. This spongy mucosal tissue lines the lumen of the otherwise muscular uterus and is responsible for the implantation of the embryo. Upon implantation, the endometrium undergoes a series of morphological and biochemical changes that promote the establishment of pregnancy by maintaining the development of the embryo and ultimately will transform to make up the maternal, or decidual, part of the placenta. These hormone-induced changes help regulate embryo implantation and invasion, thereby making the endometrium an essential component of the maternal-fetal interface during normal pregnancy. Alterations in these changes can be conducive to aberrant implantation, placentation and adverse pregnancy outcomes.[11,14,15] If no pregnancy occurs, the endometrium fails to fully decidualize due to a sharp withdrawal of P4 which induce the shedding the endometrial tissue, a process known as menstruation as discussed in detail below (Section 1.2). Thus, during a woman's reproductive life, under the influence of E2 and P4, the endometrium will undergo cyclical events that start at the onset of puberty (i.e.

menarche) that prepare the endometrium for embryo implantation monthly until reaching menopause. This cyclical pattern of endometrial priming and shedding is known collectively as the menstrual cycle.[16]

1.2 Characterization of the menstrual cycle

Traditional dating of the human endometrium dates to the 1950s and was first documented in the seminal work by Drs Noyes, Hertig and Rock that characterized the daily changes (*i.e.* dating) of the endometrium throughout the menstrual cycle.[5] This study, and other subsequent studies, have focused almost exclusively on histological changes within the endometrium, primarily on the observable changes of the glandular epithelium and the stromal compartment. As such, the idealized 28-day human menstrual cycle is divided into 3 cyclic phases that are characterized by the specific changes occurring at the anatomical, physiological and molecular level (Figure 1-1). The start of the cycle begins with the *menstrual phase* and is characterized as the first day of endometrial shedding that lasts 2-6 days. Menstruation is then followed by the *proliferative phase* which coincides with the growth and maturation of ovarian follicles. As the name implies, this phase is primarily characterized by the E2 dominant proliferation of the endometrial cells. The proliferative phase is typically annotated as days 4-13 of the menstrual cycle, but this stage can be variable in length. Although it is not directly considered a 'phase', ovulation occurs on approximately day 14 of the cycle and is indicative of the transition into the secretory phase in concert with a rise in local P4 hormone levels. The secretory phase is sometimes called the luteal phase primarily due to the formation of the corpus luteum in the ovaries, but the secretory phase receives its name from the glandular secretion of mucosal fluid and

secretion of progestational proteins by the stromal fibroblasts within the endometrium in preparation for embryo implantation. In contrast to the proliferative phase, the length of the secretory phase is highly conserved and typically lasts 14 days occurring between days 14-28. In humans, both E2 and P4 are elevated during the secretory phase; and, these steroid hormones are each responsible for inducing the necessary changes observed during this endometrial phase and are conducive to its physiological function. Key temporal processes of the secretory phase include the intrinsic temporal regulation of the potential for blastocyst implantation, also known as the "window of implantation" which occurs between days 22-24. Under the primary influence of P4, the endometrial compartment undergoes the extensive remodelling and differentiation processes necessary for the preparation of invasion of extravillous trophoblasts derived from the implanting embryo.[14,17,18] One of critical processes of successful implantation is the unique reticular differentiation of the endometrial stromal fibroblasts due to increased P4 levels known as decidualization.[19] Following the opening of the window of implantation, the timing of successful decidualization is additionally required for the establishment of pregnancy. This differentiation process is discussed in detail in Section 3.1.2. Importantly, if fertilization does not occur, the endometrium endures ischemic processes mediated by increased rates of apoptosis, increased matrix metalloproteinase (MMP) production. This inflammatory cascade results in the shedding of the upper region of the endometrium (*i.e.* the functionalis), after which, the cycle initiates again.[5,20–24] The menstrual cycle is mediated primarily by hormonal signalling that, through both direct and in-direct cellular and molecular mechanisms, govern the physiological and timely processes associated during each menstrual phase. Additional tractable changes in molecular factors have been

identified that characterize and parallel the cyclical cellular changes occurring during endometrial cycle.[1,5] The uterine endometrial tissue is particularly sensitive to endocrine, paracrine, immunological signals and, unlike other adult organs, it undergoes a cyclical pattern of growth, remodeling, and shedding approximately 400 times during a woman's reproductive life[25,26]. Thus, these multifactorial stimuli and complex tissue dynamics throughout the menstrual cycle make the endometrium highly susceptible to local and systemic reproductive tract pathological conditions. Altogether, the reproductive function of the uterus during each cycle and during pregnancy requires not only the production and appropriate responses to a diverse array of endocrine factors but also a vast complexity of well-timed inter-cellular communication. The major physiological roles of the endometrium are listed in Table 1. These are defined by the field as key specialized functions of the endometrium that ultimately promote the homeostasis of the organ and embryo implantation.[16,27,28]

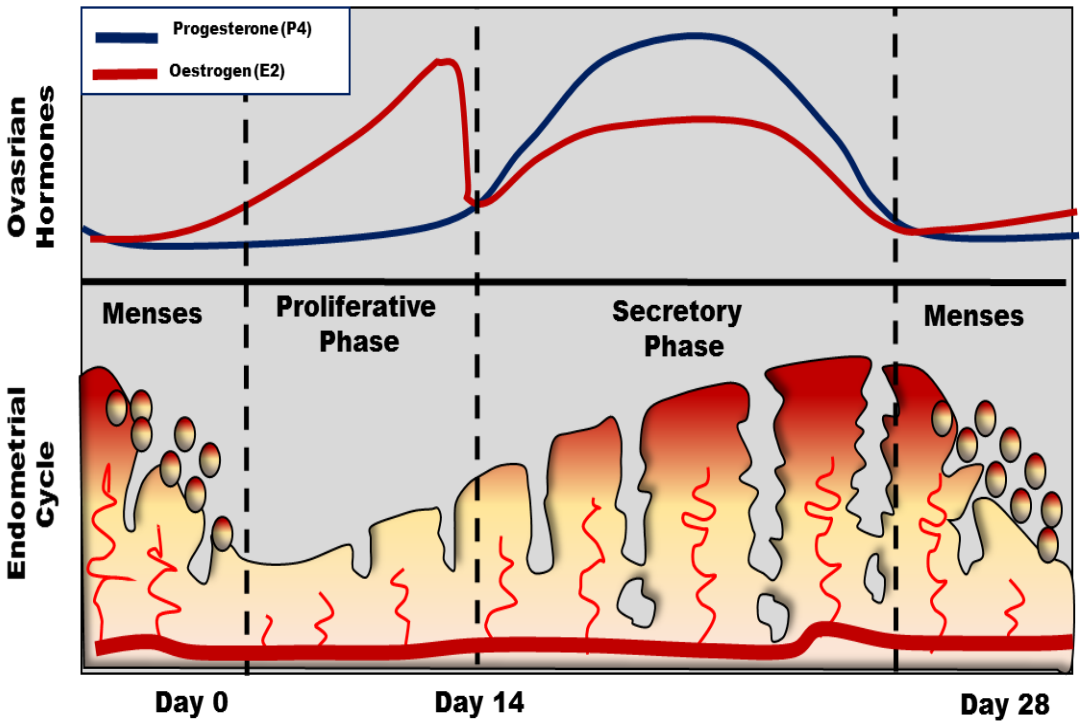


Figure 1-1. A schematic summary of the ovarian hormones changes during the endometrial cycle. The menstrual cycle can vary widely among individual women, but an idealized length is generally considered to be 28 days. During each menstrual cycle, the endometrium undergoes a proliferative phase (6-14 days) when oestrogen concentrations rise up to a level capable of triggering ovulation and the subsequent development of the corpus luteum. In the secretory phase (days 15-28), production of ovarian progesterone promotes endometrial differentiation. The endometrium later experiences a sharp withdrawal of ovarian sex steroids, resulting in an inflammatory cascade that leads to the shedding of the endometrial tissue over 3-6 days, a process known as menstruation and which marks the beginning of a new cycle (days 1-6)

1.3 The endometrial microenvironment

The tissue microenvironment is, conceptually, the collection and interactions of the cells that make up a specific organ and often includes the somatic, vascular and immune cells as well as the extracellular matrix components. Endometrial tissue homeostasis, cellular proliferation, metabolism and reproductive function are mediated by the orchestrated crosstalk between these diverse cell populations via paracrine and endocrine pathways.[29–34] Histologic analysis of the cycling endometrial microenvironment consistently reveals luminal and glandular epithelial cells supported by specialized reticular stromal fibroblasts (stroma), a vascular bed and a dynamic flux of resident or infiltrating immune cells.[5,25] The luminal epithelium of the endometrium is exposed to the external environment; and, thus, not only does it provide the first line of defense against invading pathogens, but it is responsible for becoming receptive to the implantation of an allogenic embryo during pregnancy. The epithelium also make up the glands in the inner body of the endometrial tissue which are necessary, hormone responsive cells that actively secrete key pro-gestational molecules such as glycogen-rich material, mucins, cytokines and prostaglandins.[16,35–38] Surrounding the glands, the bulk of the endometrial tissue is composed of a type of specialized reticular fibroblasts commonly referred to as a stromal fibroblasts. The stromal cell is also hormone responsive and expresses receptors for both E2 and P4. This uniquely specialized fibroblast phenotype provides structural support for the endometrial tissue, and also undergoes a transformational differentiation process in which these cells morphologically and functionally differentiate during a process called *decidualization* in response to P4. The specific topic of molecular mechanisms that drive the initiation of stromal decidualization in the human endometrium is explored in Chapter

3. In addition to specialized fibroblasts and epithelial cells, the endometrium is dependent on a complex vascular bed composed primarily of spiral arterioles and capillaries that undergo remodeling processes during the menstrual cycle to provide the nutrition and oxygen to the tissue maintaining homeostasis. Finally, the immune cell component of the endometrium is composed of both resident and recruited leukocytes that primarily include macrophages, natural killer (NK) cells, T cells and neutrophils. The relative population of these immune cells is hormonally regulated within the endometrium throughout the menstrual cycle and as mentioned above, the withdrawal of P4 induces a sharp increase in immune cell infiltration during menstruation.[5,27,39] Specifically, an influx of immune cells increases during the onset of the menstrual phase; however, they can remain as resident cells if pregnancy is established. Approximately 70% of these infiltrating and resident immune cells are NK cells ($CD56^+$), 20% macrophages ($CD14^+$) and 10% T cells ($CD3^+$).[3,27,40] The immunological axis of endometrial function plays a critical role in protecting this open tissue from infectious pathogens, while being permissive to the invasion of an allogenic embryo. Thus, the relative somatic cell numbers, proliferative states, architectural complexity and immune cell ratios within the endometrium vary significantly across each phase of the menstrual cycle further adding another level of complexity to endometrial research related to health and disease. In Section 1.6, we discuss the heterogeneity of the endometrial microenvironment in detail. Unfortunately, much of our current knowledge of the endometrial microenvironment relies on histological analysis of the endometrium and an analytical bandwidth that limits our ability to fully understand the complex multi-cellular interactions and intrinsic molecular signatures that are necessary to maintain reproductive health. Moreover, the intra-patient variability between tissue

samples have hindered the reproducibility of many studies, and thereby, investigations are often constrained to single cell *in vitro* studies, typically focusing on either the biology of epithelial cells or stromal fibroblasts (Supplementary Table 4-1), but not much on the vascular and immune components.[40–43] These experimental constraints have limited our understanding of the cellular and molecular mechanisms that drive reproductive function.

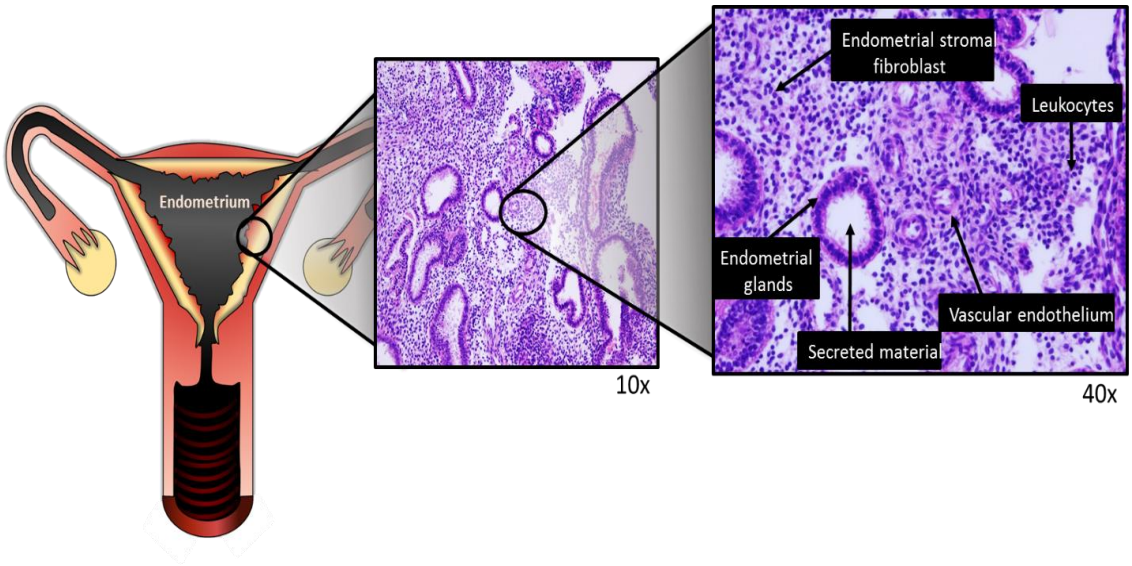


Figure 1-2. The breakdown of the endometrial microenvironment. The human female reproductive tract. (A) A schematic representation of the human endometrium. The endometrium is the inner lining of the uterine cavity and is composed of a dynamic endometrial microenvironment (B) Specifically, the endometrial microenvironment is made up of epithelial glands, reticular stromal fibroblasts, a vascular bed and infiltrating leukocytes.

As discussed above, the cyclical changes of ovarian sex steroid production dictate the timing and functional capabilities of the steroid-sensitive endometrium relative to the support of nidation. These steroids account for the distinct phases of the menstrual cycle by driving cell-specific morphological and biochemical changes. However, successful cell-specific sensitivity to systemic steroid hormones that results in an appropriate tissue-level response is locally mediated by the interactive relationship of each population of cells that make up the endometrial microenvironment. Paracrine signaling is a primary way cells can maintain a crosstalk via a number of mediators such as cytokines, chemokines, prostaglandins, nitric oxides, growth factors and MMPs. This endocrine-paracrine communication in the endometrium is important to maintain the homeostasis of the organ and disruptions of these processes may act as a driver of the pathophysiology of many endometrial disorders. Interestingly, Cunha and others revealed that endocrine response of the epithelium is largely mediated by the stromal cells.[31] This is a important finding because it suggests epithelial-stromal interactions can be reciprocal in nature during the development process of the endometrium. The stroma can regulate the epithelial proliferation, differentiation and function, but epithelial cells can synergistically regulate stromal function.[44,45] These cross-communication events are also evident in adult tissues during the decidualization response in the stromal fibroblasts as we demonstrate in this thesis (Chapter 3). Specific paracrine signals derived from additional cells within the endometrial microenvironment can regulate the stromal sensitivity to P4.[46] Furthermore, these somatic cells interact with the immune component to maintain a tightly regulated inflammatory environment that requires a bilateral paracrine circuitry. This endocrine-paracrine communication is important to maintain the homeostasis of the organ and

disruptions of this cellular dialogue, by chemical disruptors, have been shown to be conducive to the pathophysiology of many endometrial disorders (Chapter 4). Altogether, the significant contribution of the endometrial microenvironment to normal organ function is emerging as a critical component of reproductive health and underscores the need to develop translational models that recapitulate the tissue-level complexity of the human cycling endometrium.

1.4 Approaches for modeling the endometrium

1.4.1 Current techniques to model the endometrium

In this section, we briefly discuss the current methods of modeling the endometrium. These models continue to be essential to our understanding of reproductive research and have driven our understanding of reproductive health. However, we focus on the limitations of *in vivo* and *in vitro* models for reproductive research.

Animal models

The reproductive cycles of non-human mammals vary widely, particularly in the murine species which do not undergo a menstrual cycle, but rather an estrous cycle.[20,47] This issue has raised much concern with rodent models, as discussed earlier, because they are the most widely used *in vivo* model throughout drug development and toxicology. On of the reasons why human menstruation is not fully understood is the fact that this process has proved difficult to experimentally model. Menstruation only occurs in a few species including old world monkeys, anthropoid apes and, of course, humans.[48] The most similar

in vivo model of the human endometrium is currently the macaque, but high costs, low throughput and ethical issues have constricted the use of these animals for advancing reproductive biology. In contrast, the relative low cost, easy maintenance and utility of murine models has been essential to the growth of biomedical research. Although historic skepticism toward the anatomical and physiological differences between human and mice is frequently discussed, recent concerns about the reliability of these models for reproductive research have arisen due to vast differences during inflammatory processes.[49] This concern poses serious implications for reproductive biology as researcher heavily rely on mice for disease modelling and therapeutic discovery. At this juncture, whether murine findings translate to the human condition remains a red herring. For example, we rely on animal models to investigate the link between EDC and the pathogenesis of reproductive tract disorders because it is neither feasible, nor ethical to examine chemical toxicity on a human population. Historically, the Osteen/Bruner-Tran laboratories at Vanderbilt have utilized a murine model of developmental toxicant exposure, which confidently demonstrates the emergence of endometrial dysfunction. However, as discussed, these animals do not undergo menstruation, and therefore are not susepctible to endometrial diseases like endometriosis. Furthermore, despite the universal necessity of P4 in mammalian pregnancy, the role and expression patterns of specific P4 receptors vary across species.[20,47,50–52] Lastly, important differences between the murine and human inflammatory responses[49,53] may hinder the human translation of EDC toxicity studies conducted in mice. As demonstrated by Seok et al, we are in need of better model systems that recapitulate the human inflammatory axis.[49] Thus, we must accurately define the impact and mechanisms of action of environmental toxicants, such as dioxins, on women's

reporductive health. To accomplish murine/human translation it is critically important to replicate and validate murine findings using models which better recapitulate the *in vivo* human condition.[26] The need to develop more humanized murine models or robust *in vitro* human models may each provide an alternative to overcome these issues discussed above. The genetic manipulation and commercial availability of genetically engineered murine models provides a clear advantage for *in vivo* models. To improve humanized murine models, immune-deficient mouse strains such as the Rag-2γ(C)^{null} strains which have no mature and functional T and B cells, and athymic (*i.e.* nude) mice that lack T cells have been utilized in reproductive biology. Specifically, the grafting of human tissues or cells within this immunodeficiency models and control over sex steroids provides a suitable *in vivo* model for studying human endometrial processes. Xenografts[54], intraperitoneal injections of human tissue or recombinant reconstitution of human endometrial cells in a renal capsule transplantation assays are experimental methods to study endometrial physiology and pathology.[31,32,44,55–57] Furthermore, the adaptive transfer of human immune cells can enhance the immunological complexity of these models.[25,58] Unfortunately, these methods remain quite technically challenging and require both specialized strains of mice and ready access to well characterized human endometrial cells/tissues. Thus, the efficiency and reproducibility of these chimeric models remain quite limited.

Human endometrial cells

Appropriate tissue acquisition and characterization protocols are essential for clinical and basic science research settings, and, coupled with accurate histological examination, allows researchers to obtain specific cell populations for designing *in vitro* studies.[5,27,59]

Clinical endometrial research has focused almost exclusively on the histological and pathological analysis of biomarkers of disease from examination of *ex vivo* tissues[1,5]. Alternatively, isolated human cells can be used prospectively for *in vitro* culture studies. Several protocols to obtain primary human endometrial cells (*e.g.* epithelial glands and stroma), as well as endothelial cells (*e.g.* HEECs), from endometrial biopsies using enzyme digestion of the tissue have been established.[60–62]. However, the technical simplicity of current cell culture models often fail to recapitulate the interactive tissue microenvironment that cells observe *in vivo*.[9,63–65] These culture methods are often limited to oversimplified single cell type monolayers maintained in a large dilution static culture. Specifically, current models fail to fully measure the physiological changes that occur within the endometrium within an idealized 28-day menstrual cycle in response to endocrine signals and the complex inter-cellular communication that regulate endometrial function.[66,67] Prior to research reported in this thesis, an *in vitro* phenotypic model of the human menstrual cycle had not been developed.[30,68–71] The best currently used *in vitro* models include transwell assays provide for analysis of the interactions of two cell types, yet the important paracrine, autocrine and metabolic signals that occur *in vivo* are diluted by greater than 1000-fold in current culture vessels. Additionally, these co-cultures fail to integrate the multicellular and interactive paracrine networks found in the endometrial or other tissue microenvironments that corresponds to homeostasis. An extensive volume of literature critique the current *in vitro* cell culture techniques particularly demonstrating the failure to functionally replicate cellular polarization and *in vivo* physiology.[72–75] Furthermore, empirical evidence, originally led by Mina Bissell, has demonstrated that the 3D organization and micro-chemical and mechanical cues more accurately model the *in vivo*

cellular environment[76]. For example, static cultures with endothelial cells have been shown to lack essential mechanical signals such as hemodynamic forces[77,78]. In contrast, bioengineered microfluidic technologies can provide an alternative solution by introducing dynamic flow and fluidic gradients that induce relevant *in vivo* cell polarization and concentrated paracrine signals.[21, 22] The ability to develop compartmentalized chambers within a model can permit the introduction of immune cells (*e.g.* circulatory monocytes) to mirror circulating leukocyte recruitment and determine the role of inflammatory pathways to maintain uterine homeostasis using primary human cells (Chapter 4).

1.4.2 The need for more robust models of the reproductive tract

The current drug development pipeline has been hindered by high failure rates in clinical trials. Over 30% of candidate drugs prove to be toxic in humans despite the promising results seen in pre-clinical experimental studies[80]. This high attrition rate has been attributed to the oversimplified *in vitro* methods for human cell studies and reliance on murine models that lack the ability to adequately represent the human condition[7,10,49]. The NIH's National Center for Advancing Translational Sciences (NCATS), in collaboration with Defense Advanced Research Projects Agency (DARPA), has launched the *Tissue Chips for Drug Screening Initiative* to promote the development of human organotypic models capable of mimicking the structure and function of human organs. Although the goal of the initiative is instrumentation of these models to provide researchers the ability to better predict a candidate drug's xenobiotic effect in humans during drug development, this program can also drive mechanistic insights into disease progression and cell biology. Several agencies including the Environmental Protection Agency (EPA), the

Bill and Melinda Gates Foundation and NCATS have launched initiatives that promote the development of physiologic, three-dimensional (3D) organ systems from human cells. Bioengineered OoC microfluidic models mimic *in vivo* tissue architecture and physiological context that facilitate and accurately mirror key organ-level functions of the female reproductive tract.[2,26,81,82] The NIEHS and EPA are specifically interested in developing OoCs to interrogate the role of the environmental toxicants and to elucidate adverse outcome pathways of agents like endocrine disrupting chemicals (EDC). Furthermore, these microfluidic devices can be applied to the development of microphysiological systems to understand reproductive diseases. Certainly, somatic, vascular and immune components of the endometrium may be important targets of pathogenic stimuli or xenobiotics that contribute to the pathogenesis of endometrial disorders such as infertility, pre-eclampsia and endometriosis.[24] The application of OoCs to disease models may enhance our understanding of mechanism that drive pathogenesis and facilitate the identification of biomarkers, bioavailability, efficacy, and toxicity of therapeutic agents prior to entry into clinical trials. A detailed discussion of OoC technologies and applications is covered in Section 1.5.

1.5 Organs-on-Chip and microfluidic models

Multi-compartmental 3D microfluidic cell culture devices, so called OoC devices have been introduced to address limitations of current *in vitro* modelling.[6,7,76,83–85] Tissue engineered microphysiological models are designed to represent the complex microenvironment of target organs and mimic human physiology.[63,65] These OoC devices are expected to have a major impact on drug discovery, screening, and assessment of

efficacy and safety.[8,81] Such 3D platforms can recapitulate inter-and-intra cell signaling and the physiological context of tissue dynamics by compartmentalizing the major cellular components for quantitative and qualitative analysis.[6,8] Apart from their potential role in clinical pharmacology, such OoC models can also be used to study the effects of environmental insults (*e.g.*, toxicants, radiation) or infections on human cells, informing aspects of population health.[86] Organotypic culture models of the reproductive tract are being currently developed and have emerged as an innovative tool. The origin of these models stems from the concepts developed from 2D tissue culture and improved microfabrication technologies. Tissue culture was initially found to lack similarity to the human condition clinically and was a weak model for systemic diseases specifically because the cells lacked proper differentiation properties. Developments in 2D, 3D and transwell-based tissue modeling serve as a foundation for adequate cell differentiation, but these models still lack the dynamic and physiological conditions of *in vivo* biology.[7] The "organ-on-chip" technology is conceptually derived from compartmentalized microfluidics and the manipulation of the flow of small amounts of fluid in micro-fabricated porous surfaces in 3D cultures that induce proper cell differentiation and stability in order to mimic physiological processes. The strength of these innovative *in vitro* models lies in the microarchitecture of these chips that permit the recreation of tissue-level cellular interactions that replicate the organ structure and dynamic movement of human biology. Not only can the microfluidic properties of these systems provide continuous supplementation of media for these cell types, but they also provide an avenue to independently treat each cell type of interest and compartmentalize real-time effluent sampling without cell culture termination[64,87]. Developing OoC models should meet all or

most of the following key parameters: 1) multicellular architecture that represents characteristics of the tissues or organs of pathology; 2) functional representation of normal and/or diseased human phenotypes; 3) reproducible and viable operation under physiological conditions maintained up to 4 weeks in culture; 4) representation of spectrum or heterogeneity of disease presentation. Many different OoC models have been developed to mimic most human organs by many different groups, including several from Vanderbilt University. One of the most prominent examples of these types of organ-on-chip models is the "lung-on-chip" developed by the Wyss institute at Harvard University. This model follows the biomimetic basis of microfluidic channels that are lined by live human cells. The bioengineering protocol is based on a flexible membrane that separates two micro-channels while maintaining elastomeric properties.[88] This model allows for experimental treatments via the air channel or the blood channel that can conduct tissue-tissue communication and measurable pathophysiology.[88,89] Moreover, the potential of these OoCs to be interconnected in series and recreate a "human-on-chip" is expected to significantly impact basic science and biomedical research.[7,63,79,83,87] The technical advantages of OoCs are described in Table 1-2.

Table 1-2. Modeling advantages of an Organ-on-a-Chip microfluidic device
Creates a highly defined, living model of human reproductive tissues that can be maintained for days-to-weeks
The ability to define the contribution(s) of individual cell types to the immune-endocrine axis, facilitating high-resolution mapping of autocrine and paracrine signaling pathways
The potential as a mid-to-high throughput screening tool for identifying adverse outcome pathways during drug development and toxicological studies
The capacity to better model covariates such as fetal sex or race/ethnicity at the tissue level
The ability to incorporate the OoC into novel imaging tools and downstream analytics while preserving the capacity to perform longitudinal studies throughout the course of reproductive function

1.6 Scaling tissue heterogeneity of the human endometrium

1.6.1 Designing a microfluidic device

Extensive literature describes differences in organ size and physiology between animal species. However, organ size often does not scale proportionally (*i.e.* isometrically) between animals and instead obeys a number of different allometric power laws, as described elsewhere[63]. Allometric scaling developed as the early foundation for pharmacokinetic studies to measure xenobiotic delivery and activity. These studies are traditionally conducted in culture dishes and small animal models to specifically assess the absorption, distribution, metabolism, excretion, and toxicity (ADMET) of a specific compound.[90] To replicate human physiology and drug response with microfluidic tissue engineered *in vitro* platforms, it is critical that these systems are designed according to either allometric and functional parameters.[63] However, traditional models do not directly scale to the female reproductive tract, specifically the uterus. Therefore, the calculation of cellular heterogeneity within the endometrium is required for appropriate scaling to model the endometrial microenvironment. To accomplish this, a mathematical modeling index can be utilized to determine relative cell diversity throughout the menstrual cycle. The Shannon-Wiener Index (SWI) provides a useful measure of the effective heterogeneity of organs that can guide the relative cellular composition of a tissue by calculating the effective number of cell types present and the extent of their individual contribution to reproductive function (Supplementary Table 1-3).[63] We tested this approach to generate an estimation of heterogeneous populations required to model the endometrial microenvironment. Based on this approach and the literature available, we defined the

relative heterogeneity of the endometrium to represent an epithelial, stromal, vascular and immune component.[91] However, it is important to note that each cell type can be further characterized into more specific population subsets. For example, immune cells is a broad term that incorporates numerous populations of cell types and is further denoted by their activation states.[27] Furthermore, while stromal differentiation to decidua cells are not found prior to the late secretory phase, we decided to include them as a fifth cell type because they are an essential cell population and an indicative marker of hormonal response.[66] While certain organs can scale adequately (*e.g.* by weight and size), the endometrium is not as straightforward and requires supplemental physiological and functional scaling properties due to its dynamic physical growth and its cyclic differences. Thus, the necessary organ scaling for the endometrium must reflect the physiological activity and functional changes that occur within a menstrual cycle as a measure of the efficacy by which the engineered tissues can replicate human organ function. Herein, we focused primarily on modeling the vascular, stromal and immunological components of the endometrial microenvironment using functional scaling.

1.6.2 Validation a microfluidic device

The major physiological roles of the endometrium are listed in Table 1-1. These are defined by the field as key specialized functions of the endometrium that ultimately promote the homeostasis of the organ and embryo implantation.[16,27,28]. Furthermore, each physiological function is supported by congruent morphologic, cytologic, genetic, proteomic and metabolic changes within the endometrial microenvironment. Spatio-temporal changes in response to ovarian sex steroids that mirror the changes during the

menstrual cycle can be annotated using cytological and biochemical techniques. These annotated changes can be used as key parameters to design and validate our *in vitro* systems[5]. Using this microfluidic platform, we measured proliferation, barrier integrity and hormone response of the stromal fibroblasts (*i.e.* decidualization) to functionally validate our model. These results provide the potential of this microfluidic model to maintain long-term cultures, mimic physiological responses to hormones, model endothelial (or epithelial barrier function), incorporate leukocyte populations and therefore ensure sufficient sensitivity to biochemically measure secreted paracrine molecules from the conditioned media.[92]

1.7 Overall objectives

Advances in biomedical engineering are being deployed in microphysiological models geared toward mimicking multiple organs *in vitro* in many disciplines including reproductive biology.[2] By utilizing microfluidic technologies, OoC devices can provide controlled perfusion inside microfluidic structures, which can then refine cell and medium volume ratios and emulate bloodstream-like flow to continuously supply nutrients and remove wastes while mimicking hemodynamic forces.[93] One major goal is to hasten the speed and improve the accuracy of toxicity testing in preclinical drug development[2], however, such models might also be used to gain new insight into tissue level physiological processes and disease pathogenesis.[86] To date, an increasing number of organs are being modelled using these technologies; however, an OoC of the endometrial microenvironment has not been established. In this thesis, we accomplish these goals by co-culturing the somatic, immune and vascular components of the endometrium, maintain

long-term cultures that mimic the length of an idealized menstrual cycle, and functionally mirror the reproductive processes associated with the cycling human endometrium (Chapter 2). Furthermore, we demonstrate that the applications of our model extend its use as a novel robust tool for hypothesis-driven discovery research to identify the contribution of the perivascular stroma on reproductive function (Chapter 3) as well as predictive toxicology for environmental chemicals and developing drugs (Chapter 4). The ability to dissect the intercellular communication at the tissue-level *in vitro* offers the ability to examine how, for example, endocrine disrupting chemicals, may alter the gravid uterus and drive it towards a pathogenic state (17, 19).

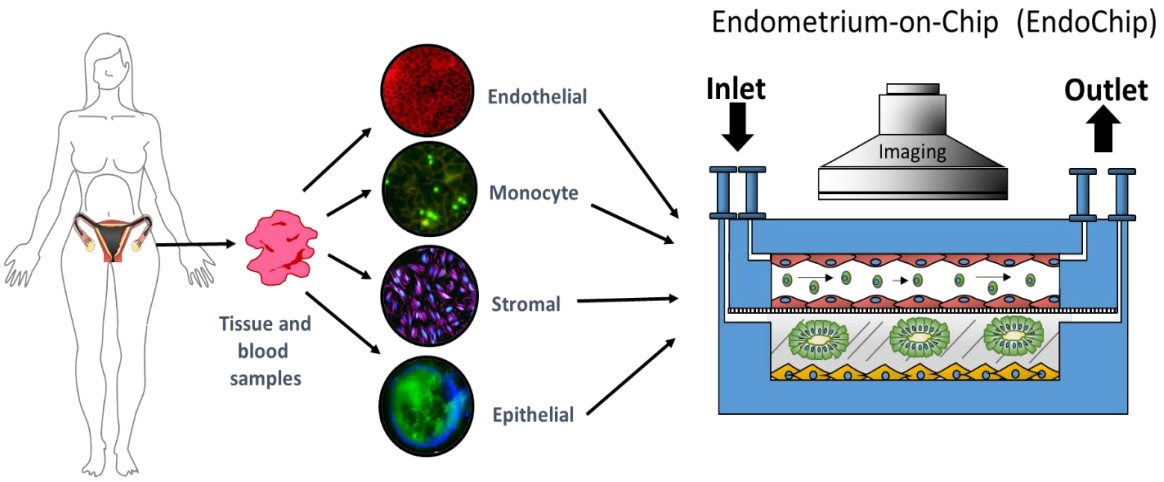


Figure 1-3. Overarching experimental approach for developing an OoC model of the endometrial microenvironment. Primary tissues will be obtained from patients and donors from hysterectomies or endometrial biopsies. Additionally, blood will be obtained to isolate peripheral monocytes. These tissues will be processed as described in the methods. A biobank will be generated using this process and isolated cells will be frozen for storage. The endometrial cells will be incorporated into a microfluidic dual-chamber device to establish a compartmentalized model of the endometrial microenvironment.

1.8 Appendix

Table 1-3. Heterogenous cell distribution in the human endometrium during the menstrual cycle.

Menstrual cycle phase	# of cell types, *N*	Cell type	%	Shannon-Wiener Index, *SWI*	Diversity Index, *DI*	P_i =1/*N* for uniform distribution of N cell types	*SWI* for uniform cell-type distribution
Proliferative phase Endometrium cells	4	Epithelium	18%				
		Stroma	65%				
		Endothelium	10%				
		Immune cells	7%				
		Decidua	0%				
		Total	100%	1.45	2.73	0.25	2.00
Early Secretory phase Endometrium cells	4	Epithelium	20%				
		Stroma	63%				
		Endothelium	10%				
		Immune cells	7%				
		Decidua	0%				
		Total	100%	1.49	2.80	0.25	2.00
Mid Secretory phase Endometrium cells	5	Epithelium	33%				
		Stroma	49%				
		Endothelium	10%				
		Immune cells	7%				
		Decidua	1%				
		Total	100%	1.70	3.25	0.20	2.32
Late Secretory phase Endometrium cells	5	Epithelium	35%				
		Stroma	10%				
		Endothelium	15%				
		Immune cells	10%				
		Decidua	30%				
		Total	100%	2.13	4.37	0.20	2.32

Note: The more monodisperse (*i.e.* less heterogeneous) is a two-cell tissue, the closer the SWI is to 0 because one cell type dominates. The more heterogeneous the tissue, then the closer SWI is to 1, since each cell type is equally represented (DI = 2). If the abundance of the two cell types is imbalanced, then the SI is intermediate between 1 and 2.

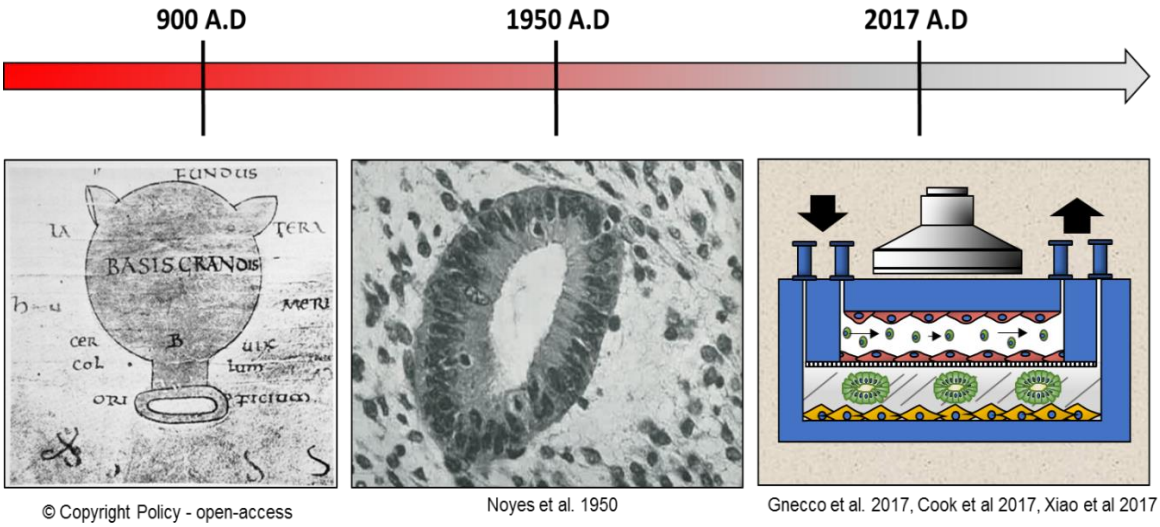


Figure 1-4. **Timeline of key events in uterine biology**. The earliest (9th century) surviving anatomical representation of uterus inspired by the studies of Soranus of Ephesus (circa 900 A.D.). Histologic dating of the endometrium in the classical paper by Noyes et al. (1950). Schematic representation of the first generation EndoChip (2017)

Supplementary table 1-4. Estimated cell ratios calculated by endometrial biopsies

	Late proliferative (N= 34)	± SD	Ovulation (N=1)	± SD	Early Secretory (N= 3)	± SD	Mid secretory (N=11)	± SD	Late Secretory (N=2)	± SD
Single Cell Stroma	6.6E+06	3.84E +06	1.7E+07	N/A	9.6E+06	3.07E +06	1.8E+07	9.44E +06	7.5E+06	1.15 E+0 6
Epithelial Gland Fragments	6.2E+03	7.29E +03	4.0E+04	N/A	1.8E+04	3.03E +03	3.8E+04	2.03E +04	2.1E+04	
Gland Fragment size (μm²)	2.7E+03	2576. 76	2.7E+03	2.5E +03	2.7E+03	2576. 76	2.7E+03	2576. 76	2.7E+03	257 6.76
Epithelial Cells/fragment	4.6E+01	1.02	4.6E+01	1.02	4.6E+01	1.02	4.6E+01	1.02	4.6E+01	1.02
Epithelial cells from isolation	2.9E+05		1.9E+06		8.2E+05		1.8E+06		9.7E+05	
Epithelium gland:Stroma Ratio	4.4E-02		1.1E-01		8.6E-02		9.6E-02		1.3E-01	
Stroma:Epithelial gland Ratio	2.3E+01		9.2E+00		1.2E+01		1.0E+01		7.7E+00	

Chapter 2

Development of an Organ-on-Chip model of the Endometrial Perivascular Stroma

Contributions: Dr. Virginia Pensabene contributed primarily as the biomedical engineering mentor for this work by providing assistance in the process of designing and fabricating the microfluidic models. This chapter appears, in part, as a manuscript published in *Annals of Biomedical Engineering*.

2.1 Background

As described in detail above (Chapter 1), during each menstrual cycle the endometrium undergoes an E2 dominant proliferative phase (4-14 days) followed by ovulation and the subsequent development of the corpus luteum. In the following secretory phase (days 15-28), production of ovarian P4 promotes endometrial differentiation and prepares the endometrium to be receptive for an implanting embryo.[94] In the absence of embryo implantation, the endometrium experiences a sharp withdrawal of ovarian sex steroids, resulting in an inflammatory cascade that leads to the shedding of the endometrial tissue over 3-6 days, a process known as menstruation which marks the beginning of a new cycle (days 1-4).[5,19,94–96] The physiologic processes that occur during an idealized menstrual cycle remain elusive. A more robust model of the cycling human endometrium should provide not only a better basic knowledge of normal steroid-mediated endometrial

function, but also holds promise to reveal how altered patterns of cell-cell communication promotes the pathogenesis of diseases, including breakthrough bleeding, infertility, menorrhagia, endometriosis, pregnancy disorders and endometrial cancer.[19,97]

Reproductive research has been driven primarily by animal models[23,25–28] and/or 2D *in vitro* cultures of human cells.[41,69,101,102] Murine models are essential to provide physiologic reproductive outcomes, such maintenance and outcomes of pregnancy.[47,97] However, the significant differences between human and rodent reproductive physiology, endocrine sensitivity, immunological responses, and key reproductive processes, including the P4-mediated decidualization response, make it difficult to interpret whether these findings translate to the human condition.[2,3,103,49,104,105] Equally important, most current *in vitro* models of the endometrium are limited to the use of monocultures, usually human stromal fibroblasts, that undermine the cellular complexity and loosely recapitulate endocrine signaling and fail to investigate tissue-level crosstalk between the multiple cell types that drives homeostatic reproductive function.[41,106] Moreover, current *in vitro* models have a limited capability for high-resolution real-time examination of functional changes. For example, the current transwell assays largely enable modelling of specific cell barriers in human organs, such as epithelium[107,108] or endothelium[109–111] with minimal quantifiable capability for evaluating the diffusion of paracrine signals and transport of molecules. Many researchers, including our group, have demonstrated an important role for stromal-epithelial crosstalk in normal endometrial function during the menstrual cycle. Likewise, dysregulated endometrial communication is associated with numerous disease processes[31,32,112,113]; however, the role of the vascular bed and the interaction between stromal fibroblasts and adjacent endothelium has received less investigative attention

(Figure 2-1).[41] To our knowledge, only a few groups have attempted to specifically model the endometrial vasculature.[20,29–31] Thus, the role of the human endometrial vasculature in reproductive function remains unclear. Albrecht *et al.* co-cultured endometrial cells (*i.e.* epithelial or stromal fibroblasts) with human myometrial microvascular endothelial cells using a transwell culture system and observed an increase in endothelial tube formation and in vascular endothelial growth factor production under the influence of E2.[41] These results strongly suggest that paracrine communication occurs between endothelial cells and endometrial cells. Unfortunately, most *in vitro* models fail to mimic *in vivo* physiological conditions that the endothelium experiences, including crosstalk between numerous cell types, and hemodynamic forces, such as laminar sheer stress.[32–38] It is currently difficult to mimic and control the hemodynamic flow conditions observed in the vasculature in transwell systems. These technical limitations of current *in vitro* models hinder our ability to understand the interactions between endometrial cell types that drive endocrine-mediated physiological function.[2] Thus, we decided to adopt a biomedical engineering approach to circumvent these technical issues using microfluidic technologies to develop an "Organ-on-a-Chip" (OoC) model of the endometrial perivascular microenvironment (EndoChip). Due to the microfluidic design, and severe lack of the models that investigate the vascular bed of the human endometrium, we focused specifically on the endothelial-somatic component interaction to design and validate the Endo-Chip.

