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Progesterone Signaling in the Endothelium:

The Role of Progesterone Receptor in Physiological Vascular Permeability and Leukocyte Trafficking

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cell, and Developmental Biology

by

Lauren Marie Goddard

2013

ABSTRACT OF THE DISSERTATION

Progesterone Signaling in the Endothelium:

The Role of Progesterone Receptor in Physiological

Vascular Permeability and Leukocyte Trafficking

by

Lauren Marie Goddard

Doctor of Philosophy in Molecular, Cell, and Developmental Biology

University of California, Los Angeles, 2013

Professor Luisa Iruela-Arispe, Chair

Progesterone is essential for all reproductive processes including cycling, embryo implantation and maintenance of pregnancy. The complex endometrial changes that occur in preparation for embryo implantation and subsequent gestation place unique functional demands on the uterine vasculature including the need for increased permeability and selective leukocyte transmigration. While these changes are assumed to be under progesterone control, the exact cellular and molecular regulation remains largely unknown. Here we identify endothelial progesterone receptor (PR) as a direct mediator of the vascular responses downstream of progesterone. Restricted expression of PR in the endothelium of veins and lymphatics ensures vessel-selectivity to

progesterone signaling, and correlates with its biological function in the endometrial vasculature. Using both genetic ablation and overexpression models combined with physiological experimentation of PR in the vasculature, we were able to demonstrate that cell autonomous PR signaling in the endothelium mediates the vascular permeability response that precedes implantation. Mechanistically, we found that PR induces a NR4A1 (Nur77/TR3)-dependent transcriptional program that broadly regulates localized and sustained vascular permeability in response to progesterone.

We also found that conditional deletion of PR from the endothelial compartment results in selective trafficking of macrophages and neutrophils into the uterus. Using unbiased transcriptome analysis in combination with a read-out of PR binding sites, we determined that PR directly downregulates the expression of the endothelial-leukocyte adhesion molecules VCAM-1 and E-selectin, as well as a select group of cytokines/chemokines including, IL-6, IL-8, CXCL2/3, and CXCL1. Thus endothelial PR enables the discrimination of leukocyte subpoulations through selective expression of molecules important for leukocyte transmigration. Together, this work has identified novel functions and molecular targets of PR In the vasculature, with a number of important implications to our current assessment of the physiological and therapeutic effects of progesterone.

The dissertation of Lauren Marie Goddard is approved.

Amander Clark

Hannah Mikkola

Peter Tontonoz

Luisa Iruela-Arispe, Committee Chair

University of California, Los Angeles

2013

DEDICATION

This dissertation is dedicated to my grandma, Anita Yelk.

Although no longer here, her strong will, resilience,

and strength always remind me that there is nothing I cannot accomplish.

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ACRONYMS

PR: Progesterone Receptor

PRE: Progesterone responsive element

PR(A/B)KO: Progesterone receptor (A/B) knock out

Cav-1: Caveolin-1

CREB: cAMP response element binding

eNOS: endothelial nitric oxide synthase

FAK: Focal adhesion kinase

FGF: Fibroblast growth factor

JAM: Junctional adhesion molecule

KLF: Krüppel-like factor

LPS: Lipopolysaccharide

NO: Nitric oxide

PDGF(RB): Platelet derived growth factor (receptor-B)

PKA/C: Protein kinase A/C

S1P: sphingosine-1-phosphate

VEGF(R2): Vascular endothelial growth factor (receptor 2)

VE-PTP: Vascular endothelial-protein tyrosine phosphatase

VVO: vesiculo-vacuolar organelle

PMN: polymorphonuclear cell

HRT: hormone replacement therapy

CVD: cardiovascular disease

WHI: Women's Health Initiative

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge my thesis advisor and mentor Dr. Luisa Iruela-Arispe. Luisa's passion for science is contagious. No matter how frustrated I was with my results or science in general, I always left her office feeling motivated and enthusiastic to move forward on my project. I truly appreciate the countless hours she has spent on my development as a scientist including providing technical advice, career guidance, and experimental support as well as the editing of all of my work. I am most thankful for her confidence in me, and although tough at times, she has challenged and pushed me more than any other person to accomplish things I never thought I had the ability to do. I could have not become the scientist and person I am today without her.

I would also like to thank the members of my doctoral committee: Dr. Peter Tontonoz, Dr. Hanna Mikkola, and Dr. Amander Clark for their valuable guidance, insight, and support. In particular, I would like to thank Dr. Tontonoz for providing very valuable reagents and mice. In addition, I would like to thank Dr. Clark for her support and mentorship during the last year of my graduate career as well as Dr. Mikkola for her knowledge and assistance of global RNA- and ChIP-sequencing.

I would like to thank the past and present members of the Arispe lab for their technical skills and advice throughout my graduate work. These include Jennifer Hofmann, Ann Zovein, Toni Torres-Collado, Ana Rivas, Saffiyah Ziyad, Anais Briot, Stephanie Lau, Tom Chen, Kirsten Turlo, Lauren Sanchez, Josephine Enciso, Carmen Warren, Huanhuan (Mahsa) He, Julia Mack, Onika Noel, Courtney Domigan, and my undergraduate students Amy Ton and Maryanne Ibrahim. I would also like to thank Liman Zhao and Michelle Steel for animal husbandry. In addition, a great thanks goes to

the UCLA Tissue Preparation Core Laboratory for their excellence in histological sectioning and staining.