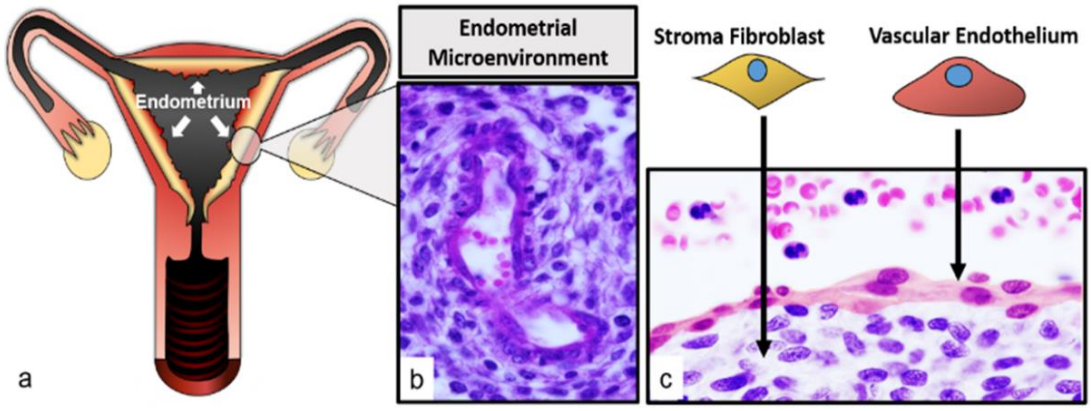


Figure 2-1. The human perivascular endometrium. (a) A schematic representation of the human female reproductive tract. (b) A histological image of the endometrial micro-environment in the secretory phase of the menstrual cycle shows decidualization of the stromal fibroblasts in regions surrounding endothelial vessels (original magnification 400X). (c) False coloring of peripheral leukocytes, and endothelial cells of the endometrial perivascular stroma (original magnification 1000X) (Haematoxylin and eosin staining, in b and c).

2.1.1 Established OoC models of the endometrium

OoCs represent robust compartmentalized, heterogeneous cell culture systems to simulate the physiology and anatomy of human organs and, thus, should enhance our understanding of the mechanisms that are otherwise difficult to study. A few first-generation microfluidic models of the pre-implantation womb have been developed and reviewed elsewhere.[92,116–118] Briefly, early attempts at modeling embryo implantation using a microfluidic 2-chamber device were presented as abstracts in 2007[118,119], yet it was not until 2009 that Kimura and colleagues developed a static, 2-chamber OoC capable of culturing a single mouse embryo on a bed of endometrial stromal cells.[93] Their device consisted of an upper polydimethylsiloxane (PDMS) chip with multiple cell trap wells and a lower PDMS chamber with a microchannel for chemical nutrient supply along with a polyester microporous membrane from a commercially available culture insert plate.[93] In 2014, Chen and collaborators developed a model for co-culturing a single embryo with a lawn of endometrial stromal cells on type IV collagen substrate using a dual-chamber device design that has an adaptable constant media flow perfusion.[120] This model examined the quality of embryo culture by utilizing microfluidic technologies. A similar approach was taken by Chang *et al*. to develop a comprehensive stand-alone microfluidic platform, "Womb-on-a Chip", that mimics the microenvironment and incorporates key parameters of embryo implantation, including some biomechanical forces.[121] This PDMS-based model also used microfluidic chambers to directly co-culture primary endometrial stromal fibroblasts with murine embryos and enabled the ability to visualize the implantation process in real time. Within the timeframe of this thesis, two OoC models specifically of the endometrium were developed that were in parallel to our EndoChip. The first is a

synthetic polyethylene glycol (PEG) extracellular matrix model to enable functional 3D endometrial epithelial and stromal co-cultures. This model developed by Cook *et al*. from Dr. Linda Griffith's laboratory at MIT and focused exclusively on the stromal and epithelial component of the endometrium as it pertains to maintaining a three-dimensional hydrogel scaffold with proteolytic capabilities of the endometrial tissue. Adapted on a perfusable transwell platform, they are able to maintain long-term cultures of an endocrine responsive tissue.[122] We have collaborated and utilized the technologies developed by this group and performed pilot studies described in Chapter 5. The second OoC model of the endometrium was developed by Dr. Theresa Woodruff's group and utilize de-decellularized *ex vivo* uterine tissues as a tissue scaffold to re-seed with stromal and epithelial cells that reconstructed glandular and stromal tissue of the endometrium.[123,124] Both of these models used comprehensive recirculation pneumatic pump platforms that maintained indirect flow-induced long-term cultures and an open microfluidic interface for organ-coupling. Altogether, these models specifically focused on the traditional somatic component of the endometrium (*i.e.* epithelium and stromal cells); however, there was an absence of published "Organ-on-Chips" models that examined the vascular and immune components of the endometrial microenvironment. As part of this thesis, we were the first to present an OoC microfluidic model that established a functional model of the menstruating endometrium with hemodynamic forces, such as laminar shear stress. Due to the feasibility of readily acquiring non-laboring term placenta umbilical cords and endometrial tissues, we utilized primary human umbilical vein endothelial cells (HUVECs) and endometrial stromal fibroblasts (stroma) as the target cell types to establish our model.

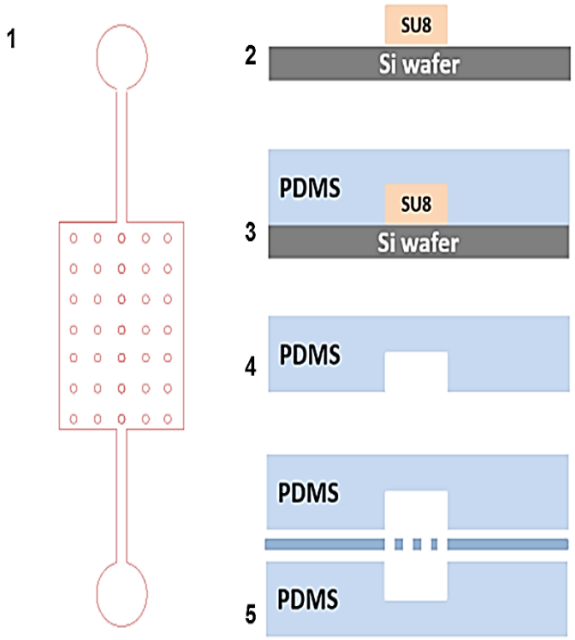


Figure 2-2. Fabrication process of a dual chamber microfluidic device (1) Mylar mask design for a single chamber, including the 35 circular pillars (diameter 100 µm); (2) fabrication of SU 8 master by photolithography; (3) liquid PDMS casting and baking on SU8 masters; (4) cured PDMS layer demolding; (5) porous membrane plasma activated and sandwiched between to PDMS layers. Soft, post exposure and final hard baking steps were done following datasheet indications**.**

2.2 Results and Discussion

The need for more robust and physiological modeling of distinct human organs has stimulated the application of innovative technologies that can be applied to basic science and medicine. Traditional OoCs are inherently miniaturized and thereby require specialized methods to reproducibly design and fabricate the individual chambers and channels.[63] Throughout the development of these models, the design of these devices must be driven primarily by the biological question to be tested while sustaining feasibility and reproducibility. Key analytical functions of OoC include the high-content imaging of a cell culture, maintenance of long term cultures (a minimum of 4 weeks), non-invasive and real-time analysis of secretion and metabolism of the individual cell types by sampling spent media from each compartment.[6,63,85,88,125] Furthermore, the microfluidic volumes of total culture media reduces the dilution of nutrients, paracrine signals, and selected test molecules within the system to provide a more robust study of the communication between the different cell types.[8,63] In order to identify potential physiologically significant interactions between the vascular and somatic components of the endometrial microenvironment, we designed the EndoChip to maintain microfluidic compartmentalization of each specific cell type. This compartmentalization also allowed us to identify cell-specific effects of biomechanical and hemodynamic forces (*e.g.* shear stress) and endocrine (steroid stimulation) cues that are externally introduced and controlled in the microfluidic system. Thus, we used soft-lithography technologies to design and fabricate a PDMS dual chamber microfluidic device that integrates a resin-based microfabricated porous semi-permeable membrane. The porous membrane allows for the compartmentalization of the chambers while maintaining bidirectional diffusion of

secreted molecules. We validated the model by examining the P4-induced pre-implantation processes of the endometrium by mimicking endocrine changes associated with each individual phase of the menstrual cycle. With the EndoChip model of the perivascular microenvironment, our group and other researchers will be able to accurately examine and analyse the morphological and biochemical aspects of the interactive roles of endometrial stromal and endothelial cells under the influence of sex steroid regulation throughout an experimental menstrual cycle.

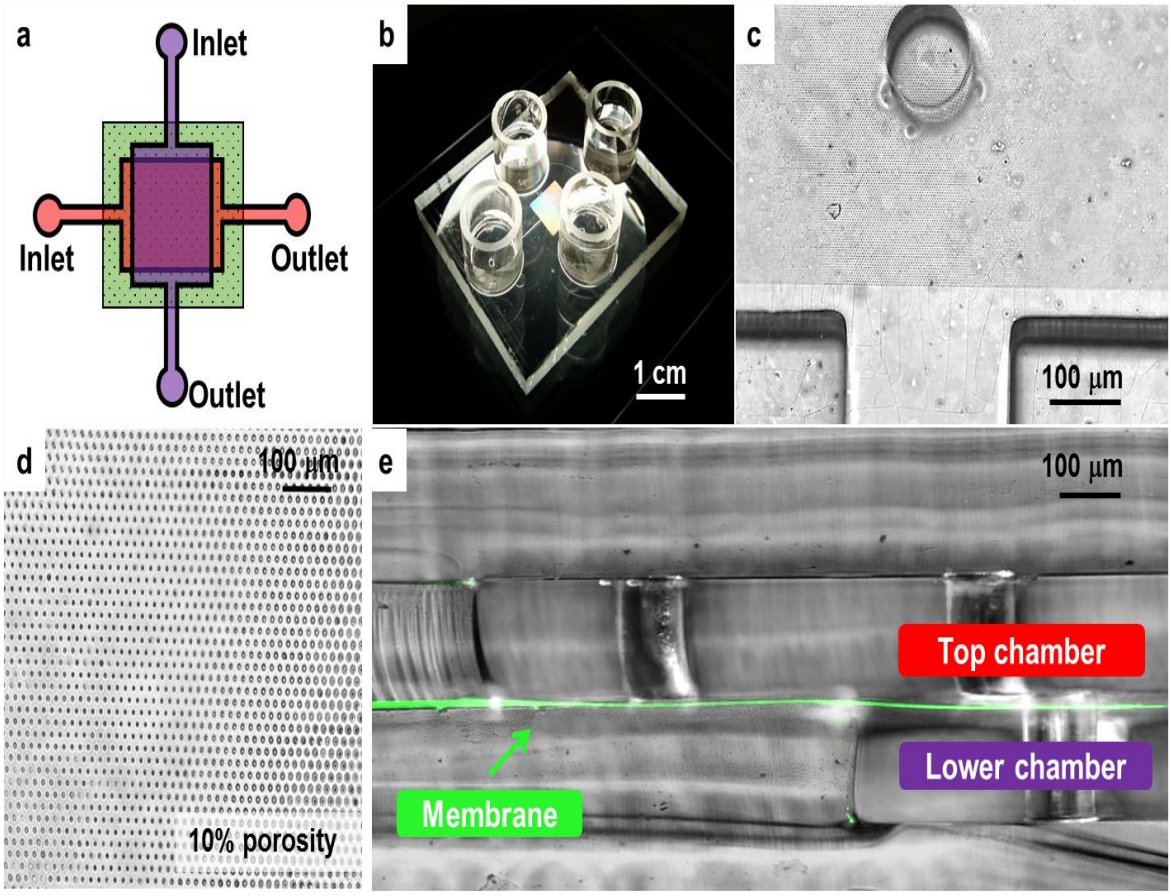


Figure 2-3. Design and characterization of the dual chamber microfluidic device with a high-resolution membrane. (a) Schematic of the two chambers design. (b) A photograph of the assembled PDMS device with four reservoirs corresponding to inlets and outlets: the porous membrane appears translucent. (c) A top view of a chamber with bonded porous membrane: the border of the porous region on the top can be distinguished by the lower non-porous region, and thus easily aligned with the chamber wall and the circular pillars; one of the 5 pillars appears on the top, in the exact center of the chamber. (d) Top view image of the 1002F membrane with 10% porosity inside device. (e) A cross section of the assembled device: the two chambers and the cylindrical pillars can be visualized as well as the green coloured 1002F membrane (enhanced background fluorescence was used to image the membrane (green)).

2.2.1 Design and fabrication of a dual-chamber microfluidic device with a high-resolution porous membrane

We designed the EndoChip as a modification of previously developed models including the Harvard University Wyss Institute's "Lung-on-a-Chip" compartmentalized dual chamber model[88,89], and Vanderbilt University's Neuro-Vascular Unit (NVU) designed by Dr. John Wikswo.[126] Our device consists of 2 orthogonal microfluidic chambers, each one providing 29.45 mm^2 of cell growth area and a total volume of 4.7 µL (Figure 2-2 and Supplementary Figure 2-9A). We generated a dual-chamber device using PDMS micropatterned chambers and semi-porous membranes. The chambers are divided by a 1002F resin-based membrane provided by our collaborator at the University of California Irvine, Dr. Elliot Hui.[127] The two chambers are separated by the semi-permeable membrane and are sealed by plasma bonding (Figure 2-2). The 1002F membrane is biocompatible, has a 6-µm thickness (as measured with a DekTak3 surface profilometer) and 2-µm circular pores that allow soluble factor communication and cellular contact between cells cultured on the two sides.[127] The patterned membrane fully spans the chamber to provide a compartmentalized region, suitable for cell co-culture assays (Figure 2-3, Supplementary Figures 2-8, 2-9). An advantage of utilizing the transparent 1002F membrane is the capability to distinguish the porous regions from non-porous regions (Figure 2-3C, Figure 2-6A). This simplifies layer alignment during device assembly and aids in determining the cell density ratios following device seeding. Traditional transwell inserts rely on polyester and polycarbonate membranes that are opaque in bright field, but may contribute to high autofluorescence. In comparison, resin-based microfabricated membranes and PDMS devices result in a completely transparent apparatus, which does

not limit high resolution imaging analysis and enhances data acquisition. As such, we are able to characterize real time cell maintenance inside the device as described below and perform low background immunofluorescent imaging. However, as part of the establishment of this model, we also additionally compared the fabrication of the EndoChip using alternative microfabricated membranes[128] and commercially available 2-and-3 μm polycarbonate and polyester (PCTE, PETE, respectively) track etched membranes from different vendors and characterized fluorescent imaging and cell viability within our model (unpublished, Supplementary Figure 2-10). As expected, we observed reduced visibility in bright field and a lower resolution for fluorescent imaging, although these alternative membranes functionally supported cell growth and sufficient image analysis. These findings suggested that commercially available polycarbonate membranes could be bonded and utilized for the fabrication of the EndoChip as an alternative to the 1002F membranes. In addition, as a part of the design, we incorporated a series of pillars within the chambers to provide structural support to the flexible membrane (unpublished, Supplementary Figure 2-9 C-D). While it is important to note that these pillars may alter dynamic flow profile inside the chamber, we always observed homogeneous cell distribution during loading and uniform proliferation and polarization throughout the chamber. Furthermore, the density and size of membrane pores does not affect the cell loading and the flow dynamic inside the device. Repetitive loading of cells in the two chambers did not show leakage of cells through the pores (which was expected as the 2-μm diameter is smaller than the size of the individual cell) and clear contact with the 1002F resin membrane (Supplementary Figure 2-9 D).

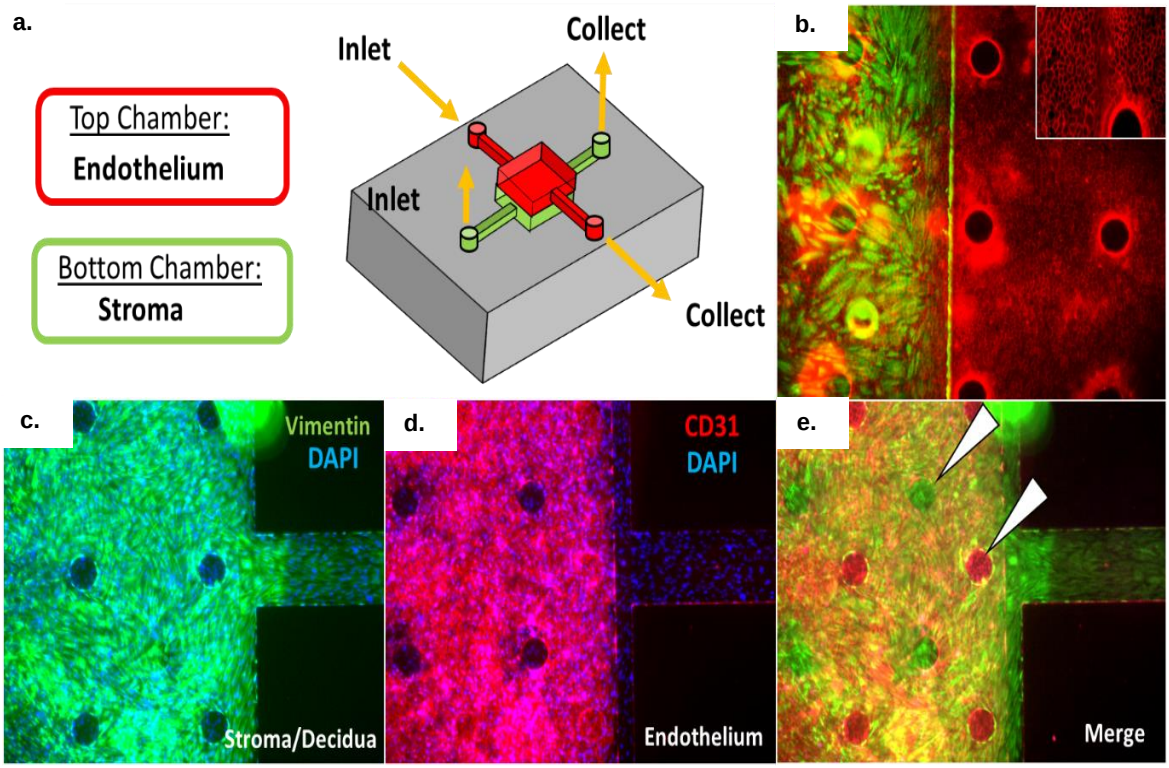


Figure 2-4. Characterization of co-culture of endometrial stromal fibroblasts (stroma) and HUVECs in the two-chamber device. (a) Schematic of the perivascular stroma model. (b) Characterization of compartmentalization and morphology: the stromal cells were cultured in the lower chamber and stained for vimentin (in green), while HUVECs were cultured in the top chamber and stained for CD31 (in red) (40X with inset 40X). (c) Stroma compartment showing the confluent layer of cells. (d) Endothelial compartment with confluent layer of CD31 stained HUVECs. (e) By merging the three channels (DAPI, FITC, CY5) it is possible to visualize the identity of each cell layer growing within the two compartments. As a consequence of the circular pillars (pointed by arrows), it is possible to visualize a single cell type at a time (DAPI for nuclei staining in blue, 4X for c, d, e).

2.2.2 Characterization of the microfluidic model of the endometrial perivascular stroma

To evaluate the cellular growth inside the device, we seeded both primary human endothelial cells and primary human endometrial stromal cells in the top and bottom chambers of the device, respectively. Both cell types were loaded simultaneously at a concentration of $1x10^6$ cells/mL, leading to a final cell seeding density of 5000 cells/mm^2. Static culture conditions inside the OoC were maintained for up to 28 days, corresponding to the length of an idealized menstrual cycle. In order to confirm the compartmentalization of the two cell types in the dual chamber device, the vascular monolayer was selectively stained for the endothelial cell marker CD31, while the stromal compartment was stained for vimentin (Figure 2-4, Figure 2-6). We observed a clear compartmentalization of the two cell types throughout the device. Each cell type was observed to adopt characteristic morphologies similar to those seen in cells cultured on traditional polystyrene cell culture dishes, with distinct cobblestone morphology exhibited by the endothelial monolayer and a confluent layer of the stromal fibroblasts that initially exhibited a striated morphology prior to decidualization. Furthermore, as confirmed by CD31 immunofluorescent (IF) staining, there were no distinguishable morphological differences in the endothelial layer between porous and non-porous regions of the membrane (Figure 2-6A). The 1002F served as a biocompatible substrate for high-resolution imaging with minimal background fluorescence. Upon reaching confluence, there are approximately $30\text{-}50x10^3$ cells per chamber that can be rescued using trypsin dissociation. Over time, endothelial cells were able to colonize and establish a confluent layer at the top and at the bottom of the chamber (Figure 2-6D).

2.2.3 Validation of the microfluidic model of the endometrial perivascular stroma

We validated the model biochemically by examining the expected vascular and stromal physiological processes induced throughout a menstrual cycle. Endometrial stromal fibroblasts are specialized cells that are uniquely different from other types of mesenchymal cells in the human body. Specifically, these cells play a fundamental role in the endocrine-mediated function of the endometrium and are highly responsive to the changing pattern of ovarian sex steroids across the menstrual cycle.[95] They express both E2 and P4 receptors and under the influence of P4 the stromal cells undergo a differentiation process called decidualization which is required for successful embryo implantation.[19,129] The process of decidualization can be characterized by the morphological changes in stromal cells from the typical spindly shaped fibroblast to a cuboidal and glycogen rich decidual cells.[5] Additionally, biochemical changes are associated with the differentiating stromal cells that include the active secretion of several progestational proteins that accurately serve as biomarkers of the decidualization process.[130–132] Therefore, to validate both the physiological function and the sensitivity of our microfluidic perivascular stroma co-culture model, we mimicked an idealized, complete physiological menstrual cycle. To mirror the changes of endocrine signals, we supplemented the cell culture medium for the first 2 weeks with E2 alone and subsequently another 14 days with E2 and medroxyprogesterone acetate (MPA), a synthetic progestin that exhibits more stability under culture conditions than natural P4. A shift in the morphology of the stromal cells toward a round and cuboidal shape was observed after 10 days of MPA treatment (Figure 2-5. A-D) as expected from other *in vitro* studies.[19,108] For a quantitative analysis of biochemical markers of decidualization in response to different steroids treatments, we

measured prolactin (PRL) secretion in spent media collected daily from gravity fed co-cultures using ELISA. A continuous increase of PRL over time was measured from stroma stimulated with E2 and MPA compared to the stroma treated only with E2, which exhibited consistently low production of PRL after 2 weeks of treatment (Figure 2-5E). An additional perfused device was treated with E2 + MPA with exogenous 8-Br-cAMP, a potent, intracellular driver of stromal decidualization that is frequently utilized for *in vitro* studies of P4 action in endometrial cells and cell lines.[129,131] As expected, we observed significantly higher PRL production from cultures exposed to 8-Br-cAMP, approximately one order of magnitude higher after 4 days of treatment (Supplementary Figure 2-11). These findings additionally demonstrate the utility of our device to discriminate changes in the rate of *in vitro* decidualization between cultures exposed to MPA alone versus culture exposed to MPA with cAMP. Importantly, our results equally demonstrate the capability of the model to mimic each phase of a full 28-day menstrual cycle, with continuous sampling capabilities for quantitative biochemical analysis. Discriminating among biological agents that affect endometrial decidualization at the perivascular interface will be important for future studies using our OoC device to identify potential agents that influence P4 action within the endometrium. This notion is explored mechanistically in Chapter 3.

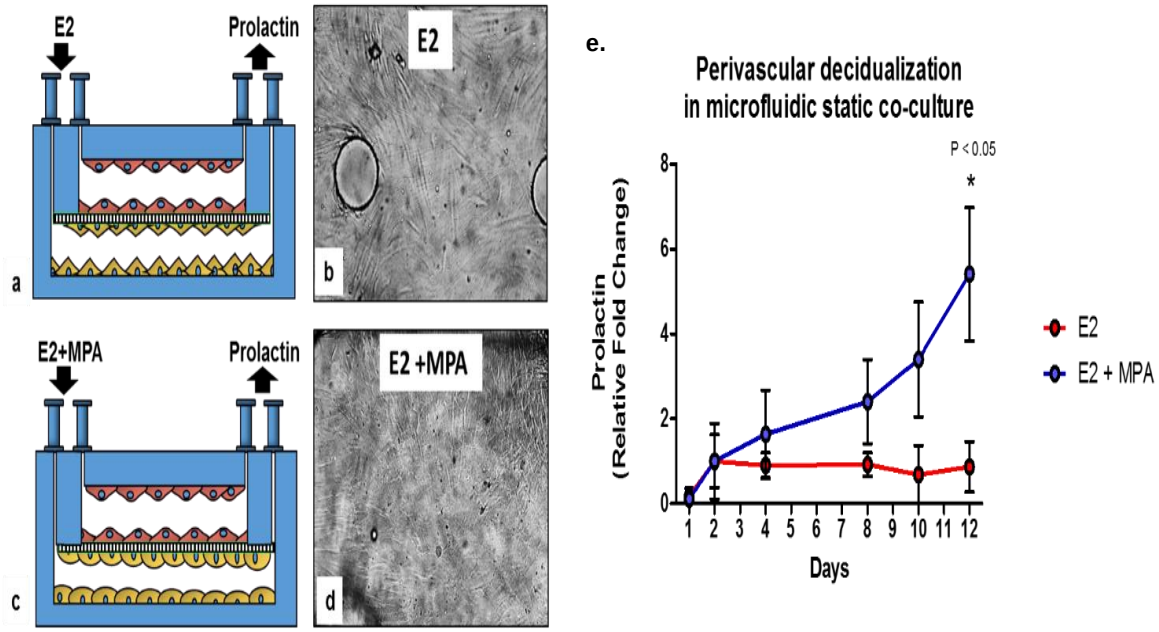


Figure 2-5. Phenotypic validation of the perivascular stroma to endocrine cues. (a) Schematic of the experimental design of the co-culture device. (b) Bright field image of co-culture with stromal cells directly cultured with estradiol (E2) alone for 14 days. (c) Schematic of the stimulation settings of the co-culture device with E2 and MPA with the corresponding changes of morphology for the stroma from fibroblast-like to cuboidal shape, characteristic of the decidualized cells. (d) Bright field image with stroma cells cultured with E2 + MPA for additional 14 days shows morphological changes (10X). (c) Prolactin (PRL) production measured by ELISA increases over time under the influence of E2 + MPA, but not when cultured with E2 only ($p < 0.05$).

Next, we examined the role of flow perfusion of the vascular chamber on endothelial remodeling. First, we characterized the growth on HUVECs on dual chamber devices. We mimicked a hemodynamic condition of the perivascular endometrium within our OoC by continuously perfusing the endothelial chamber. The stroma cells were maintained under static conditions since this cell type does not undergo direct laminar shear stress forces in physiological conditions within the endometrium. We utilized morphological polarization as a marker of culture conditions exhibiting physiologic shear stress. The endothelial cells were allowed to proliferate to confluence in full medium in static conditions and subsequently exposed to flow conditions at 1 μL/min for a minimum of 4 days. We observed endothelial polarization and remodeling of cell shape from a polygonal to ellipsoidal morphology (Figure 2-6 E-F) compared to static (balanced gravity fed reservoirs) conditions. This hemodynamic stimulation resulted in the alteration of F-actin filaments and reorientation of stress fibers in the cytoskeletal structure. Next, we functionally validated the vascular chamber by measuring endothelial barrier integrity. The dual-chamber capability of the device also permits analysis of the functional integrity of the endothelial monolayer in forming a barrier to diffusive transport. We evaluated the permeability of the endothelial monolayer by measuring the transfer of fluorescently labelled dextran (150 kD MW) between the two chambers of the device. Compared to an unpopulated device, there was a significant reduction in the permeability coefficient of the membrane once the endothelial layer was confluent (Figure 2-7). These findings confirm both the permeability of the membrane and the establishment of a confluent and tight endothelial monolayer working as barrier to macromolecule transport. The comparison of single cultures of stromal cells in the bottom chamber, compared to co-culture of both

endothelial and stromal cells, suggests that the endothelium is specifically promoting the barrier capabilities. These findings are supported by the formation of tight junction between cells as observed by positive ZO-1 staining (Figure 2-6). In a pilot experiment of the co-cultures of endothelial and stromal cells we observed that E2 and MPA treatment for 14 days increased diffusion of FITC-Dextran and loss of endothelial barrier integrity (unpublished, Supplementary Figure 2-12). These findings supports previous work that indicates that P4 induces vascular permeability in the human endometrium.[133,134]

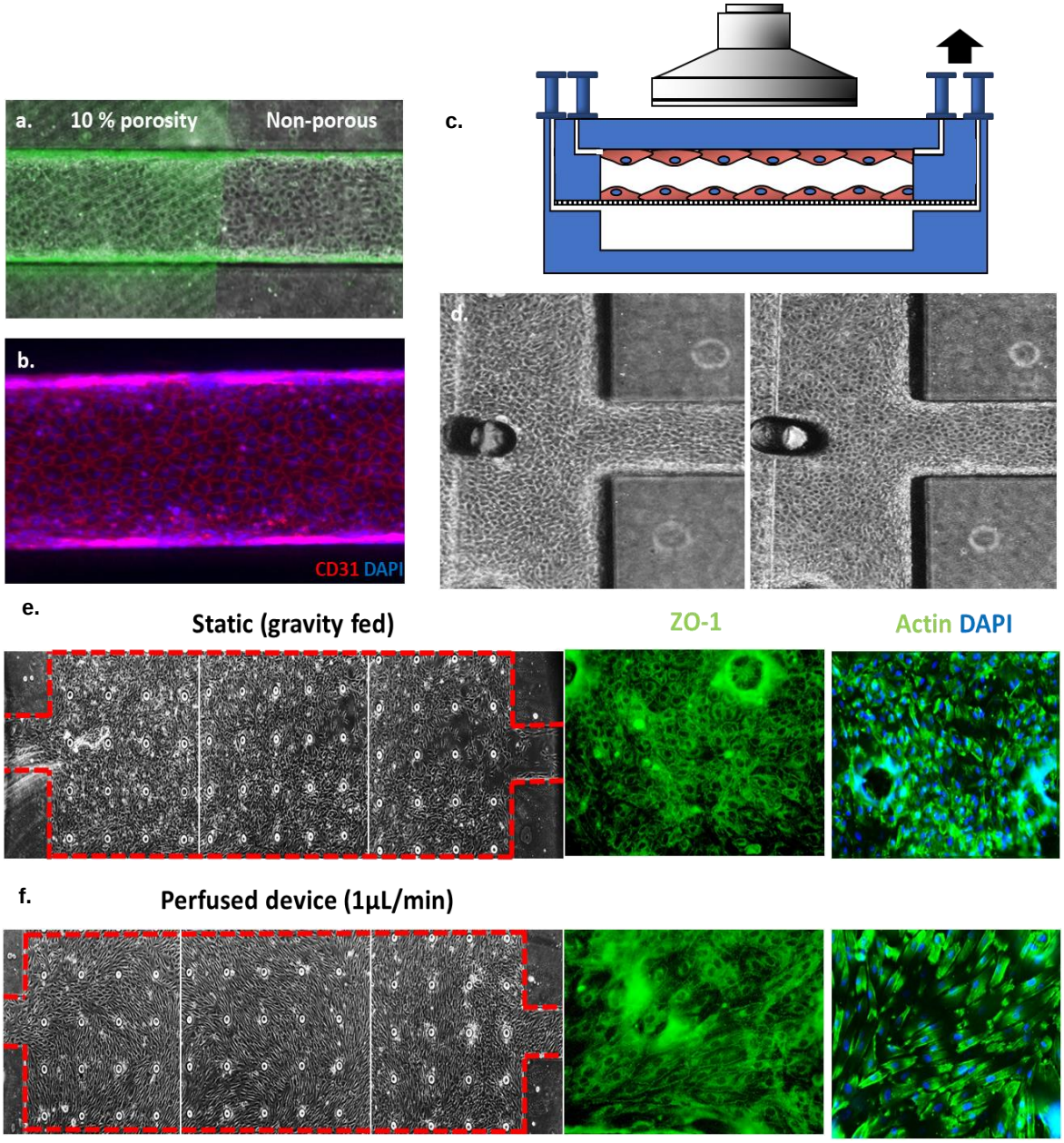


Figure 2-6. Phenotypic Validation of endothelial cells inside the device. (a) Vimentin staining of stroma cultured on the 1002F membrane inside the microfluidic chamber: morphology and density of cells are not affected by the presence of pores in the substrate (10x). (b) CD31 staining of HUVECs shows a confluent and tight cell layer (10x). (c) A schematic of the microfluidic device loaded with HUVECs in the top chamber: the cells form two confluent layers, one on top and one at the bottom of the chamber. (d-e) By focusing at different z-levels, corresponding to top and base of the circular supporting pillar, it is possible to focus on the two confluent layers inside the chamber. (f) Without shear stress, cells showed a non-directional and disorganized cytoskeleton. (g) In the presence of flow, cells elongated and aligned with the flow (from left to right). Right panels show ZO-1 (green) and F-actin filaments staining (green with blue nuclei stained in blue for contrast) in the two conditions (Bright field images at 40X, fluorescent images at 100X).

2.3 Materials and Methods

2.3.1 Fabrication of the 1002F membranes

A transparent, semi-permeable membrane was used in the device to separate the cell types within two microfluidic chambers. The membranes are fabricated by using a photolithographic method with biocompatible resin, EPON 1002F (Miller-Stephenson, Sylmar, CA).[127,135] Briefly, the 1002F resin and a photoinitiator (UVI-9676, purchased from Dow Chemical, USA) were dissolved in γ-butyrolactone (GBL, from Sigma Aldrich, USA) at a ratio of 10:1:10 by weight. A 100-mm silicon wafer was first treated with plasma (2 min, O_2, 13.3 Pa) and then coated with a sacrificial layer of soap (2% Micro-90, International Products, Burlington, NJ, USA). An 8-µm layer of 1002F (diluted 1:1 in GBL) was deposited by spin coating for 10 s at 500 rpm followed by 40 s at 2000 rpm and then soft baked for 10 m at 65°C and 20 m at 95°C. The wafers were then exposed through a 125-mm chromium–quartz photomask (10 mW/cm^2, 14 s) (Supplementary Figure 2-8) After a post-exposure bake, the patterned resist was developed in propylene glycol methyl ether acetate. Each membrane is patterned with a 6.5x6.5 mm^2 array of 2-µm pores enclosed within a 15-mm diameter circle. The porosity, calculated as the ratio of the combined area of the pores to the total area of the patterned region, is 10%.

2.3.2 PDMS layer design and fabrication

The device was assembled using two 4.75 mm by 6.2 mm microfluidic chambers separated by a semi-permeable 1002F membrane. The complete device was fabricated by soft lithography in polydimethylsiloxane (PDMS, Sylgard® 184, Dow Corning, MI,

USA).[136] Briefly, a bas-relief master was fabricated in a Class 100 clean room with SU8-2100 negative photoresist (Microchem, MA, USA). 4" silicon wafers were spin coated with SU8 (30 s at 2000 rpm, final thickness ~150 μm). The resist layer was exposed to UV light through a 40,000 dpi printed Mylar mask (Infinite Graphics Incorporated, MN, USA) and finally processed with SU-8 developer to removed unexposed resist (Figure 2-2).

Liquid PDMS (prepolymer to curing agent ratio 10:1) was cast and cured on the SU8 master to obtain two equal layers. Ports to access the microfluidic chambers were opened by punching 1/16'' holes in the top layer with a stainless steel round punch (Integra Myltex, NJ, USA). The Mylar mask design included 35 round pillars (100 μm in diameter) to provide structural support for the membrane and served as a reference for the alignment of the chambers (Supplementary Figure 2-9).

2.3.3 Assembly of the two-chamber microfluidic device

The 1002F membranes were released from the silicon master by dissolving the sacrificial Micro-90 soap layer using DI H_2O for a minimum of 5 minutes. The membranes were carefully removed and inspected for quality prior to bonding (Supplementary Figure 2-8B). After release, the membranes were kept in sterile H_2O to remove residual soap and to prevent drying. The top PDMS layer and the 1002F membrane were then oxygen-plasma treated (600 mTorr, 100 W, 45 s) and bonded together. Since both PDMS and 1002F are optically transparent, alignment of various components was completed under a stereomicroscope. The second layer was bonded with the same method on the 1002F membrane orthogonally to the top PDMS layer.

Oxygen-plasma treatment renders the exposed surfaces hydrophilic. Hence, once

assembled, the devices were immediately filled with deionized sterile water and stored at 4°C until used. For static experiments, 500 µL Pyrex cloning cylinders (Fisher Scientific, Pittsburgh, PA, USA) were bonded with liquid PDMS to the inlet/outlet regions of each channel to form small reservoirs for the cell media.

2.3.4 Acquisition of human tissues

The Vanderbilt University Institutional Review Board approved the procedures for collection of all primary cells used and tissue acquisition was performed only after patients gave informed consent. Primary human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord[137], obtained from de-identified term placenta collected from patients who underwent elective caesarean section between 37 and 39 weeks of gestation. For this phase of establishment, optimization and characterization of the OoC, HUVECS were selected since they represent the most common model based on human derived primary endothelial cells.[138] After isolation, we consistently observed ≥95% purity of endothelial cells, validated morphologically and by immunofluorescent staining for CD31 (DAKO, USA) before loading in the devices. Cells were cultured in EBM-2 medium supplemented with EGM™-2 Single Quot® growth factors (Lonza, USA), maintained at 37°C in a saturated humidity atmosphere containing 95% air / 5% CO_2, and they were sub-cultured before reaching 60–70% confluence (approximately every 2 days) up to passage 5. For acquisition of endometrial stromal cells, surgically excised uterine tissues were collected from consented donors (ages 18–45) exhibiting predictable menstrual cycles and undergoing a hysterectomy for benign leiomyoma not associated with any additional inflammatory ovarian or endometrial disease. Endometrial stromal cells were isolated by

enzymatic digestion and filter separation.[62] As with our endothelial cell preparation, the purity of the stromal cell isolation was above 95% and was quantified by morphological assessment and positive staining for vimentin as previously described.[62] Stromal cells were maintained in phenol red-free DMEM/F-12 with 10% charcoal-stripped calf serum, 1nM 17-β estradiol (Sigma Aldrich, USA) and 1X antibiotic-antimycotic solution (stromal complete growth medium). As required for experimental objectives, some stromal cell cultures received treatments with 0.5 mM medroxyprogesterone acetate (MPA, Sigma Aldrich, USA) and/or 8-bromoadenosine-3',5'-cyclic monophosphate (cAMP, 0.5mM, Sigma Aldrich, USA) to induce a decidualization response over a period of 14 days.

2.3.5 Cell culture, maintenance, collection and analysis in device

As noted above, isolated stromal and endometrial cells were initially expanded within 75 mm flasks before transfer to our microfluidic devices. Upon achieving approximately 80% confluent monolayers, both endothelial and stromal cells were separately trypsinized, pelleted by centrifugation (220 rcf), resuspended in full medium ($1x10^6$ cells/mL) and subsequently loaded into each chamber using a 1 mL syringe. To enhance cell adhesion inside the device we utilized a thin coating of 1:50 dilution of Matrigel (BD Bioscience, USA) on both chambers to provide an extracellular matrix substrate. The cells were allowed to adhere for a minimum of 30 minutes inside the incubator and then 300 µL of EBM-2 medium or complete growth medium, was added to the endothelial top chamber or stromal bottom chamber, respectively. Spent media from the reservoirs was collected and replaced with fresh media every day throughout all static experiments.

For dynamic flow experiments, the endothelial chamber was perfused by using a syringe pump (PicoPlus, Harvard Apparatus, Cambridge, USA) and by connecting Tygon® tubing (Cole-Parmer) directly into the inlet port of the device. Shear stress conditions were induced when the cells reached 60% confluence inside the chamber. The final value of 1 µl/min was defined with this protocol as minimal value for the cells to form a tight and reoriented endothelium. The stromal cells were maintained under the same static culture conditions through all experiments. Wall shear stress in the center of the chamber was calculated as in Sung et al.[139] by treating the chamber as a relatively flat channel (with width >> height). Bubble formation was limited by loading the microfluidic chambers with DI water immediately after plasma treatment and by equalizing the temperature of the media in the syringe and inside the device before starting the perfusion.

The devices were cultured initially for 14 days in either complete growth medium, or complete EBM-2 medium supplemented with oestradiol (1 nM) until both cell monolayers reached 80% confluence. The complete growth medium for the stromal chamber was then supplemented with MPA (0.5 nM) and media was collected and changed daily (300 µl) for an additional 14 days from both inlet and outlet. Collected effluents were then analysed by measuring prolactin production, a marker of decidualization, using an enzyme-linked immunosorbent assay (ELISA Duoset, R&D systems). The ELISA was performed according to the manufacturer's instructions using 50 µL samples. The plate was read using an absorbance microplate reader (GloMax® Multimode Readers, Promega). The results are representative of three different experiments. Due to the patient-related variability among experiments, we normalized prolactin concentration to the minimal mean prolactin production at day 2. Statistical analysis was performed by a 2way ANOVA using

a Bonferroni correction. Statistical significance was calculated as $p<0.05$.

2.3.6 Immunofluorescence imaging

Mouse monoclonal anti human CD-31 primary antibody (DAKO, USA) was used at a 1:50 dilution and goat anti-mouse IgG Cy3 conjugate (Jackson Immuno Research, USA) was used as the secondary antibody (1:200). Tight junction expression of zona occludens-1 (ZO-1) was measured with a mouse anti-human FITC-conjugated antibody (1:50, Invitrogen, USA). A rabbit antibody against human vimentin (1:200, Abcam, USA) was used with a FITC conjugated goat anti-rabbit antibody as a secondary antibody (1:100, Jackson ImmunoResearch, USA). Standard fixation and immunofluorescence staining protocols were performed as described in the product datasheet. The cell nuclei were stained with 4’6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) for 1 minute and then washed with 1X PBS.

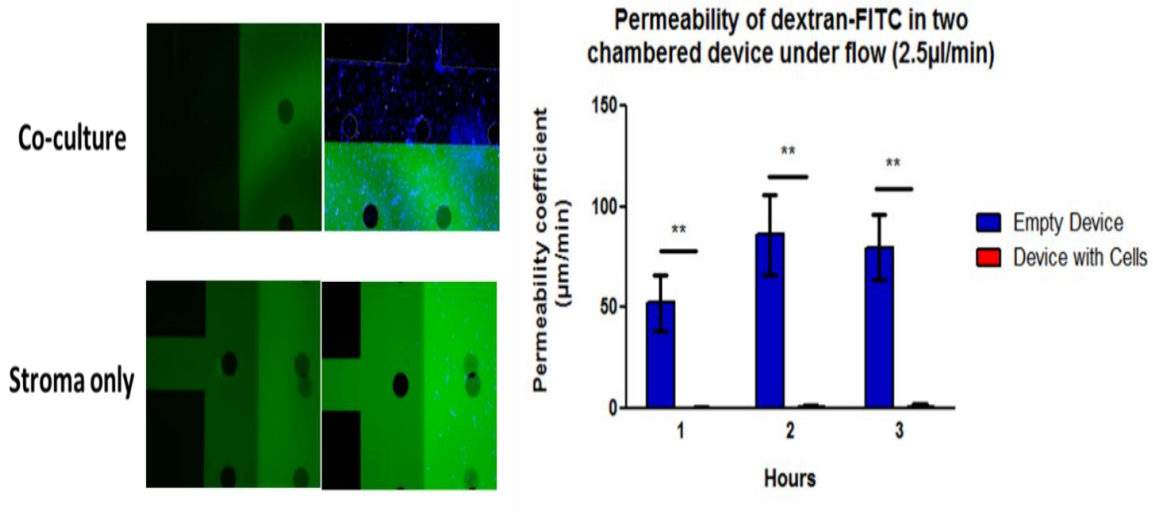


Figure 2-7. Assessing the vascular barrier integrity of the perivascular stroma. Green fluorescent dextran crosses the permeable membrane when only the stroma cells are cultured in the device (left column). When both cell types are cultured in the system, and once the endothelial cells form a tight layer, the FITC dextran did not cross the membrane and was confined in the endothelial top chamber. The permeability was quantified by measuring the intensity of the fluorescent in the different samples collected form the two compartments. Permeability coefficient of membrane in two-chamber platform calculated using macromolecule diffusion (FITC-dextran) shows endothelial cells making a tight barrier. Statistical analysis was performed using a Student t-test of 3 different empty devices against 3 different co-cultures ($p = 0.0098$).

2.3.7 Membrane permeability assays

A 3 mL syringe was filled with a 2.5 mg/mL solution of FITC-dextran (150 kDa MW, Sigma Aldrich, USA) and mounted on a syringe pump. The syringe was connected to the top chamber inlet with microbore tubing, while 200 µL of 1X PBS were added to the outlet reservoir and to the inlet and outlet of the bottom chamber. Perfusion was run at 2.5 µL/min. 100 µL samplings were obtained from each of the reservoirs and replaced with 100 µL of PBS. Samples were collected at 1, 2 and 3 hours intervals. Fluorescence intensity in the collected effluent was measured using a fluorescence microplate reader (GloMax® Multimode Readers, Promega) at 470 nm excitation and 520-550 nm emission range. Knowing the concentration of FITC-dextran in the initial solution (50mg/mL), we first generated a standard curve with a serial dilution of the solute for calibration and to correlate the measured fluorescent intensity with the concentration. The amount of transported solute (ΔQ) was obtained by summing the transported solute mass over time. The permeability coefficient was calculated using the equation $P_{coeff} = (\Delta Q/\Delta T)/(A \times Co)$, where $\Delta Q/\Delta T$ is the slope of the linear portion of the compound transported vs. time curve, A is the area of the membrane, and Co the initial concentration of FITC-dextran (mg/mL).[110] This simplified equation was obtained considering the diffusion of solute negligible when compared to bulk flow of fluid across the hydrophilic membrane, with 2 µm pores, not covered by the cells. The solute reflection coefficient, σ, was as well assumed ~0, considering the large pores and the hydrophilic properties of the membrane once equilibrated with full medium. Statistical analysis was performed using a Student t-test of 3 different empty devices against 3 different co-cultures.

2.4 Conclusions

In this chapter, we described the development of a novel a dual-chamber microfluidic OoC device for endometrial tissue modeling (EndoChip). We applied resin-based high-resolution porous membrane and soft-litography microfabrication technologies to design and fabricate the first microfluidic model of the perivascular stroma of the human endometrium. The fabrication process protocols are well characterized and remain the gold-standard for OoC development. The microfabricated membrane used in these experiments represents an interesting alternative to polycarbonate etched membranes, thicker PDMS based membranes[85,88] or more fragile vitrified membranes.[111] The high-resolution, and microfabricated design of the 1002F membrane is advantageous for imaging analysis, however, it remains limited due to its costs, availability and batch-to-batch variability.[127] It is important to note that PDMS has an ability to absorb hydrophobic/lipophilic molecules, specifically hormones; however, until materials with improved absorption behaviour become widely available, PDMS remains the most commonly used and accepted material for prototyping biomedical microfabrication technologies. Moreover, the results that we obtained herein from our PDMS device replicate previous observations in traditional *in vitro* polystyrene culture dishes in terms of hormone responsive biochemical and morphological decidualization, suggesting that absorption of steroids by the membrane was not an impediment to cellular response.

Within our model, for the first time to our knowledge, primary human endothelial and endometrial stromal cells were co-cultured in a microfluidic and compartmentalized device separated by a porous membrane and maintained in both static and perfused conditions for 28 days, spanning the physiological period of an idealized human menstrual

cycle. The biological responses to endocrine signalling were examined in this paper by mimicking the E2 and P4 changes that accompany the transition from the proliferative to the secretory phase *in vivo*. Importantly, the co-culture system described herein allows simultaneous analysis of both stromal decidualization and endometrial vascular function under controlled physiological conditions as functional phenotypic outcomes. Specifically, endothelial cells exhibited remodelling inside the device and formed an intact vascular barrier and is responsive to endocrine stimuli (Figure 2-7 and, unpublished Supplementary Figure 2-12). Our results demonstrate that the device provides sufficient sensitivity for assessment of biochemical changes that also reflect phenotypic changes that are amenable to imaging analysis. Together, these findings validate the ability of the microfluidic model to phenotypically recapitulate and examine a physiological reproductive process. We quantitatively and qualitatively measured the functional capability of the perivascular stroma to undergo decidualization. Early evidence of stromal/decidual transformation, which was induced after 10 days of combined treatment of these hormones in the OoC, was observed by a shift in the morphology of the stromal cells (Figure 2-5). This visual morphological transformation can be utilized in future studies by us or others as an established marker of endometrial viability under controlled conditions like hemodynamic forces. Since decidualization is a critical process for embryo implantation, the ability to explore the earliest stages of decidualization inside the device will provide a unique screening measurement for fertility studies. Clinically, our OoC model may also have relevance since similar histological changes have been observed *in vivo* after hormone therapy for prevention of postmenopausal morbidity or treatment of endometrial hyperplasia and carcinoma.[140]

These experiments position our model as a novel method to determine the normal homeostatic function of the vascular system in the endometrium under hormonal regulation. Furthermore, the model can subsequently be used by us and others to begin to dissect key elements of endothelial-stromal crosstalk and the impact of this communication on inflammation (Chapter 3). Furthermore our EndoChip model can potentially provide insight into disease-related reproductive processes such as endometriosis and abnormal bleeding.[141] In this regard, our OoC model was designed to not only control perfusion of the endothelial compartment but also allow the introduction of immune cells into the microfluidic co-culture device to establish a model of peripheral leukocyte recruitment into the endometrium (Chapter 4). The vascular endothelium acts as the interface between peripheral circulating immune cells and endometrial tissue. Hence the loss of anti-inflammatory action of P4 during the latter part of the secretory phase is both directly and indirectly linked to phenotypic changes within the endothelium that affect immune cell migration.[3,50,138,142] Thus, the *in vitro* model system we describe herein has been engineered to be compatible for future studies of each of the interactive cell types that help trigger menstruation and inflammatory endometrial diseases (*i.e.* epithelial, stromal, endothelial and leukocytes). Modelling the human endometrial microenvironment at the immuno-endocrine inflammatory axis offers the capability to better understand complex biological and pathogenic factors that act to support or disrupt endometrial function and reproductive success.

2.6 Appendix

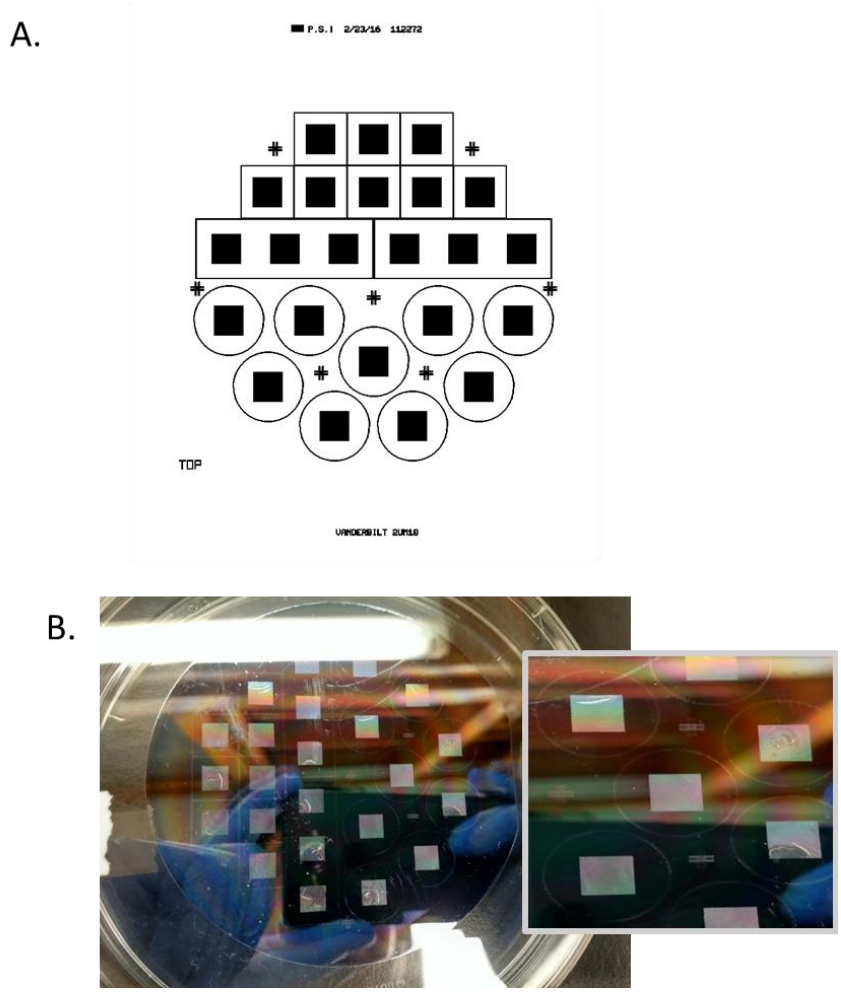


Figure 2-8. Designing the 1002F membranes for increased production of two-chamber devices. (a) CAD rendering of the design of the 2µm, 10% porosity membranes obtained from our collaborators in UC Irvine. Rectangle membranes can be broken into individual membranes or for a high throughput device. (b) Received wafer with membranes. Individual membranes are collected by dissolving a soap layer with sterile H_2O. (unpublished data)

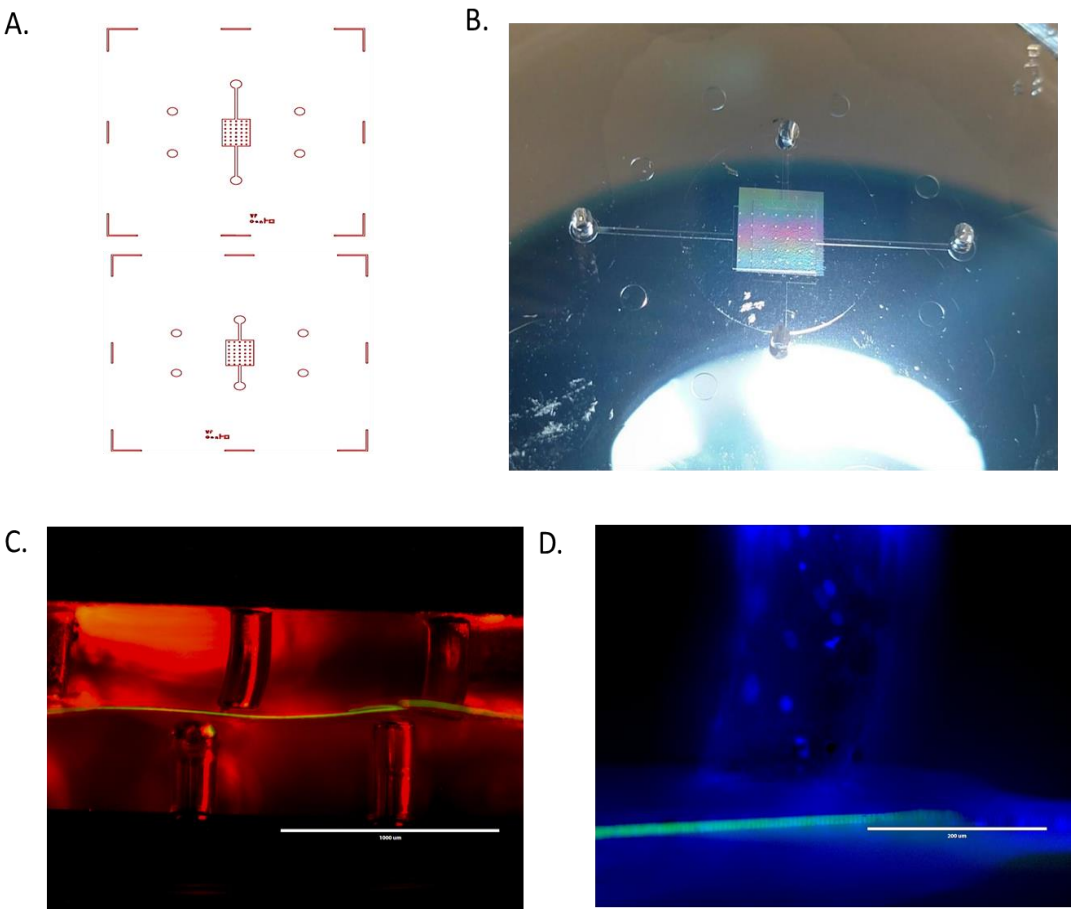


Figure 2-9. Assembly and bonding of the dual chamber model. (a) CAD rendering of the design of the top (long channels) and bottom (short channel) chambers in the final design of the two-chamber device. (b) Bonding of the 1002F membrane on the dual chamber device using a plasma cleaner. Distinct porous regions cover and compartmentalize the chambers. (c) Cross-section of the device using enhanced background fluorescence to identify the pillar-membrane interactions. Pillars maintain stability of the flexible membrane. (d) Higher magnification of the pillars demonstrates and DAPI staining demonstrates crawling of cells on pillars. (1002F membrane, green). (unpublished data)

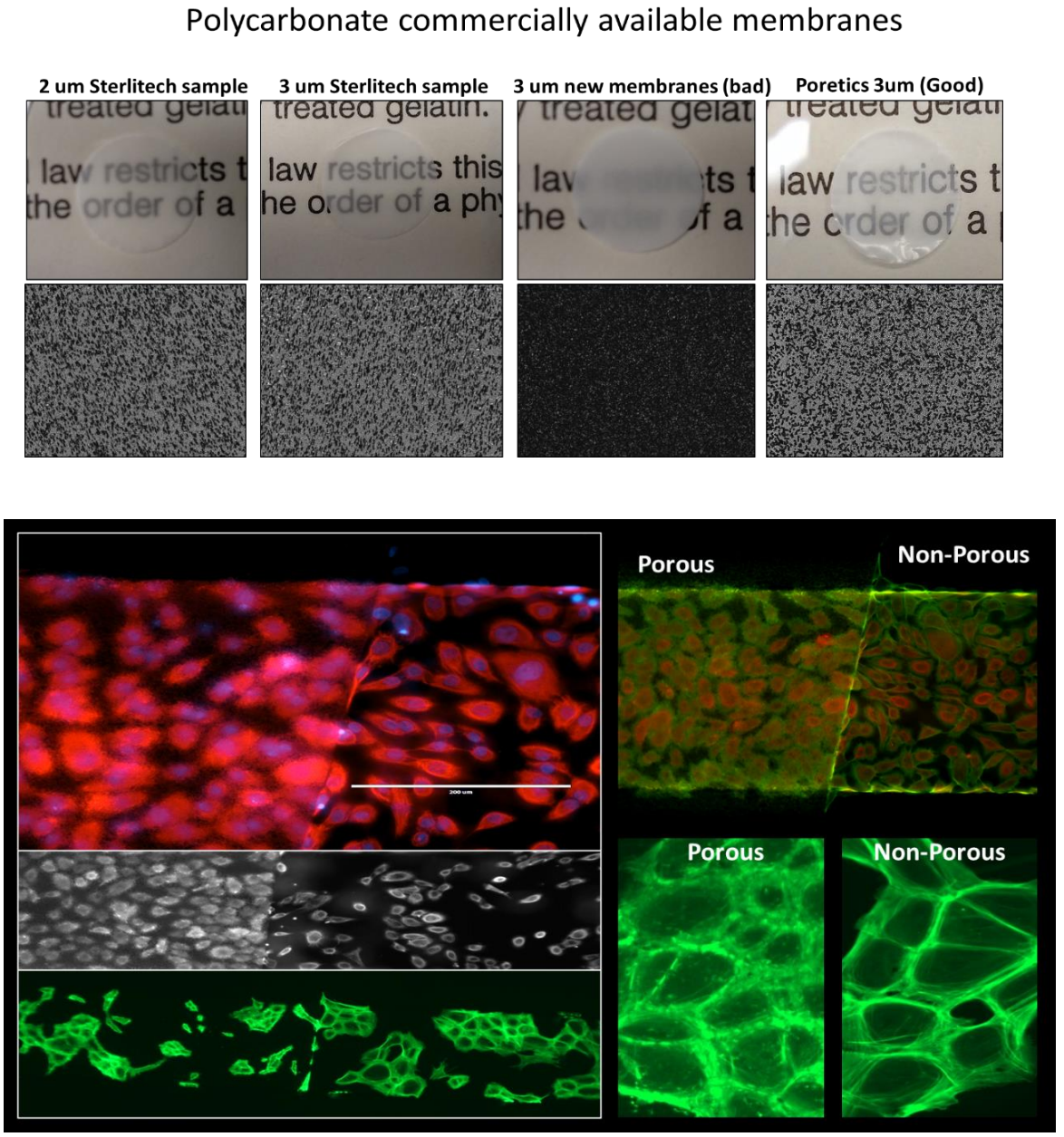


Figure 2-10. Characterization of alternative membranes used in the OoC device. (a) Different commercially available membranes were obtained from Sterlitech including polycarbonate (PCTE) and polyester (PETE) track etched membranes. Visibility was poor on both compared to the 1002F resin-based device. (b) Cells were adherent and viable to the polycarbonate membranes if the membranes were coated with a poly-L-Lysine and a thin layer of collagen IV as a substrate. Amniotic epithelial cells cultured in the two-chamber device are shown (CK7 in red and F-Actin in green) to demonstrate the visibility and viability with the PCTE 2um membranes. There is reduced resolution for immunofluorescence, but it is functional as an alternative membrane. (unpublished data)

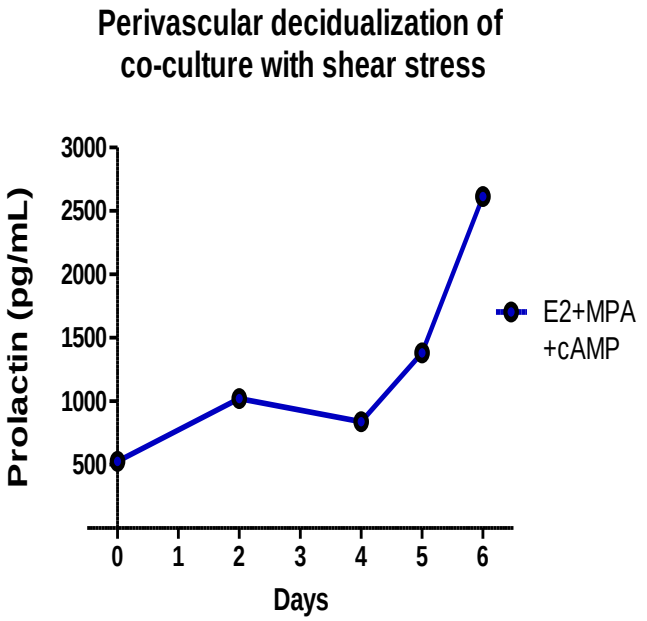


Figure 2-11. Validation of physiological response of the perivascular stroma to endocrine cues. PRL production measured by ELISA increases over time under the influence of E2 + MPA and cAMP (0.5nM).

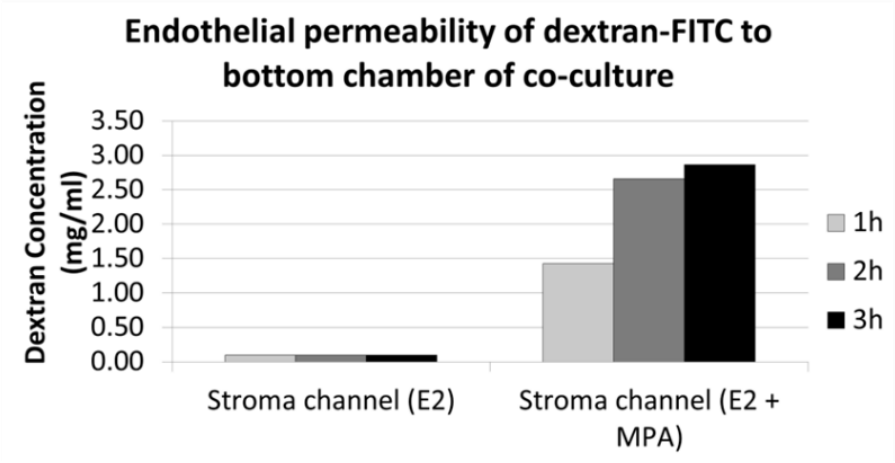


Figure 2-12. Validation of the response of the vasculature to endocrine cues. Fluorescently tagged dextran (FITC-Dextran, 150kDa) was used to measure permeability in co-cultures of HUVECs and stromal cells. A permeability assay shows an increase in permeability with treatment of E2 and MPA at the end of 14 days. (N=1, unpublished)

Chapter 3

Applications of the EndoChip:
Identifying the role of the vasculature
on normal reproductive function

In this chapter, we utilized the microfluidic dual-chamber device developed in Chapter 2 to test the hypothesis that *the endometrial microvascular endothelium regulates the initiation of key reproductive processes*. The importance of the vasculature in the female reproductive tract has been exemplified by extensive studies in numerous animal models described elsewhere.[143,144] Specifically, the vascular bed is an essential component of the endometrial microenvironment to maintain tissue growth and reproductive function. However, whether the vascular endothelium contributes to regulating specific reproductive processes in humans remains elusive[41,46,132]. Decidualization is a cellular and biochemical differentiation process of the human endometrial stromal fibroblasts under the influence of P4 that is required for the establishment and maintenance of pregnancy. Among mammals, the human and primate endometrium is unique in that decidualization originates within stromal cells directly surrounding the microvascular endothelium during the late secretory phase of the menstrual cycle. Furthermore, while most mammals require an implanting conceptus to initiate decidualization, in the human, it can occur in the absence of a deciduogenic stimulus. These uniquely localized events suggest that the vascular bed undergoes specific spatial and temporal processes during the menstrual cycle; however, the mechanisms that drive this localized histological observation remain to be identified.

Herein, we utilized a microfluidic Organ-on-Chip model of the endometrium to examine the crosstalk between the microvascular and somatic components of the human endometrial microenvironment under hormonal and physiological conditions approximating the secretory phase of the menstrual cycle. We induced decidualization in the model and measured the biochemical and morphological response of stromal cells when co-cultured with uterine microvascular endothelial cells (UtMVEC). Significantly enhanced stromal decidualization response was observed when the endothelial cells were stimulated with hemodynamic forces (*e.g.* laminar sheer stress) derived from controlled microfluidic perfusion. To identify the mechanism, we examined the secreted paracrine factors between the cell types and demonstrated that endothelial-derived prostanoids, specifically prostacyclin (PGI_2) and prostaglandin (PG)E_2, but not PGD_2, significantly accelerated the decidualization process of the adjacent stromal cells. Furthermore, to confirm these findings, we demonstrated that hemodynamic forces acting on the vascular endothelium actively induce COX-2, promote prostaglandin secretion and act via G-protein coupled receptor signaling pathway on stromal cells to regulate inflammatory mediators during steroid-driven differentiation. Altogether, these translational findings show that the endometrial vascular endothelium plays a key physiologic role during paracrine-mediated initiation of decidualization at the perivascular stroma in the human endometrium. The therapeutic targeting of uterine vasculature and specific prostaglandins support their clinical implications for reproductive disorders such as infertility, endometriosis, preeclampsia and poor pregnancy outcomes.

3.1 Background

Our understanding of the role that the vascular bed plays in the human endometrium remains limited primarily due of a lack of comprehensive models that recapitulate the human physiological condition.[41,69,97–102] However, it is clear that, in animal models, vascular changes in response to the implanting embryo contribute to uterine receptivity.[145,146] Specifically, changes in vascular permeability, angiogenic events and local increased expression of molecules that enhance reproductive function suggests that the vasculature plays a critical role in regulating endometrial receptivity.[147–150] Unfortunately, it is unclear whether animal studies translate to the human condition. Moreover, most *in vitro* human vascular models fail to recapitulate the *in vivo* physiological conditions that the endothelium experiences, such as the crosstalk of the cells in the microenvironment and hemodynamic forces from peripheral blood flow perfusion.[32–38] As discussed in Chapter 2, OoCs represent robust compartmentalized, heterogeneous cell culture systems that enhance our modeling of complex organs and can help dissect the molecular interaction between distinct cell populations. Furthermore, these models provide essential discovery tools for testing hypothesis-driven questions. We have previously developed and established an OoC microfluidic model of the perivascular stroma using HUVECs and primary endometrial stromal fibroblasts. Herein, we adapt this model to introduce and examine the cellular crosstalk between primary uterine microvascular endothelial cells (UtMVECs) and stromal cells under hemodynamically perfused conditions. Importantly, this microfluidic perivascular stroma model responds appropriately to hormonal changes and can be subjected to laminar perfusion, mimicking the hemodynamic forces derived from endometrial blood flow (Supplementary Figure 3-9).

The microfluidic design provides both spatial and temporal characterization of the paracrine communication between endometrial cellular components, allowing the exploration of the earliest stages of decidualization (Figure 3-1. C). The studies performed on the OoC model of the endometrial perivascular microenvironment revealed that the perfused-vascular endothelium enhances the sensitivity of the stromal fibroblast to P4, thereby enhancing decidualization. Moreover, the compartmentalized design of this microfluidic model demonstrated that hemodynamic forces regulate decidualization via an endothelial-derived paracrine mechanism. Specifically, we identified that laminar shear stress modulates endometrial function via the specific endothelial-derived prostaglandins PGE_2 and PGI_2.

3.1.1 The vasculature in the human endometrium

The human endometrium is a unique adult organ that undergoes approximately 400 cycles of steroid-driven growth, differentiation and tissue breakdown during a woman's reproductive life. While endometrial growth is largely controlled by estradiol (E2), it is exposure to progesterone (P4) during the secretory phase that preserves endometrial integrity in support of the establishment of pregnancy, thus avoiding menstruation.[48] Significantly, at the end of each menstrual cycle ending in menstruation, the endometrial vascular bed also breaks down, then regrows in coordination with other endometrial cell types in support of the next chance to establish pregnancy.[151] Early human histological studies carefully detailed the changing architecture of the uterine vasculature,[12,36,152] determining that the endometrial microvascular bed is derived from radial and arcuate arteries that traverse the myometrium.[153] Following menstruation, the

subsequent development of small spiral arterioles and branching capillaries supply blood within the stroma and provide the oxygen and nutrients during tissue repair, growth and differentiation. The unique coiling of the arterioles within the functionalis region of the secretory phase endometrium is the result of extensive vascular remodeling and angiogenic expansion that occurs throughout the menstrual cycle (Figure 3-1. A). Systemically, we now understand that the endothelium is an extremely important biologically active cell, producing a myriad of mediators that can regulate vascular smooth muscle tone and prevent against the development of cardiovascular diseases. Thus, the endometrial vasculature is equally subject to cyclical changes of ovarian sex steroid production primarily represented by ovarian E2 and P4 production which dictates the timing and functional capacity of the differentiated endometrium to support nidation. In all women, these sex steroids account for the distinct phases of the human menstrual cycle by driving cell-specific morphological and biochemical changes that occur over the idealized 28-day menstrual cycle.[5] To sustain the biological functionality of the newly developing endometrium, in addition to steroid, the spatial and temporal expression of local mediators, including cytokines, chemokines and eicosanoids, are tightly regulated during the menstrual cycle via direct and indirect mechanisms.[96,149] These key paracrine signals are particularly necessary for both physiologic function and tissue stability that are necessary for receptivity of an embryo during the secretory phase of the menstrual cycle.[19,154,155] While it is evident that sex steroids regulate the overarching timing of these events, specific cell populations produce these inflammatory mediators that mediate intercellular communication and are necessary to promote receptivity in the uterus for implantation during the menstrual cycle.[19,154,155]

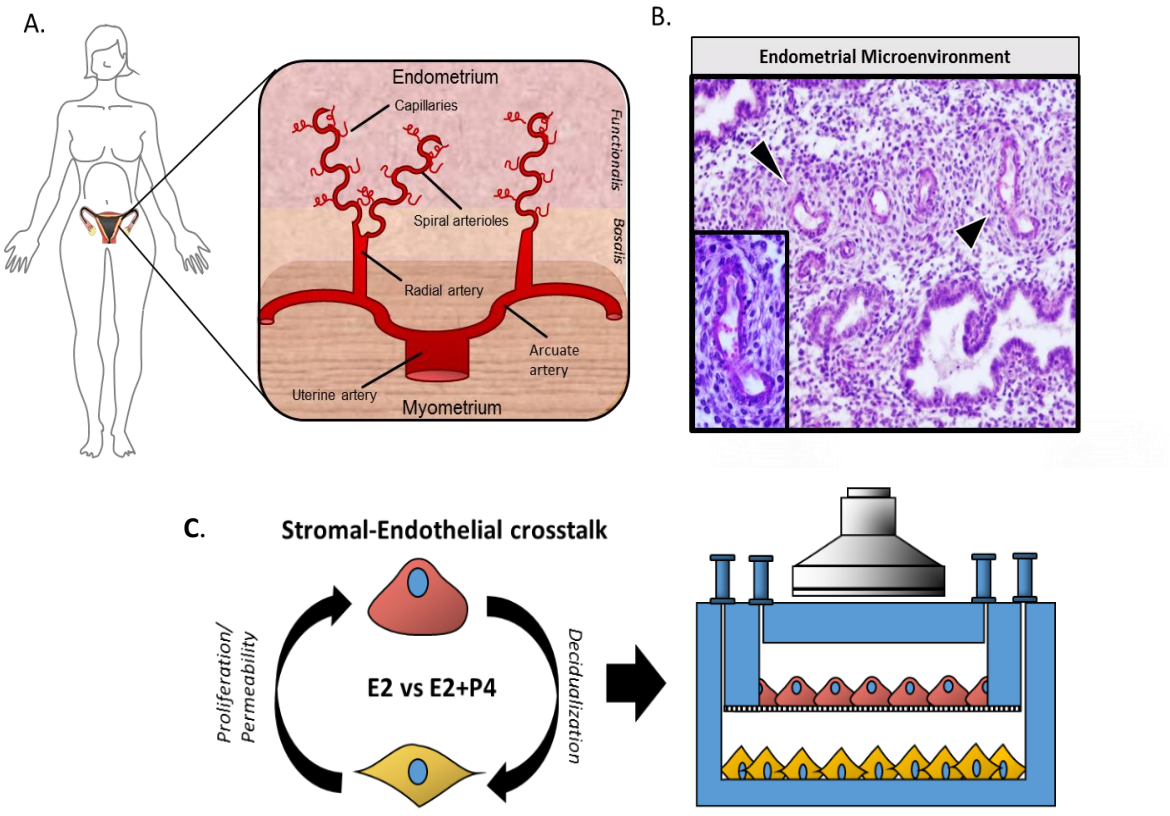


Figure 3-1. Breakdown of the human endometrial vascular bed. (A) Schematic characterization of the architecture of the vasculature in the human endometrial microenvironment. Remodeling of branching spiral arterioles provide the perfusion for the functionalis region of the endometrium. (B) Histological characterization of the endometrial vasculature in the human endometrium during the late secretory phase of the menstrual cycle. Arrows indicate the localized initiation of decidualization surrounding the spiral arterioles that is unique to the human endometrium. (c) Hypothesized crosstalk between stromal cells vs co-culture with UtMVEC during the cyclical changes of sex steroids during the menstrual cycle. Schematic design of the previously established dual chamber microfluidic device. Red cells (top) are representative of endothelial cells and yellow cells (bottom) are representative of stromal fibroblasts

3.1.2 The unique and localized reproductive processes of the endometrial perivascular stroma

As a critical component of endometrial preparation for pregnancy, specialized endometrial stromal fibroblasts undergo a unique differentiation process called decidualization. Proper decidualization is recognized clinically as necessary not only for the successful establishment of pregnancy but also for maintenance of pregnancy to term.[15,19] Defective decidualization has been implicated in infertility and pre-eclampsia-induced infertility.[11,15] This stromal transformation process is characterized morphologically and biochemically by changes from a undifferentiated fibroblast-like cell to an epithelial-like cuboidal cell capable of secreting pro-gestational proteins including prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1).[156] As mentioned, in humans and old-world primates, the onset of decidualization occurs in the mid-to-late secretory phase of the menstrual cycle in the absence of an implanting embryo and full decidualization only occurs in association with pregancy.[19,132] Although decidualization has long known to be a P4 dependent process, less is known about the role of locally produced factors that may serve in the *initiation* of stromal differentiation. Specifically, in humans, decidualization originates in the absence of implantation in stromal cells adjacent to the terminal spiral arterioles approximately 8-9 days after ovulation that will then spread throughout the endometrium if pregnancy is established.[5,19,96,143] Although this histologic observation was first recognized in the 1950s and is well accepted, the regulatory mechanisms that promote this localized decidualization response have not been extensively investigated. In the rodent, blastocyst implantation is initially associated with an increase in endometrial vascular permeability followed by

decidualization.[145,146] Relevant to the current study, numerous studies suggest that the rise in ovulatory P4 is not the only factor capable of initiating the decidualization response[19,149,157] It has been well-established, primarily in animal models, that prostaglandins can regulate the decidualization process.[96,146,158,159] Herein, we explored the proximal interactions between perivascular endometrial stromal cells and their adjacent vascular endothelium in an effort to understand early triggers of decidualization in the human endometrium (Figure 3-1.B, arrows). The crosstalk between the endometrial cells and microvascular endothelium may play a role in regulating reproductive function.

3.2 Results and discussion

3.2.1 Continuous Perfusion of the Vascular Endothelium Enhances Decidualization of the Stromal Fibroblast

The concurrent changes that occur at perivascular space suggest an interactive role for microvascular endothelial cells and the endometrial stromal fibroblast during reproductive decidualization.[143,160,161] To delineate the interaction between these two cell types in the endometrium, we first adapted the perivascular stroma model by co-culturing primary stromal fibroblasts and UtMVEC together under static and continuously perfused conditions. Compared to the model described in Chapter 2, we obtained a more relevant endothelial cell line in order to physiologically mimic the microvascular component of the human endometrium. Furthermore, in this chapter, we decided to utilize a commercially available semi-permeable track etched polycarbonate (PCTE) membrane to replace the 1002F resin-based membrane. This was necessary to reduce intra-variability between

devices and increase the throughput of device production. However, these changes did not alter our previously validation of the microfluidic model (Figure 3-2. A-B). We observed a clear compartmentalization of the two cell types that replicates what we have previously shown.[92] Each cell type adopted the characteristic morphologies akin to those seen in cells cultured on traditional polystyrene cell culture dishes, with distinct cobblestone morphology exhibited by the endothelial monolayer and a confluent layer of the stromal fibroblasts. Furthermore, we functionally compared primary HUVECs and UtMVECs in microfluidic applications by measuring the sensitivity to hemodynamic forces (Supplementary Figure 3-13).

OoCs were treated with E2 and MPA (*i.e.* EP) over 14 days to induce decidualization (Figure 3-2. C-F). To identify an additional role of the vascular endothelium on this process, we assessed stromal decidualization in the OoC, monitored morphologically and biochemically, in cells maintained alone or in co-culture with endothelial cells. In all experiments, we observed increased PRL secretion in cells treated with E2+MPA compared to E2 only; however, under static conditions, no statistically significant differences were apparent between stromal single cultures and the co-cultures (Figure 3-1. D). However, decidualization was significantly enhanced when co-cultures with endothelial cells were maintained under continuous laminar perfusion throughout the duration of the 14-day experiment as measured by the increased secretion of PRL and IGFBP-1 (Figure 3-2. E-F). Furthermore, co-cultures under perfused conditions effectively promoted a significantly more robust decidualization response compared to single culture of stromal cells, further suggesting that these effects are mediated by the flow-stimulated endothelium (Supplementary

Figure 3-11).

Next, to determine whether the observed enhanced stromal decidualization response is mediated via a flow-dependent paracrine mechanism, we examined the effect of E2 + MPA-primed (EP) conditioned media (CM) obtained from monocultures of UtMVEC in single chamber devices that were perfused at increasing flow rates (Figure 3-3. A). Stromal cells that were supplemented with perfused endothelial CM, at 1 and 3 µl/min, exhibited significantly elevated PRL secretion in a flow rate dependent manner compared to static endothelial CM (Figure 3-3. B). Altogether, these results confirm our findings and suggest that endothelial-derived paracrine signals from perfused conditions were mediating the enhanced decidualization response of the stromal cells.

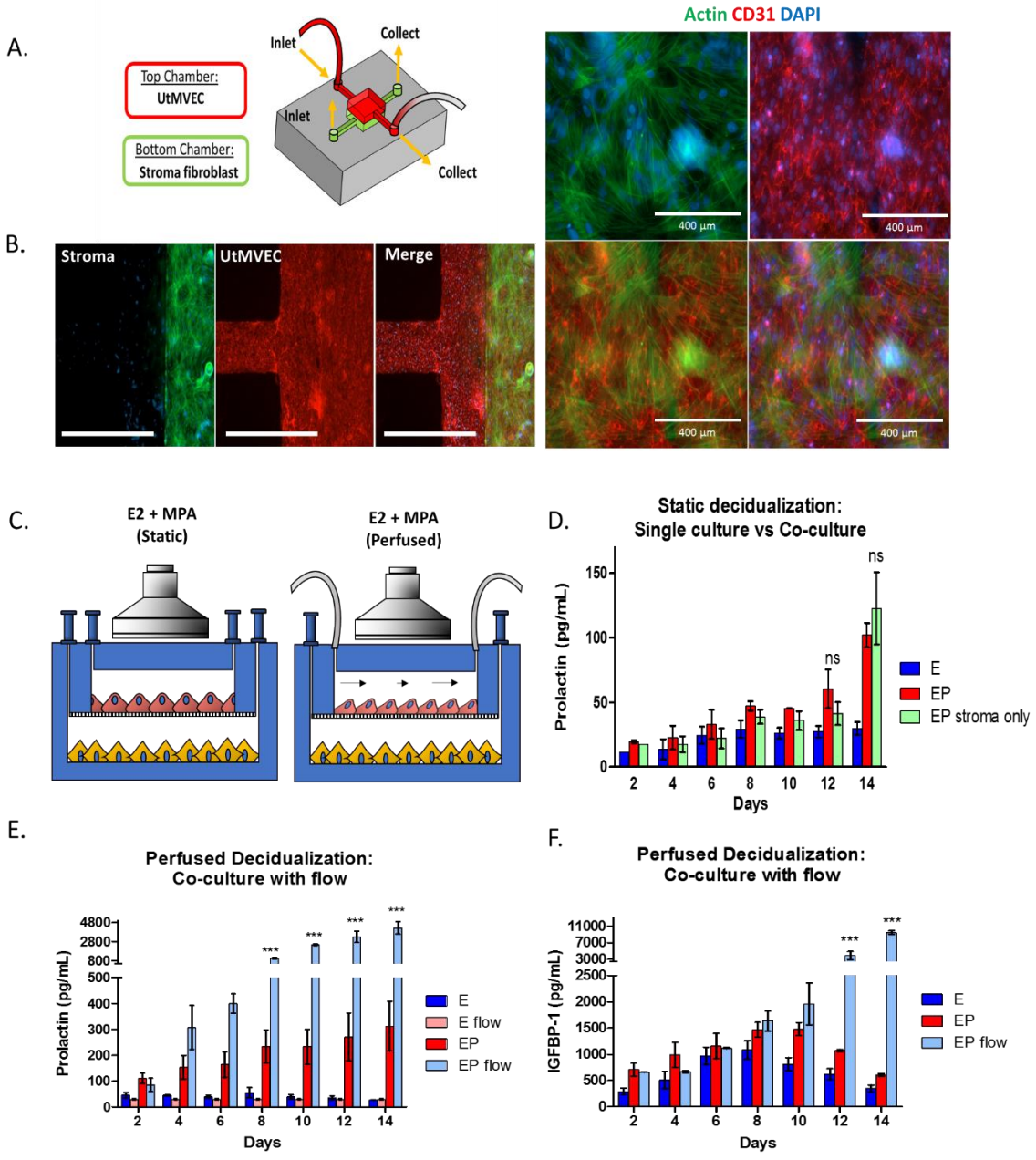


Figure 3-2. Characterization of primary endometrial fibroblasts (stroma) and uterine microvascular endothelial cells (UtMVEC) co-cultures in a microfluidic model during decidualization in static and perfused conditions. (A) Schematic of model of perivascular stroma model using a design established device with UtMVEC and an experimental perfusion set-up. (B) Immunofluorescence staining shows individual stromal (green, actin) or UtMVEC (red, CD31) monolayers in co-cultures with adequate characteristic morphologies during long-term cultures. (b, scale bar= 100um). (C) Schematic of experimental set up comparing static (i.e. gravity fed) vs flow perfusion during a 14-day experiment under the influence of a synthetic progestin (MPA). (D-F) Daily samples of spent media were analyzed biochemically for decidualization markers. (D) Static co-culture of the perivascular model had no difference in PRL secretion compared to single culture of stromal cells. (E) Prolactin and (F) IGFBP-1 were significantly enhanced in the perfused model compared to static co-cultures. (N=7) E=E2 only, EP=E2 + MPA, Flow = Perfused at 1µL/min

3.2.2 Endothelial-Stromal Co-Cultures with Laminar Shear Stress Enhanced Decidualization via COX-2 Induction

Hemodynamic forces can act as a stimulus in endothelial cells to induce prostaglandin generating cyclooxygenase (COX) enzymes.[162–165] Therefore, we characterized whether the enhanced stromal cell decidualization is due to laminar shear stress forces via activation of the endothelial COX-2 signaling pathway in our microfluidic model.[162,166] Using single chamber devices, we stimulated UtMVEC with perfusion and observed morphologic and molecular responses to increasing flow rates associated with shear stress. Under perfused conditions, we observed endothelial re-orientation of the actin filaments toward the direction of the flow (Figure 3-2. C) with a concomitant increased activation of COX-2 (Figure 3-2. D, Supplementary Figure 3-13. C). COX-2 is the rate-limiting enzyme for the generation of terminal prostanoids, thus, we demonstrated that the specific prostaglandins, prostaglandin E2 (PGE_2) and PGI_2 (prostacyclin) were secreted in the CM of perfused endothelial cells on a rate-dependent manner. (Figure 3-3. E). Altogether, these findings confirm that laminar shear stress forces are inducing the prostaglandin pathway in our microfluidic model.