I want to especially acknowledge Dr. Josephine Enciso who I have worked so closely with over the last 5 years. Josephine has been an integral part of many of the projects presented in this thesis, but has also become a close friend. Additionally, I want to thank my undergraduate Amy Ton, who has helped immensely on these projects by making reagents, running experiments, and editing a lot of my writing. Helping Amy become a scientist has been one of the most rewarding experiences of my graduate career and taught me a lot about my own skills as a teacher and mentor as well as helped me to become a better scientist. I would like to also acknowledge my good friends Jennifer Hofmann, Courtney Domigan, Michaela Patterson, Onika Noel, and Ben Van Handel who have all been a strong support system, great listeners, and most importantly kept me sane both inside and out of the lab.

I would like to thank my friends and family for their continued support and love. My parents, Alvin and Susan Goddard, are always there when I need them. They have become amazing listeners and have supported and encouraged me through every step of this process. My brother Matt, for always reminding me to have fun and to never be afraid to try new things, meet new people, and take advantage of every opportunity that comes my way. My family away from home, my Aunt Ellen, Uncle David and my three “sisters” Julia, Catherine, and Tricia, for always making me feel welcome and apart of their family. I would not have made it this far without any of you.

Lastly, I would like to acknowledge the people who contributed to the experiments and the work contained within this dissertation. Chapter 2 is version

Goddard LM, Iruela-Arispe ML. Cellular and Molecular Regulation of Vascular Permeability. Thrombosis and Haemostasis. (2013) 109: 407-414. Permission to reprint this review was granted by Schattauer GmbH (Copyright Permission No 72/42013). I would like to thank Courtney Domigan and Dr. Luisa Iruela-Arispe for critical reading of the manuscript.

Chapter 3 is a version of the manuscript entitled Physiological Vascular Permeability Requires Induction of NR4A1 by Progesterone Receptor, which is currently under review at Cell. The PRLacZ mouse used in this paper was generated by Dr. Lauren Sanchez, Dr. Minako Hashimoto-Partyka and Nancy Allen. I would also like to acknowledge Dr. Tonis Org for his guidance in ChIP-sequencing and his analysis of our ChIP-seq and RNA-seq datasets. In addition, a great thanks goes to Dr. Tom Murphy who initially determined that progesterone regulates vascular permeability through the examination of PRKO mice (Figure 3.1). Dr. Lily Chao from Dr. Tontonoz's lab was also instrumental in reagent generation and gifted many important Nur77 constructs and mice used in this work. I would lastly like to thank Carmen Warren for assistance with the Evans Blue experiments, the UCLA Vector Core for generation of viral constructs, and the contribution of the Tissue Procurement Core Laboratory Shared Resource.

Chapter 4 was completed in collaboration with Drs Josephine Enciso and Tonis Org, and my undergraduate Amy Ton. Dr. Enciso and myself ran all of the flow cytometry experiments together on both the PRKO and PR^{ECKO} mice. These were intense and time-consuming experiments that could not be completed without her help. Amy Ton assisted in genotyping of the animals and ran most of the qPCR and Westerns that validated the targets from the RNA-sequencing data. Dr. Tonis Org also contributed

by analyzing the RNA- and ChIP-sequencing data. Mouse models used in this study were generated by Dr. Minako Hashimoto-Partyka.

Chapter 5 is a version of the manuscript, Selective suppression of endothelial cytokine production by progesterone receptor, which is under review at Vascular Pharmacology. This work was completed with the help of Dr. Tonis Org and Amy Ton. Amy validated all of the cytokine targets using qPCR and assisted in the flow experiments (Figures 5.4-5.6). Dr. Tonis Org analyzed the RNA- and ChIP-sequencing data, and along with Dr. Hanna Mikkola, thoroughly reviewed the manuscript.

All of the research contained in this thesis was supported by the Vascular Biology training grant (Ruth L. Kirschstein National Research Service Award T32HL69766) from 2008-2011, the American Heart Association Predoctoral Fellowship (AHA 11PRE7300043) from 2011-2013, and the Iris Cantor Women's Health Center pilot funding from 2012-2013

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PUBLICATIONS AND PRESENTATIONS

Goddard LM, Enciso JM, Sanchez LA, Iruela-Arispe LM. The Role of Progesterone Receptor in Vascular Smooth Muscle Cells. 91st International Endocrinology Conference. June 2009, Washington D.C. Abstracts on CD

Goddard LM, Enciso JM, Sanchez LA, Champion C, Kelly KA, Iruela-Arispe LM. The role of progesterone receptor in the inflammatory response in vivo. MCDB Retreat Oct. 2009, Lake Arrowhead, CA

Goddard LM, Enciso JM, Hashimoto-Partyka M, Sanchez LA, Allen N, Ton A, Iruela-Arispe LM. Progesterone receptor is expressed on murine uterine endothelial cells and may function to regulate leukocyte trafficking in aged mice. International Vascular Biology Meeting UCLA June 2010 at Los Angeles, CA

Lauren M. Goddard. Progesterone Signaling in the Vascular Endothelium Regulates Leukocyte Trafficking in the Uterus. Natural Sciences Seminar Series Feb. 2011 at Pepperdine University, Malibu, CA

Lauren M. Goddard. Progesterone increases vascular permeability by direct regulation of endothelial cells. MCDB Retreat Dec. 2011 at Lake Arrowhead, CA

Goddard LM, Murphy TJ, Enciso JM, Hashiomoto-Partyka M, Sanchez LA, Allen N, Iruela-Arispe LM. Progesterone signaling within endothelial cells results in increased vascular permeability and platelet adhesion. ATVB Conference. April 2012, Chicago, IL

Lauren M. Goddard. Progesterone Signaling in Endothelium may Underlie Sex Specific Differences in Cardiovascular Disease. The Cleveland Science Initiative April 2012 at Grover Cleveland High School, Reseda, CA

Lauren M. Goddard. Progesterone Signaling Results in Blood Vessel Leakage. Iris Cantor Women's Health Center Executive Board Luncheon Feb. 2013 at UCLA, Los Angeles, CA