Next, we conducted a similar experiment using the OoC co-culture of endothelial cells and stromal cells and confirmed that endothelial perfused conditions at a flow rate of 1µL/min did enhance the decidualization response and was associated with hemodynamic activation of endothelial COX-2. As shown in Figure 3-4. A, immunofluorescence (IF) analysis of endothelial cell COX-2 protein expression was evident in the OoC co-culture model. We also observed significantly increased secretion of PGE_2 and prostacyclin in the perfused models throughout the 14-day treatment

compared to static cultures (Figure 3-4. B). Furthermore, to determine whether the COX-2 pathway is mediating the enhanced stromal decidualization in the perfused OoC co-culture, we examined the impact of indomethacin, a non-steroidal anti-inflammatory drug (NSAID). Treatment of endothelial cells with indomethacin suppressed the production of PGE_2 and prostacyclin and resulted in a concomitant loss of the perfusion-induced enhanced decidualization response (Figure 3-4. C-D). These findings suggest that COX-2 signaling is mediating the effects of the perfused endothelial cells and supports previous findings in animal models that administration of COX-2 inhibitors suppresses decidualization.[154,155,167,168]

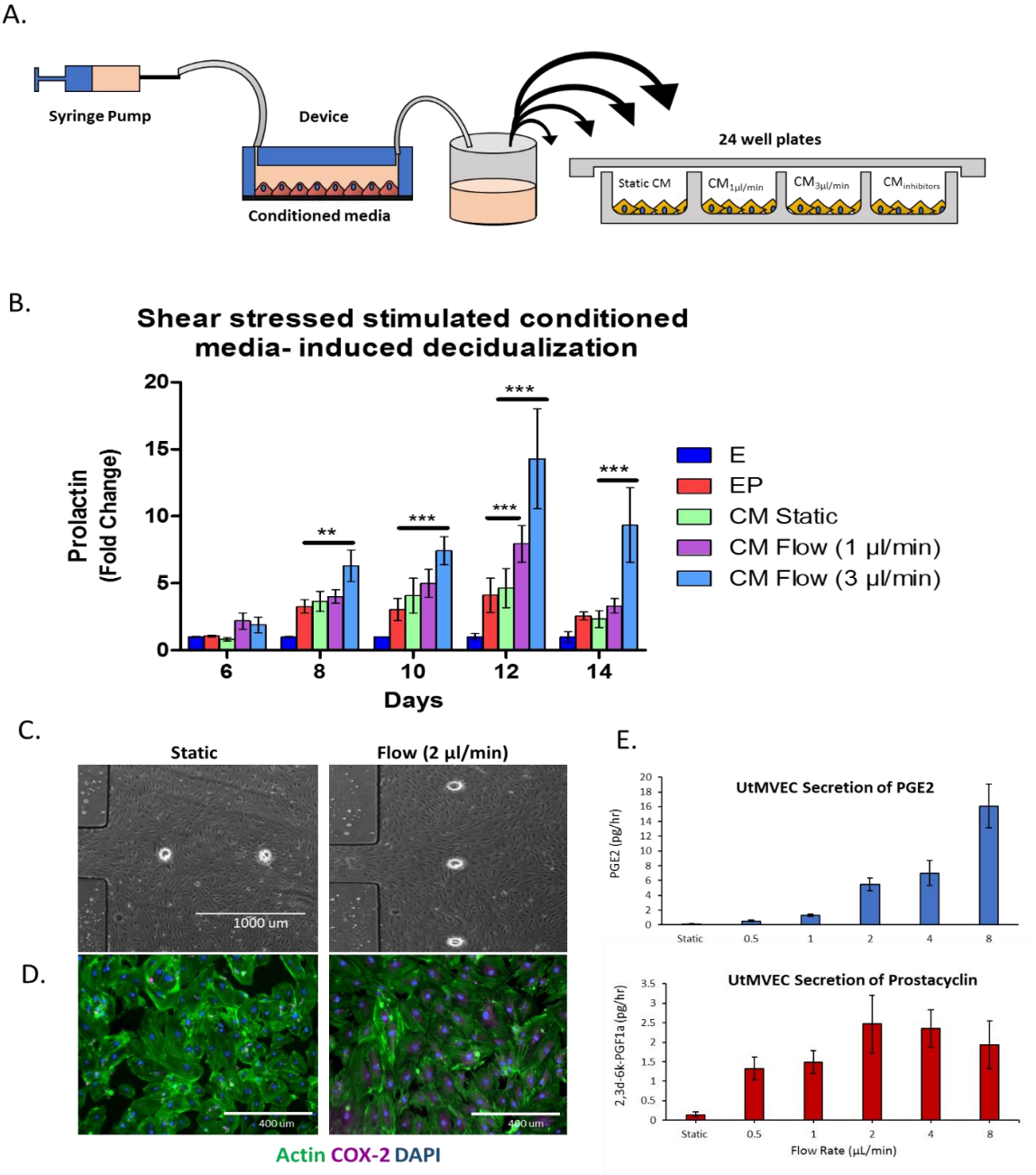


Figure 3-3. Vascular endothelial cells enhance decidualization via a paracrine mechanism mediated by hemodynamic activation of COX-2 pathway. (A) Schematic of experimental approach for perfused conditioned media (CM) assays. (B) Perfused endothelial CM accelerated PRL secretion on a flow-rate dependent manner compared to static endothelial CM (N=7). (C-E) Characterization of shear stress activation of the UtMVEC in the microfluidic perfused model. (C) Bight field remodeling of UtMVEC under flow conditions in single chamber devices in response to microfluidic flow perfusion. (D) Morphological remodeling and orientation of the cytoskeleton in direction of flow (actin, green) and shear stress-induced activation of COX-2 (purple). (E) Validation of shear stressed secretion if endothelial derived prostaglandins. Conditioned media from single chamber shear stressed endothelial cells was collected and measured for PGE_2 and prostacyclin during step wise increases in flow rates (N=4). CM = endothelial conditioned media.

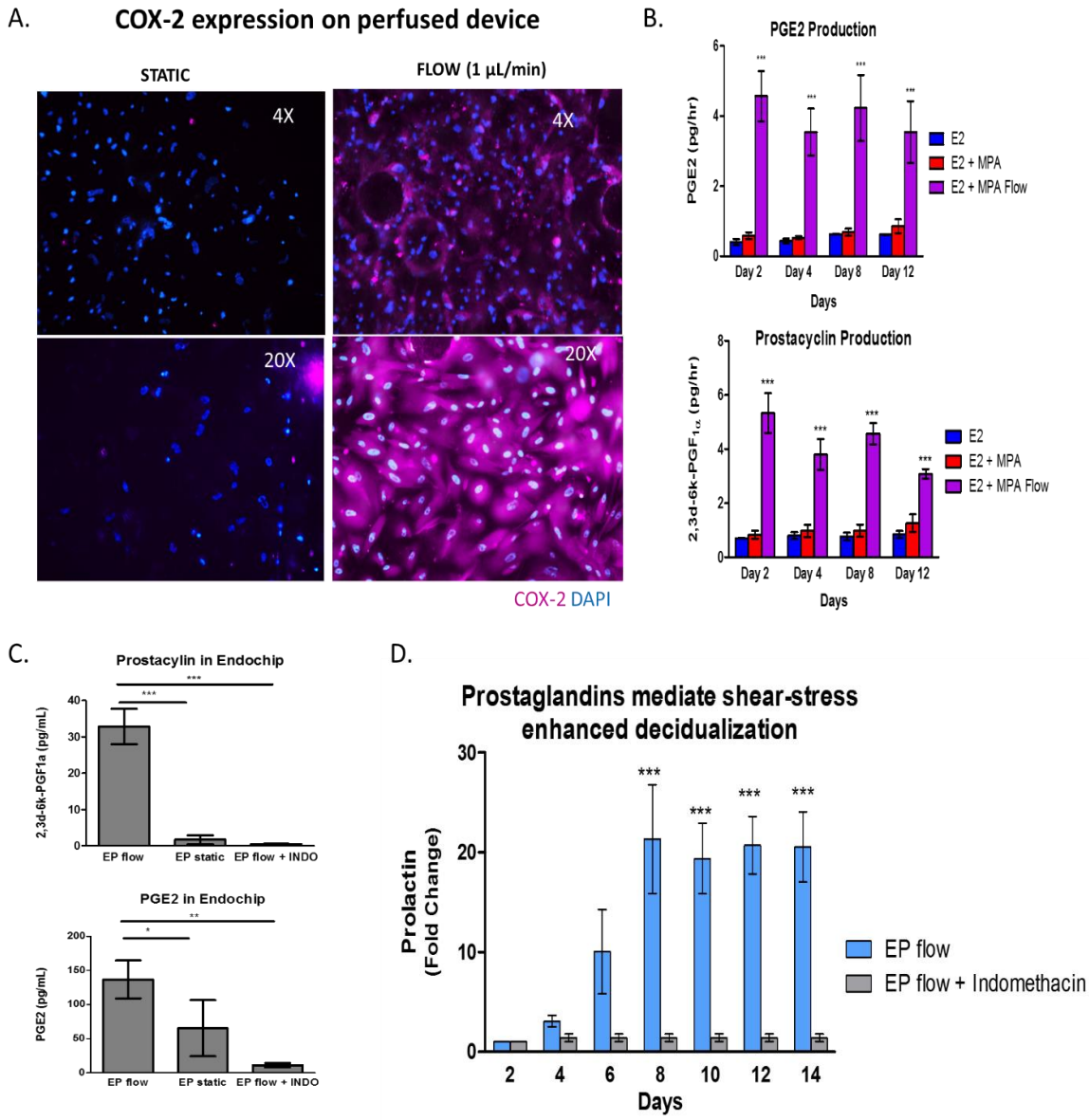


Figure 3-4. The COX-2 pathway in an OoC co-culture model mediates perfusion-enhanced stromal decidualization. (A) Immunofluorescent staining for COX-2 in perfused (1µL/min) microfluidic co-culture devices demonstrates increased activation of COX-2 in the endothelial cells compared to static conditions in perivascular model (Original magnification 4X and 20X). (B) Subsequent secretion of prostaglandins such as prostacyclin and PGE_2 in microfluidic co-cultures under flow compared to static conditions. (C-D) Enhanced decidualization can be inhibited with administration of indomethacin (INDO, 100 µM) in the endothelial chamber of co-culture perfused model throughout the 14-day experiment. (C) Indomethacin treated co-cultures have reduced prostaglandins and (D) result in suppression of perfused enhanced PRL secretion.

3.2.3 Endothelial-derived prostacyclin, and prostaglandin E2 promotes the initiation of decidualization in the endometrial perivascular stroma

Prostanoids are separated into four major types of bioactive prostaglandins; prostaglandin E2 (PGE_2), prostacylin (PGI_2), prostaglandin D2, (PGD_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and, as such, hold multifaceted roles in both homeostatic functions as well as mediating pathogenic inflammatory responses. Specifically, some of these prostaglandins can act as intermediates in the cyclic adenosine monophosphate (cAMP)-mediated signaling pathway, a secondary messenger necessary in the decidualization process.[130,131,169] Next, we examined the response of human endometrial stromal cells to the specific prostanoids PGE_2, PGD_2 and prostacyclin since they are known to be potent activators of intercellular cAMP and have previously been implicated in reproductive function.[102,129,131,132,170–173] EP media supplemented with PGE_2, PGD_2 and iloprost, an analog for prostacyclin, was administered on cultured stromal cells in order to identify specific endothelial-derived terminal prostaglandins that may promote decidualization. As shown in Figure 3-5, we observed that both PGE_2 and iloprost, but not PGD_2, significantly enhanced PRL secretion (Figure 3-5. A). As expected, this was associated with increased stromal cell cAMP production (Figure 3-5. B). Although PGE_2 induced a maximal response nearing that of exogenous 8-Br-cAMP, iloprost appeared to induce decidualization at an accelerated response (Figure 3-5. A, C). Importantly, while a number of cells produce multiple prostaglandins, prostacyclin is a primarily a product of stimulated vascular endothelial cells,[174] suggesting a potential role in regulating reproductive function in the perivascular space.[163,175] As expected from the literature, cultured stromal cells in our studies were confirmed to express the receptors for PGE_2 (EP2, EP4), and prostacyclin

(IP), but not PGD_2 (DP-1), at the protein level *in vitro* (Figure 3-5. D, Supplementary Figure 3-15), likely explaining the lack of response for PGD_2. Additionally, co-treatment with a receptor antagonist of prostacyclin completely blocked the effect of iloprost, while inhibitors for EP2, EP4 receptors individually had no effect in reducing the enhanced PRL production by the stroma unless used in combination (Figure 4. C). Taken together, these findings suggest a temporal role of prostacyclin during the initiation of decidualization in the human endometrium.

We also demonstrated that both PGE_2 and prostacyclin were generated by shear stressed (3μL/min) endothelial cells and that this CM enhanced decidualization of stromal cells *in vitro.* Furthermore, CM-induced stromal cell differentiation could be blocked with the addition of specific prostaglandin receptor antagonists *(*Figure 3-6. A*)*. Although we observed strong suppression of enhanced decidualization by blocking either IP or EP2 and EP4, their effects were modestly additive when used in combination (Figure 3-6. A). Morphological assessment confirmed suppression of decidualization by inhibitor treated CM (Figure 3-6. B).

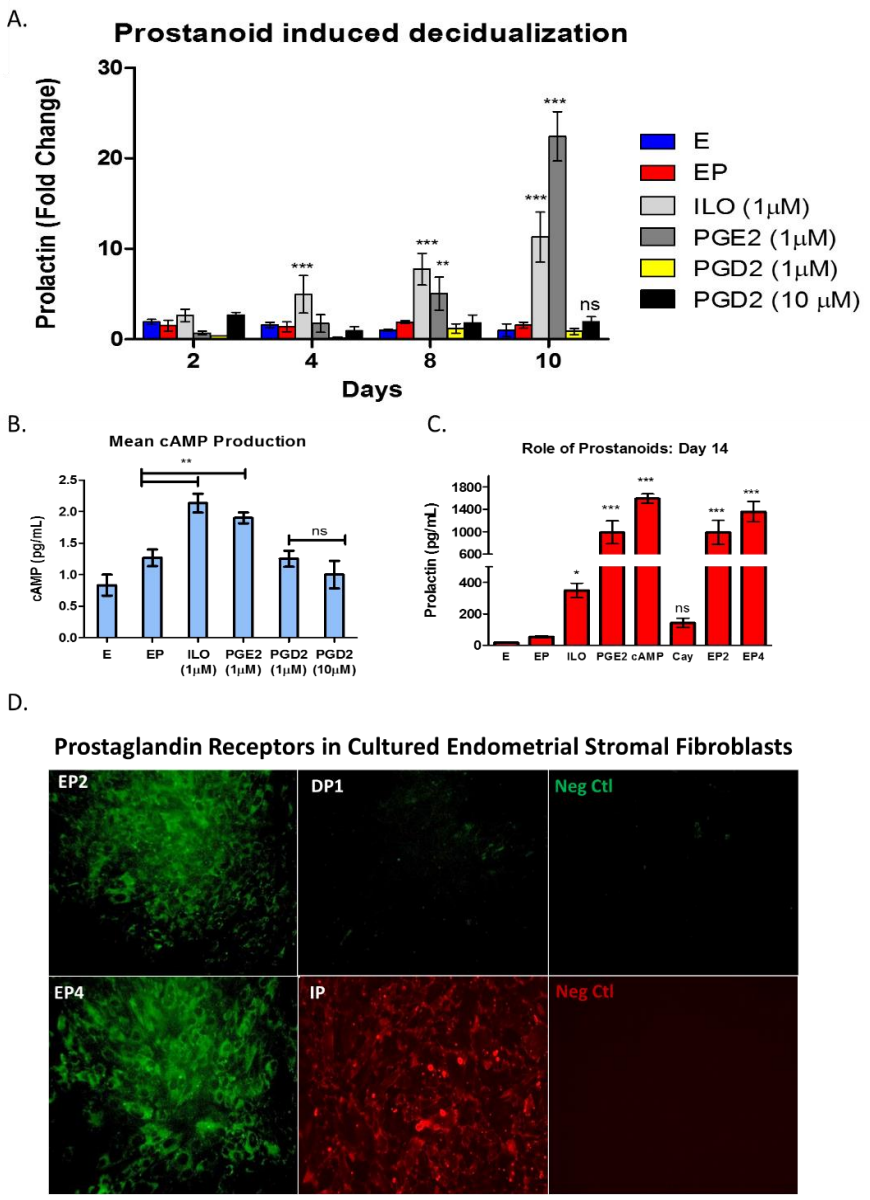


Figure 3-5. Prostacyclin and PGE_2, specifically enhance the decidualization response of stromal cells via activation of the cAMP pathway. (A) Exogenous prostaglandins can enhance the decidualization process of endometrial stromal fibroblasts in vitro. The addition of PGE_2 (1μM) and Iloprost (ILO, 1μM), but not PGD_2 (1 or 10μM), enhance the decidualization response. (N=6 in duplicate). Administration of 8-Br-cAMP (0.5mM) can maximize the decidualization response of the stromal cells. Administration of prostacyclin receptor (CAY, 10 μM) can block this effect. Blocking individual PGE_2 receptors (EP2, 20 μM and EP4, 20 μM) fail to block PGE_2 unless used in combination. (D) Representative immunofluorescence images of prostaglandin receptor expression in the cultured stromal cells. IP = prostacyclin receptor, EP2, EP4 = PGE_2 receptor isoforms DP1 = PGD_2 receptor.

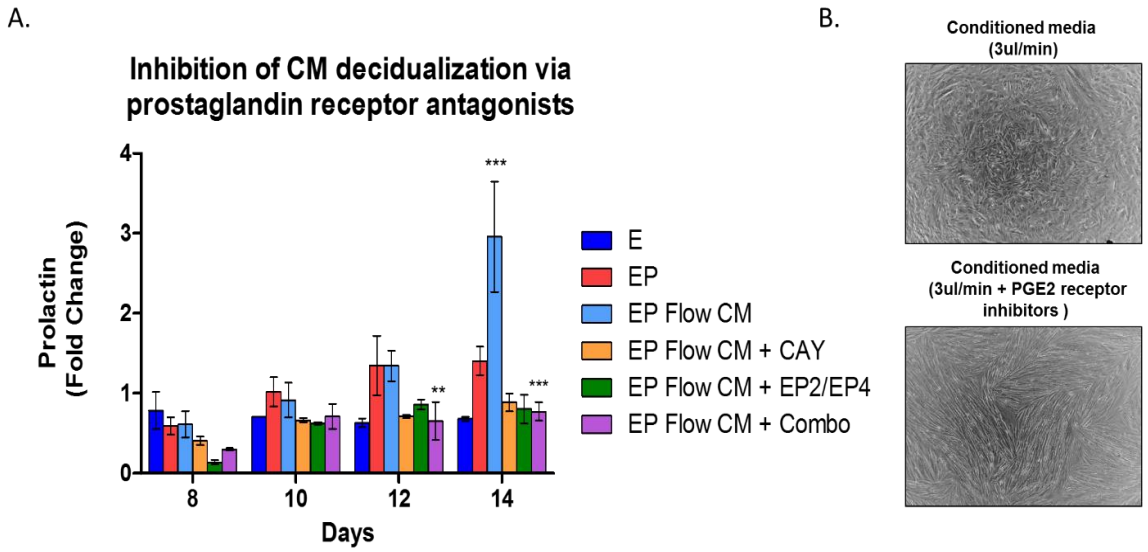


Figure 3-6. Shear stress-induced endothelial-derived prostaglandins enhance decidualization via stromal prostanoid receptors. (A) Accelerated decidualization was observed on a flow rate dependent manner compared to static endothelial conditioned media using conditioned media from perfused endothelial cells. CM-induced decidualization from shear stressed endothelial cells (3µL/min) can be blocked with co-treatment with prostanoid receptor antagonists. (C) Representative images of CM stimulated stromal decidualization and blocking with combinatorial prostaglandin receptor antagonists. CAY = IP inhibitor (CAY10441), EP2 and EP4 = specific PGE_2 receptor inhibitors. Combo = CAY + EP2 + EP4. Experiments were performed with an N=6 in duplicate.

3.2.4 COX-2 and Endometrial Decidualization in the Human Tissue

Lastly, we demonstrated a similar role of COX-2 *in vivo* by examining human late secretory phase[5] endometrial surgical samples that clearly exhibit decidualization at perivascular sites (Figure 3-1. B). Immunofluorescence analysis of COX-2 and the endothelial cell marker ULEX, revealed co-localization in the vascular endothelial cells in the human endometrium. Importantly, decidualization of these same tissues was observed only in cells surrounding the spiral arterioles, while stromal cells distal to vessels did not exhibit morphologic changes (Figure 3-7.*V*). In comparison, residing immune cells and epithelial glands predominately expressed COX-2 in proliferative phase tissues, but do not necessarily promote stromal cell decidualization (Supplementary Figure 3-14. A-B), suggesting a cycle dependent expression of specific terminal prostaglandin synthases and their respective receptors.[155,176,177]

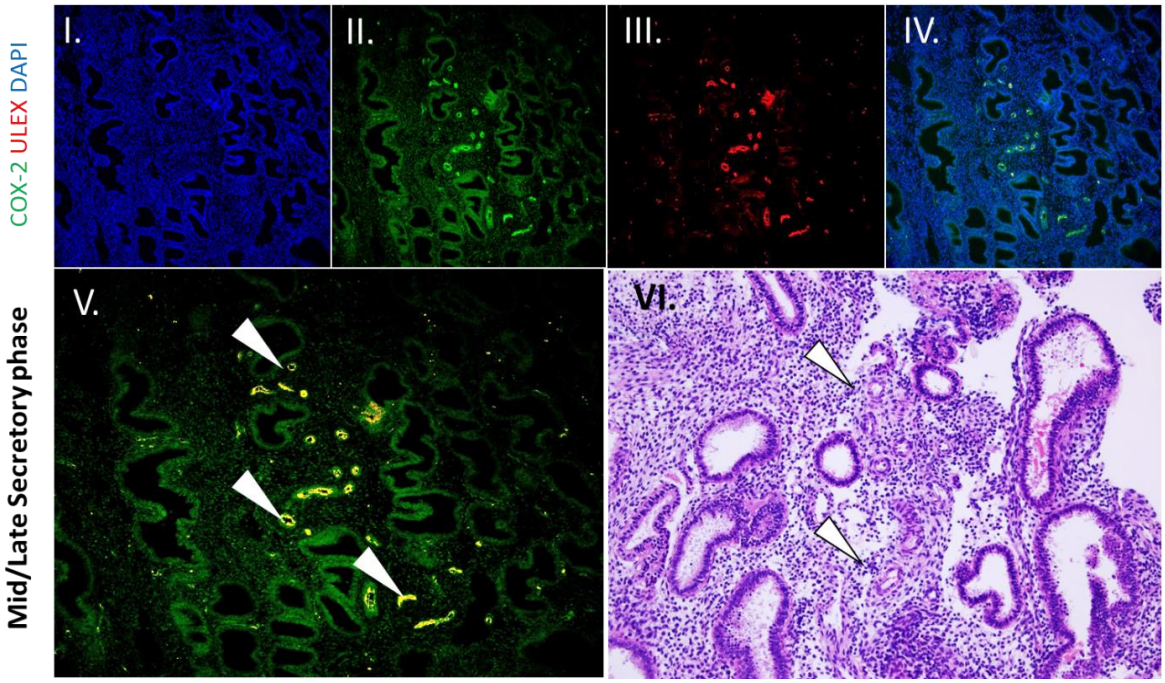


Figure 3-7. COX-2 co-localizes in the human spiral arterioles in the secretory phase of the menstrual cycle. Histologic section of an endometrium beginning to decidualize around the perivascular space (see background, H&E). (I) DAPI, (II) COX-2, (III) Ulex europaeus agglutinin I (ULEX) was used to identify endothelial cells, (V) Merge of the images. Arrows depict co-localization of staining (yellow, 20x) (VI) Matched H&E of mid secretory phase human endometrium undergoing decidualization response at the perivascular stroma.

3.2.5 Shear Stress-Induced, but Not Pro-Inflammatory Cytokine-Induced, Activation of Endothelial-Derived Prostaglandins Enhances Decidualization

Inflammatory mediators such as prostanoids are strictly regulated to maintain reproductive function and protection against invading pathogens. Therefore, we examined whether alternative agonist-mediated stimulation of these inflammatory mediators is sufficient to confer the enhanced decidualization response observed in our perfused model. We tested the CM from pro-inflammatory cytokine-activated endothelial cells in static conditions and its effect on the decidualization response of stromal fibroblasts. As compared to shear stress-mediated COX-2 activation, we observed that pro-inflammatory cytokine activation of endothelial COX-2 with IL-1β suppressed the decidualization response although elevated concentration of PGE_2 and prostacyclin were present (Figure 3-8). Although the stromal cells were indirectly exposed to an IL-1β, the endothelial cells likely secrete other pro-inflammatory molecules in addition to prostaglandins that promote this negative effect.

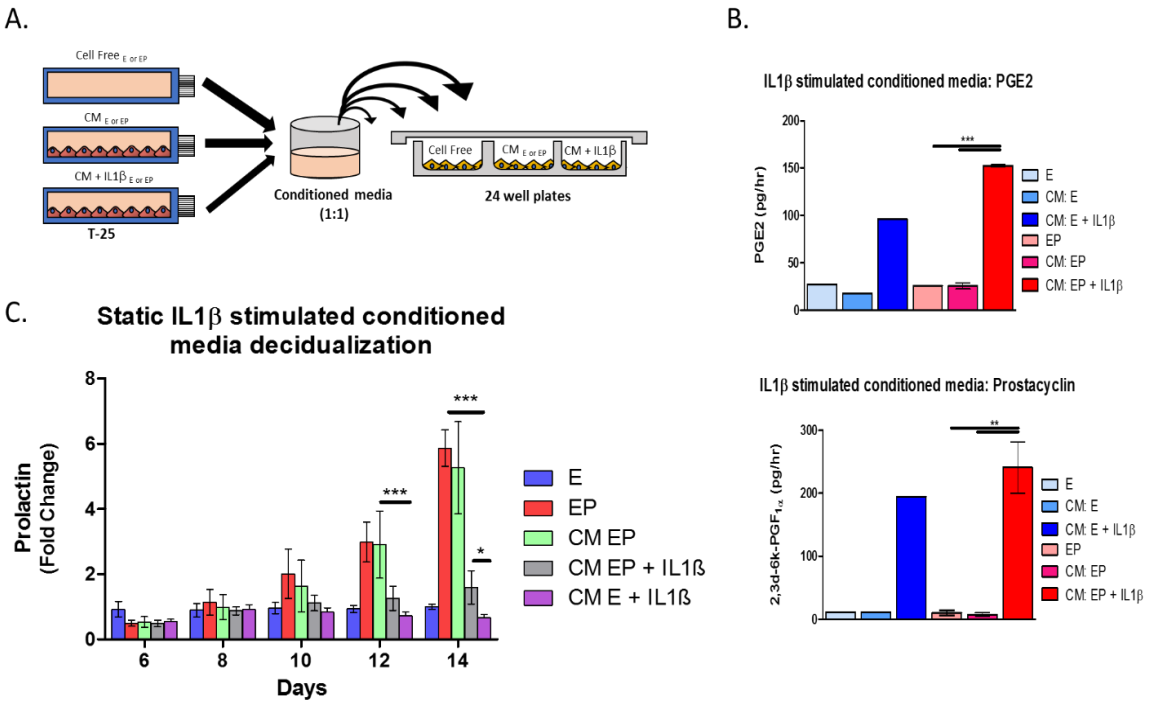


Figure 3-8. Characterization of static conditioned media assays and COX-2 activation by pro-inflammatory stimuli. (A) Schematic of the experimental design for static Il-1β stimulated conditioned media (CM) experiments. Endothelial cells were stimulated with IL1β for 24 hours, washed and replenished with fresh media for collection. Stromal cells were then cultured for 14 days supplemented with the conditioned media in a 1:1 dilution. (B) Production of prostaglandins (PGE_2 and PGI_2) were confirmed in these stimulated conditions compared to unstimulated. (C) Decidualization was suppressed in the IL1β stimulated cells compared to EP only or unstimulated CM with EP.

3.3 Materials and Methods

3.3.1 Acquisition of Human Tissues and Isolation of Stromal Cells

The Vanderbilt University Institutional Review Board approved the procedures for collection of all primary human cells used and tissue acquisition was performed only after patients gave informed consent. For acquisition of primary endometrial stromal cells, surgically excised uterine tissues were collected from consented donors (ages 18–45) exhibiting predictable menstrual cycles and undergoing a hysterectomy for benign leiomyoma not associated with any additional inflammatory ovarian or endometrial disease. Some samples were also obtained by a pipelle biopsy from voluntary endometrial donors with no history of reproductive disorders nor on any hormonal contraceptive medication from the late proliferative phase. All endometrial stromal cells were isolated by enzymatic digestion and filter separation as previously described.[178] The purity of the stromal cell isolation procedure utilized was above 95% and was quantified by morphological assessment and positive staining for vimentin as previously described.[178] Stromal cells were maintained in phenol red-free DMEM/F-12 with 5% charcoal-stripped calf serum, 1nM 17-β estradiol (E2, Sigma Aldrich, USA) and 1X antibiotic-antimycotic solution (ThermoFisher Scientific, USA) (stromal complete growth medium). As required for experimental objectives, some stromal cell cultures received treatments with 0.5 mM of the synthetic progesterone medroxyprogesterone acetate (MPA, Sigma Aldrich, USA) and/or 8-bromoadenosine-3',5'-cyclic monophosphate (cAMP, 0.5mM, Sigma Aldrich, USA) over a period of at least 14 days. Primary human uterine microvascular endothelial cells (UtMVEC) (Lonza, Cologne, Germany) were purchased and cultured as described by

the manufacturer.[137] Briefly, the cells were cultured in EGM™-2MV BulletKit™ (Lonza, USA), maintained at 37°C in a saturated humidity atmosphere containing 95% air/5% CO_2, and they were sub-cultured before reaching 60–70% confluence (approximately every 2 days) up to passages 8–10. Upon experimental set up, complete endothelial media was supplemented with E2 (1nM) or E2 + MPA (0.5mM).

3.3.2. Fabrication and assembly of microfluidic two-chamber device

The microfluidic organ-on-a-chip device was assembled using our previously described protocol.[92] Briefly, liquid polydimethylsiloxane (PDMS, Sylgard® 184, Dow Corning, USA) was mixed at a 10:1 prepolymer to curing agent ratio was cast and cured on the SU8 master to obtain two equal layers. Ports to access the microfluidic chambers were opened by punching 1/16'' holes in the top layer with a stainless-steel round punch (Integra Myltex, USA). Porous polycarbonate membranes (PCTE, 3μm, 13mm, Sterlitech, USA) were used as the semi-permeable membrane to separate the two chambers. The PDMS layers and the membrane were then oxygen-plasma treated (Plasma Cleaner 600 mTorr, 100 W, 45 s) and bonded together. For seeding and static maintenance, 500 μL Pyrex cloning cylinders (ThermoFisher Scientific, USA) were bonded with liquid PDMS to the inlet/outlet regions of each channel to form reservoirs for the cell culture.

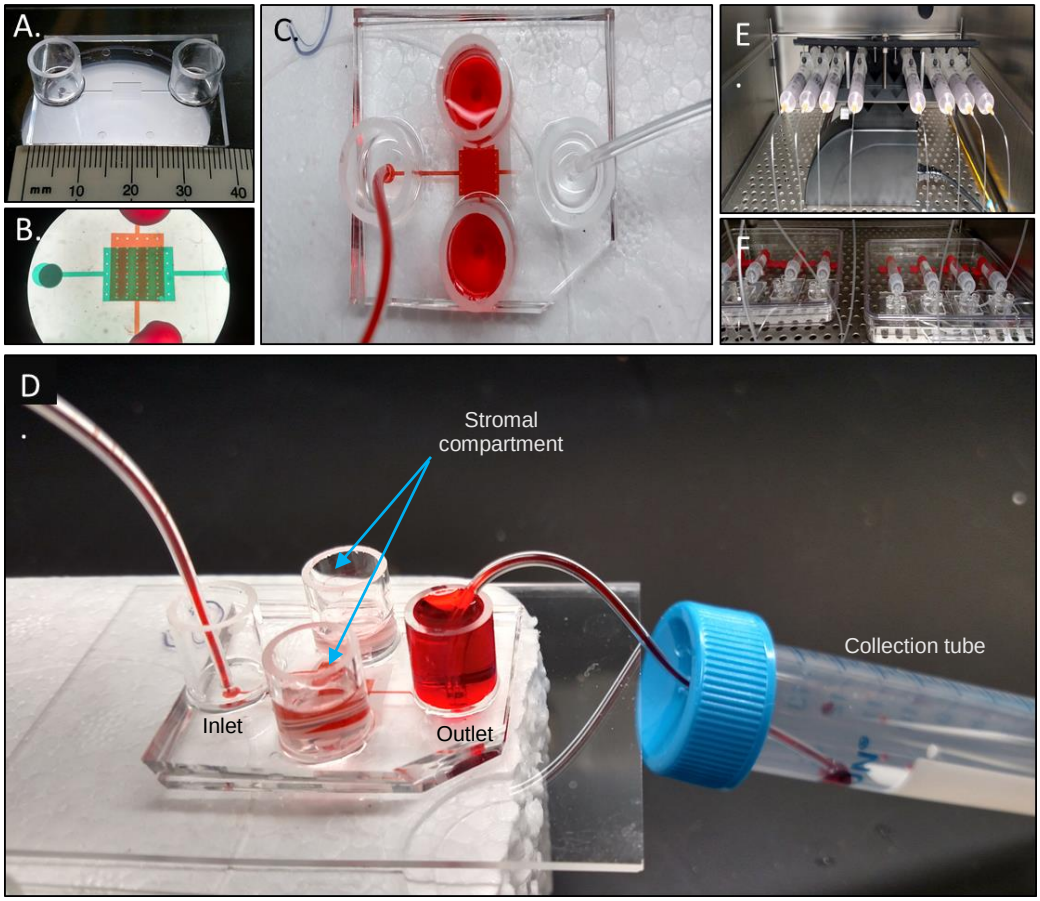


Figure 3-9. Experimental microfluidic perfusion design in OoC co-cultures. (A) Photograph of single chamber devices bonded to glass were used to culture UtMVEC and perfused for collection of shear stressed conditioned media (CM). (B) Photograph of representative compartmentalized dual chamber device (red: vascular chamber; green: stromal chamber). (C-D) Top and side view of the microbore tubing for perfusion of the vascular chamber. (E-F) Photograph of the devices being perfused using a Multi-Rack syringe pump.

3.3.3. Cell culture and maintenance in microfluidic device

Isolated primary stromal and endometrial cells were seeded and cultured using the procedure described elsewhere[92] with a few experimental adaptations. Briefly, the assembled, empty microfluidic device was sterilized by UV exposure for 12 hours on both the top and the bottom by flipping the device. Poly-L-lysine (Sigma Aldrich, USA) was used to charge the PDMS for 20 minutes, washed with DPBS (ThermoFisher Scientific, USA) and coated with a thin layer of collagen type IV (10 µg/cm^2, Santa Cruz, USA) on both chambers and washed with PBS (3X). All reservoirs were aspirated and using a 1 mL syringe (Monoject, Sherwood Medical, USA), the endothelial cells were seeded on the top chamber (2 x 10^6 cells/mL) and stromal cells on the bottom chamber (1.5 x 10^6 cells/mL). The cells were allowed to adhere for a minimum of 30 minutes at 37°C and then 350 µL of EBM-2 medium or complete growth medium, was added to the endothelial top chamber or stromal bottom chamber, respectively. Spent media was collected and replaced with fresh media every 48 hrs throughout all static experiments. Continuous laminar shear stress (perfusion) of the endothelial compartment of the microfluidic device was performed with a programmable PHD Ultra syringe pump (Harvard Apparatus, USA) with a 6 X 10 Multi-Rack adapter using 20 mL luer-lock disposable plastic syringes (Monoject, Sherwood Medical, USA) filled with complete endothelial media (EBM-2). Syringes were adapted with a blunt end needle (SAI, Infusion Technologies, USA) and connecting microbore Tygon® tubing (0.02"ID X 0.06"OD, Cole-Parmer, USA) to the needle and the other to directly into the inlet port of the device. Shear stress conditions were induced when the cells reached 60% confluence inside the chamber and treatment was run for 14 days. The arbitrary rate of 1 µl/min was defined within this protocol as minimal value for the cells to

morphologically and metabolically become shear stressed. Wall shear stress in the center of the chamber was calculated as in Sung et al.[179] by treating the chamber as a relatively flat channel (with width >> height). The stromal cells were maintained under the same static culture conditions through all experiments. Collection of the effluent from perfused devices was performed using 5 mL disposable tubes connected to the outlet of the devices via microbore tubing. The height of the outlet and collection tubes must be exactly the same height to avoid a syphon effect by the pump. Media was collected from the bottom chamber and the collection tube and frozen at -80°C every other day for analysis. Pharmacologic inhibition of COX-2 was performed by supplementing indomethacin (100μM, Sigma Aldrich, USA) in the syringe and perfused as described above throughout the 14-day experiment. Administration of exogenous prostaglandins on stromal cells cultured in polystyrene dishes was performed to measure their individual effect on decidualization during 14-days. Spent media was collected every 48 hrs. Iloprost (Cayman Chemicals, USA) was utilized as a prostacyclin analogue and exogenous PGE_2 and PGD_2 (Sigma Aldrich, USA) were used as prostaglandin agonists.

3.3.4. Analysis of decidualization and prostaglandins

Sample collection was performed from confluent single or two chamber devices containing stromal or UtMVEC were cultured for 14 days in their respective medium containing estradiol (1 nM) and/or MPA (0.5 nM). In static cultures, media was collected and replaced (300 μl) from all reservoirs every other day. Sample collection from perfused devices was performed by taking 600 μl from the collection tube connected to the vascular chamber outlet. The total volumes dispersed by the pump was recorded for each

experiment for downstream analysis. Sample collection from the static stromal compartment was performed as described above. Collected effluents were frozen at -80°C until the termination of the experiment. Biochemical measurements of PRL and IGFBP-1, were performed using Human PRL and IGFBP-1 enzyme-linked immunosorbent assays kits (ELISA Duoset, R&D Systems), respectively. The ELISAs were performed according to the manufacturer's instructions and expanded to 384 formats, using 23 μL per sample. Prostaglandins were measured using the Prostaglandin E2 ELISA Kit – Monoclonal (Cayman Chemicals, USA) and urinary prostacyclin ELISA kit (Enzo Life Sciences, USA) according to the manufacturer's protocol. Absorbance was measured using an absorbance microplate reader (GloMax® Multimode Readers, Promega). Validation of diffusion of prostaglandins between the vascular (top) and stromal (bottom) chambers were confirmed (Supplementary Figure 3-12).

3.3.5. Fluorescence microscopy

For *in vitro* immunostaining, samples were fixed with PBS containing 4% (wt/vol) paraformaldehyde (PFA). Fixed cells were blocked with 10% (vol/vol) normal goat serum and incubated with either primary fluorescence-labeled mAb or unlabeled mAb, followed by fluorescence-labeled secondary antibody, or the goat anti-mouse ReadyProbes™ AlexaFluor 594 Reagent Secondary (ThermoFisher Scientific. USA) for 30 minutes at room temperature. F-actin probe ActinGreen™ 488 ReadyProbes™ Reagent (ThermoFisher, USA) was used for staining for actin. For endothelial cells, we used mouse monoclonal anti human CD-31 primary antibody (1:50, DAKO, USA) and as the secondary antibody. Mouse anti-human COX-2 antibody conjugated to AF647 (1:50, Santa

Cruz, USA) was used. To identify stromal cells, a rabbit antibody against human vimentin (1:400, Abcam, USA) was used with an AF647 conjugated goat anti-rabbit antibody as a secondary antibody (1:200, Jackson ImmunoResearch, USA). Rabbit anti-human antibodies for EP2, EP4, DP1 (1:100, Cayman Chemicals), and the mouse anti-IP (1:100, Santa Cruz, USA) were utilized as primary antibodies for IF with the appropriate secondaries.

Immunohistochemical (IHC) localization of selected proteins was conducted in our laboratory using commercially available antibodies and the Vectastain Elite ABC kit (Vector Laboratories Inc, USA) according to the manufacturer's protocol. Antibodies and concentrations used were: Rabbit anti-human antibodies for EP2, EP4, DP1 (Cayman Chemicals), and the mouse anti-IP (Santa Cruz, USA). The cell nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) for 1 minute and then washed with 1X PBS. Commercially available, species-specific biotinylated secondary antibodies were used per the manufacturer's recommendation. Co-localization studies were performed using mouse anti-human COX-2 antibody conjugated to AF647 (1:50, Santa Cruz, USA) and rhodamine labeled Ulex Europaeus Agglutinin I (ULEX, Vector Laboratories Inc, USA) according to the manufacturer's recommendation. ProLong® Gold antifade reagent with DAPI (ThermoFIsher Scientific) was used to prior to adding the coverslip. All slides were viewed using an Olympus BX51 microscope system and images captured using an Olympus DP71 digital camera.

3.3.6. Conditioned media assays

Endothelial cell conditioned media was collected using single chamber devices

bonded to glass. UtMVEC were seeded at 1.5 x 10^6 cells/mL and were cultured until they reached 80% confluence. BD-luer lock (12mL, BD, USA) syringes were pre-loaded with media and warmed in the incubator overnight at 37°C to acclimate the media and prevent bubble formation within the device. The syringes were connected to a Harvard Apparatus PHD pump and set at the desired flow rates; a collection tube was connected to the outlet of the single chamber device. A stepwise increase in flow rates was applied to the cells. After collection the conditioned media (CM) was supplemented with E2 and MPA, centrifuged at 300 RPM, to remove particulates, and frozen at -80°C in aliquots for experiment supplementation and analysis. The CM was utilized by diluting it in 1:1 in fresh complete stromal media (complete DMEM/F12 media). To avoid dilution issues, three single chamber devices with endothelial cells were serially connected and pooled to increase the physiological response and secreted factors. Is for collection of CM or for its use? Static conditioned media was acquired from cells grown in maintained in gravity-fed conditions. Static activation of COX-2 was performed by stimulating the endothelial cells with rhIL1β (10ng/mL, R&D, USA) for 24 hours followed by washes with PBS (3X) and replacement with fresh media for an additional 24 hours. Endothelial media supplemented with hormones was used as cell-free controls. Inhibitory assays of CM-induced decidualization was performed with CM derived from endothelial cells perfused at 3μL/min for 24 hrs supplemented with specific prostaglandin receptor antagonists: EP2 (AH6809, Sigma Aldrich, USA), EP4 (AH23848, Sigma Aldrich, USA), IP (CAY10441, Cayman Chemicals) and DP1 (BAY u3405, Cayman Chemicals, USA).

3.3.7. Reagents used

A list of the reagents used in this study with annotated concentrations used is provided in Table 3-1.

3.3.8. Statistical Analysis

All experiment were performed from a total of 7 primary control stromal cells derived from both endometrial biopsy donor and surgical hysterectomies. It is important to note that the sensitivity to hormones varied between stromal cells acquired from hysterectomies or pipelle biopsies. Representative example of the differences is provided (Supplementary Figure 3-16). The raw data for fold change comparison for individual endometrial cells are provided in the Appendix of this chapter (Supplementary Figures 3-17, 3-18). Data were analyzed using a Student t test when comparing numerical variables between two groups and one-way analysis of variance followed by Bonferroni's post-test for comparisons between more than two groups. Statistical analyses were performed using GraphPad Prism software. $P < 0.05$ was considered statistically significant.

Table 3-1. Reagents used in this chapter.

Details	Item	Cat. #	Source	Working concentration(s)
	Antibodies			
Rabbit	EP2	101750	Cayman Chemicals	1:100
Rabbit	EP4	101775	Cayman Chemicals	1:100
Rabbit	DP1	101640	Cayman Chemicals	1:100
Mouse	IP	sc515139	Santa Cruz	1:100
N/A	ULEX- conjugated Rhodamine	RL-1062	VECTOR	1:100
Mouse	COX-2 conjugated AF647	sc-376861	Santa Cruz	1:50
Mouse	ICAM-1	353102	Biolegend	1:100
Mouse	PR	ab199224	Abcam	1:50
	ActinGreen™ 488 ReadyProbes™	R37110	ThermoFisher	1 drop/mL
	CD31	M0823	DAKO	1:50
	vimentin	ab45939	Abcam	1:400
Goat	goat anti rabbit-AF647	AnaSpec, Inc	AS-61056-05-H647	1:200
Goat	Ready probes GAM-AF594	R37115	ThermoFisher	1 drop/mL
Goat	Biotinylated IgG	HK335	Biogenex	1 drop/mL
	Prostanoid Agonists			
	Iloprost	18215	Cayman Chemicals	25 µM, 2.5 µM, 1 µM, 500 nM, 250 nM, 100nM,
	PGE_2	p0409	Sigma	1 µM, 500nM 100 nM, 10nM
	PGD_2	p5172	Sigma	10 µM, 1 µM
	8-Br-cAMP	B7880	Sigma	0.5 mM
	Prostanoid Antagonists			
Cox-2 inhibtor	Indomethacin	I7378	Sigma	100 µM
DP1 inhibitor	Bay u3405	10156	Cayman Chemicals	n/a
IP inhibitor	CAY10441	10005186	Cayman Chemicals	10 µM
EP2 inhibitor	AH6809	A1221	Sigma	20 µM
EP4 inhibitor	AH23848	A8227	Sigma	20 µM
	rhIL1β	201-LB/CF	R&D	10ng/mL
	ELISAs			
	Human prolactin	DY682	R&D	384 well format
	Human IGFBP-1	DY871-05	R&D	384 well format
	PGE_2	500141	Cayman	96 well format
	Prostacyclin	ADI-900-025	Enzo	96 well format

3.4 Conclusions

The successful establishment of human pregnancy is dependent on the ability of P4 to initiate the process of decidualization within the specialized stromal compartment of the endometrium.[19,157,169] Decidual transformation can be characterized initially by an acute-phase inflammatory process, which is followed by a profound anti-inflammatory response.[19,157] The rather long delay between exposure to post-ovulatory P4 and the onset of stromal decidualization, suggests that locally produced factors in addition to P4 are likely necessary for an appropriate early decidualization response.[19] The uniquely localized origin of decidualization suggest an interactive crosstalk between the stromal and adjacent endothelial cell. However, the specific identity and regulatory roles of these factors remains speculative due to a lack of appropriate, physiologically relevant *in vitro* models of the human perivascular endometrium.[154,177,180,181] At this juncture, animal studies have provided the best evidence that vascular events are essential for endometrial receptivity,[145–150] and only a few groups have attempted to model the human endometrial vasculature.[20,29–31] Thus, our understanding of the role that the vascular bed plays in human endometrial function remains limited. In this chapter, we utilized an OoC model to demonstrate that the microvascular endothelium contributes to reproductive function by inducing a timely and profound endometrial decidualization via a paracrine mechanism (Figure 3-10).

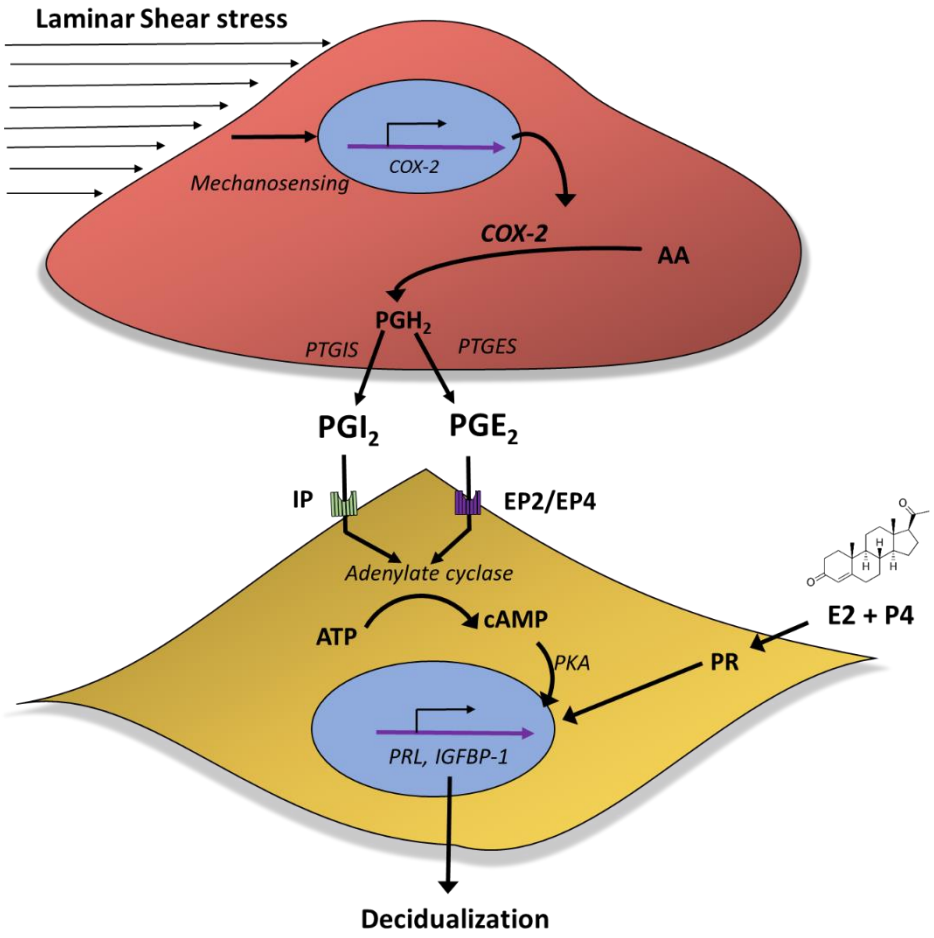


Figure 3-10. Proposed mechanisms driving localized decidualization in the cyclin human endometrium. Prostaglandins can enhance the decidualization process in the human endometrium. In vivo, decidualization originates in the stroma surrounding the vascular endothelium. Activation of endothelial cells by physiological cues such as shear stress forces can induce the prostaglandin pathway in endothelial cells and promote the secretion of prostaglandins. Specifically, prostacyclin (PGI_2) and to a certain extent PGE_2, but not PGD_2, can accelerate decidualization in adjacent endometrial stromal cells via their respective prostaglandin receptors. These events lead to the enhanced response to progesterone via cAMP activation that enhance sensitivity to P4.

3.4.1 Vascular contribution to reproductive function

Evidence from animal studies have clearly identified critical vascular processes that is essential for reproductive function. For example, the localized increases in microvascular permeability in response to P4 during the early steps in embryo implantation suggests that vascular tone may be contributing to uterine receptivity.[133,143,146] Outside the reproductive tract, a large body of *in vivo* and *in vitro* studies has defined the role of hemodynamic forces in the regulation of vascular function.[33,182] Specifically, laminar shear stress can stimulate endothelial cells via mechanoreceptors that activate intracellular signaling pathways that modulate numerous vascular processes.[162] For example, shear-stress forces can stimulate vasodilation/vasocontraction signaling via locally acting eicosanoids on neighboring smooth muscle cells (SMC).[163,183] Herein, we demonstrated for the first time that decidualization of primary human endometrial stromal fibroblasts is enhanced when cells were co-cultured with uterine microvascular endothelial cells (UtMVECs) under continuously perfused conditions that mirrors *in vivo* blood flow perfusion. We demonstrated that shear-stress activation of endothelial COX-2 and the subsequent secretion of specific prostaglandins mediate this observation. Either blocking COX-2 activation with NSAIDs in the perfused microfluidic co-cultures or blocking specific prostaglandin receptors downstream suppressed endothelial-mediated enhancement of the decidualization response of stromal cells. Our findings support previous *in vivo* animal models in which pharmacological or genetic inhibition of the COX-2 pathway can delay or block localized decidualization and implantation in a variety of species.[145,146,158,168,184] In turn, exogenous addition of specific prostaglandins in similar models acts to directly reverse the effects of blocking COX-2.[185,186] Our studies demonstrated a similar effect.

Taken together with our human results, these findings may help explain the association between reduced fertility and NSAID use in reproductive age women.[187,188]

Our results support a role for the vasculature in reproductive success and further suggest that the initiation of decidualization at perivascular sites reflect the contribution of endothelial-derived paracrine mechanisms. Specifically, we demonstrated that specific shear stress-induced prostaglandins are centrally responsible for mediating the focal process of perivascular decidualization. The prostanoid family includes numerous short-lived lipid mediators which exert diverse biological actions and hold multifaceted roles in both homeostatic functions as well as mediating pathogenic inflammatory responses.[130,131,164,165,169] Prostaglandins exert their effects via G protein-coupled receptors (GPCRs)[189] and regulate a range of intracellular signaling pathways within the reproductive tract. Most notably, activation of EP2, EP4, IP and DP1 receptors can induce adenylyl cyclase activity and increase intercellular concentrations of cAMP, a known modulator that is required to promote decidualization.[159,185,190] Thus, developing a better understanding of the specific prostaglandins that are critical for reproductive function of both the gravid and non-gravid uterus is necessary. Kennedy and others have provided substantial evidence for the role of prostaglandins related to uterine function and reproductive success, specifically prostacyclin and PGE_2, primarily using murine models[146,168,184,191,192] In the current study, we examined the effects of cAMP-stimulating prostaglandins, PGE_2, PGI_2 and PGD_2, on the decidualization response in primary human endometrial stromal cells. We demonstrated PGE_2 and prostacyclin, but not PGD_2, significantly promoted decidualization via increased intercellular cAMP. Although PGE_2 promoted a more robust decidualization response, prostacyclin consistently modulated the

early steps in the initiation and promoted a more rapid decidualization response compared to PGE_2, albeit at a lower magnitude. The timing, stability, relative concentrations of specific prostaglandins, as well as the expression of their receptors, may explain the differences in response we observed with exogenous administration of prostaglandins. For example, although prostacyclin can exert its effects through alternative nuclear receptors such as the peroxisome proliferator-activated receptor (PPARγ),[193,194] we demonstrated that prostacyclin associated action can be blocked by inhibiting the stromal cell IP receptor. Nevertheless, it will be necessary in future studies to investigate the likely effects of redundancies of prostaglandin signaling during the human menstrual cycle.[158] Equally important, although we showed in the current study that the local production of prostaglandins by endothelial cells can act to enhance initial stages of decidualization of stromal cells, pro-inflammatory cytokine-induced prostaglandins can act to also suppress decidualization. These findings suggest that the context of chronic COX-2 stimulation related to an infectious stimulus may be different than the localized effect of physiological stimulation via hemodynamic forces. These potential differences will need to be further explored in future studies.

3.4.2 Limitations

It is important to note that in the human uterus, the localized differences in vascular remodeling that include structural and physical changes in arterial impedance and tortuosity throughout the menstrual cycle may be related to different rates of shear stress that the endometrial microvascular endothelial cells experience. Thus, precise shear stress rates in the human endometrium and have been difficult to calculate.[153,195,196] However,

Raine-Fenning *et al* show correlation between serum P4 levels and increase in endometrial vascular flow index post-ovulation (Supplementary Figure 3-19).[196] Altogether, these findings suggest that shear stress activation of endothelial COX-2 mediates enhanced decidualization in stromal compartment. We utilize our model as a controlled system to experimentally examine the biological effect of laminar shear-stress rates (Supplementary Table 3-2). Moreover, indirect mechanical stimulation of the stromal cells may also promote the secretion of prostaglandins that act in an autocrine mechanism to regulate decidualization. However, we demonstrate that there is a clear contribution of the endothelial cells and furthermore we specifically identify prostacyclin, which is a major product of shear stress stimulated endothelial cells. The effect of stromal cells on vascular function will be discussed in Chapter 4. In addition to these limitations, the vascular bed is associated with additional cells that retain and help regulate dynamic vascular processes including vascular tone. Specifically, vascular smooth muscle cells (VSMC) maintain the integrity of vascular contractibility and permeability.[143] While different vascular beds throughout the body have different composition and distribution of specific cell populations, they must be considered when studying the vasculature. In reproductive biology, VSMC for example, play a role in the development of the placenta as by interacting with invading trophoblasts and residing uterine NK cells that drive the remodelling processes of the vasculature. The role of VSMC have been implicated in the pathophysiology of disorders and reduced proliferation and abundance of these cells have been implicated in the development of spiral arterioles and thereby incidence of menorrhagia.[197] Herein, we did not include the contribution of VSMCs to the model of the cycling endometrial perivascular stroma; however, the continuing development of these

models that integrate the role of this cell types will further enhance our understanding of the endometrial vasculature.

3.4.3 Clinical correlate

Altogether, these results show a role for hemodynamic forces to maintain the homeostatic role of the vasculature in the human endometrium. However, dysregulated endometrial vascular function and associated alterations in paracrine factor production and action can promote infertility, as noted in reproductive disorders including endometriosis and pre-eclampsia.[48,154,198,199] Unexplained infertility has been associated with impaired endometrial blood flow perfusion.[195] Furthermore, increased PGI_2 is observed in women suffering from menorrhagia, or excessive menstrual bleeding, compared with those with normal menstrual blood loss.[48,154,199] Alterations in the expression of this molecule may alter the menstrual bleeding in the non-gravid uterus and placental health in the gravid uterus. Patients with menorrhagia, have increased endometrial pressure that may further reduced blood flow resulting in anoxia. Other disorders like endometriosis are characterized by the highly inflammatory microenvironment driven by pro-inflammatory cytokines such as IL-1β, TNFα and PGE_2. Thus, the perfused vasculature system described in our study revealed the identity of key prostaglandins during early decidualization, potentially offering an opportunity to target these molecules for therapeutic development. Targeting specific prostaglandin signaling, such as specific receptors for prostacyclin or PGE_2, may provide putative nonsteroidal solution to alleviate pain and chronic inflammation for certain reproductive disorders, while maintaining endometrial receptivity.[200–202] This model sets a new translational tool for examining adverse outcome

pathways in the human endometrium. Revisiting the role of specific prostaglandins is necessary for both physiological and pathological conditions. A physiologic model system of the vasculature provides a tool to better understand normal steroid-mediated endometrial function, but also holds promise to reveal how altered patterns of cell-cell communication promotes the pathogenesis of diseases, including breakthrough bleeding, infertility, menorrhagia, endometriosis, pregnancy disorders and endometrial cancer. Lastly, in these studies, we characterized the source of the endometrial cells and identified a divergence between endometrial biopsies and cells acquired from hysterectomies (Supplementary Figure 3-16). While both cell types were sensitive to hormonal cues, at least temporarily, the sensitivity of the biopsies is about 100-fold higher to E2 + MPA compared to hysterectomies. We provide an example of this analysis and this difference explains why we observed such high-interpatient variability. This emphasizes the importance and relevance of personalized devices to calibrate to each patient's sensitivity to stimuli.

3.5 Appendix

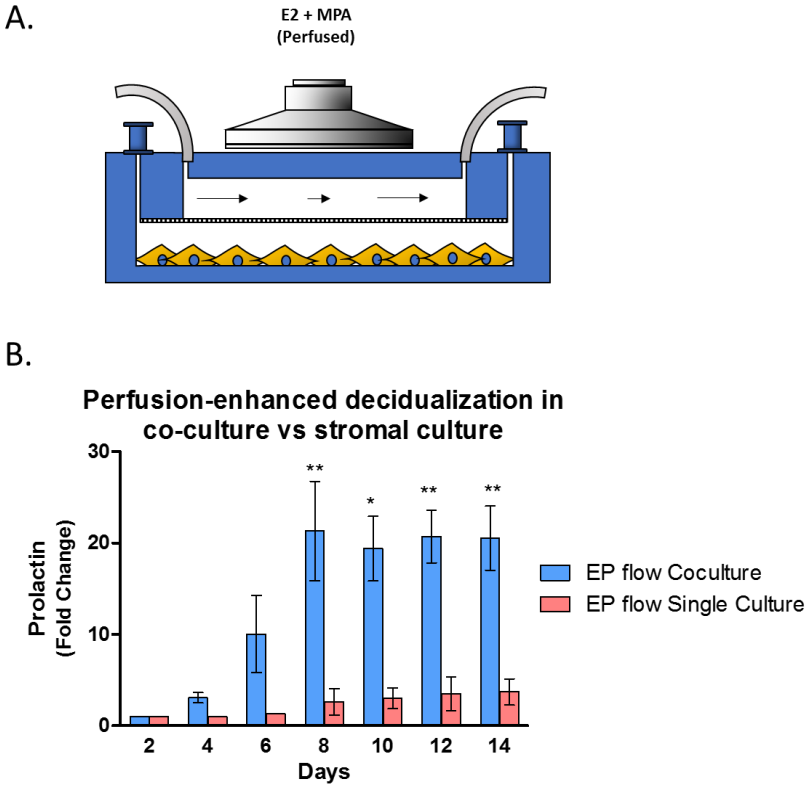


Figure 3-11. Perfusion enhanced decidualization is mediated by the endothelial vascular cells. Decidualization under flow conditions was observed to be significantly enhances in the co-culture model with UtMVEC compared to stromal cell single culture perfused with an empty endothelial chamber. (N=3)

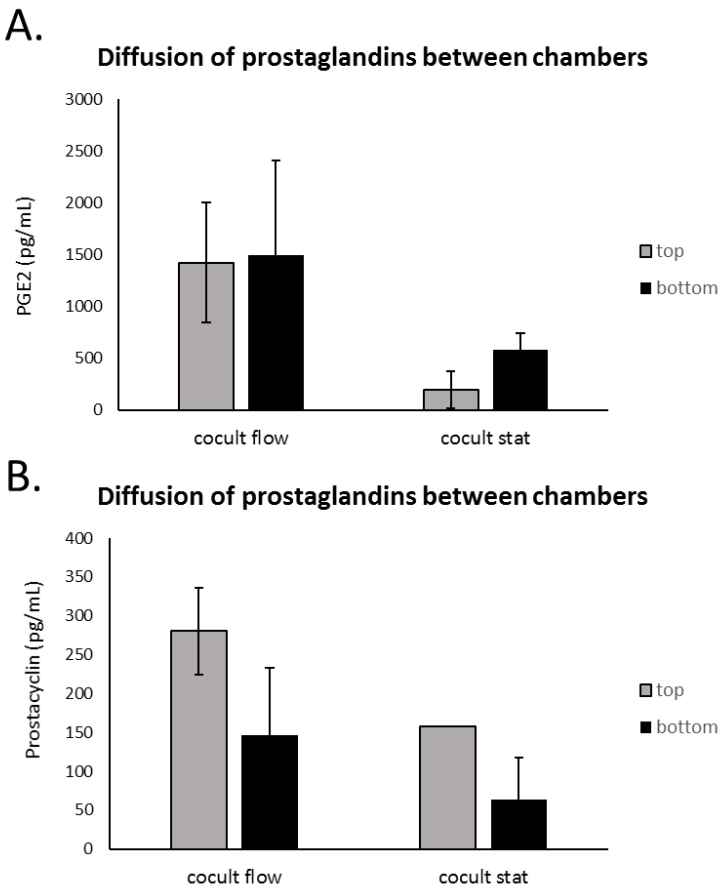


Figure 3-12. Validation of bi-directional communication in the OoC dual-chamber model. Prostaglandins (PGE_2 and prostacyclin) were measured in both the top (vascular) and bottom (stromal) chamber of the model. Accumulation of prostenoids were measured in both compartments and were higher in perfused (flow) co-culture than in static (stat) conditions.

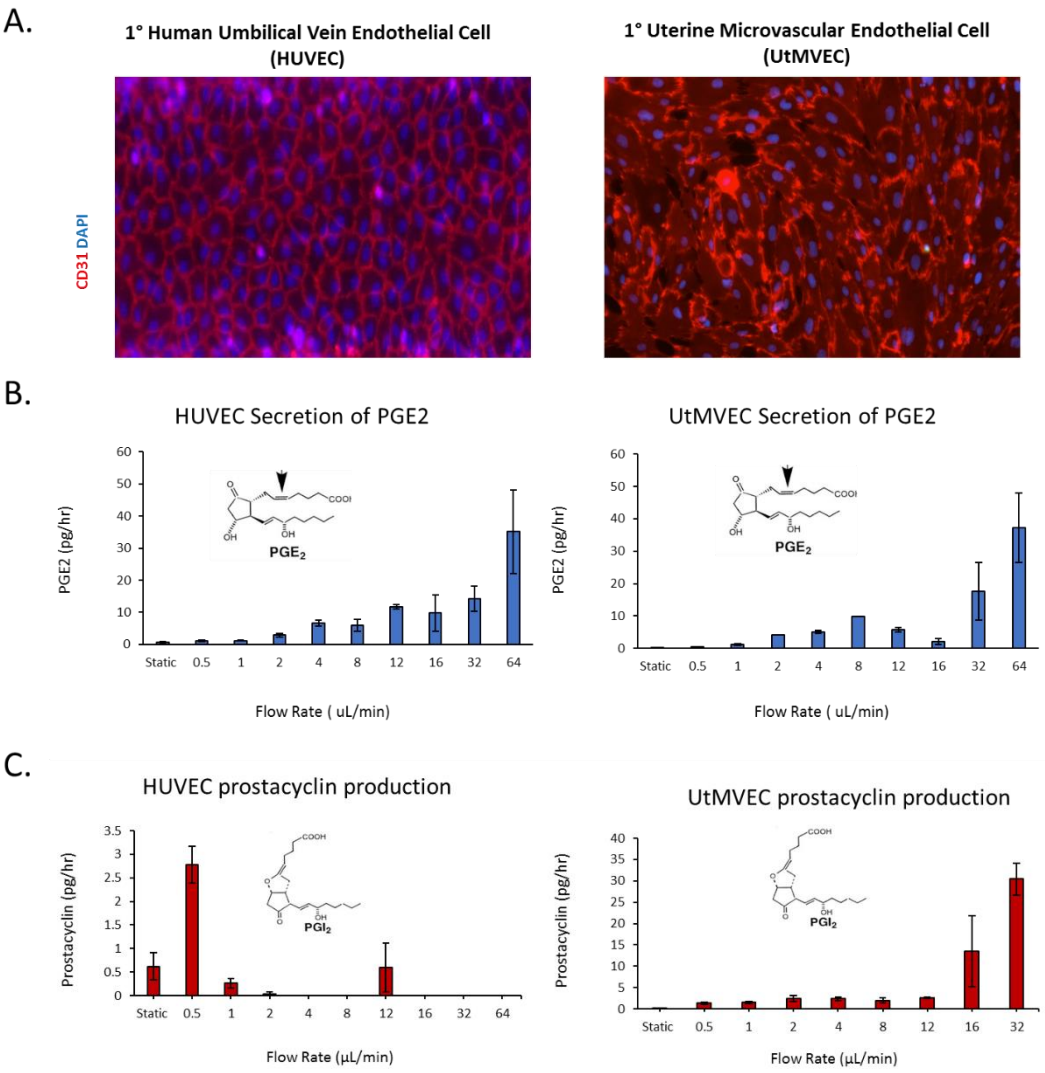


Figure 3-13. Characterization of primary endothelial cells utilized in this study. Primary human umbilical endothelial cells (HUVECs) were compared to uterine microvascular endothelial cells (UtMVEC) both morphologically (A) and phenotypically (B-C). CD31 immunofluorecnece demonstrated differences in morphology, but not in viability. (B-C) Step-wise increases in flowrates were performed on these cell types and measure the specific prostaglandins secreted. An increase in prostaglandins was evident with the exception of the production of prostacyclin by HUVECs suggesting that UtMVEC may mirror a more adequate physiological model of the endometrial perivascular stroma.

Table 3-2. Experimental Shear Stress Rates

Flow rate (µl/min)	Flow rate (mL/min)	Flow rate (mL/s)	Shear stress (dyn/cm²)
0.25	0.00025	4.17E-06	1.40E-03
0.5	0.0005	8.33E-06	2.80E-03
0.75	0.00075	1.25E-05	4.19E-03
1	0.001	1.67E-05	5.59E-03
1.5	0.0015	2.50E-05	8.39E-03
2	0.002	3.33E-05	1.12E-02
2.5	0.0025	4.17E-05	1.40E-02
3	0.003	5.00E-05	1.68E-02
3.5	0.0035	5.83E-05	1.96E-02
4	0.004	6.67E-05	2.24E-02
4.5	0.0045	7.50E-05	2.52E-02
5	0.005	8.33E-05	2.80E-02
6	0.006	1.00E-04	3.36E-02
7	0.007	1.17E-04	3.91E-02
8	0.008	1.33E-04	4.47E-02
9	0.009	1.50E-04	5.03E-02
10	0.01	1.67E-04	5.59E-02
12	0.012	2.00E-04	6.71E-02

Table 3-2. Experimental Shear Stress Rates. Shear stress inside the OoC was calculated as discussed above. Briefly, The equation, $T= 6\mu Q/wh2$, was used to calculate the experimental shear stress in the OoC device where Q = flow rate (mL/s), µ= viscosity (0.0068 dyn·s/cm²), w = width of the channel (0.475 cm), h = height of the channel (0.0160 cm), and T = wall shear stress (dyn/cm²).

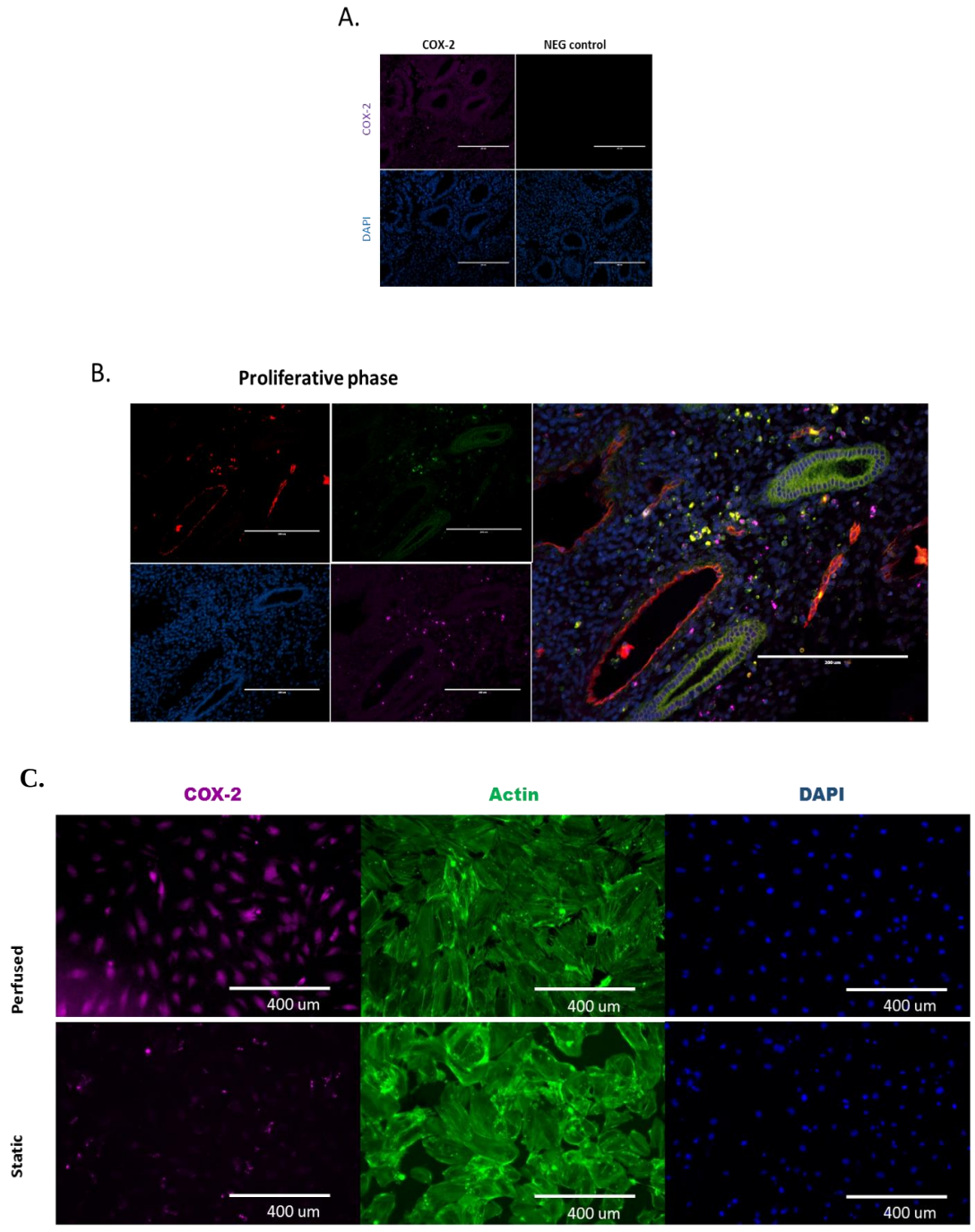


Figure 3-14. Immunofluorescence of COX-2 in the ex vivo and in vitro endometrium. (A) Characterization of negative controls in immunofluorescence in tissues. (B) Co-localization of endothelial cells (ULEX) and COX-2 expression in the proliferative phase endometrium. COX-2 does not localize to the vascular endothelium (red), but is primarily expressed in the epithelium and the local immune cells. (C) Cultured endothelial cells under flow perfusion at 1 µL/min have induced COX-2 expression and reorganization of actin filaments in the direction of the flow (Actin, green).

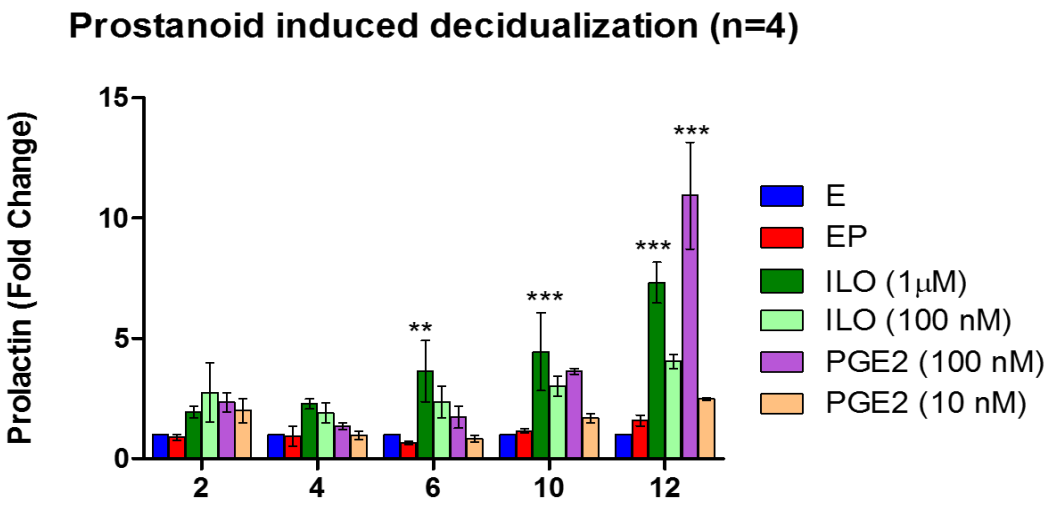


Figure 3-15. Iloprost dose response on stromal decidualization rapidly induced the decidualization response. Time and dose dependent stimulations of decidualization with prostaglandins comparing PGE_2 and prostacyclin. (N=4)

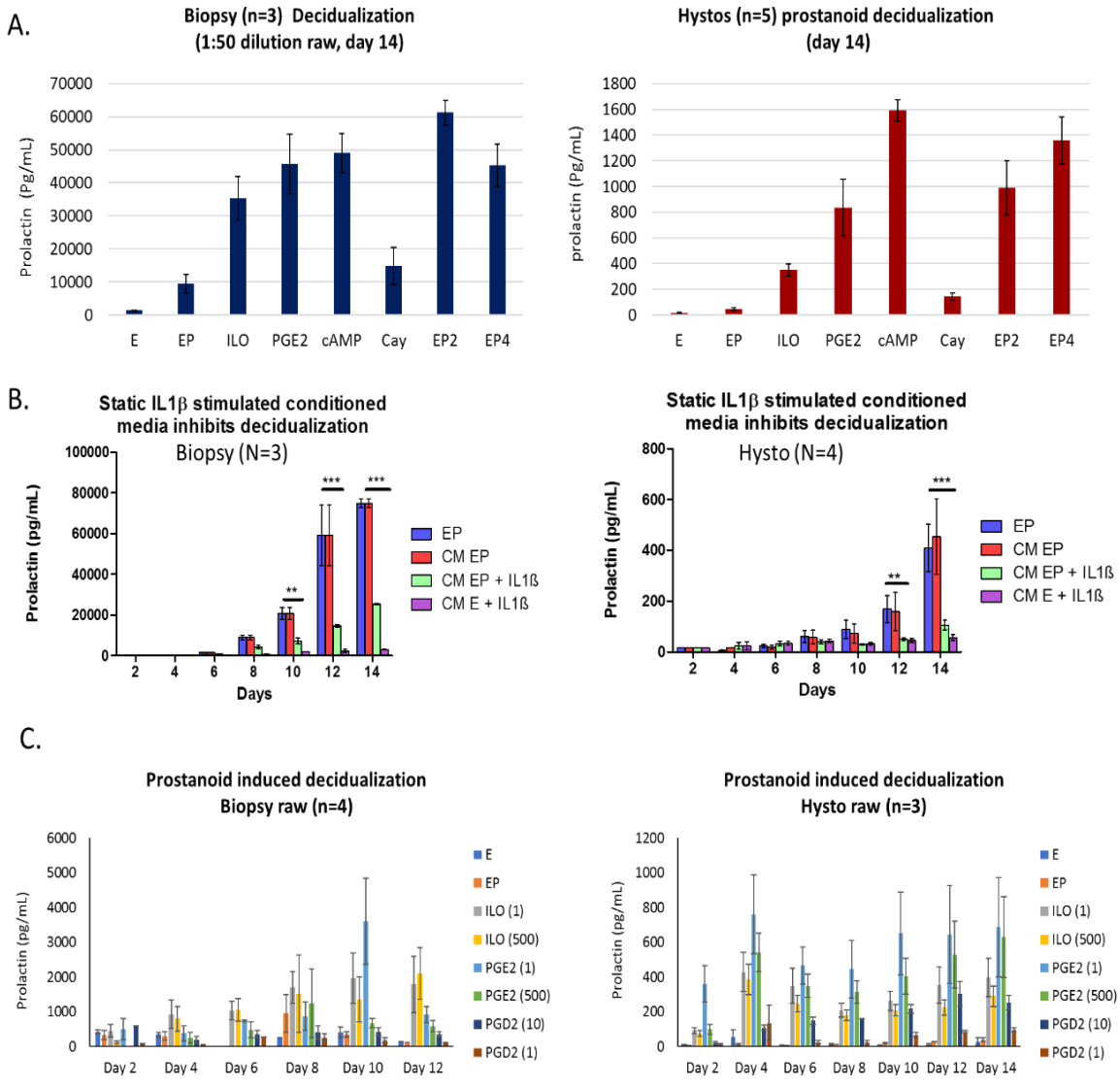


Figure 3-16. Characterization of differences between primary stromal cells acquired from biopsies or hysterectomies. Representative raw data results from key experiments described in the body of the manuscript. (A-C) prostanoid induced decidualization, (B) CM suppression of decidualization. Key differences demonstrate that cells from timed endometrial pipelle biopsies are more sensitive to E2 and MPA compared to cells obtained from hysterectomies. Note that the left and right panels are graphed to different scales due to the vast difference in response between the tissue origin.

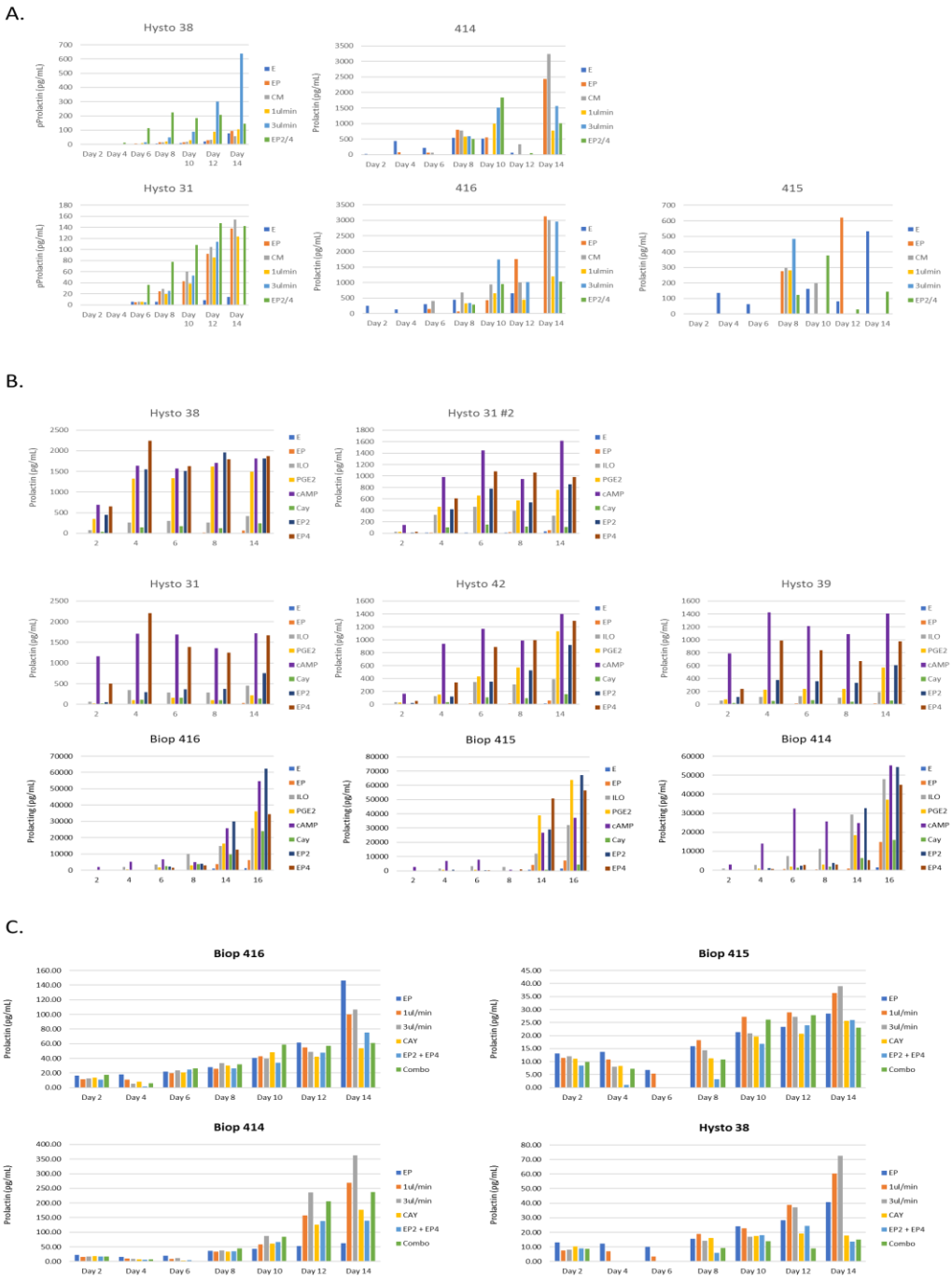


Figure 3-17. Representative raw data from each endometrial sample from CM experiments. Three different experiments representative of the raw data obtained. Biop and Hysto = sample ID.