Goddard LM and Iruela-Arispe ML. (2013) Cellular and Molecular Regulation of Vascular Permeability. *Thromb Haemost.* 109: 407-15

Goddard LM, Ton AN, Org T, Mikkola HK, Iruela-Arispe ML. (2013) Selective suppression of endothelial cytokine production by progesterone receptor. *Vascul Pharmacol.* 59: 36-43

Goddard LM, Murphy TJ, Org T, Enciso JM, Hashimoto-Partyka MK, Warren CM, McDonald AI, He H, Domigan CK, Sanchez LA, Allen NC, Chao LC, Tontonoz P, Mikkola HKA, Iruela-Arispe ML. (2013) Physiological Vascular Permeability requires induction of NR4A1 by progesterone receptor. *Cell. In Review.*

Chapter 1-Introduction: Physiological Progesterone Signaling and its Role in the Vasculature

Section 1.1: Introduction

Progesterone, a steroid hormone predominantly secreted by the ovary, is well known for its role in coordinating the many cellular and molecular events required for successful implantation, maintenance of pregnancy, as well as ovulation [1]. The menstrual cycle is governed by highly coordinated changes in the levels of estrogen and progesterone, which produce varying responses in diverse tissues and organs (Figure 1.1). Changes imposed by these hormones prepare the endometrium for implantation and continue to be essential during the subsequent post-implantation phases to ensure a successful pregnancy [2,3]. Estrogen is expressed at high levels during the proliferative phase where it stimulates growth and expansion of the endometrium. Following ovulation, in the secretory/luteal phase, progesterone initiates the process of decidualization, a series of physiological and molecular changes in the endometrium (subsequently called the decidua) that specifically include an increase in physiological edema, stromal cell differentiation, and the alteration of matrix proteins [4]. These changes are critical for successful implantation and successive survival of the embryo.

Two biologically important and cyclically controlled processes in the decidua during the progesterone-predominant secretory phase are vascular permeability and the regulation of immune cell trafficking. Although both are assumed to be under the control of progesterone, the exact mechanism of progesterone action remains elusive. While cellular and molecular changes to the vascular endothelium underlie permeability and immune responses, a role for PR in the endothelium during decidualization is poorly understood. Much of the vascular function of PR has been attributed to paracrine effects

via PR signaling in other cell types, yet few studies have addressed the biological function of progesterone directly on vascular cells.

In this chapter we will review progesterone signaling and its physiological function in the reproductive tract. Furthermore, we will expand upon current knowledge on progesterone regulation of vascular function, particularly its role in vascular permeability and immune trafficking.

Section 1.2: Progesterone Receptor Signaling

The large majority of progesterone's biological actions are mediated through the progesterone receptor (PR), a member of the evolutionarily conserved nuclear receptor superfamily of transcription factors. Similar to other nuclear receptors, PR contains three structural domains including a ligand-binding domain, DNA binding domain, and an N-terminal transactivation domain capable of both inhibitory and activating functions [5] (Figure 1.2). The latter domain aids in PR interaction with coregulators and other transcription factors important for the regulation of gene expression. There are two predominant isoforms of PR, PR-A and PR-B, each of which is transcribed from alternative promoters within the same gene [6,7]. While both are structurally related, the transactivation domain of PR-B is 164 amino acids longer than that of PRA, and it contains an additional activation domain (AF-3) [8,9]. These appear to be small differences, however they underlie remarkably distinct biology [10].

In the absence of ligand, PR is localized to both the cytoplasm and nucleus and is bound by a multi-protein complex consisting of heat-shock proteins [11,12]. Upon progesterone binding, PR becomes phosphorylated, dissociates from chaperone

proteins, dimerizes (either hetero- or homotypically) and completely localizes to the nucleus [13] (Figure 1.3). In the canonical (genomic or classical) PR signaling, PR either directly binds to DNA through progesterone response elements (PRE) or indirectly through tethering interactions with other transcription factors (AP1, SP1, STATs) [14]. Interestingly, PR isoforms do not appear to be functionally redundant, as they regulate distinct subsets of target genes and control unique reproductive functions [10,15]. While non-canonical (nongenomic) signaling through secondary messenger cascades has been implicated in a subset of progesterone functions, the mediators of these signaling events, whether classical PR, or putative membrane progesterone receptors, remain unclear [16].

PR activity is regulated by a series of posttranslational modifications including phosphorylation, sumoylation, methylation, and ubiquitination [17-19]. Functionally, these modifications alter transcriptional activity, promoter selectivity, binding interactions, and receptor stability, further enhancing the specificity of PR signaling [19]. Interestingly, basal phosphorylation of PR occurs in the absence of ligand, but it is enhanced following ligand binding, implicating phosphorylation as a key mechanism in controlling the activation state of PR [5].

Section 1.3: PR Loss of Function Studies

Genetic and pharmacological studies in humans and mice have demonstrated the vital role for progesterone in many reproductive processes including pregnancy, cycling, and implantation. Administration of the PR antagonist mifepristone (RU486) renders the uterus unreceptive to implantation and initiates the breakdown of the endometrium reminiscent of menstruation [20,21]. If administered post-implantation,

mifepristone results in the abortion of the conceptus, demonstrating the critical role for progesterone in the maintenance of pregnancy [22].