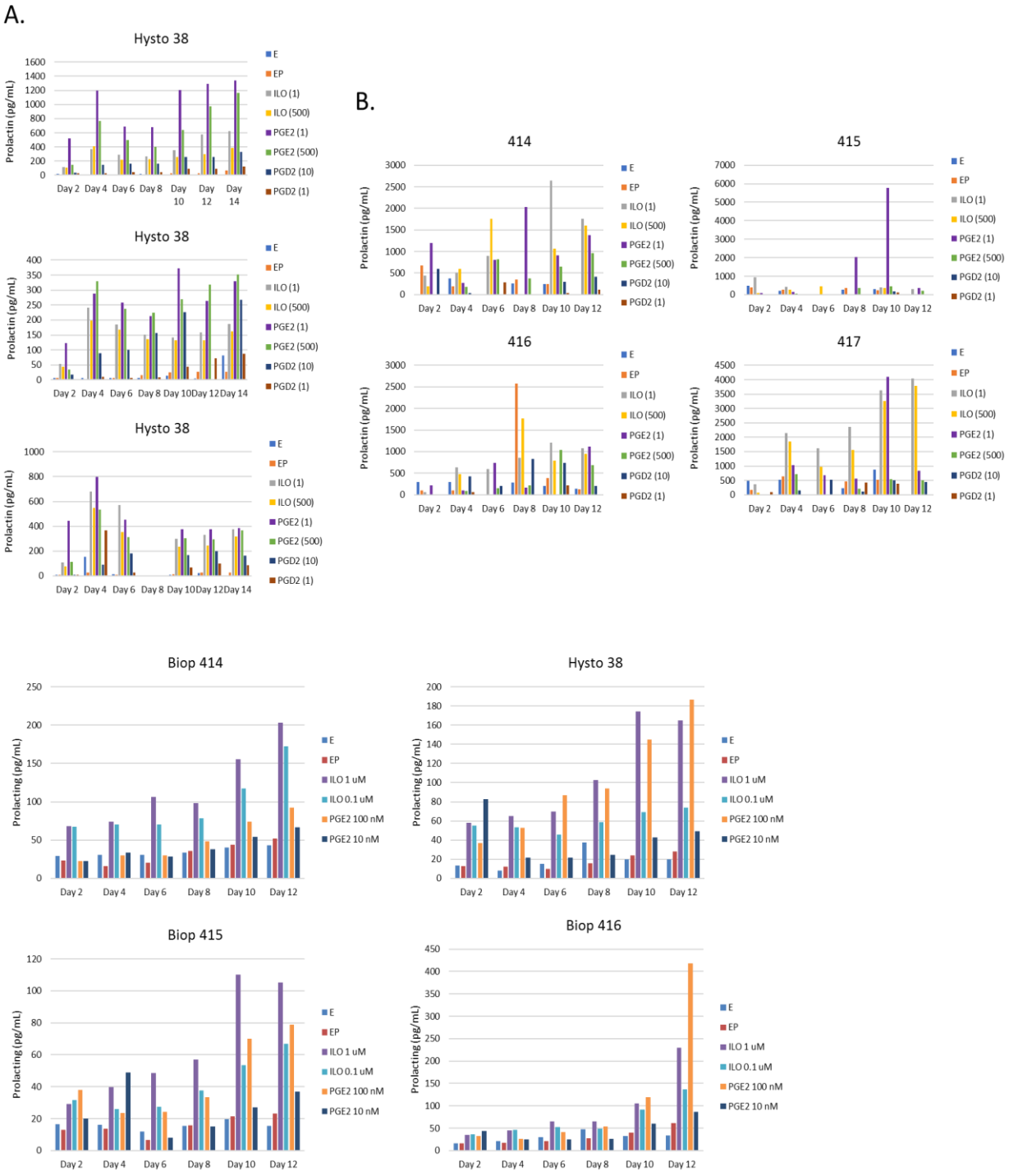
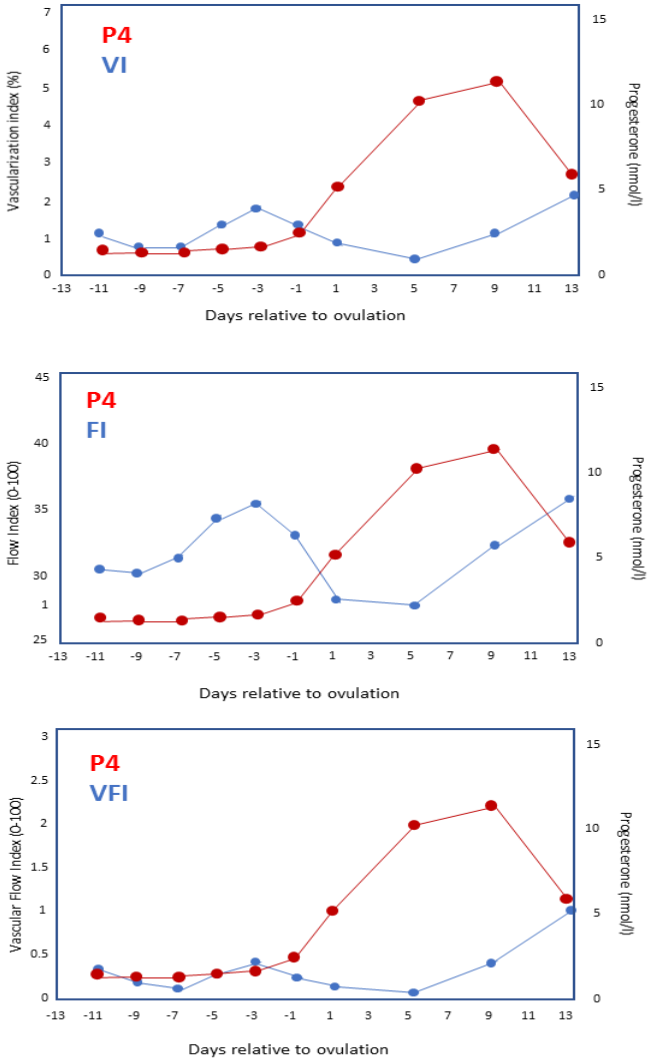


Figure 3-18. Representative raw data from each endometrial sample from prostanoid results. Experiments representative of the raw data obtained. Biop and Hysto = sample ID.



Adapted from Raine-Fenning, N. J., et al. *Human Reproduction* (2004)

Figure 3-19. Relationship between progesterone and flow dynamics in the endometrium. Temporal variation in the three-dimensional power Doppler indices throughout the menstrual cycle relative to ovulation defined as day zero and indicated by the central vertical line. The blue line represents data from the endometrium, and the red line represents relative levels of serum progesterone. [196]

Chapter 4

Applications of the EndoChip: Immunological origins of toxicant-mediated endometrial pathogenesis

Sex hormones are key for maintaining the homeostasis of the female reproducive tract. Therefore, any disruption of endocrine signaling could be conducive to the pathophysiology of many reproductive disorders. An example of a disease associated with endocrine disruption is endometriosis, an endometrial disorder characterized by the dislocation and implantation of endometrial tissue outside of the uterine cavity. Endometriosis is considered a chronic inflammatory disease resulting in reverted cellular cross-talk from a stromal dominant to an epithelial dominant pattern of communication.[203–205] Increased resistance to P4's anti-inflammatory properties is now recognized as a hallmark of the pathophysiology of endometriotic tissues.[142] Furthermore, P4-sensitive somatic cells interact with the immune system to maintain appropriate and cyclical migration of circulatory leukocytes and inflammatory pathways which requires a tightly regulated paracrine network[3,27]. Within the cycling endometrium, immune-endocrine crosstalk is a crucial, tightly regulated component of cell-cell communication that contributes to reproductive function. As such, the immunological axis plays a critical role in relation to gene-environment interactions that may promote disease development. Endocrine disrupting chemicals (EDC) exert their effects on disease pathology by altering either individual cell behaviors within the microenvironment of the endometrium or

systemic components of immune-endocrine communication.[206] Throughout the body, immune cell populations, especially macrophages, must appropriately secrete cytokines, prostaglandins and chemokines that induce and suppress the immune and inflammatory responses in order to regulated normal wound repair and angiogenesis.[151,207–209] As an open organ system that is susceptible to invading pathogens, the endometrial mucosal immune system confers a protective response, yet is permissive to the implantation of an embryo. The interactions of the embryonic trophoblasts, and the vast remodeling of the endometrial tissue to accommodate implantation, is one of the most physiologically invasive processes in vertebrate organisms and thus makes the endometrium a uniquely vulnerable tissue for disease. Unfortunately, our understanding of the immunological processes and the mechanisms that control endometrial physiology is limited. In this chapter, we introduce the capability to utilize the EndoChip as a tool to examine immune-endocrine interactions in the context of endometrium. We accomplish this by incorporating naïve immune cells (*e.g.* circulating monocytes) to our existing model of the perivascular stroma (Chapter 2 and 3). Furthermore, we identify that disruptions to the endocrine system by environmental toxicants can result in a hyperinflammatory microenvironment characterized by increased immune cell infiltration and enhanced expression of proinflammatory cytokines. Specifically, we have previously shown, using murine models, that the endocrine disruptor 2,3,7,8-tetrachlorodibenzodioxin (TCDD or dioxin) alters reproductive function and is associated with the development of experimental endometriosis.[26,210,211] We utilized this toxicant as a test agent to demonstrate that these murine studies translate to the human condition. Moreover, we identify an inflammatory mechanism by which TCDD may contribute to endometrial disease pathogenesis.

4.1 Background

Controlled inflammatory processes are necessary for successful uterine function and reproductive success. As we demonstrated in chapter 3, some inflammatory mediators like specific prostaglandins are necessary to regulate reproductive function (Chapter 3). However, chronic and hyper-inflammatory conditions within the endometrium can compromise the homeostatic milieu of the tissue microenvironment and drive it towards a pathogenic state. Leukocyte infiltration has been characterized to play a role in the key inflammatory processes that dictate endometrial function and, as such, the temporal leukocyte recruitment is tightly regulated by sex steroid-mediated endocrine signaling.[25,50,206,212–214] P4 has been demonstrated to be a key steroidal mediator of immune-endocrine control within the endometrium by exerting anti-inflammatory effects in addition to inducing key reproductive processes necessary for the establishment of pregnancy.[212] P4 action is responsible for regulating leukocyte recruitment by modulating cytokines, chemokine and cell adhesion molecules (CAMs) that act on the vascular interface.[27,212,215] However, the local mechanisms of cell-cell communication that regulate these events are not well understood.[3,103,215] Disruptions in these processes within the cycling or gravid endometrium may be conducive to chronic and hyperinflammatory environments associated with disease pathogenesis.[206,216–218]

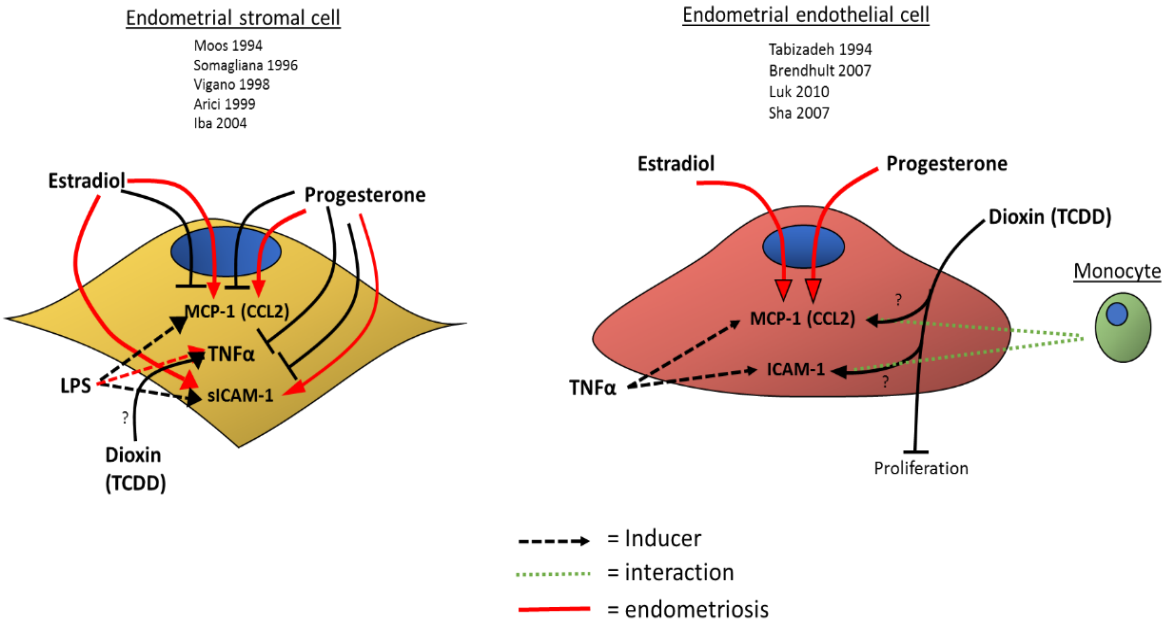


Figure 4-1. Brief review on endometrial stromal and endothelial cells inflammatory responses to sex hormones, dioxin, and in endometriotic disease. Our current, albeit limited, understanding of the endometrial regulation of inflammatory processes and vascular function under hormonal pathways.

Due to this tightly regulated immune-endocrine system, the endometrium is uniquely vulnerable to chemical toxicants within the environment. In particular, endocrine disrupting chemicals (EDC) are capable of promoting uterine dysfunction.[2] Sex steroids help maintain the inflammatory tone of the endometrium by regulating the intercellular crosstalk between heterogeneous cell populations. Although the regulatory role of P4 to suppress leukocyte recruitment into the endometrium during the menstrual cycle is widely accepted, the mechanisms of leukocyte control are not well understood[212]. However, recent empirical evidence demonstrated that E2 and P4 can mediate their effects on immune cell populations within the endometrium through indirect mechanisms[31,32,44]. Since neither immune cells nor endothelial cells express PR, an indirect signaling mechanism may explain the regulation of P4 on leukocyte recruitment in the endometrium. The anti-inflammatory action of P4 are mediated in part by the hormone sensitive stromal fibroblasts, cells that are critical to endometrial function. As we proposed in a recent review, reduced P4 sensitivity and alterations in stromal cell function and immune cell recruitment may collectively promote the inflammatory microenvironment of reproductive diseases such as endometriosis, infertility, and abnormal bleeding.[26,206,211,219]

4.1.1 Endocrine disrupting chemicals and reproductive dysfunction

Historically, our laboratory has focused on how exposure to environmental toxicants such as EDCs contributes to the development of reproductive disorders.[26] The National Institutes of Health National Toxicology Program estimates that >80,000 chemicals have been released into our environment over the past few decades and, yet, limited data exist that mechanistically assesses the potential risk of these toxicants to human populations.[220]

One of the most potent and persistent endocrine disrupting chemicals relative to reproduction is the ubiquitous environmental toxicant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD or dioxin is a member of the halogenated aromatic hydrocarbons (HAHs) and is produced primarily as a by-product of industrial processes and combustion.[221] Since TCDD and other HAHs are highly resistant to degradation, with an estimated half-life >7.1 years[222], they bio-accumulate by contaminating the soil and groundwater and eventually enter the food supply.[223] The toxicological adverse outcomes associated with this chemical are not entirely clear and is an area of high investigation. At this juncture, TCDD has been linked by us and others to the pathogenesis of several endometrial disorders, including endometriosis, preterm birth and infertility[141,203,224,2255,34,36,89–91]. Significantly, TCDD exposure promotes an "endometriosis-like uterine phenotype" in toxicant exposed animals and in toxicant-exposed human endometrial cells cultured *in vitro* by reducing PR expression levels.[229] Consequentially, we observed in our *in vivo* studies that nearly 50% of female mice were infertile in an murine model of TCDD toxicity and, among mice achieving pregnancy, 40% exhibited spontaneous preterm birth.[206,210] TCDD can promote a hyper-inflammatory uterine phenotype and alters normal reproductive function; however, our molecular understanding of the breadth of TCDD toxicity to drive endometrial microenvironment from homeostasis to pathogenesis is not clear. Furthermore, to accurately identify the mechanisms of toxicant induced endometrial pathogenesis in humans is a challenge in part due to physiologic inter-species reproductive differences in cell specific immune-endocrine responses and variable sensitivity to endocrine disrupting toxicants.[26,206] The development of endometriosis appears to require interacting pathways involving the vascular, endocrine and immune components of the endometrial

microenvironment; however, TCDD may directly alter any of these components to promote endometriosis. The adverse outcome pathways of TCDD on human endometrial vascular function has not been extensively studied (Figure 4-1). Thus, in this chapter we examined the adverse effects of endocrine disrupting toxicants at a human tissue-level by modeling the endometrial immune microenvironment.[31,44,230,231] Specifically, we focus on the extent of stromal and vascular dysfunction using the OoC model of the perivascular stroma. Identifying the specific cellular targets of TCDD toxicity that subsequently promote inflammation and disruption of normal endocrine-immune interactions can enhance our understanding of reproductive pathogenesis and should allow the development of more effective targeted therapeutics. Herein, we examine the effect of TCDD on vascular function, leukocyte recruitment and pro-inflammatory pathways using a microfluidic OoC *in vitro* approach.

4.1.2 Endometriosis as a paradigm for toxicant-mediated endometrial dysfunction

Endometriosis is a common gynecologic disorder characterized by the presence of ectopic implants of endometrial tissue dispersed in extra uterine locations and manifests itself with severe and debilitating pelvic pain.[142] Comorbidities associated with endometriosis include, infertility, abnormal bleeding and spontaneous preterm birth.[232] The prevalence of endometriosis is 5-10% in women of reproductive age, and increases to 20-30% in women with subfertility and to 40-60% in women with pain and infertility.[142,233] Endometriosis represents the single major clinical cause for hysterectomy in reproductive age women in the U.S. with an estimated societal cost of ~$69.4 billion per year.[234] Despite its prevalence and socio-economic implications this disease remains one of the most poorly

understood conditions affecting women's reproductive health.[142] Pathologically, endometriosis is characterized by the growth of endometrial tissue-composed of epithelial glands and stroma-outside the uterus[233] and manifests itself through chronic, and often, debilitating pain in patients along with infertility.[142] Although endometriosis has been clinically described over 100 years ago[235] it continues to be considered a "black box" in medical practice and is one of the most poorly understood gynaecological conditions affecting women. Several theories and hypothesis have been proposed to explain the etiology of ectopic endometrial lesions. The most prominent of these include mechanical dissemination through retrograde menstruation,[236,237] activation of embryonic cell rests,[238] coelomic metaplasia theory,[239,240] and contribution by adult stem cells.[241] Sampson's theory of retrograde menstruation is currently the most widely accepted hypothesis to explain the translocation of endometrial tissue into the peritoneal cavity. However, since up to 90% of healthy women have a prevalence for retrograde menstruation, it does not explain the nearly 10% prevalence of disease. These clinical observations are strongly suggestive of inherited or acquired traits in endometrial properties as a risk for developing endometriosis. While the etiology of endometriosis may be multi-factorial, several hallmarks of disease have been, and continue to be identified. These include an altered steroid biosynthesis and sensitivity as noted by increased estrogen receptor β (ERβ) expression, increased aromatase expression and decreased P4 sensitivity[242]. These clinical phenotypes likely contribute to the E2 dependency and P4 resistant phenotype observed in women with endometriosis.[142] Increased invasiveness and vascularization classifies as another hallmark of endometriosis as several lines of evidence demonstrate alterations in MMP networks and enhanced angiogenic signals, respectively.[218,242] Another hallmark of endometriotic cells

include their ability to evade immunological clearance by peritoneal macrophages, thus resulting in ectopic lesions.[25,39,40] The inflammatory axis has been identified to play a critical role in the establishment of endometriosis and may contribute to the pain associated with endometriosis and the linkage of this disease to co-morbidities such as infertility and peritoneal adhesive disease.[206,219,243] Increased levels of proinflammatory cytokines have been associated with the endometriosis.[244] Among endometriosis researchers, it is becoming increasingly clear that the unprecedented complexity of this disorder is multifactorial and underlies a contributory effect by the same endocrine, immunological and paracrine signals that normally regulate endometrial function. Whether endometriosis contributes to or is the result of a pre-existing alteration in endometrial homeostasis is still unknown. To address this question, we must first understand that the histologic presentation of this disease reveals a complex and dysregulated microenvironment. [245] Thus, immune cells, the vasculature and subsequent inflammatory cytokine networks play a critical role in the pathophysiology of endometriosis (Figure 4-3A).

It has become clear that the phenotype of the eutopic endometrium of women with endometriosis is often intrinsically different from disease free women.[246–250] Historically, most research has focused on characterizing the major somatic cells (*i.e.* the epithelial glands and the specialized reticular stromal fibroblasts) that contribute to endometriotic disease. However, research by several groups have identified that the eutopic microenvironmental milieu of multiple cell-types that populate the endometrium may each play a significant role in disease pathogenesis.[56,208,244,246,249] Specifically, Kahn *et al*. have shown that in endometriosis this milieu is altered and demonstrates a heightened influx of pro-inflammatory $CD68^{+}$ macrophages in the eutopic endometrium of endometriosis

patients.[246] While, cytokine network analysis of peritoneal fluid from women with endometriosis demonstrates pro-inflammatory cytokines, specifically IL-1β, TNFα, MCP-1, and others are critical components in order to characterize disease pathogenesis.[244] Moreover, patients with endometriosis have a greater tissue aggregations of pro-inflammatory macrophages in their eutopic endometrium.[237,251] In support of this disease characterization, leukocyte recruiting chemokines (*e.g.* MCP-1) and cell adhesion molecules (*e.g.* ICAM-1) are altered in endometriosis patients and correlate with disease severity.[252–254] Herein, we focused on determining whether these endometriosis-associated pathways are dysregulated by EDC and may be conducive to inflammatory disease.

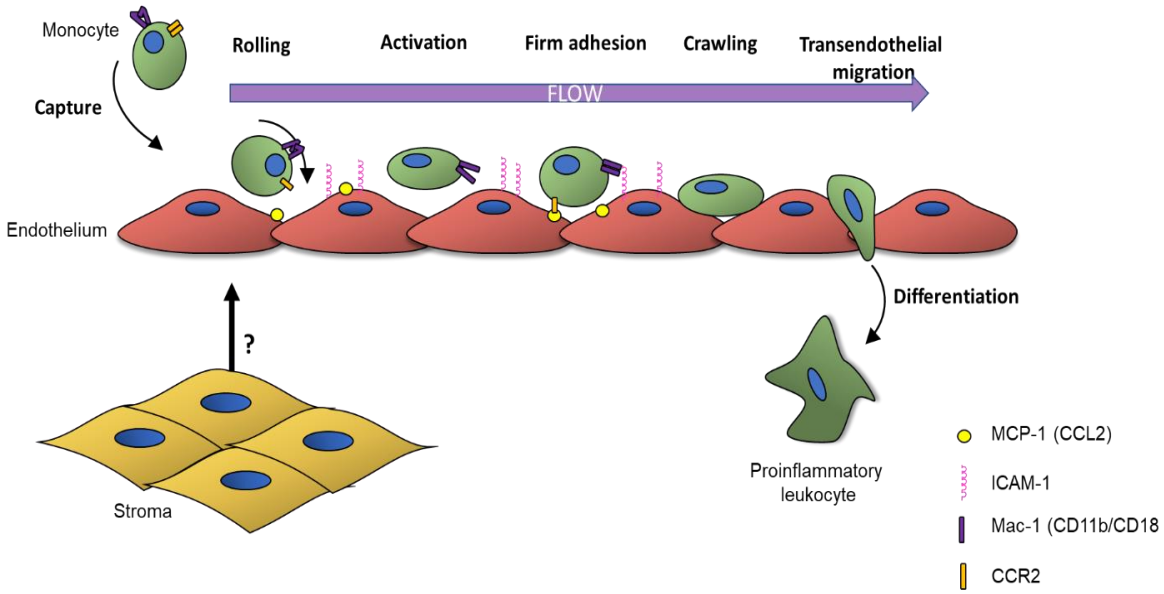


Figure 4-2. Schematic of leukocyte diapedesis during immune cell recruitment. The vascular endothelium acts as the interface between circulating immune cells and tissues. The molecular mechanisms by which circulating leukocytes such as monocytes is mediated by chemokines, cell adhesion molecules and cytokines, that regulate the rolling, tethering, firm adhesion and trans-endothelial migration in response to an inflammatory stimuli. Firm adhesion is a rate-limiting step in diapedesis, yet the endocrine mechanism that regulate leukocyte recruitment remains elusive. Furthermore, the role of the endometrial stromal fibroblasts, as a progesterone responsive cell, to regulate this process is unknown.

4.2 Results and discussion

Within the endometrium, the events leading to the recruitment of leukocytes are tightly regulated by endocrine (E2 and P4) signals. Furthermore, the endometrial vascular bed acts as the interface between peripheral circulating white blood cells, such as monocytes, and the somatic tissue[255]. Therefore, an inadequate or untimely infiltration and activation of inflammatory macrophages may be conducive to disruptions throughout the menstrual cycle. Studies suggest the endometrial vasculature is regulated by sex steroids; however, the consensus remains that endometrial endothelial cells lack endocrine receptors (PR) and, thus, are not directly responsive to signaling by this anti-inflammatory steroid.[133,134,214,256,257] Likewise, it has been suggested that the immune-endocrine regulation of endothelial function may be mediated through an indirect mechanism, yet this concept has not been investigated. Developing a better mechanistic understanding the cellular processes that dictate vascular-leukocyte crosstalk would help identify the cellular targets in the endometrial microenvironment that regulate normal and pathologic inflammatory processes.[3,258,259–262] Once infiltrated into the endometrial stromal compartment, microenvironmental cues can activate the differentiation of monocytes to macrophages which can subsequently further promote the secretion of pro-inflammatory molecules (*e.g.* IL-1β, IL-6, IL-8 and TNFα). In response to inflammatory stimuli, such as lipopolysaccharide (LPS) a membrane component of gram negative bacteria, or cytokine mediators such as tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β), the endothelial cells are induced to express adhesion receptors, including E- and P-selectin, and cell adhesion molecules (*e.g.* ICAM, VCAM) that mediate a rapid forward kinetic inflammatory cascade (Figure 4-2). The expression of chemoattractant molecules

(chemokines) specifically support rolling and firm immobilization of immune cells that eventually undergo transendothelial migration via integrin adhesion receptors in a process known as diapedesis.[263,264] Inflamed tissues secrete leukocyte chemoattractants, such as monocyte chemotactic protein-1 (MCP-1), which normally acts in the recruitment of monocytes to maintain homeostasis; however, unresolved and chronic inflammation may result in pathogenic disorders. Dysregulation of endothelial function has been associated in the etiology and pathophysiology of several reproductive diseases, including toxicant mediated disorders.[218,265–267]

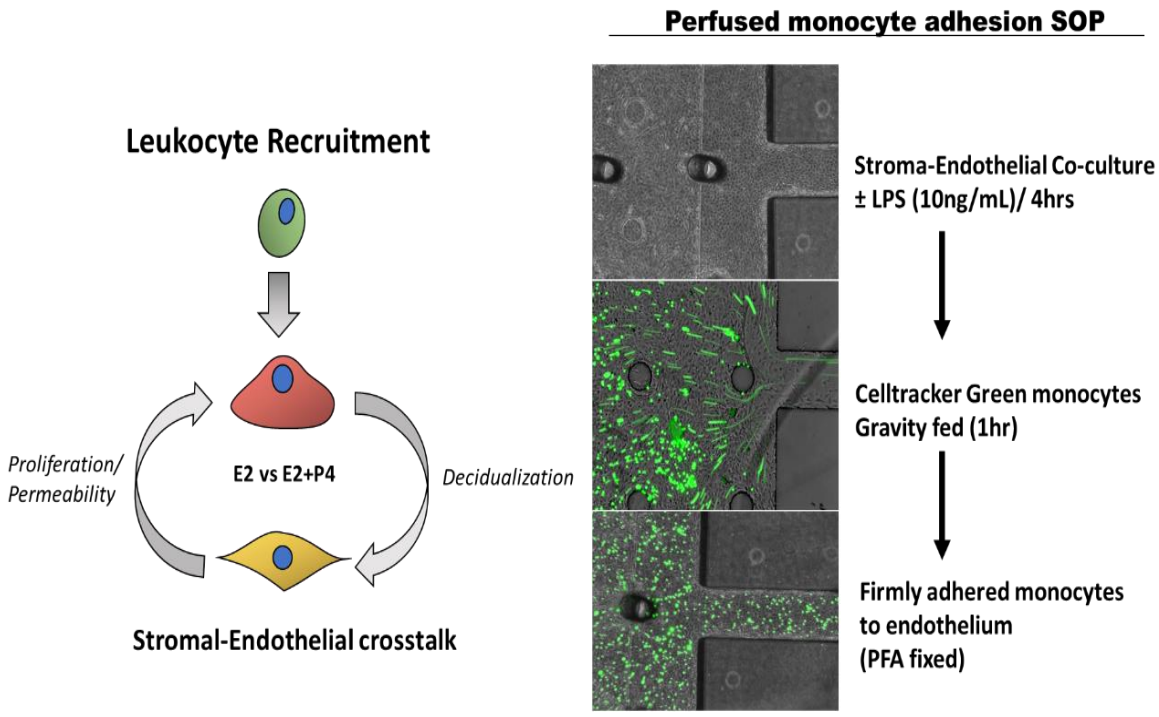


Figure 4-3. Experimental approach and standard operating protocol for modeling leukocyte recruitment in the EndoChip. (a) Schematic of the crosstalk between the immune, vascular and somatic components of the endometrium and their contribution to inflammatory and reproductive processes. (b) Single culture of endothelial cells in a single chambered EndoChip are maintained and challenged with inflammatory stimuli. Fluorescently tagged monocytes (THP-1) are introduced to the inlet of the vascular chamber and permitted to flow under gravity –fed conditions. After several washes, firmly adhered monocytes are quantified by counting the number of green cells adhered to the monolayer of endothelial cells in at least three different fields of view. Quantification was performed using ImageJ**.**

4.2.1 Establishing a microfluidic model of leukocyte recruitment

As discussed, the interactions among endocrine responsive somatic cells, the vascular endothelium and circulating leukocytes are necessary for maintaining tissue homeostasis in the endometrium. As such, inflammatory processes, including cytokine networks, immune cell activation, and subsequent reproductive processes, are highly regulated by intercellular crosstalk. To address immune cell communication with somatic cells, we adapted our microfluidic model of the perivascular stroma to establish a standard operating protocol (SOP) in order to qualitatively and quantitatively measure monocyte recruitment in the human endometrium. We introduced monocytes under gravity perfusion to mimic the flow of immune cells through blood circulation. As a proof of principle, we utilized an immortalized monocytic cell line (THP-1) as an analog of immune cells and assessed ability to perfuse our device so that we could quantitatively measure their interactions within the vascular chamber. First, we established a co-culture model of stromal cells and HUVECs (as described in chapter 2) and we induced decidualization for a minimum of 14 days under the influence of E2 or E2 and MPA. At day 14, we introduced fluorescently tagged $1x10^6$ THP-1 cells/mL to track the cells and observe their perfusion and the firm adhesion to the vascular chamber in the dual-chamber device. Upon perfusion, an even distribution of the monocytes throughout the chambers was visibly observed and the THP-1 cells compartmentalized exclusively within the vascular chamber (Figure 4-3B). Under basal conditions, minimal THP-1 cells were observed to firmly adhere to the endothelial layer; however, an increase in THP-1 adhesions was observed when an inflammatory stimulus was administered. Specifically, in order to incite an inflammatory event, we utilized LPS (10 ng/mL) as an experimental challenge to mirror a bacterial

infection. We observed a significant increase in number of firmly adhered monocytes after four hours of LPS stimulation (Figure 4-4). A tri-culture of endometrial stromal fibroblasts, endothelial cells and monocytes was established, and selective staining allowed a quantitative analysis of firmly adhered monocytes (Figure 4-4A). We performed both direct exposure of LPS to the endothelial cells as a positive control that mirrors endotoxemia, but focused primarily on indirect stimulation (*i.e.* only challenged the stromal compartment) when comparing treatment groups (Section 4.2.2). Using this SOP platform, we obtained preliminary evidence to support and validate the concept that P4 confers anti-inflammatory effects in the endometrium compared to devices only treated with E2 (Figure 4-4.D). These human cell-based OoC findings support similar results obtained from *in vivo* rodent models. Specifically, genetic suppression of the P4 receptor in the murine model results in increased infiltration of immune cells in the endometrium.[212]

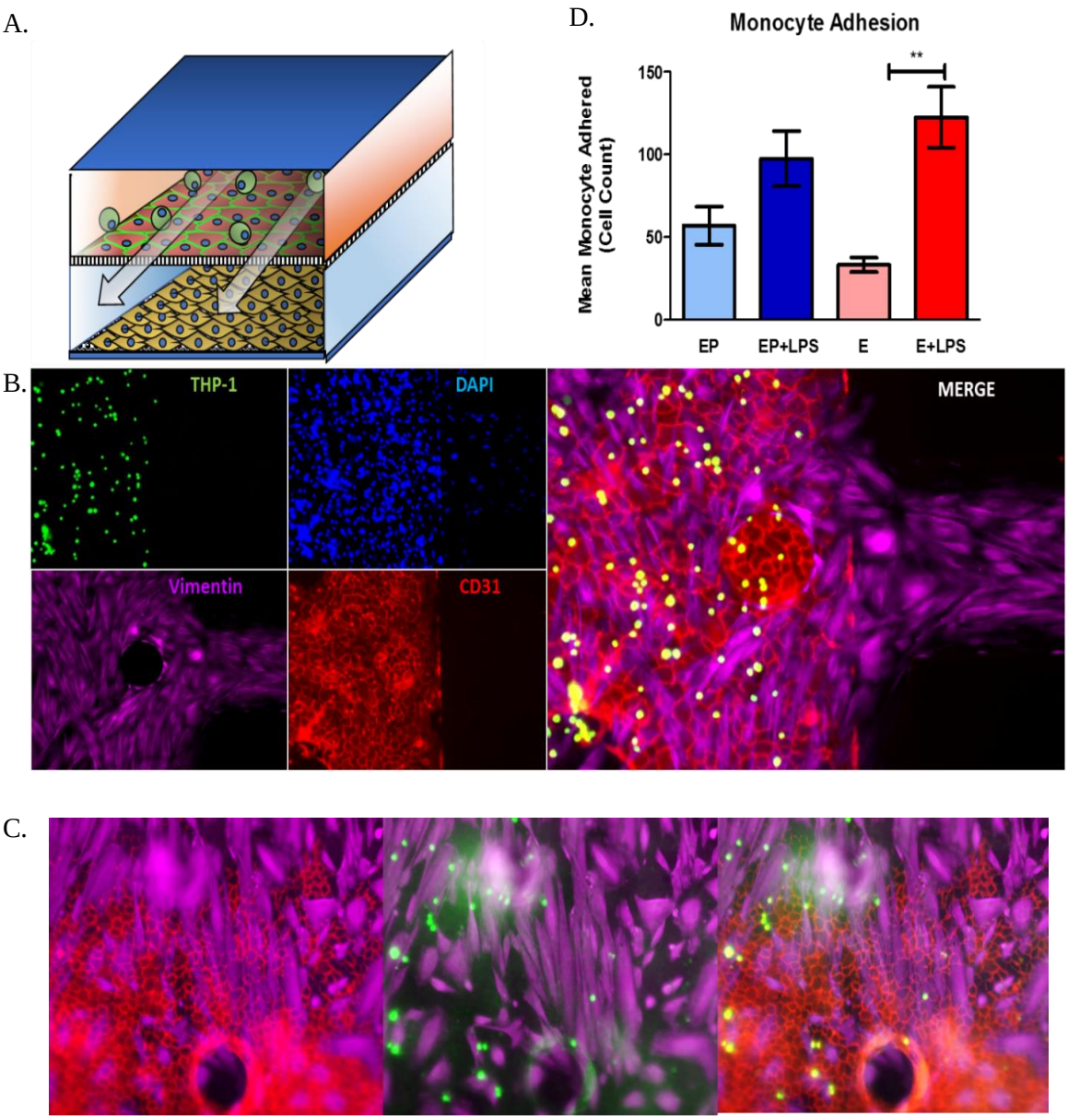


Figure 4-4. Establishment of a quantitative model of monocyte adhesion. (a) Schematic of introduction of monocytes to the vascular chamber in the EndoChip perfused under gravity-fed conditions. (b) Representative images of monocyte adhesion on co-culture of endometrial stromal and HUVECs. Green are the tagged monocytes. Selective adhesion of the monocytes is constrained to the endothelial chamber. (c) Additional images of the tri-culture model. Clear compartmentalization and selective staining for the three cell types is evident and can be quantified. (d) Quantification of firmly adhered monocyes under indirect stimulation with LPS (10ng/mL, 4 hrs) to the stromal chamber. Significant activation of the endothelial cells can be observed with LPS stimulation compared to untreated devices.

4.2.2 TCDD dysregulates progesterone action in the human endometrium

We have previously demonstrated in murine models that endocrine disruptors, specifically TCDD can induce an endometriosis-like phenotype in the uterus of female mice.[25,26,206] These findings were supported mechanistically by a robust loss of P4 sensitivity that was driven by the loss of P4 receptor (PR) in endometrial tissue.[26,229] To further translate our murine findings, we examined whether TCDD would similarly alter P4 modulation of reproductive processes in our established microfluidic model of the perivascular stroma. In these experiments, we utilized the same experimental protocol described in Chapters 2 and 3, and added a toxicant treatment group that was primed with TCDD (10 nM) for the duration of the 14-day experiment in static conditions. We collected and measured the biochemical and morphological changes associated with stromal decidualization. We observed and demonstrated that TCDD significantly inhibited the decidualization process (Figure 4-5). Morphologically, the stromal cells maintained their spindly, fibroblast-like morphology in the TCDD treated group as if the cells had only been treated with E2 (Figure 4-5. A). Biochemical analysis of prolactin secretion confirmed a significant suppression in the secretion of prolactin in the model (Figure 4-5. B). Mechanistically, we found a reduced expression of PR, via immunofluorescence imaging, in the stromal cells that were treated with TCDD compared to vehicle treated controls (Figure 4-5. C). These findings corroborate our murine findings (Supplementary Figure 4-8) and validate that our model supports translational toxicological research in our OoC model.

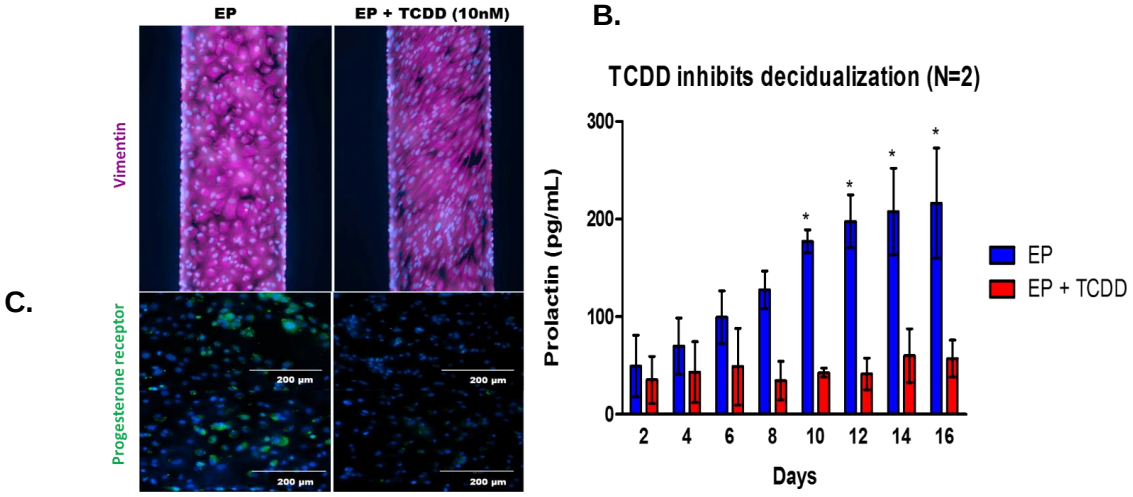


Figure 4-5. TCDD dysregulates reproductive function by suppressing PR expression. Co-cultures of endothelial (HUVECs) and endometrial stromal cells were primed first with TCDD (10nM) for 48hrs with E2 only followed by 14 days with E2 and MPA (EP) with co-treatment of TCDD. EndoChips treated with TCDD conferred reduced decidualization. (a) Morphologically, TCDD treated stromal cells failed to undergo an epithelial-like differentiation toward a decidual cell compared to vehicle treated devices. (b) Biochemically, production of PRL in response to progesterone is significantly reduced in the devices treated with EP + TCDD. (c) Disruption in reproductive sensitivity to progesterone by TCDD is mediated by a reduction in progesterone receptor (PR) compared to vehicle treated devices. These findings validate our previous in vivo findings using murine models, where we observe similar results. (N=2)

4.2.3 TCDD enhances monocyte recruitment in the endometrium

Next, we applied the monocyte adhesion SOP described in Section 4.2.1 for the TCDD experimental approach described in 4.2.2. To date, technical limitations in modeling the human endometrial microenvironment have hindered our ability to identify the key early events in immune-endocrine targets of TCDD toxicity. However, our previous *in vivo* murine models of TCDD toxicity have confirmed an influx of inflammatory $CD45^+$ leukocytes in the omentum and the eutopic endometrium of TCDD exposed mice (unpublished work, Supplementary Figure 4-8). This preliminary observation supports the findings by several groups that show both increased chemokine secretion and subsequent increased macrophage infiltration in several distinct tissues in response to TCDD.[218,266] Our group has previously shown that mice with an early life exposure to TCDD develop an adult-onset endometriosis-like uterine phenotype. This phenotype includes: a loss of P4 sensitivity, increased local infiltration of leukocytes and a hyper-inflammatory endometrial microenvironment[141,142]. Furthermore, the TCDD-induced endometrial microenvironment acted to increase sensitivity to secondary infections that negatively affected the adult reproductive function. We attributed this effect to the loss of anti-inflammatory P4 action by which leukocytes might be aberrantly recruited and activated to promote a hyper-inflammatory uterus. Herein, we utilized our established OoC model to test whether TCDD disrupts the inflammatory microenvironment of the endometrium by enhancing recruitment of peripheral monocytes at the perivascular stroma. As described, we quantified the number of firmly adhered monocytes to the endothelial cells following an LPS challenge (10 ng/mL) in decidualized devices that had been primed with TCDD for 14 days. In this pilot experiment the experimental approach was designed

to test the “second-hit hypothesis” that TCDD enhances the inflammatory response of cells and tissues that is driven by the loss of PR as demonstrated in 4.2.2. We observed a significant increase number of THP-1 cells in the co-treatment group compared to the LPS stimulated devices (Figure 4-6B) and quantified these results from 3 random fields of view (Figure 4-6A). To explain this increase in immune cell recruitment we used immunofluorescence to examine proteins involved in leukocyte recruitment. We found that intercellular cell adhesion molecule (ICAM-1) and we observed an increased ICAM-1 expression in the endothelial cells that were pre-treated with TCDD (Figure 4-6C). Moreover, we observed increased secretion the chemokine MCP-1 (Supplementary Figure 4-9A). In addition to the increased number of infiltrating monocytes, we suspected that the resulting inflammatory cytokine secretion was increased. Indeed, we measured an inflammatory cytokine panel using a Luminex multiplex protein magnetic bead–based assay in the effluent from these devices, and we have preliminary evidence to suggest that the double-hit with LPS enhances the secretion of IL-6, IL-8, and TNFα thereby enhancing the pro-inflammatory microenvironment of the endometrium OoC (Supplementary Figure 4-9). Altogether, these toxicant-mediated effects may drive the chronic inflammation that is characteristic of women with endometriosis. While these results are preliminary, they should be pursued to identify mechanism and specific molecular targets for therapeutic development.

4.3 Materials and Methods

4.3.1 Acquisition of human cell populations

The Vanderbilt University Institutional Review Board approved the procedures for collection of all primary cells used and tissue acquisition was performed only after patients gave informed consent. Acquisition of primary endometrial stromal cells and HUVECs were performed as described in Chapter 1. THP-1 cell lines were purchased from ATCC and maintained according to the instruction of the distributer. Suspension THP-1 cells were maintained at 37°C in a saturated humidity atmosphere containing 95% air / 5% CO_2 in complete media containing 10% FBS. THP-1 cells were cultured and maintained in suspension. All endometrial stromal cells were isolated previously described.[62] Peripheral blood samples were obtained from all endometrial biopsy donor samples. Peripheral blood mononuclear cells from whole blood were matched to stromal donors. Isolated PBMCs were frozen in 90%FBS and 10% DMSO at $1x10^7$ cells/mL and used for monocyte isolation at the day of the experimental timeframe. Naïve monocytes were isolated by enriching a CD14+ cell population using a magnetic isolation purification (Miltenyi Biotec MACS magnetic cell separation system) and yielded approximately 1 million viable monocytes per 10 million PBMCs. Stromal cells were maintained in phenol red-free DMEM/F-12 with 5% charcoal-stripped calf serum, 1nM 17-β oestradiol (E2, Sigma Aldrich, USA) and 1X antibiotic-antimycotic solution (stromal complete growth medium). HUVECs were maintained using EGM-2 media as described in Chapter 1 and monocytes were maintained using a complete RPMI basal media supplemented with 10% FBS, 1X antibiotic/antimycotic (ThermoFisher). As required for experimental objectives, some

stromal cell cultures received treatments with 0.5 mM medroxyprogesterone acetate (MPA, Sigma Aldrich, USA) and TCDD (10nM) over a period of at least 14 days. Vehicle (DMSO) controls were utilized for the non-TCDD treatment groups.

4.3.2. Fabrication and assembly of microfluidic two-chamber device

Fabrication and maintenance of the devices were performed as described in chapters 2 and 3. Briefly, dual-chambers were generating by sandwiching a polycarbonate membrane (3μm pores) between the two PDMS-based chambers and plasma bonding the layers. All cell cultures were maintained in static conditions

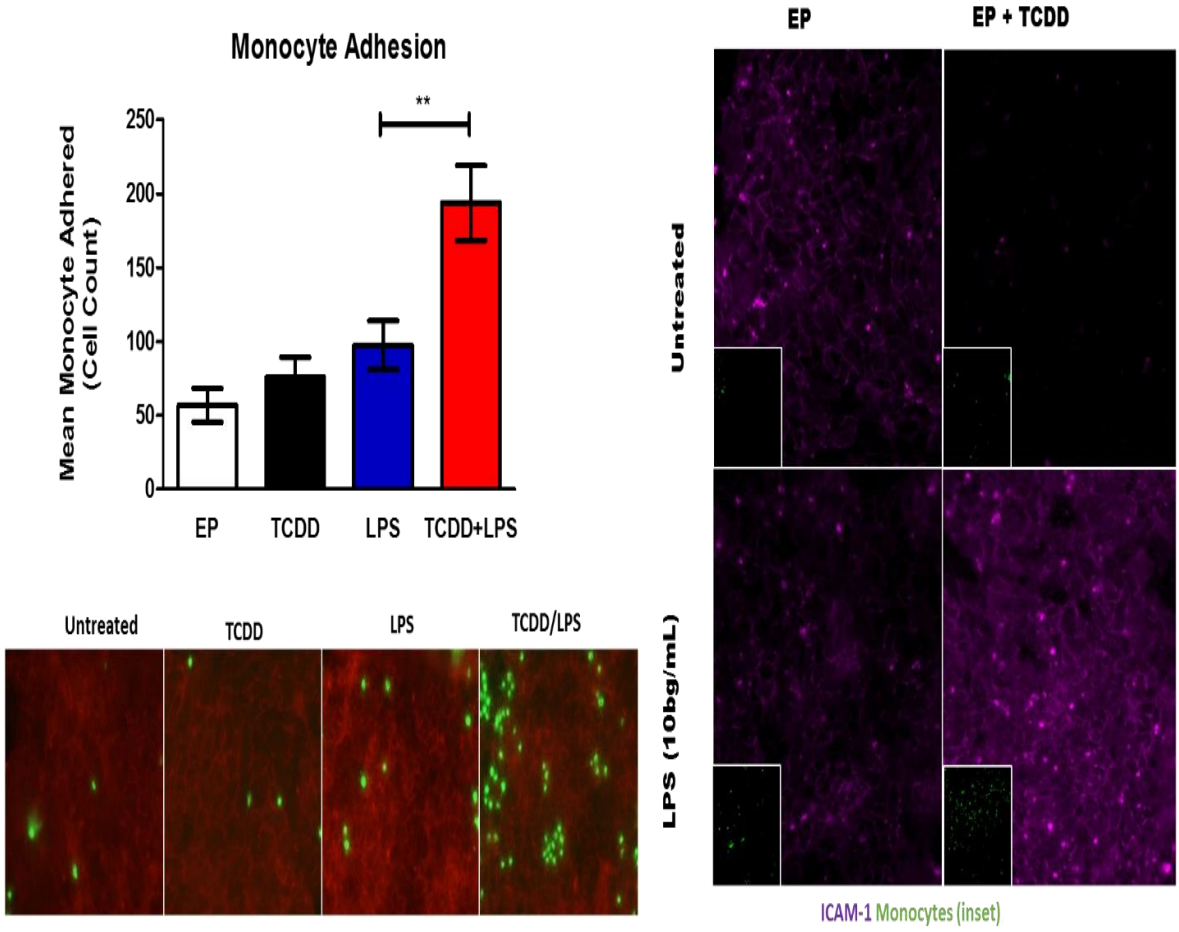


Figure 4-6. TCDD dysregulates the immunological processes of the endometrial microenvironment by enhancing sensitivity to inflammatory stimuli. Monocyte adhesion was performed in the TCDD vs vehicle treated devices. (a) Representative images of enhanced monocyte (green) recruitment by TCDD in response to an inflammatory challenge (LPS 10ng/mL, 4 hrs). Enhanced immune cell recruitment is observed in the double hit (TCDD/LPS) compared to LPS only treated devices. (b) Quantification of mean monocyte adhesion. (n=3). (d) Increased ICAM-1 expression in the endothelial cells from the EndoChip co-culture. Representative and matched monocyte adhesion is included in the inset. TCDD enhances endothelial activation to inflammatory stimuli and enhances monocyte recruitment via ICAM-1. (n=3)

4.3.3. Cell culture and maintenance in microfluidic device

Isolated primary stromal and endometrial cells were seeded and cultured using the procedure described in Chapter 2 with the exception of a few adaptations.[268] The somatic cells were seeded and allowed to adhere for a minimum of 30 minutes inside the incubator and then 350 µL of EBM-2 medium or complete growth medium was added to the endothelial top chamber or stromal bottom chamber, respectively. Spent media from the reservoirs was collected and replaced with fresh media every other day. All experiments were performed under static conditions for a minimum of 14 days. Once confluent layers were observed, a 48-hour pre-treatment with E2 (1 nM) only and TCDD (10nM) was initiated prior to inducing decidualization. After 48 hours, the media was appropriately discarded and replaced with E2 with or without MPA (0.5 nM) to model the secretory phase and induce decidualization. Treatment was run for 14 days. Collection of the effluent from the devices was performed to analyze secreted proteins such as cytokines and prolactin. Media was collected from each chamber and frozen at -80°C for analysis. Collected effluents were then analyzed for expression of inflammatory proteins using a magnetic-bead 27-plex array panel Bio-Plex Pro™ Human Cytokine, Chemokine, and Growth Factor Assay (Bio-Rad). Specific hits were validated with individual enzyme-linked immunosorbent assay (ELISA Duoset, R&D systems). The ELISA was performed according to the manufacturer's instructions and expanded to 384 well format, using 23 µL per sample. The results are representative of three different experiments. Statistical analysis were performed by a 2way ANOVA between treatment groups using a Bonferroni correction. Statistical significance was considered $p<0.05$.

4.3.4. Monocyte perfusion and adhesion quantification

Following the treatment experimental approach described above, at day 14 of treatment we introduced THP-1 monocyte cell suspensions to the system. To observe the monocytes, we labelled the monocytes with CellTracker™ Green CMFDA (12nM, 5-chloromethylfluorescein diacetate, ThermoFisher) following the manufacturer's suggested staining protocol. A 400 μL suspension of fluorescently tagged monocytes ($1x10^6$ cells/mL) were loaded to one of the reservoirs of vascular chamber. The reservoirs of the bottom chamber (stromal chamber) were maintained with 350 μL of media. The devices were immediately visualized using an EvosFL microscope to observe gravity-fed perfusion of the monocytes through the vascular chamber for 1 hour. The devices were then washed at least three times with warm fresh media to remove non-adhered monocytes. To measure firm monocyte adhesion, LPS (10ng/mL) was utilized as an inflammatory stimulus to activate the endothelial cells directly, and indirectly, prior to adding the monocytes. LPS was introduced to the stromal chamber for 4 hours to stimulate the cells and then washed (3X) with fresh media to remove residual LPS. As a positive control and a model of endotoxemia, LPS was added to both chambers. After washing the non-adhered monocytes to the endothelial layer, the model was fixed with 4% paraformaldehyde (PFA) for 15 minutes and washed with fresh PBS. Quantification of adhered monocytes was assessed by image analysis in 4 random fields of view and quantified by cell count using ImageJ (NIH). Areas of monocyte adhesion between treatment groups were obtained from regions of confluent endothelial monolayers. The results are representative of three different experiments. Statistical analysis between treatment groups was performed by a 2way ANOVA using a Bonferroni correction. Statistical significance was considered $p<0.05$.

4.3.5. Fluorescence microscopy

For immunostaining, samples were fixed with PBS containing 4% (wt/vol) paraformaldehyde. Standard fixation and immunofluorescence staining protocols were performed as described in the product datasheet. Fixed cells were blocked with 10% (vol/vol) goat serum and incubated with either primary fluorescence-labeled Ab or unlabeled Ab, followed by secondary antibody, or the F-actin probe ActinGreen™ 488 ReadyProbes™ Reagent (Invitrogen). Briefly, once the 4 hrs time period was completed, the cells were washed three times with 1X PBS and fixed with 4% paraformaldehyde (PFA) solution for 15 mins at room temperature. The cells were then permeabilized with 0.5% Triton X-100 (Sigma) in 1X PBS for 30 mins at room temperature. Cells underwent several washes in PBS and were blocked with goat serum solution (5% goat serum and 2.3 % glycine in PBS) for 1 hr at room temperature. The cells were then stained with the primary purified anti-human CD54 (I-CAM1, HA58, Biolegend) at a 1:100 concentration in blocking solution for 1 hr at room temperature followed by several washes in PBS. Cells were then stained with the ReadyProbes secondary antibodies conjugated with Alexa Fluor™594 dyes goat anti-mouse antibody (R37121, ThermoFisher Scientific) by using one drop per mL of PBS and incubated for 30 mins at room temperature followed by several washes in PBS. Mouse monoclonal anti human CD-31 primary antibody (DAKO, USA) was used at a 1:50 dilution and detected with ReadyProbes™ Alexa Fluor® 594 Donkey Anti-Mouse IgG antibody (ThermoFisher). A rabbit antibody against human vimentin (1:200, Abcam, USA) was used with an Alexa Fluor® 647 conjugated goat anti-rabbit antibody as a secondary antibody (1:100, Jackson ImmunoResearch, USA). Progesterone receptor antibody conjugated to FITC was used (1:50, Abcam) to visualize

PR expression in the devices. The cell nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) for 1 minute and then washed with 1X PBS.

4.3.6. Statistical Analysis

All data are reported as mean values ± SD and were analysed by Student's t test. Group comparisons were performed using a 2-way ANOVA and results were deemed significant for two-tailed unpaired p values below 0.05. All experiment were performed from a total of 7 primary control stromal cells derived from both endometrial biopsy donor and surgical hysterectomies. Data were analyzed using a Student t test when comparing numerical variables between two groups and one-way analysis of variance followed by Bonferroni's post-test for comparisons between more than two groups. Statistical analyses were performed using GraphPad Prism software. $P < 0.05$ was considered statistically significant.

4.4 Conclusions

The most extensive toxicological studies must include developmental and reproductive toxicological assessments (DART). These studies are often solely conducted using murine models even though they frequently fail to predict the human toxic response (http://toxnet.nlm.nih.gov). Cumulatively, it has been suggested that the limitations of both of these *in vitro* and *in vivo models* have hindered the drug development pipeline by failing to replicate the human condition in the preclinical development phase, resulting in false positives in mice models and ultimately subsequent failures in the first and second phases of clinical trials. In particular, the immunological contribution to disease pathogenesis is

highly divergent between humans and rodents and immune-endocrine interactions are poorly understood in the reproductive tract. Nevertheless, as demonstrated by our OoC studies, interactions between immune-endocrine pathways is clearly a major toxicological target in the human endometrium. Endocrine disrupting chemicals, such as TCDD, likely promote reproductive dysfunction by altering these inflammatory pathways.[206,217,266,267]

A lack of a robust experimental models limits our understanding of the pathophysiology of many endometrial disorders, thus limiting the design of therapeutic options for patients with these conditions. A better understanding of the etiology of these diseases is critically needed to design appropriate medical interventions. Here, we introduce an immunological model of monocyte recruitment into the endometrium to examine the initial steps of inflammatory disease processes, specifically the firm adhesion to the endothelium during diapedesis. As discussed in Chapter 3, inflammatory processes are a two-edged sword for reproductive function. We identified that specific inflammatory mediators, such as prostaglandins, are instrumental in promoting the initiation of decidualization. However, hyper-and-chronic inflammatory conditions may be detrimental to endometrial function and promote disease pathogenesis. In this chapter, we examined the toxicological effect of TCDD on reproductive sensitivity to P4 to determine if we could recapitulate our previous murine studies in a translational OoC model of the human endometirum. We confirmed that TCDD is disrupting P4's anti-inflammatory action by suppressing PR expression. A limitation of this analysis remains the characterization of specific PR isoforms and their expression patterns. However, these issues may be overcome as more specific and confocal microscopy improves high content imaging. We observed in a pilot study that TCDD can promote a hyper-inflammatory endometrial

microenvironment by enhancing the sensitivity to secondary inflammatory challenges and increase the inflammatory mediators involved. This “second-hit” hypothesis is supported by animal models that demonstrate increased infiltration of pro-inflammatory producing leukocytes in TCDD exposed organs. Furthermore, we observed that in *ex vivo* punch biopsies of other reproductive tissues, such as the fetal membrane, acute TCDD treatment can significantly shift the effective dose of LPS and enhance the production of pro-inflammatory cytokines such as IL-1β and TNFα (data not shown). To investigate the mechanisms that drive the toxicant-mediated immune cell infiltration, we examined the initial steps of leukocyte recruitment during diapedesis from circulation. Thus, our model mirrors the recruitment of monocytes from circulation, and we measure the rate limiting step before extravasation and activation into the tissue. Furthermore, we demonstrate that this process is at least partially mediated by increased expression in vascular ICAM-1, a molecule necessary to establish firm adhesion between endothelial cells and peripheral monocytes. As such, we can begin to dissect the direct targets of TCDD toxicity and identify novel targets for developing therapeutic interventions. Moreover, our model can serve as a tool for testing efficacy of drugs in development (As discussed in detail in Chapter 5). It is important to consider that in our model, we are not including the epithelial cell, a major somatic component of the endometrium that is capable of secreting cytokines, chemokines and other inflammatory mediators. Whereas, the contribution of the epithelial cells is not experimentally considered in this thesis these cells would be incorporated in future approaches. Due to the hydrophobic nature of PDMS-based microfluidic models, such as ours, it is important to acknowledge that absorption rates by PDMS complicate toxicant pharmacokinetic/pharmacodynamic (PK/PD) analysis from these devices. This is

problematic for lipophilic compounds, such as certain hormones and TCDD, which express high partition coefficients (LogP) values. Herein, we report that despite this potentially confounding variable, we observe a biological effect on the cells within our model, likely due to the frequent media changes and/or saturation of the PDMS with the target compound. Although PDMS remains the gold standard for OoCs, future studies must address these technical and pharmacological issues.

Lastly, as a clinical correlate, our findings support TCDD and inflammatory mediators may be conducive to endometrial disorders, specifically endometriosis. The molecular contribution of the immune and vascular components to its development is essential to understand the pathogenesis of endometriosis. As a parallel model, we can utilize cells acquired exclusively from women diagnosed with endometriosis and compare their phenotypes in an OoC model. We have preliminary evidence to support that endometriotic cells confer a substantial inflammatory environment and this translates to the *in vitro* condition (Supplementary Figure 4-7). Cells acquired from control patients demonstrate enhanced monocyte adhesion to the stromal cell when primed with TCDD and challenged with LPS. However, endometriotic cells have increased sensitivity to the secondary challenge. While this is a preliminary experiment, it supports our hypothesis that TCDD induces an endometriosis-phenotype in the stromal cells and results in an inflammatory microenvironment. As part of this model we utilized the suspension monocytic cell line THP-1s as a proof of principle to establish the tri-culture. However, it is important to acknowledge the limitations of using such a cell line for biological and clinical implications. THP-1 monocytes are derived from an acute monocytic leukemia patient and confer some of the principal characteristics associated with monocytes

including their differentiation to adherent macrophages and dendritic cells and secretion of inflammatory molecules. Agonist-stimulated differentiation using compounds such as phorbol 12-myristate 13-acetate (PMA) can induce their activation to macrophage-like cells and can be driven toward specific macrophages polarities (*e.g.* M1 vs M2 phenotypes). Altogether, these characteristic, the reproducibility and commercial availability of THP-1s confer utility of this cell line in an experimental setting. However, they retain limitations and differences compared to primary monocytes or monocyte-derived macrophages. THP-1s sustain phenotypic programing due to their cancer-origin phenotype and should not be considered to be an analog for disease-free and naïve monocytes. Alternatively, when applicable, we can obtain primary monocytes from matched endometrial donors isolated from peripheral blood mononuclear cells to retain a more direct source for patient derived immune cells. The inherently difference between these cell types may dictate a more relevant and physiological response compared to THP-1s, specifically for their immunological transformation to inflammatory macrophages.

4.5 Appendix

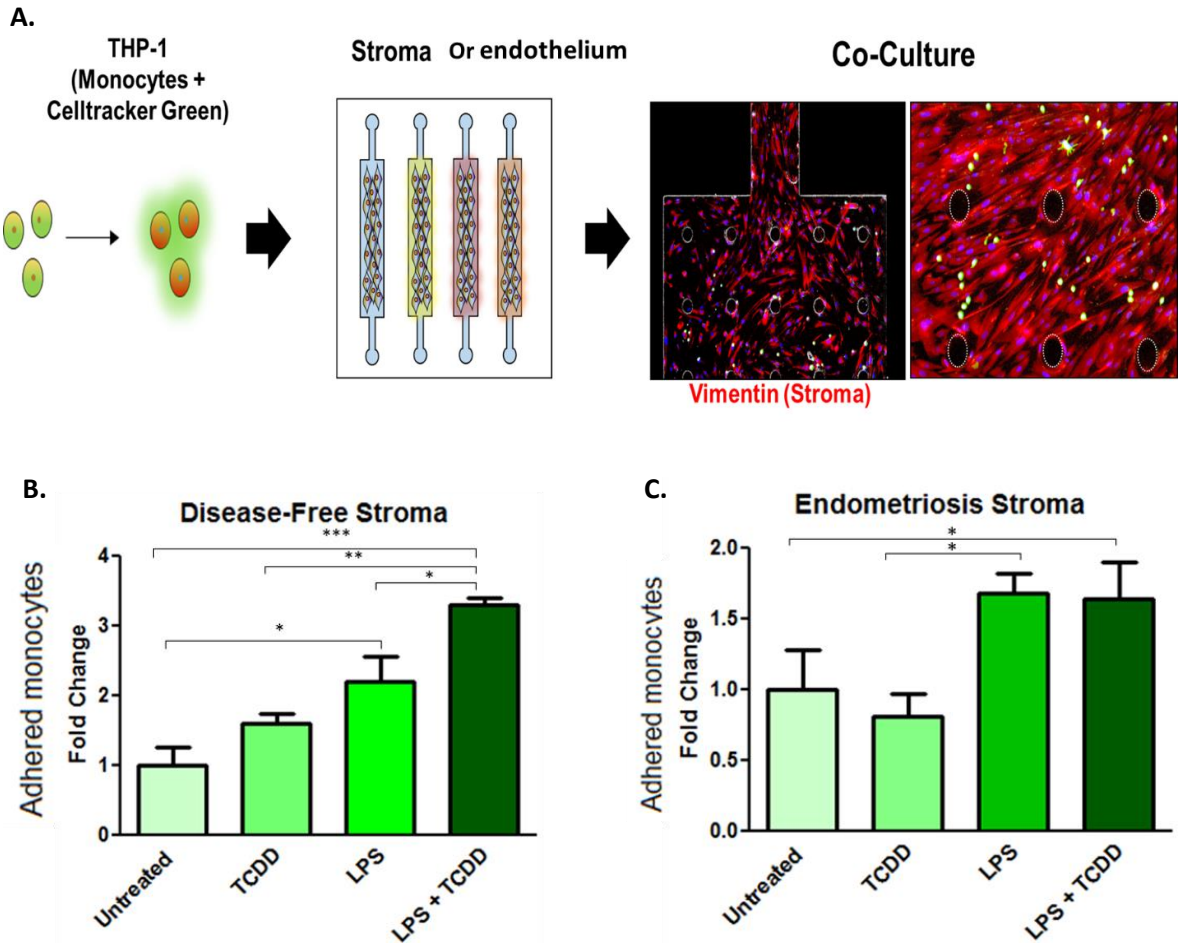


Figure 4-7. Endometriotic disease alters the immune cell recruitment in the human endometrium. (a) Experimental approach for co-culture between stromal fibroblasts and monocytes (THP-1). Firm monocyte adhesion was determined between treatment groups. (b) In a control patient LPS enhanced the number of firmly adhered monocytes, however TCDD significantly enhances this effect. (c) Endometriotic cells (isolated from a patient with endometriosis) demonstrates that TCDD does not significantly enhance monocyte adhesion compared to LPS treated cells and suggests an inherently hyperactive stromal cell.

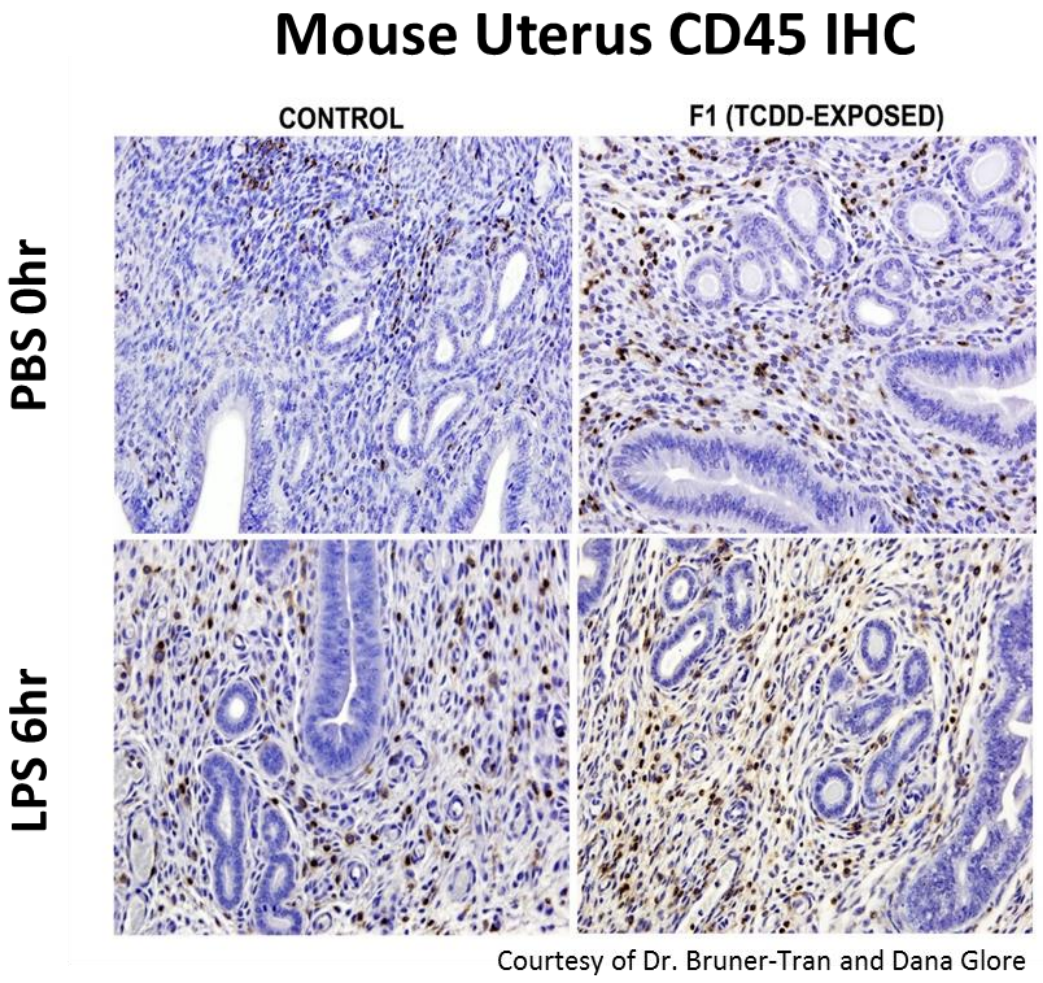


Figure 4-8. Representative IHC of the murine endometrial microenvironment from developmental TCDD exposure. IHC staining for CD45, a marker for leukocytes, demonstrates an increased influx of immune cells in the endometrium of the TCDD exposed mice. This increased flux in immune cells is enhanced in response to an I.P. stimulation with LPS.

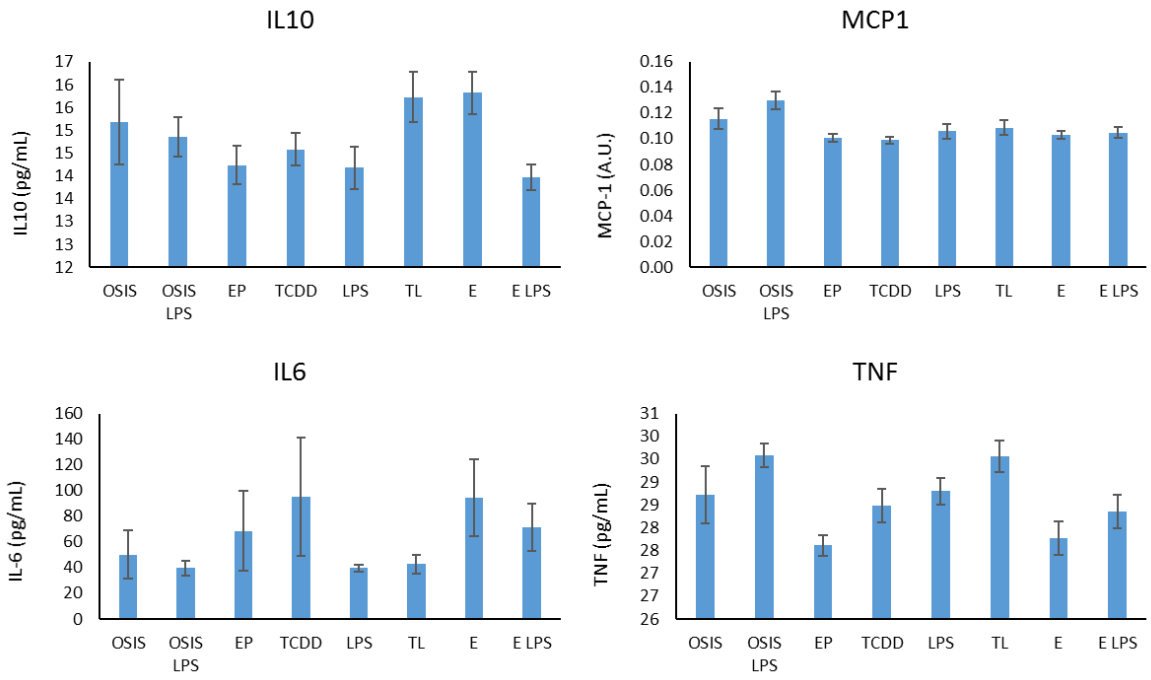


Figure 4-9. TCDD enhances inflammatory cytokines in EndoChip. Sampled effluent from devices treated with TCDD or from cells acquired from patients with endometriosis (Osis). TCDD (10nM), LPS (10 ng/mL), TL = TCDD + LPS, E = E2, EP = E2 + MPA. (N=12)

Chapter 5

Conclusions and Future Approaches

Contributions: This chapter appears, in part, as a literature review published in *Current Pharmaceutical Design*. The references cited herein that were primarily relevant to OoC models of the gravid female reproductive tract were obtained by searching the MEDLINE database for English language articles using PubMed (United States National Library of Medicine (Bethesda, MD)) for all years available. The following search terms or combination of terms were used: “endometrium”, “embryo”, “microfluidic”, “organ on chip”, “placenta”, “pregnancy”, and “reproductive tract”. Additional references were obtained from manuscripts that were reviewed through December 2018.

To summarize, as a major goal of this thesis, we designed, developed and utilized an OoC microfluidic model of the human endometrial perivascular stroma (EndoChip) that incorporates the major somatic, vascular and immune cell components of the endometrial microenvironment. We demonstrated that this OoC model remains responsive to steroids for a 28-day culture period, corresponding to the length of an idealized menstrual cycle. The EndoChip also provided sufficient sensitivity to assay stage-specific physiological reproductive processes associated with menstruation, such as endocrine mediated peripheral immune cell recruitment, vascular permeability and stromal decidualization. Our model is equally conducive to understanding temporal and spatial complexity of the immune-endocrine pathways that regulate inflammatory processes occurring within the menstruating endometrium and during disease pathogenesis.

The potential to examine the somatic, immune and endocrine components of the human endometrium in an EndoChip holds potential as a powerful tool for pre-clinical and toxicological applications in biomedical research. We specifically designed the EndoChip as a robust pre-clinical tool to advance reproductive drug development by: (1) elucidating human *in vitro* data sets that mirror the scale of organismal complexity that most severely confound translational predictions *in vivo*; and (2) by elucidating the adverse outcome pathways of potential agents from cellular to tissue-level responses. Furthermore, our dual-chamber device offers the capability to generate additional OoCs representative of specific disease states and other organs or tissues of the female reproductive tract.[92,268] Although this thesis primarily focused on the non-pregnant (*i.e.* non-gravid) cycling endometrium and its role in the establishment of pregnancy, the study of the gravid uterus is equally important to understand pregnancy-related complication. Thus, OoCs provide an innovative method to identify key molecular targets and test the potential toxicity or efficacy of newly developed therapeutics.

In this final chapter, we first briefly describe the utility of our OoC device for the development of an instrumented fetal membrane on a chip (IFMOC) to investigate toxicant and infection-induced preterm birth.[268] Next, we discuss the limitations and future approaches of our OoC models of the human endometrium (EndoChip). Lastly, we conclude by examine and discuss the overacrching impact our EndoChip may have on biomedical research

5.1 Development of OoC models of the gravid uterus

5.1.1 Preterm birth background

Maternal-fetal health is a field of research that is difficult to study due to both complex ethical and physiological dilemmas, which have undermined our understanding of pregnancy related disease processes, including preterm birth. Throughout this thesis, we have primarily focused on the cycling endometrium; however, we feel that it is important to discuss the physiology and pathology associated with a gravid uterus. We discussed how P4 primes the endometrium to be receptive for the implanting embryo. If there is no embryo capable of implantation, then there is a sharp withdrawal of ovarian steroid support with leads to the process of menstruation. However, if pregnancy is established, then P4 levels continue to increase until ovarian P4 production shifts to the placenta which then subsequently maintains production of this steroid throughout the duration of the pregnancy. The maternal responses to P4 during both early and late pregnancy control key physiological responses and physical remodeling that supports the invasive establishment and subsequent function of the placenta, a relationship that is critical for fetal growth and development. At term, numerous well-orchestrated inflammatory processes regulate the initiation of labor and the timing of birth. In humans, a functional loss of P4 action acts as a trigger for cervical ripening, increases in uterine prostaglandin mediators and eventual rupture of the fetal membrane. However, the timing for the onset of labor leading to birth is critical for the development and survival of the fetus. Annually, nearly 15 million preterm births (PTB) occur worldwide[270], making prematurity the leading cause of death in neonates and the second-leading cause of death in children under 5 years of age.[271,272]

However, our understanding the endocrine and immune mechanisms that drive PTB remain limited. While there are many causes of PTB, it is frequently associated with subclinical infections of the membranes that surround the developing fetus and extend from the placenta, a condition referred to as infectious chorioamnionitis (CAM). This pregnancy outcome is usually the result of bacteria ascending from the vagina to invade the fetal membranes.[273] Defining the host-microbial interactions within the fetal membrane at a cellular and molecular level are needed to reveal actionable targets for early diagnosis, prevention and treatment of CAM. As many as 70% or more of preterm births are associated with CAM, particularly when the delivery occurs before 30 weeks of gestation.[274] Babies exposed to CAM *in utero* are at increased risk for neonatal sepsis, necrotizing enterocolitis, bronchopulmonary dysplasia, cerebral palsy and retinopathy of prematurity.[275] Unfortunately, CAM is often asymptomatic and difficult to diagnose in time to prevent maternal and fetal adverse outcomes. Furthermore, subsets of pregnant women with microbial contamination of amniotic fluid carry their pregnancy to term, suggesting host factors likely influence the risk for CAM-associated PTB.[276] Additionally, antibiotic therapies have shown discrepancy between populations of women and have failed, for the most part, to prevent preterm birth.[277,278] A common pregnancy complication that stems from CAM, and is a major contributor to the burden of PTB, is preterm premature rupture of the fetal membranes (PPROM). Although fetal membrane rupture is an essential part of the normal delivery process, PPROM at less than 34 weeks of gestation is responsible for approximately 25% of premature births.[279] Our limited understanding of the early steps involved in fetal membrane rupture related to disease pathogenesis impedes solutions to this immense problem. However, PPROM is a major contributor to prematurity

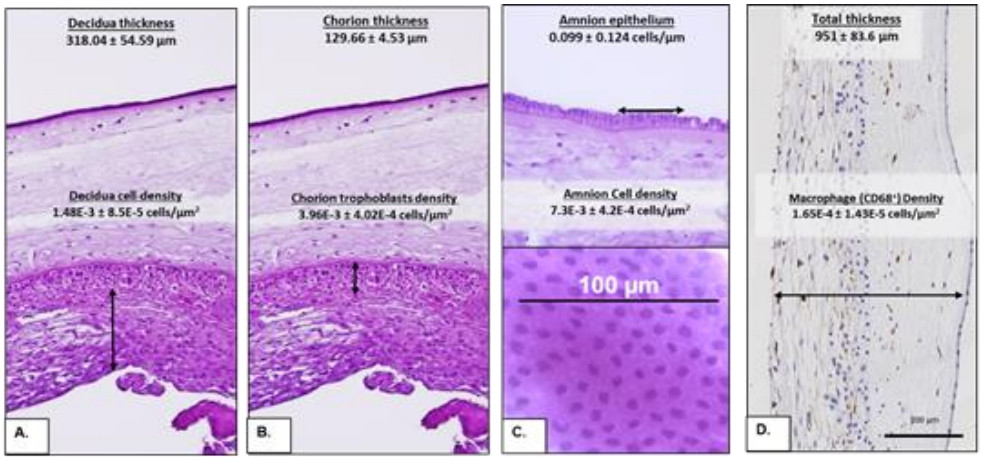
and accounts for approximately 25%of all PTBs.[280] To facilitate our understanding of the pathophysiology of these diseases, we must identify the interactive contributions of the major cell types that comprise the microenvironment of the fetal membrane.

5.1.2 Fetal membrane biology

As mentioned above, the fetal membrane plays an integral physiologic function to create a barrier and encapsulate the embryo to form a maternal-fetal interface. The fetal membranes are primarily composed of three structural layers, including the fetal-derived amnion and chorion and the maternal-derived decidua.[281] Fetal membranes are a deceptively simple tissue structure[268,281,282], composed primarily of decidual stromal cells, chorionic trophoblasts, fibroblasts (mesenchymal cells), a monolayer of amniotic epithelial cells, resident immune cells and a collagen-rich extracellular matrix. While the exact fetal membrane thickness and cell densities are variable between individuals, the overall cell ratios and histologic analysis between the somatic cells demonstrate a consistent composition. Specific cell populations and the relative cell ratios comprising the fetal membrane are depicted in Figure 5-1. Despite this relatively simple organ structure, little is known about how fetal membranes participate in immune defense or how microbes evade these defenses. Like the endometrium, there is a significant deficit in tractable model systems of human fetal membranes. Most studies of human fetal membrane immunology employ traditional cell and *ex vivo* tissue culture models[282,283] and are limited by either loss of the biological context or an inability to maintain *ex vivo* tissues for prolonged periods. These traditional tissue culture models also lack the capability to dissect the roles of individual cell types within the context of a tissue microenvironment. Typically,

independent cell and tissue culture experiments are conducted at a limited number of experimental intervals that are terminated at discrete time points. These standard tissue culture models do not accurately convey the real-time and cell-specific molecular pathways that are constantly changing throughout the course of infection. This disjointed approach creates challenges for understanding the dynamic host-microbial relationship. Animal models are beneficial for physiologic studies, but the placenta and fetal membranes have tremendous differences in anatomy and physiology amongst mammalian species. These biological differences among placental phenotypes limits progress towards translational solutions.[284,285] Thus, there is an essential need to develop robust models of human CAM that can eliminate species-specific differences, incorporate all relevant cell subtypes to accelerate research in immunology and microbiology.[49,286]

A.



Cells/µm, [a]Approximation, [¥]Total FM thickness
Note: Mesenchymal fibroblasts are not included

B.

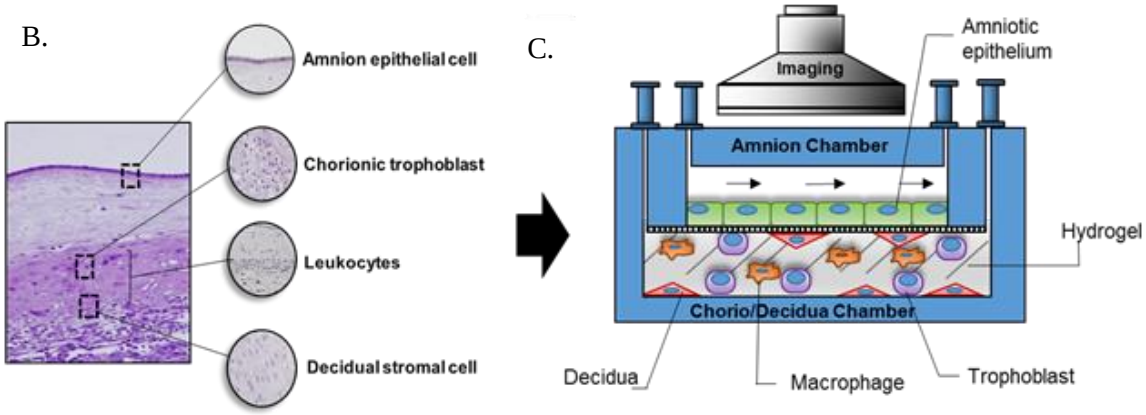


Figure 5-1. A prototype of the first generation IFMOC. (a) Fetal membranes are primarily composed of amnion epithelial cells, chorion trophoblasts, residing leukocytes and decidual stromal cell. Our interest in macrophages stems from a sub-hypothesis to examine their role in inflammatory processes of the fetal membrane, but it is important to note, that any immune cell of interest can be incorporated within this system. (b) A schematic of the development of the first generation IFMOC using a two-chamber microfluidic device for analysis of inflammatory networks and membrane barrier integrity.

5.1.3 Instrumented fetal membrane on a chip (IFMOC)

As we have discussed in detail throughout this thesis, microfluidic OoC models can provide an alternative and innovative solution to the technical limitations in modeling complex human organ systems, including fetal membranes.[2,268,287] Emerging approaches, incorporating OoC and microfluidic technologies represent innovative technologies to enhance the *in vitro* modeling of human organs and tissues and supplement current *in vivo* models. In recent years, significant interest has been put forth by several agencies including the Environmental Protection Agency (EPA), the Human Placenta Project (National Institutes of Health)[288,289] and the National Center for Advancing Translational Sciences (NCATS) to develop these organotypic models of the reproductive tract, including the gravid uterus. Herein, we describe a parallel work funded by the Environmental Protection Agency (EPA) to develop a first generation OoC model of the fetal membrane to recapitulate anatomical, biochemical and reproductive processes of the fetal membrane that physically separates maternal and fetal components (Figure 5-1C and Supplementary Figure 5-3). These fetal membrane OoC devices can be leveraged to shed new light on many physiological and pathophysiological processes, including the host-microbial interactions that occur during CAM and PTB. For example, we use PPROM as an example of a gestational membrane PTB-related process that can be examined using this OoC model. We envision that the IFMOC will model important biological variables such as fetal sex and/or race/ethnicity at the tissue level and be capable of incorporating novel imaging tools as well as downstream analytics. These additional capabilities will allow longitudinal studies fetal membrane studies throughout the course of an infection: from colonization to mechanical loss of membrane integrity. Akin to *ex vivo* fetal membrane

models, an IFMOC will provide a "living" model of the fetal membrane, including tissue polarity, tissue and cell specific paracrine networks, and easily measurable membrane barrier functions. Together, these functional, quantitative and qualitative outcomes will facilitate our understanding of bacterial colonization and transmittance mechanisms from the maternal side to the amnion. Although infections are common causes of PTB, only a subset of women develop CAM-induced PTB, suggesting that other stressors, including environmental agents such as endocrine disrupting chemicals, may be at play. This possibility is currently being actively explored by several reproductive toxicology-related projects in our laboratory and our IFMOC design has been shown to be experimentally compatible with toxicology testing.

By applying the same, basic microfabrication and engineering technologies developed in Chapter 2 to the maternal and placental cells that make up the fetal membrane, we can begin to integrate the first generation of the IFMOC to model the compartmentalization of decidua, chorion, and amniotic components of the fetal membrane *in vitro*. The primary advantage of the IFMOC is that this device significantly reduces the total culture media volumes and cell numbers required to establish a co-tri-and-quad-culture (Figure 5-2, supplementary Figures 5-5, 5-6). As mentioned earlier, this reduction in culture media among the various cells types provides a stronger signaling network between compartmentalized cells and thus a more robust crosstalk as occurs *in vivo*. In the first series of IFMOC experiments, we have established quad-cultures of primary amniotic epithelial cells, decidual cells, a trophoblasts cell line (HRT-8SV/neo) and a phorbol myristate acetate (PMA)-induced macrophage cell line (THP-1) using the two-chamber device we described in Chapter 2 (Figure 5-2). Importantly, the capability to introduce

other key immunological cells (*e.g.*, neutrophils) is equally feasible within this microfluidic platform. The experimental goal remains to compartmentalize each cell type to assess their individual contribution to fetal membrane homeostasis and function, perhaps using existing multi-chambered devices as in the work by Sticker and colleagues.[84] Initially, we have experimentally focused the IFMOC on identifying the possible interactive roles of the choriodecidua in regulating amniotic epithelial barrier integrity in response to bacterial infections during CAM-induced PPROM.