PR knockout (PRKO) mice, lacking both PR-A and PR-B isoforms, also display pleiotropic reproductive abnormalities including the inability to ovulate, impaired implantation, and lack of a decidual response following hormonal stimulation [23]. When PR-A and PR-B specific null mice (PRAKO and PRBKO, respectively) were generated, only PRAKO females recapitulated the reproductive phenotype observed in PRKO mice, indicating that PR-A is critical for reproductive functions. While PRBKO mice were fertile, they did have defects in mammary gland morphogenesis, further demonstrating the unique biological roles of individual isoforms [10,24-28]

Aside from the acknowledged role of PR in the reproductive tract, PR is also required for the normal physiology of a myriad of other organs including the thymus, immune system, and vasculature [29-31]. PRKO mice have impaired thymic involution, an important requirement for normal fertility. Further analysis demonstrated that PR signaling in thymic stromal cells was critical for paracrine mediated inhibition of T cell development [29]. Moreover, PRKO uteri were found to have increased immune cell infiltrate following hormonal stimulation, further linking PR signaling and immune regulation [30]. In addition, treatment of wild-type animals with progesterone worsened the response to carotid injury while PRKO mice were unaffected, demonstrating a potential protective affect of progesterone signaling in the vasculature [31].

More recently, the generation of PR conditional knockout mice has enabled a better understanding of the cell specific functions of PR. Using this approach, it was determined that loss of PR from the epithelium alone could prevent embryo implantation

[2]. Further analysis on conditional knock out mice will reveal important cellular functions that will aid in unraveling the complex nature of progesterone's multifarious actions.

Section 1.4: PR Expression *in vivo*

Although progesterone is systemically distributed, only tissues/cells that express PR are capable of responding. Therefore, much attention has focused on understanding the transcriptional regulation of PR and in which cells and under what context PR is expressed. Both isoforms of PR are predominantly upregulated by estrogen signaling through the estrogen receptor (ER) [32,33]. PR-A and PR-B are expressed at different levels depending on the cell type and the physiological status of the tissue, yet it is unclear how this isoform ratio is controlled. In addition to estrogen regulation, the transcription factors cyclinD1 and CEBPβ were found to increase PR expression in certain reproductive cells, while PR itself can negatively autoregulate its own expression, particularly in glandular and the luminal epithelium during pregnancy [14] The fact that multiple mechanisms exist for PR regulation reminds us of its high physiological importance and the exquisite requirement for tight control of its expression.

In mice and humans, PR expression has been assessed at both the protein and mRNA level. Using a PR-B specific LacZ reporter mouse, PR was described to be highly expressed in the uterus, ovary, mammary gland and pituitary gland, which correlates well with its known physiological functions [34,35]. At the cellular level, PR was detected in stromal, smooth muscle, and epithelial cells of the uterus, while expression in the vasculature seemed to be restricted to the smooth muscle cells. As PR-A is the

predominant isoform for reproductive function, these mice may not offer a thorough representation of total PR promoter activity. Therefore, further analysis of PR expression in the mouse may be needed to enrich these findings. In humans, aside from reproductive expression, PR is also found in other organs including the nervous system, pancreas, immune system, bone, and the vasculature [36-43]. Thus, the ubiquitous and varied expression of PR highlights its potential widespread physiological effects throughout the body.

Although somewhat debated, several reports have clearly demonstrated PR expression in different human vascular beds [39,44-48] including endothelial cells of human atherosclerotic vessels [38]. Functionally, progesterone inhibits endothelial cell proliferation in vitro and it reduces aorta re-endothelialization in response to experimentally induced injury [31,38]. Additionally, progesterone decreases endothelial expression of the endothelial-leukocyte adhesion molecule, VCAM-1, as well as the cytokines IL-8 and MCP-1, linking the vascular endothelium to immune regulation [49,50]. Therefore, progesterone is capable of signaling within the vasculature, yet its functional significance in vivo is currently unclear.

Section 1.5: Hormonal Control of Uterine Vascular Responses

The endometrial vascular bed actively responds to the cyclical nature of estrogen and progesterone. Unique properties of the endometrial vasculature associated with the endometrial cycle include angiogenesis, regression, hemostasis, nutrient supply, and tissue fluid balance. During the estrogen-dominant proliferative phase of the menstrual cycle, there is a peak in endothelial proliferation, as angiogenesis of both venous and

arterial vessels into the endometrium expands following menstrual repair [51]. During this time, large arteries supplying the uterus divide into basal and spiral arterioles in the endometrium. Basal arterioles innervate the basal endometrial zones adjacent to the smooth muscle rich myometrium, while spiral arteries grow upwards towards the luminal epithelial where implantation occurs (Figure 1.4).

During the luteal/secretory phase, spiral arteries continue to coil and further branch into an extensive capillary network as the endometrium progressively expands in preparation for successful implantation [51]. The venous system parallels that of the arteries, with larger veins branching into relatively smaller and venules. These venules become dilated and form venous lakes that lie in close proximity to the capillary plexus in the subepithelial space. While these smaller arterioles and venules are shed during menstruation, their larger counterparts in the myometrium remain intact and are used for regrowth of vessels during the proliferative phase following menstruation.

Regulation of these vascular events is assumed to be under the control of female sex hormones, but due to the conflicting and equivocal information on receptor expression in the vasculature, many have assumed that these vascular events are governed by paracrine signaling initiated by surrounding endometrial stromal cells. As there is little definitive evidence to support this hypothesis, it has become necessary to attain a more comprehensive understanding of PR function in the vasculature.

Section 1.6: Progesterone and Permeability

In addition to angiogenesis, a second unique feature of endometrial vessels is cyclic alterations in vascular permeability. Increases in permeability result in the

recurrent formation of physiological edema during the secretory phase, a time when progesterone levels peak [1]. Leakage of blood-borne proteins into the functional endometrium is needed for the support and invasion of trophoblastic cells and the survival of the blastocyst upon implantation [52].

Much of the cellular and molecular understanding of vascular permeability comes from studies on pathological permeability responses in tumors, during wound healing and following an inflammatory reaction. While the mechanisms of physiological vascular permeability are unclear, the response is most likely controlled by mechanisms distinct from those affecting pathological permeability. As increases in edema occur during times of high circulating progesterone levels, it is likely that progesterone mediates the vascular leakage. In support of this, women taking progestin-only contraceptives tend to have breakthrough bleeding and thrombosis, two consequences of enhanced vascular permeability [53-55]. However, it remains unclear whether progesterone modulates vascular permeability by acting directly on vascular cells or indirectly through paracrine mechanisms.

VEGF, a potent permeability mediator, has been an attractive candidate for the paracrine regulation of vascular permeability. Although several studies have examined stromal VEGF secretion in response to progesterone stimulation in vivo and in vitro, only moderate increases in VEGF levels were reported [56-58]. Even so, no study has conclusively demonstrated that increased VEGF levels *in vivo* directly control vascular permeability in the uterus. Therefore, it remains plausible that progesterone exerts its actions directly on the vasculature itself, independent of VEGF signaling.

Section 1.7: Immune trafficking and progesterone regulation

Changing hormone levels also parallel fluctuations in the trafficking of leukocyte subpopulations into the uterus [59,60] (Figure 1.5). Physiologically, many reproductive events, such as implantation, menstruation, and labor, are associated with local inflammatory events with increased recruitment of inflammatory cells including T-cells, natural killer (NK) cells, macrophages and mast cells [61-64]. Interestingly several lines of evidence have demonstrated marked changes in the severity of various diseases during different stages of the menstrual cycle [59,65,66]. Furthermore, inflammation contributes to the susceptibility and progression of many diseases that exhibit gender-based differences in prevalence [67-69]. Hence, it is believed that hormones may play an important role in systemic immune regulation.

PR is generally assumed to play an anti-inflammatory role in these processes as the influx of immune cells into the endometrium parallels progesterone withdrawal [59,62]. Furthermore, localized suppression of the immune system during gestation of the semi-allogenic embryo is another essential role played by progesterone [70]. However, these findings are correlative and do not provide a molecular link between PR action and suppression of inflammation. Several studies have demonstrated PR expression on particular human leukocyte subpopulations indicative of direct regulation by progesterone [60,65,71]. However, these findings do not account for leukocytes that do not express PR, yet are under hormonal control. Thus there must be other mechanisms in place that, under the control of hormones, regulate immune trafficking either indirectly through cell-cell interactions or via paracrine signaling.

The vascular endothelium is an active participant in immune cell trafficking and is an important barrier in the regulation of leukocyte extravasation into tissues [72]. While there is evidence of PR expression in the endothelium a role for endothelial PR in physiological leukocyte trafficking is unsuspected.

Section 1.8: Goals of the Dissertation

The aim of this dissertation was to determine the cell specific physiological role of progesterone signaling within the vascular endothelium, particularly with respect to vascular permeability and immune regulation, with the hopes of determining the downstream targets of PR in these responses. Chapter 2 is a version of a review on the cellular and molecular regulation of vascular permeability that was published in *Thrombosis and Haemostasis* in 2013. The goal of this review was to obtain a greater grasp on the field of vascular permeability in an attempt to better understand our findings with relation to permeability control by progesterone as demonstrated in Chapter 3.

Chapter 3 is a version of a manuscript compiled for submission to *Cell* and it is currently under revision. Here we explored the contribution of endothelial PR to regulation of physiological vascular permeability in the uterus. Using a loss-of-function approach, we were able to determine that PR signaling in the vascular endothelium was required for the physiological permeability that precedes implantation. We also show that this effect was specific to the venous and lymphatic endothelium, as PR expression was devoid from arterial endothelial cells. Integration of global RNA- and ChIP-sequencing revealed that progesterone directly upregulates the orphan nuclear receptor

NR4A1 (Nur77/TR3). Genomic inactivation studies in mice demonstrated that Nur77 was required for progesterone-mediated vascular permeability, indicating a link between PR and NR4A1. Activation of NR4A1 was found to suppress the expression of cell-cell junctional proteins leading to a sustained vascular permeability response.

In Chapter 4 we shifted our focus towards determining whether PR signaling in the endothelium was important for physiological immune trafficking. Global loss of PR resulted in an increase in several immune cell types (T cells, PMNs, and macrophages) into the uterus. Interestingly, when PR was specifically deleted from the endothelium, only PMNs and macrophages were found at higher numbers in the uterus, demonstrating that endothelial PR directly regulates either the trafficking or the retention of a specific subset of leukocytes in the uterus. Using RNA- and ChIP-sequencing we determined that progesterone directly downregulates the expression of the endothelial-leukocyte adhesion molecules E-selectin and VCAM-1. We further demonstrated that the effects of progesterone on the endothelium also occurred in the presence of pathological activation by lipopolysaccharide, implicating progesterone not only in modulation of physiologic immune trafficking, but also during an acute inflammatory response.

In Chapter 5 we assessed the paracrine secretion of cytokines by the endothelium in the presence of progesterone. As a significant portion of immune cells does not express PR, we explored the possibility that progesterone may affect immune regulation by directing expression of paracrine mediators from the endothelium. This study determined that progesterone selectively inhibits the expression of a small group of cytokines, which are known to selectively recruit monocyte and neutrophils. This work

was recently compiled into a manuscript submitted to *Vascular Pharmacology* and is currently under review. Together, the results from these three chapters are the first to demonstrate the importance of progesterone signaling in the endothelium and its contribution to physiological vascular responses *in vivo*. We will discuss the significance of these findings Chapter 6.