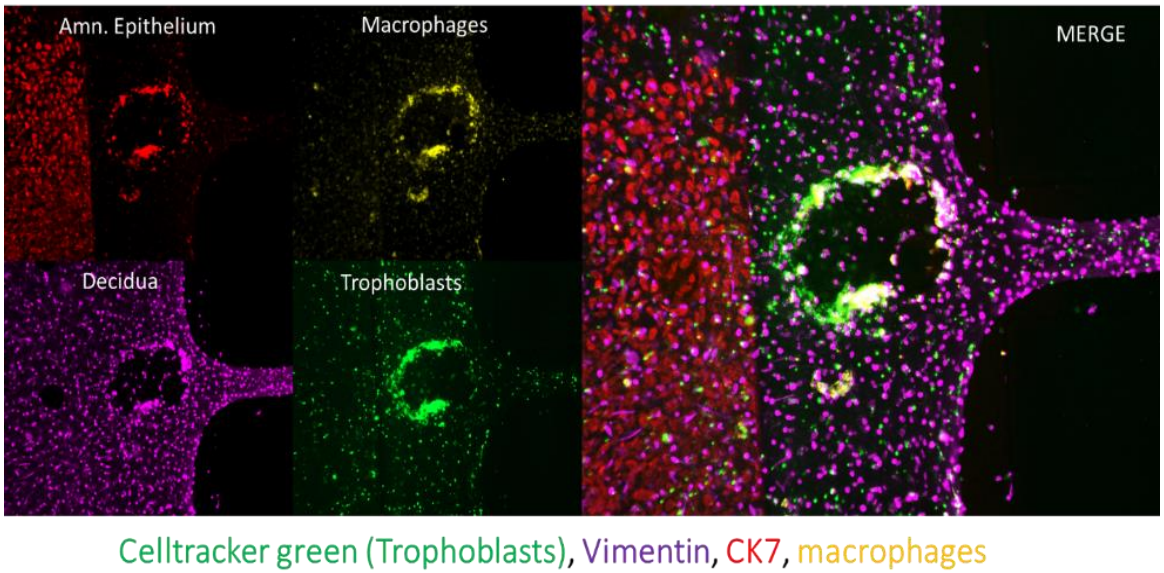


Figure 5-2. Development of a first generation IFMOC: Quad-culture. The idealized IFMOC should incorporate most, if not all, cell types and extracellular matrix that makes up the fetal membrane tissue in relative and physiological ratios. We have developed a co-, tri-, and quad-culture model that introduces the decidua stromal cell (vimentin, purple), a trophoblast cell line (actin green), primary amnion epithelium (CK7, red) and PMA-induced THP-1 macrophages (CD45, yellow). The colocalization demonstrates a viable 3D model for the fetal membrane and the establishment of an idealized IFMOC.

5.1.4 Additional gravid OoC models: the placenta

Preterm birth is a complicated disorder that can be mediated by a variety of inflammatory stressors within reproductive tissues. Although the fetal membrane plays a crucial role in successful, full-term birth, disruptions in placental function can also be an integral part of disease pathogenesis leading to PTB. The human placenta is an understudied organ that has long been recognized as playing a major pathophysiologic role in complications of pregnancy, including prematurity, infection, intrauterine growth restriction, (pre)eclampsia and gestational diabetes.[290–293] More recently, our research group and others have focused on the placenta based on this organ's potential role in the developmental origins of health and disease (DOHaD), a paradigm that relates early life exposures (including gestational health) to lifespan and disease risk in offspring.[294,295] The possibility that placental pathology could be a critical root mechanism for major causes of morbidity and mortality in children and adults,[296,297] including cardiovascular disease, obesity, diabetes, and neurocognitive problems, creates a new imperative for better defining normal placental structure and function relative to disease-related placental pathology. Knowledge of placental molecular and cellular biology lags behind that of other organs; however, this is not surprising, since it is not feasible to sample an individual human placenta at multiple time points throughout pregnancy. Thus, there is a need for new, experimental models of placental biology that can be applied to understanding both normal and disease states.

Recently, two research groups have published placenta OoC models.[298,299] The placental chip system reported by Lee, *et al.* was developed using a technique known as soft lithography, resulting in a microfluidic system made of two PDMS chambers separated

by a thin extracellular matrix membrane. An immortalized trophoblast cell line (JEG-3) combined with primary human umbilical vein endothelial cells (HUVEC) were used to represent two of the major non-immune cell types of the placenta in the Lee device. These cell-types were seeded onto the opposite sides of an extracellular matrix membrane and subsequently cultured under dynamic flow conditions. This design allowed confluent layers of trophoblasts and endothelial cells to exist in close apposition to mimic the human placental maternal-fetal interface.[299] Under conditions of flow, functional validation of this system was performed by measuring glucose transport across the trophoblast-endothelial interface over time. The permeability of the barrier was analyzed and compared to that obtained from acellular devices and additional control groups comprised of either epithelial or endothelial layers alone.[299] The OoC model developed by Blundell *et al*., was very similar to the design of Lee's group, utilizing a two-chamber PDMS device generated via soft lithography and populated with a primary vascular cell type (human primary placental villous endothelial cells (HPVECs)) and a placental choriocarcinoma epithelial cells (BeWo, ATCC), separated by a porous membrane coated with extracellular matrix components.[298] As in the Lee study[299], the Blundell study also demonstrated functionality of the device in terms of permeability, hormone production and nutrient transport.[298] Both models of the trophoblast-vascular interface represent important advances in microscale modeling of the human placenta. However, a limitation of these OoC models of the placenta is their over-simplified cellular community structure, lacking immune cells and other relevant non-immune cells such as maternal-derived decidualized stromal cells. Thus, at this juncture, these placental models only represent the initial steps needed in order to fully represent the more robust models that will be needed to fully represent this important,

and transient reproductive tissue. Going forward, it is anticipated that new generation of placental OoCs will further advance our understanding of maternal-fetal nutrient and waste transport, drug toxicity, immunology/tolerance, and the pathogenesis of infectious diseases as well as other complications of reproduction. For example, in a more advanced model, Sticker *et al.,* developed a multi-chambered microfluidic device that further enhances the development of complex *in vitro* cell cultures for placental research. These advanced models will be essential for developing a culture system that provides the opportunity for individual assessment of each relevant placental cell type.[300,301] Professor Peter Erlt's group fabricated a 4-chamber device using a photosensitive thermoset (OSTEMER 322-40) as a porous membrane and used it to establish a compartmentalized tri-culture of human umbilical vein endothelial cells (HUVEC), BeWo cells, and adipose tissue-derived human mesenchymal stem cells (adMSCs).[300] In addition to these placenta-based models, the endocervix model has been developed that can undergo the hormone-induced events during an idealized 28-day menstrual cycle.[302] Altogether, these organs-on-chips offer an opportunity to enhance the multicellular *in vitro* models of complex tissues for pre-term birth research, including the placenta and fetal membranes, and demonstrates the increasing complexity of *in vitro* tissue modeling. Altogether, these models represent the current driving interest among many laboratories in the development of OoCs of the gravid uterus, as an on-going objective, to better understand maternal-fetal interactions.

5.2 Limitations

Microfluidic and Organs-on-Chip technologies are an innovative method of modeling *in vivo* organ phenotypes that has the potential to revolutionize the way we

conduct basic translational science. However, it is important to note that like all experimental models, OoCs do have conceptual and technical limitations. These models cannot fully reproduce all biological and physiological processes that occur *in vivo* (*i.e*. an EndoChip cannot become pregnant). However, OoC models do represent important phenotypic models to experimentally test biological questions using human cells. In our first-generation models, we focused on specific cell types that play critical roles in both physiological and pathological processes of reproductive function. However, additional or alternative cell types, such as specific immune cells or sub-populations of somatic cells or stem cells, can be incorporated within our existing platform to characterize their potentially unique contributions. Currently, one important technical limitation involves the chemical properties of PDMS, the prototypical material from which many of these devices are fabricated, including our first generation OoCs. Although PDMS remains the gold standard for microfabrication due to its biocompatible and air permeable properties, its hydrophobic nature is prone to absorb lipophilic molecules.[92,268,314,315] As briefly discussed in this chapter, this limitation may hinder our current ability to examine detailed pharmacokinetic/pharmacodynamic (PK/PD) analysis of certain drugs, toxicants or molecules. Lastly, the design our microfluidic device primarily investigates how the crosstalk among cells mediates reproductive function via paracrine mechanisms; however, we must also consider that cell-cell contact, and cell-ECM play critical roles in mediating cell and tissue homeostasis. Specifically, the EndoChip is not immediately intended to replicate the gross changes occurring in the endometrium including tissue growth and menstrual shedding, but rather provides a model of the cell-cell communication that is conducive for normal physiological function.[316–318] Different models may be required to

accurately model vascular events such as angiogenic tubule formation, perhaps including vascular smooth muscle cells (VSMC) that may impact endothelial function. Furthermore, in this thesis, we abstained from introducing the epithelial component of the microenvironment to establish a novel model focused on the uterine vascular bed. However, working with collaborators, the next steps involved will be to utilize 3D hydrogels[38] in order to incorporate and maintain viable polarized epithelial glands to generate a complete EndoChip, as described below. We have had some preliminary success in generating this more complete OoC model, but these efforts are outside the scope of this thesis (Supplementary Figure 5-9, unpublished data). While current iterations of our EndoChip models may not be able to replicate all physiologic conditions that are found *in vivo*, OoCs remain an innovative, emerging technology that promise to revolutionize future human *in vitro* studies by generating a more comprehensive experimental model of tissue-level homeostasis and disease pathogenesis. Thus, while it is unlikely that OoC models will ever completely replace *in vivo* animal models, OoCs serve as complimentary translational tools that will likely enhance both basic science and promote the preclinical identification of the most effective therapeutics that are constantly emerging from the drug-development pipeline. Moving forward, we foresee and expect that the continuously evolving Vanderbilt OoC models, described above, will expand our understanding of physiological and pathological human conditions in reproductive health. As models develop, and emerging technologies become more readily available, the design complexity and elegance of analytical capability of these microfluidic models will also continue to expand.

5.3 Future directions for EndoChip models of the human endometrium

5.3.1 Incorporation of innovative analytical tools to the EndoChip

Currently, numerous complementary tools and platforms are being developed to support and enhance analytical capability of the OoC models. These technologies include embedded ceramic-based electrodes to continuously analyze real-time changes of key metrics such as electrical impedance as a measurement of transendothelial electrical resistance (TEER), and measurements of cell metabolic processes.[81,303–305] Moreover, incorporation of biosensors for reporter genes will be essential to promote real-time, non-invasive analysis rather than end-point measurements. Transfected cell-based biosensors are being developed for specific genes that would be very applicable to the studies described in this thesis. Some of these targeted reporter genes include, but are not limited to, aryl hydrocarbon receptor (AhR), caspase-3, and the progesterone receptor (PR). These biosensors will allow accurate measurement of important aspects of TCDD-associated toxicity, such as cellular apoptosis and sex hormone signaling, respectively. Additionally, with the advancements in high content imaging, we expect significant improvements in the analytic capability for data acquisition within these OoCs. Sample measurements using a combination of multiplexing and proteomic analysis via mass spectrometry can provide unsupervised analysis of protein network systems. These improvements in the analytic capability of reproductive tract OoCs are underway both at Vanderbilt at other universities. Altogether, we expect that as these improvements become embedded into the second generations of OoC models and technological advances promote reduction in costs, we will see a comprehensive integration of these models into the basic science benchtop.

5.3.2 Collaborative work for modeling the endometrium

As mentioned in Chapter 2, the development of an endometrium microfluidic model capable of dissecting the molecular changes that occur during a menstrual cycle has been an initiative taken by several distinct research teams.[92,122,123] To accomplish these mutual goals, we have been fortunate to develop a continuous dialogue with Dr. Linda Griffith's group at MIT and establish a collaborative effort with the mutual goal of generating a model to investigate endometriosis. Specifically, Dr. Griffith's group have generated a 3D model of the endometrium using a PEG synthetic hydrogel that is specifically formulated to support endometrial cell growth and differentiation.[122] They have demonstrated that they can maintain a hydrogel culture for the idealized length of a menstrual cycle and measure endocrine mediated molecular networks including progestational molecules, cytokines and matrix metalloproteinases. As part of the collaborative effort, the Griffith and Osteen laboratories have maintained an open exchange of these technologies. We have tested their synthetic hydrogel in the Osteen laboratory and have observed successful recapitulation of their results (Figure 5-3). Furthermore, we applied their hydrogel *in vivo* as a substrate for endometrial xenografts and observed long-term survival of the hydrogel, cellular viability and even the formation of functional vascular structures within the hydrogel (Supplementary Figure 5-8, unpublished data). These preliminary findings suggest that the PEG hydrogel is an adequate material for long-term endometrial studies and can be applied for both *in vitro*, and *in vivo* endometrial applications. Furthermore, we are utilizing this synthetic gel within our microfluidic model of the perivascular stroma as a durable hydrogel to incorporate the 3D epithelial glands into the EndoChip (Supplementary Figure 5-9, unpublished data). Current biologic hydrogels, such as collagen type I and Matrigel,

cannot be maintained for long-term cultures (>28 days) and analysis can be confounded by additional factors within the Matrigel matrix such as growth hormones, that can introduce variability on a batch-to-batch basis. A key advantage to the MIT system is two-fold; first, it provides an alternative to PDMS-based models that suffer from absorption issues, and second, the MIT system allows the integration of additional organ models up-stream and down-stream of the endometrium. This platform is continuously perfused via recirculation through controlled pneumatic pumps to other organs including the gut, liver, brain and others. Other groups have designed a similar platform that specifically couples the organs and tissues that make the female reproductive tract.[124] Therefore, a durable and strictly defined synthetic hydrogel provides a compelling alternative for endometrial research and may be applicable as the ECM component in the next generation of the EndoChip.

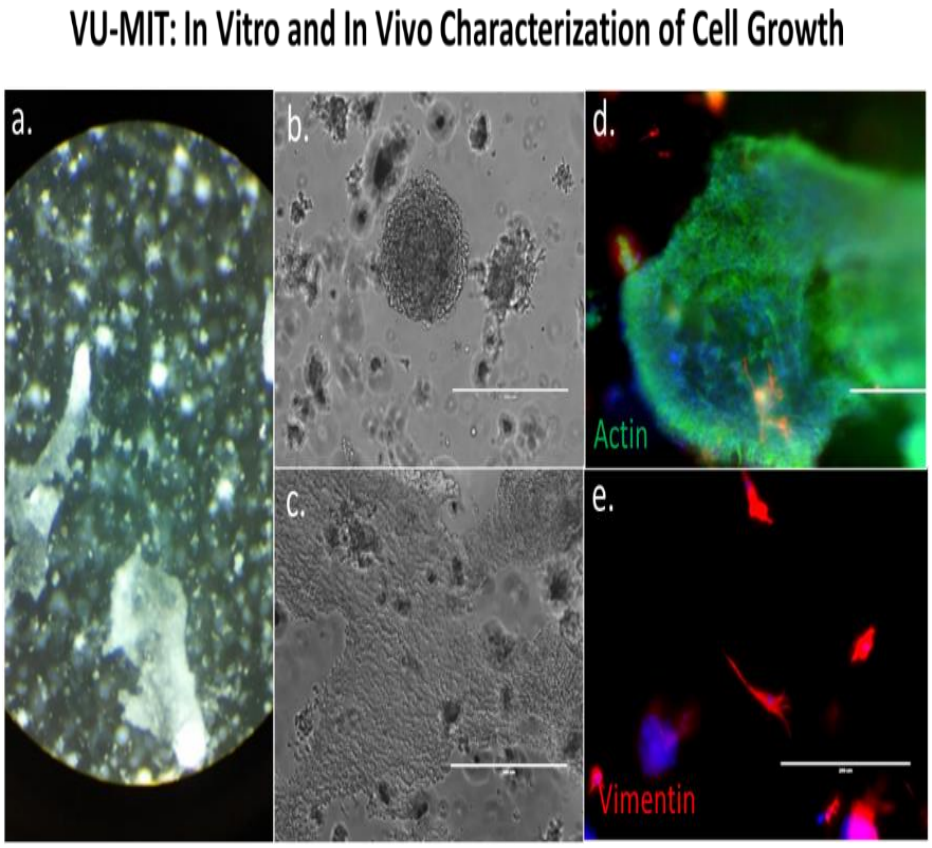


Figure 5-3. The application of synthetic PEG hydrogels in endometrial research. In a collaboration with the Griffith group at MIT, we performed pilot experiments to validate their technologies to model the endometrium in a xenograft model in a murine model. (a) Gross images of the cell growth of epithelial glands and stromal fibroblasts after 28 days with 14 days of E2 only and 14 days of E2 + P4. These cells were grown intraperitoneally in a immunodeficient mouse. (b-c) Bright field images of the tissue growth. (d-e) Immunofluorescence of fixed hydrogels with cells. Selective staining for vimentin (red) and actin (green) shows 3D morphologic viability and formation of epithelial glands.

5.4 Impact and applications of the reproductive OoCs on biomedical research

5.4.1 Applications in personalized medicine

Clinically, current treatment options for many reproductive diseases, such as endometriosis consists of hormonal therapies that reduce the impact of E2 by the administration of synthetic progestins.[269] Unfortunately, most endometriosis patients present resistance to these medical treatments and therefore rely on surgical excision of ectopic lesions. Recurrence of endometriosis is common, and many patients often resort to total removal of the uterus.[142] Our limited understanding of the basic etiology of this disease and hindered identification of better therapeutics has been driven by the lack of physiological pre-clinical models that recapitulate the human endometrial microenvironment. In this thesis, we have demonstrated a pre-clinical model of the endometrium primarily focusing on the normal physiologic function. However, these models offer the potential to provide clinical insight into the pathogenesis of numerous reproductive disorders by mirroring specific disease conditions. To generate a disease model of endometrial disorders, we can utilize the microfluidic device using primary cells acquired from patient populations.

Traditionally, to reduce the burden of intra-sample variability associated with the experimental use of primary human cells, commercially available cell lines are often used in traditional basic science research. However, substantial problems have recently been identified related to the use of certain cell lines. Some major issues include common misclassification of cells or contamination with the incorrect cell type. These cell line issues are also evident in cells often utilized in the reproductive field, as exemplified by

endometrial cell lines such as an endometrial epithelial cell line (*i.e.* HES) which actually identified to primarily be HeLa cells, a cervical cancer cell line.[309] Additionally, Ishikawa epithelial cells line are reproducibly used for reproductive science as control cells; however, it is the origin of this cell line is derived from an endometrial adenocarcinoma and, as such, exhibits an intrinsically altered cellular programing. Issues of altered cell identity, can lead to unreliable results, and hinder the discovery of effective and reproducible treatments for reproductive diseases.[310] Furthermore, tert-immortalized endometrial stromal fibroblasts (tHESC) have been experimentally utilized as a key experimental cellular component of the endometrial microenvironment.[311] However, we and others have identified a reduced physiological sensitivity of these cells to endocrine signaling, specifically P4, as a result from reduced PR levels in this cell line.[20] From our experience, we observed that tHESC are able to undergo decidualization, however they require the supplementation with exogenous cAMP (8-Br-cAMP) in addition to P4. This artificial induction of decidualization bypasses much of the P4-mediated circuitry within these cells. In comparison, primary cells derived from control endometrial donors offer increased sensitivity to P4 and can decidualize, even in the absence of exogenous cAMP. Thus, utilizing primary cells obtained from these control tissue donors or patients can be implemented to a distinct advantage within OoC models to generate specific disease models with improved fidelity to human biology.

Compared to other human organs and tissues, the endometrium provides unique opportunities to harvest whole tissue or primary cells using minimally invasive biopsy methods.[62] Throughout this thesis, we have generated a biobank of primary endometrial cells isolated from either surgical hysterectomies or endometrial biopsy donors that can be

frozen and readily used for cell culture. As we move into more comprehensive disease models and hypothesis-driven applications of these models, we can employ isolated monocytes, epithelial, and stromal cells from endometriosis donors that we have collected to begin addressing their immunological contribution to the disease processes that drive the development and progression of diseases such as endometriosis. Similarly, fetal membrane OoCs could also be designed to model either healthy maternal-fetal communication or disease-associated alternations that lead to abnormal pregnancies. Primary cells could be utilized, for example, from fetal membranes or endometrial tissues obtained from preterm labors or other patient groups at known risk for PTB, respectively and used to study specific disease pathogenesis.[15] Using primary cells acquired from tissue donors can better support translational research related to both basic research and personalized medicine.

As our ability to incorporate primary cells representing various diseases into OoCs grows, this emerging technology will support both precision and personalized medicine initiatives. By recapitulating patient specific endometrial tissues *in vitro,* we may imagine a world where an individual patient's cells can be readily incorporated into a personalized OoC to examine whether their disease-altered cells would react favorably to an available therapeutic agent. Furthermore, induced pluripotent stem cells (iPSCs) could be obtained and differentiated into tissue specific cell types for use in an OoC.[82] Genetic engineering tools, such as CRISPR/Cas9, can be harnessed in the future to manipulate the contribution of specific genes to reproductive biology. Thus, an effective OoC model might be modified to use diverse cell types with specific gene editing in order to gain a deeper insight into disease pathogenesis. Similarly, the same concept can be utilized to identify the negative impact of environmental toxicants on individual women or on specific populations.

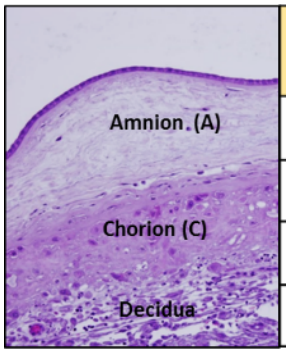
5.4.2 Applications for predictive toxicology

Our laboratory and others have demonstrated in animal models that environmental toxicants may cause disruptions of immune-endocrine pathways during infection-related processes that contribute to both infertility and poor pregnancy outcomes, although, human epidemiological data has been less conclusive.[210,280,293,312,313] Thus, it is important to develop better models using human cells to investigate gene-environment interactions of various toxicants (*e.g.* dioxins) and the mechanisms by which these endocrine disrupting chemicals alter the individual and systemic components of the microenvironment of the endometrium.[206] Clearly, intentional exposure of reproductive age women to specific toxicants such as TCDD or mixtures of other EDCs is neither ethical nor feasible. Animals models have shown that early life exposures to environmental toxicants, including the potent endocrine disputing chemical TCDD, can alter inflammatory processes in adult animals.[26,210,228] However, the cellular mechanisms behind this phenomenon remain elusive. In chapter 4, we provided an example of the toxicant-mediated vascular dysfunction in our model of the endometrial perivascular stroma. Specifically, we introduced the applications of microfluidic organotypic model systems for toxicity testing using TCDD as a test compound to recapitulate our previous murine and human findings. However, OoC models are expected to have an even greater impact on drug discovery, toxicant screening, and assessment of efficacy or safety.[81] The ability to perform precise pharmacologic studies and interconnect different human organs (*e.g.* liver) in serial will provide a strong impact on adverse outcome pathways. A major advantage of OoCs is the microfluidic design also offers the possibility to interconnect different OoCs in tandem to order to mimic the systemic communication between organs. As an example, we are

currently coupling the EndoChip or IFMOC downstream of a liver organotypic model (*e.g.*, liver-on-a-chip) to identify a robust physiologic toxicant response by modelling how xenobiotics pass through the liver and become metabolically active prior to reaching the target organ. Moreover, we can begin to assess the role of sex hormone signaling, and likewise, the distal activation of immune cells within a circulatory system. These serial interactions between organs may continue to enhance the complexity of *in vitro* systems and provide innovative modeling avenues for translational research that extends to systemic toxicological research.[81] As mentioned, this approach is being pursued by several groups at Vanderbilt and elsewhere. Thus, the scientific community is beginning to recognize the utility of OoCs as phenotypic screening tools for revealing the negative impact of reproductive toxicants.

In turn, these devices equally support multiple aspects of medical interventions in reproductive health, including contraceptives development. Going forward, these microfluidic OoC models will continue to serve as a platform to test and screen therapeutic agents in order to identify high efficacy candidates prior to testing in animal models, [81] prehaps eventually eliminating the need for animal testing.

5.5 Appendix



Fetal membrane cell types		Cell density (cells/µm²)	Tissue thickness (µm)	Tissue composition[a] (%)
Amniotic epithelium		7.3E-03 ± 4.2E-04	0.099 ± 0.124*	0.6%
Chorion trophoblasts		3.96E-03 ± 4.02E-04	129.66 ± 4.53	45%
Immune cells (e.g. Mϕ)		1.65E-04 ± 1.43E-05	951 ± 83.6¥	13 %
Decidual cells		1.48E-03 ± 8.5E-05	318.04 ± 54.59	41%

Cells/µm, [a]Approximation, ¥Total FM thickness
Note: Mesenchymal fibroblasts are not included

Table 5-1. Characterization of the cellular composition of the human fetal membrane microenvironment. Breakdown of the tissue composition of the fetal membrane and the relative cell density, gross tissue thickness and percent composition. Immune cells are specifically outlining the estimated number of resident macrophages ($CD68^+$).

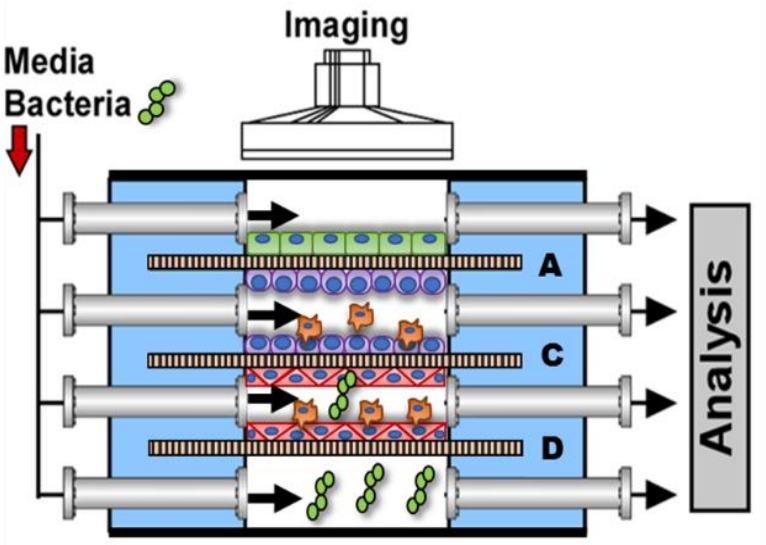


Figure 5-4. An idealized IFMOC. The idealized design of an instrumented fetal membrane on a chip (IFMOC). Four chambers compartmentalize each cell type and real-time measurements of ascending bacterial infection can be assessed. A= amnion, C=Chorion, D=Decidua.

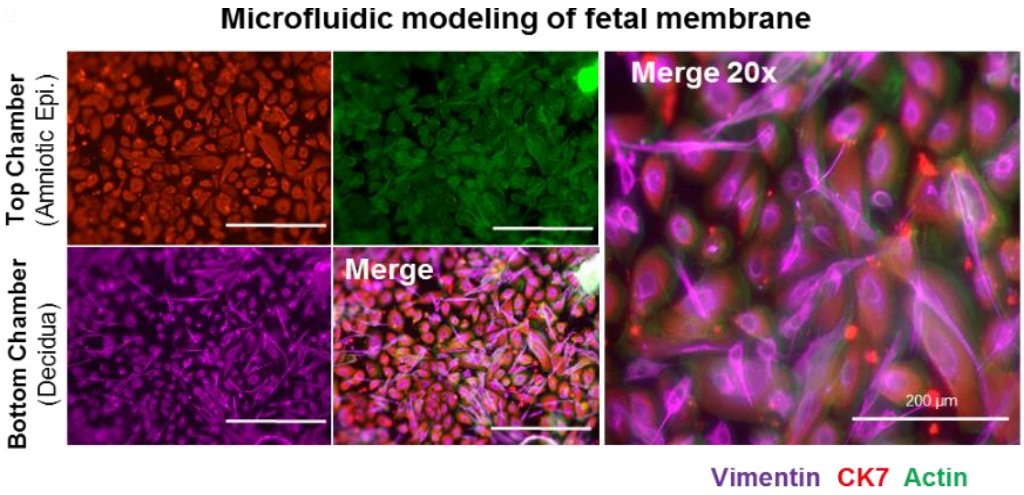


Figure 5-5. Development of a first generation IFMOC: Co-culture. Using the dual chamber device, we co-cultured and characterized the growth and confluence of amnion epithelium and the maternal decidua. This co-culture is the simplest compartmentalized model for investigative toxicology. Distinct layers form with each cell types.

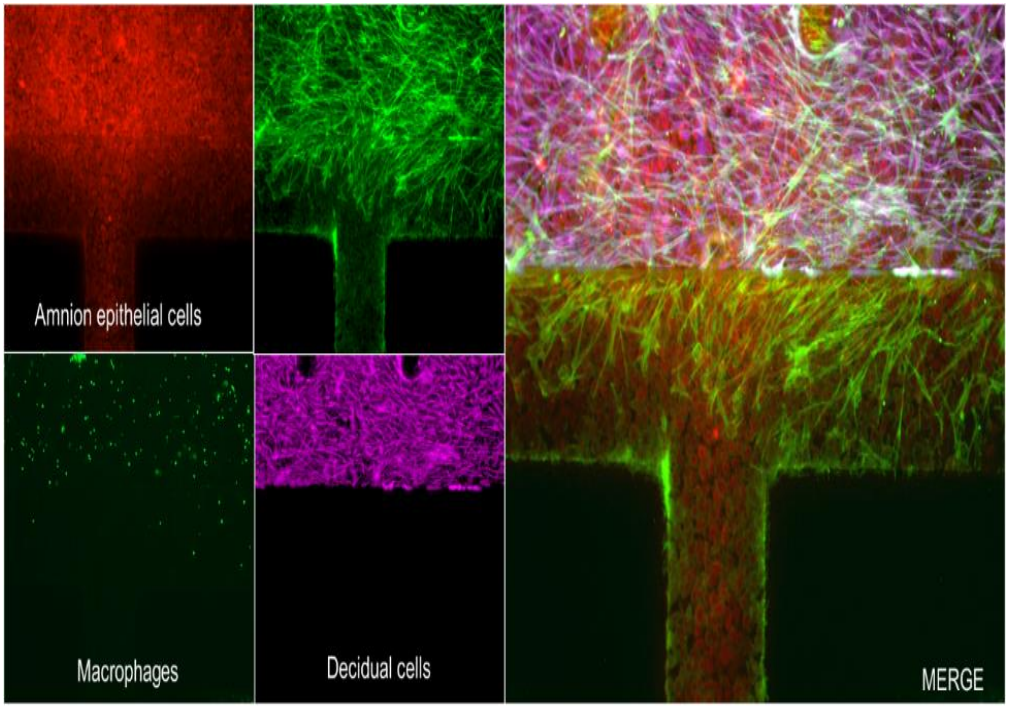
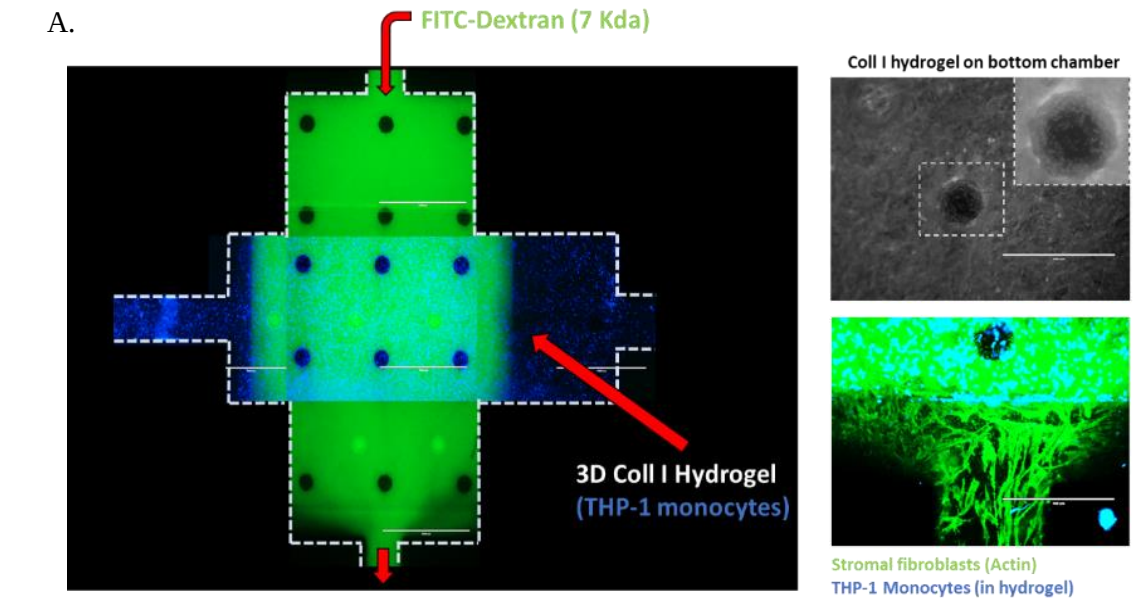


Figure 5-6. Development of a first generation IFMOC: Co-culture: Tri-culture. We adapted the devices shown in figure 5-5 and introduced an immune component to the co-culture. Specifically, we utilized a collagen I hydrogel in the bottom (Chorio/decidua chamber) to introduce the 3D structure of the decidua and embedded macrophages that were labelled with CellTracker Green.



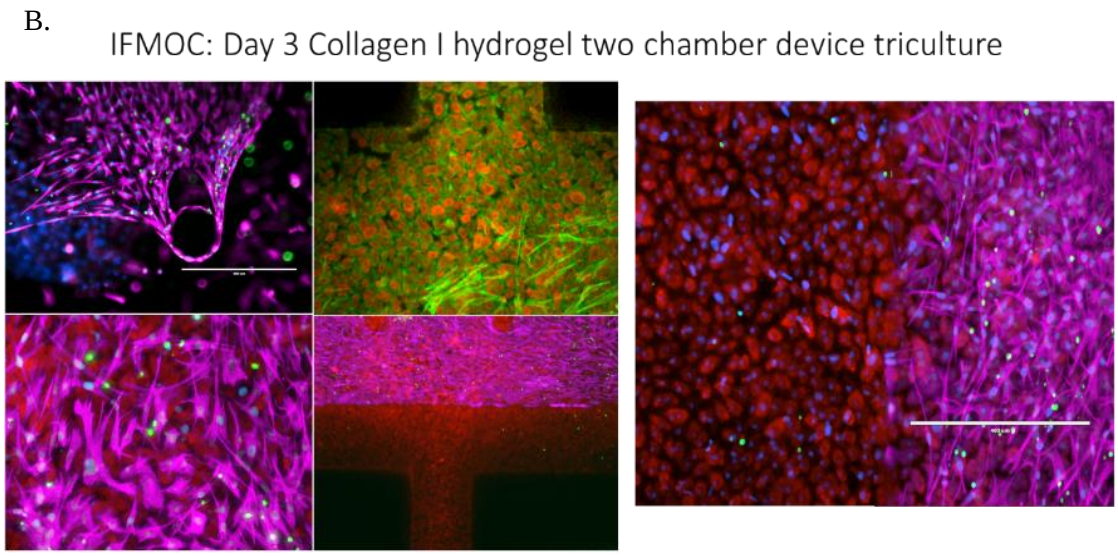


Figure 5-7. Applications of collagen I hydrogels for 3D modeling in a compartmentalized microfluidic model. (a) Collagen I hydrogel (10ng/mL) embedded with cells (decidua and macrophages) were seeded in the bottom chamber of the dual chamber devices. We observed an even distribution of hydrogel within the chamber and a distinct compartmentalization without leakage into the ulterior chamber. FITC-labelled dextran demonstrates the separation between chambers and diffusion in the hydrogel. (b) Characterization of cell viability within the hydrogel in the microfluidic devices in a tri-culture. Distinct separation between cells and selective staining demonstrates that hydrogels can be an effective way to seed cells in a 3D microenvironment and mirror the extracellular matrix within a tissue.

A. B.

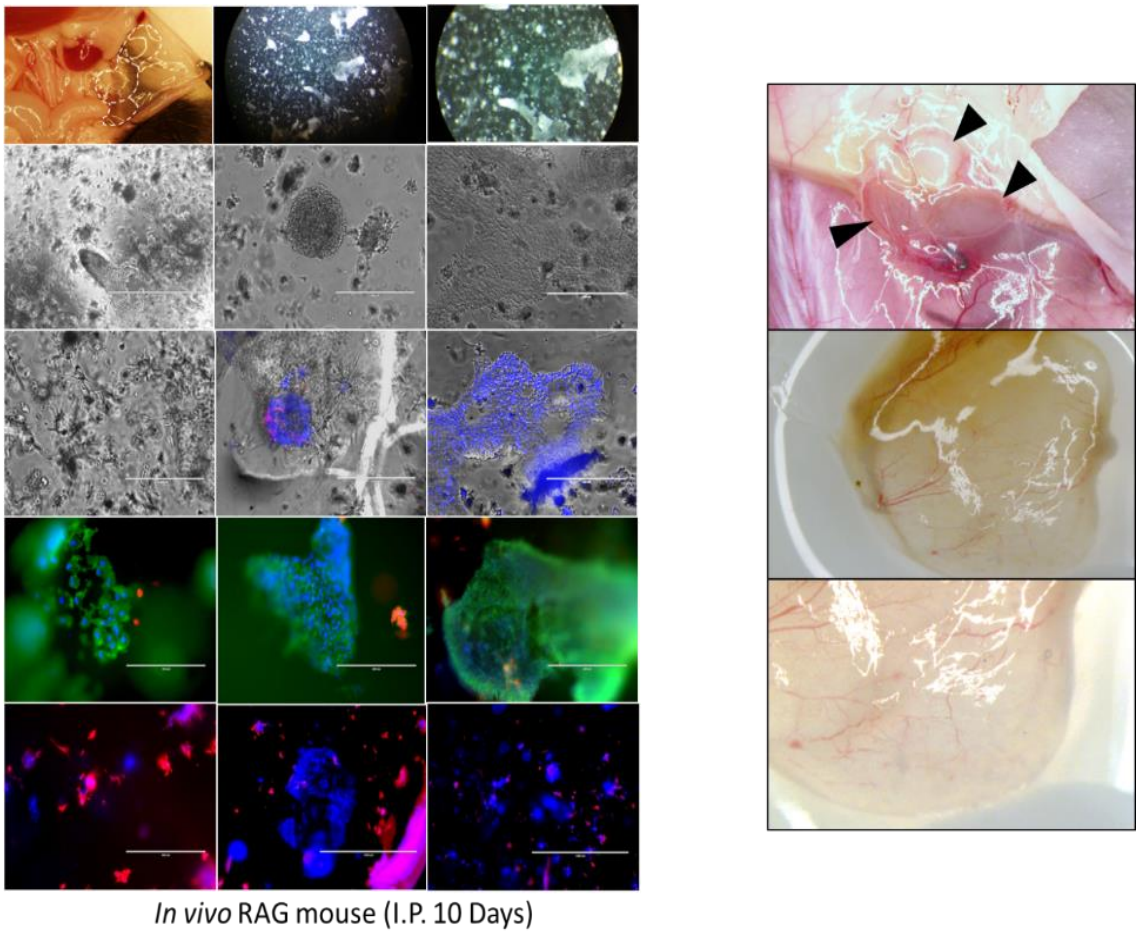


Figure 5-8. Pilot experiment with MIT's "Magic Matrix" synthetic PEG hydrogel for endometrial tissue modeling in an *in vivo* environment. Primary endometrial cells were co-cultured in the PEG hydrogel (12µL dollop) and introduced in an intraperitoneal surgery (a) and subcutaneously (b) in an immunodeficient murine model. The gels were cultured for 28 days following the hormonal changes of an idealized menstrual cycle (2 weeks of E2 and 2 weeks of E2 and P4). Gels maintained rigidity during this time in both I.P. and subcutaneous conditions. Furthermore, the cells were characterized for growth of stromal and epithelial components (a). (Actin green, vimentin red, DAPI blue, and bright field). (b) Subcutaneous growth of the hydrogel underwent angiogenesis that embedded into the hydrogel (gross imaging).

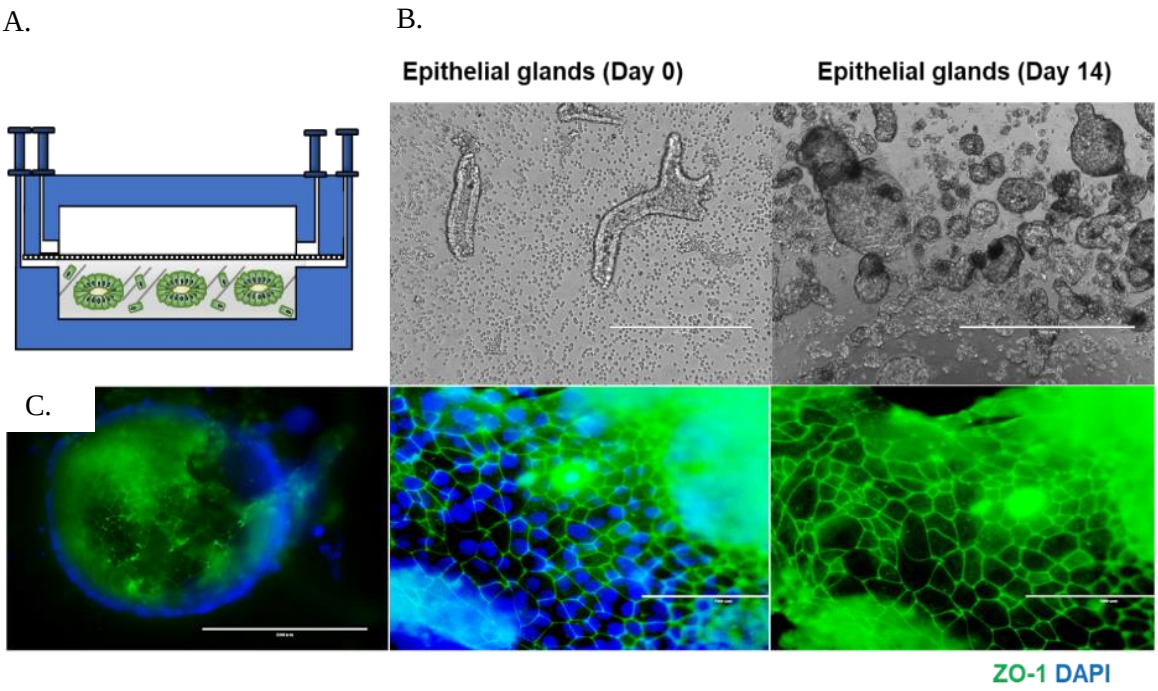


Figure 5-9. Introduction of primary epithelial glands into the EndoChip generates 3D glandular structures. (a) Schematic of the applications of hydrogels in the "somatic" compartment of the first generation EndoChip. The hydrogel will promote epithelial growth and polarization to maintain a physiological growth condition. (b) Collagen type I 3D hydrogel and epithelial glands inside the EndoChip. Epithelial glands (day 0) are directly isolated or frozen at passage 0. After 14 days of culture, the glands begin to develop round organoids. (c-e) Characterization of tight junction formation (ZO-1, green) and morphology of epithelial cells after 14 days of culture in EndoChip shows 3D architecture and tight junction formation. The protocols used were adapted from Eritja et al. 2010 using a 3% solution of liquid Matrigel.

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