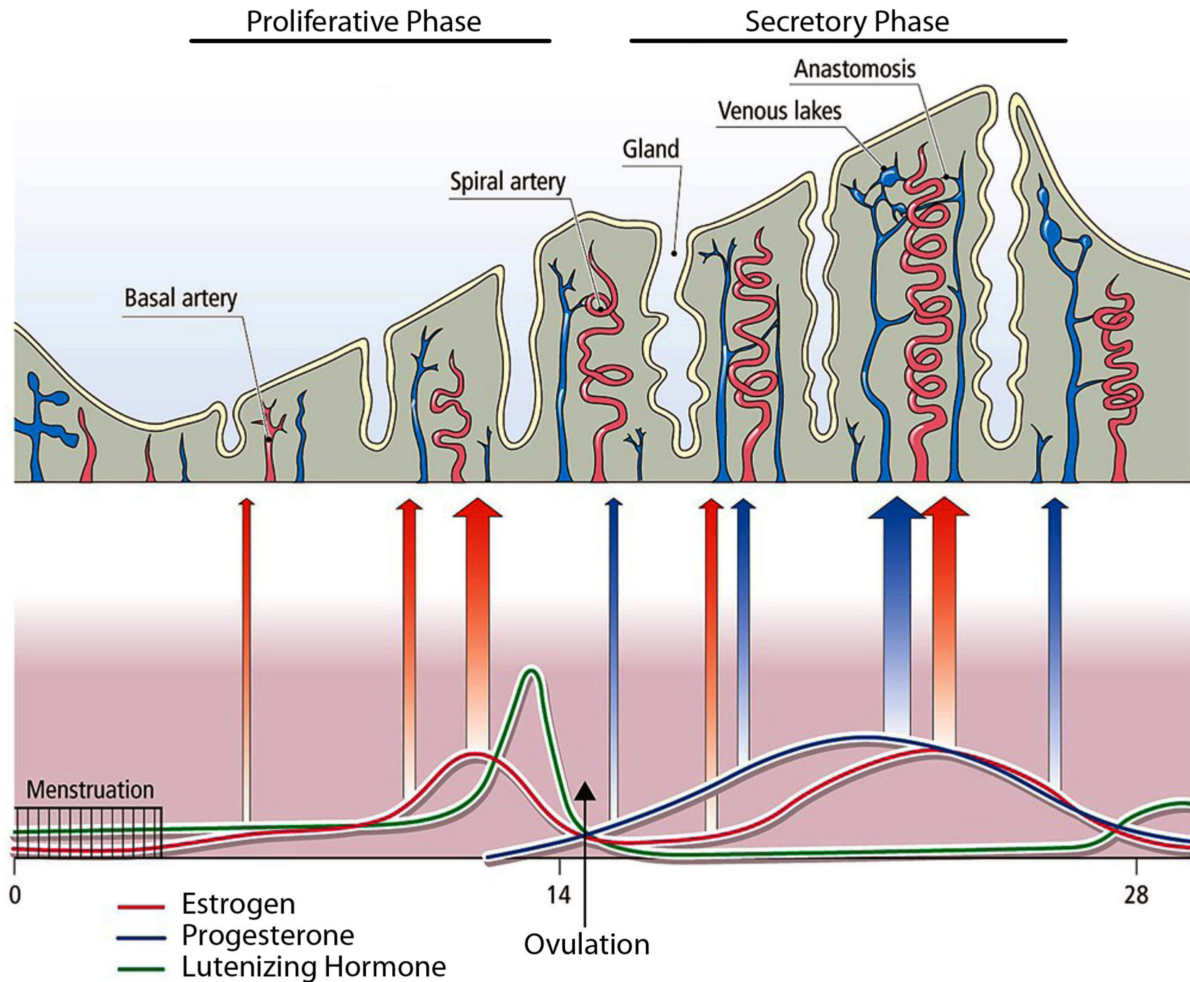


Figure 1.1. Changes in the uterine endometrium during the female menstrual cycle
Estrogen levels predominate during the proliferative phase, a time when growth and expansion of the endometrium occurs following menstruation. Following ovulation, increased progesterone secretion from the ovary characterizes the secretory phase, a time of continued endometrial growth and cellular changes important in the preparation for embryo implantation. Adapted from *Essential Reproduction* [73] with permissions.

Figure 1.2. Progesterone Receptor Structure
PR isoforms, A and B, contain three structural domains, the N-terminal domain (red), the DNA binding domain (DBD; blue) and the ligand binding domain (LBD; green). There are three activation domains, two of which are in the N-terminus (AF-3 and AF-1) and one in the LBD (AF-2). The site of isoform dimerization, termed the dimerization domain (DD), is located in the most C-terminal region of the LBD. Overlapping this area is the site of heat shock protein (HSP) binding. ID=inhibitory domain; H=hinge region

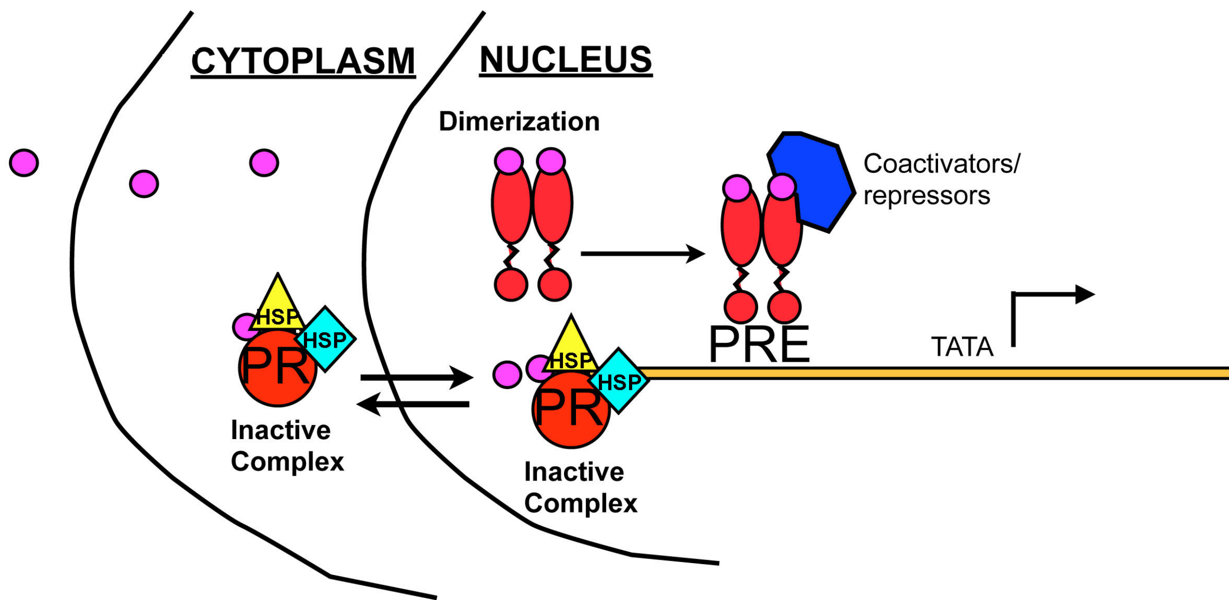


Figure 1.3. Canonical PR signaling
Progesterone, a lipophilic steroid, can transverse the cell membrane where it can bind PR. Upon binding by progesterone, PR undergoes a conformational change, enabling the dissociation from chaperone proteins (HSP), dimerization, and binding to progesterone response elements in the nucleus. PR interacts with a host of coregulators and other transcription factors to regulate gene expression.

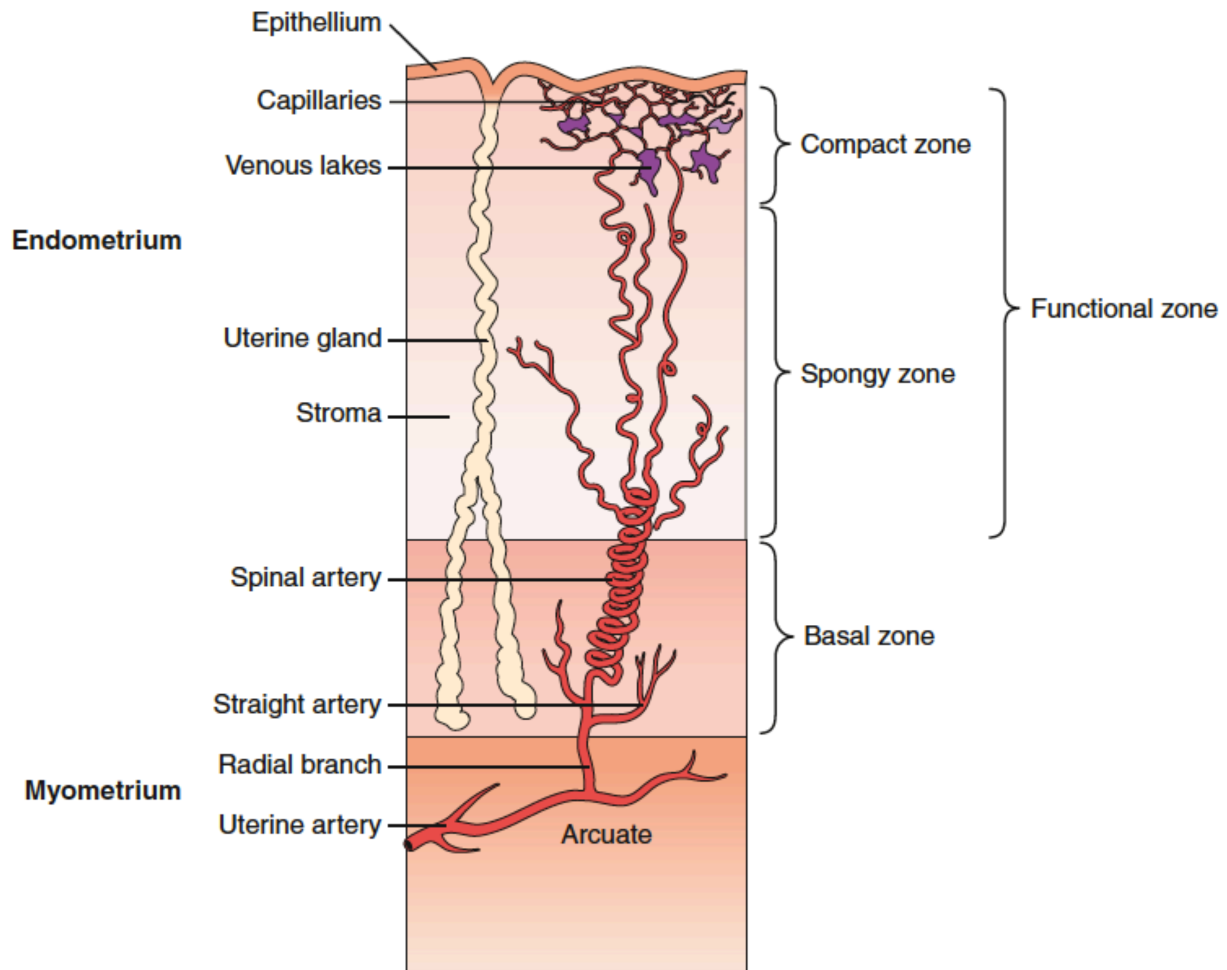


Figure 1.4. Histological organization of the human endometrium during the secretory phase
Vessel growth of both arteries and veins towards the subepithelial space continues in the secretory phase. Arcuate arteries originating from the smooth muscle rich myometrial layer grow and divide into basal/straight and spiral arteries in the basal zone. Beneath the luminal epithelium, a complex capillary network branches from the spiral arteries and drains into venules that compose dilated venous lakes. Adapted from *Yen and Jaffee Reproductive Endocrinology* [1] with permission.

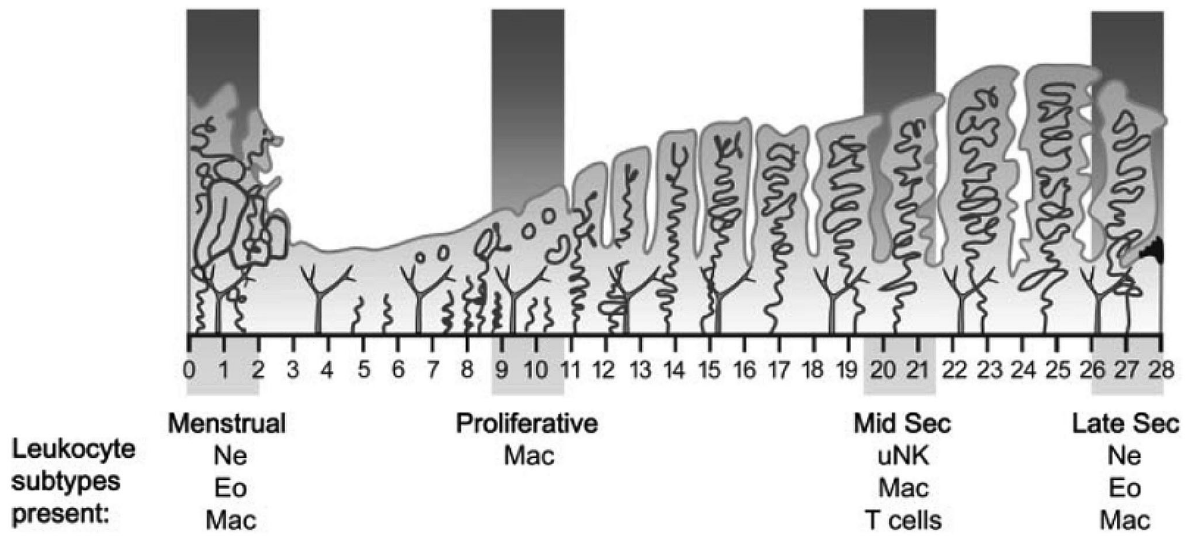


Figure 1.5. Cyclical fluctuations in immune cell populations in the endometrium
During different times of the menstrual cycle, unique leukocyte subpopulations are seen in the endometrium. During the proliferative phase the predominant leukocyte present in the endometrium are macrophages (Mac). When progesterone levels peak in the secretory phase, there is a recruitment of uterine natural killer (uNK) cells, T cells, and macrophages. Following a drop in progesterone levels at menstruation, a large influx of neutrophils (Ne), and eosinophils (Eo) enter the endometrium. Adapted from *Jones et al.*[74] with permission.

References:

1. Jerome F Strauss I, Robert L Barbieri MD. Yen and Jaffe's Reproductive Endocrinology. Elsevier Health Sciences; 2009.

2. Franco HL, Rubel CA, Large MJ, Wetendorf M, Fernandez-Valdivia R, Jeong JW, et al. Epithelial progesterone receptor exhibits pleiotropic roles in uterine development and function. FASEB J 2012;26:1218–27.

3. Wetendorf M, DeMayo FJ. The progesterone receptor regulates implantation, decidualization, and glandular development via a complex paracrine signaling network. Mol. Cell. Endocrinol. 2012;357:108–18.

4. Large MJ, DeMayo FJ. The regulation of embryo implantation and endometrial decidualization by progesterone receptor signaling. Mol. Cell. Endocrinol. 2012;358:155–65.

5. Hill KK, Roemer SC, Churchill MEA, Edwards DP. Structural and functional analysis of domains of the progesterone receptor. Mol. Cell. Endocrinol. 2012;348:418–29.

6. Conneely OM, Maxwell BL, Toft DO, Schrader WT, O'Malley BW. The A and B forms of the chicken progesterone receptor arise by alternate initiation of translation of a unique mRNA. Biochem. Biophys. Res. Commun. 1987;149:493–501.

7. Conneely OM, Kettelberger DM, Tsai MJ, Schrader WT, O'Malley BW. The chicken progesterone receptor A and B isoforms are products of an alternate translation initiation event. J. Biol. Chem. 1989;264:14062–4.

8. Tung L, Abdel-Hafiz H, Shen T, Harvell DME, Nitao LK, Richer JK, et al. Progesterone receptors (PR)-B and -A regulate transcription by different mechanisms: AF-3 exerts regulatory control over coactivator binding to PR-B. Mol. Endocrinol. 2006;20:2656–70.

9. Sartorius CA, Melville MY, Hovland AR, Tung L, Takimoto GS, Horwitz KB. A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. Mol. Endocrinol. 1994;8:1347–60.

10. Conneely OM, Mulac-Jericevic B, Lydon JP, De Mayo FJ. Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. Mol. Cell. Endocrinol. 2001;179:97–103.

11. Kost SL, Smith DF, Sullivan WP, Welch WJ, Toft DO. Binding of heat shock proteins to the avian progesterone receptor. Mol Cell Biol 1989;9:3829–38.

12. Carson-Jurica MA, Lee AT, Dobson AW, Conneely OM, Schrader WT, O'Malley

BW. Interaction of the chicken progesterone receptor with heat shock protein (HSP) 90. J. Steroid Biochem. 1989;34:1–9.

13. Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu. Rev. Biochem. 1994;63:451–86.

14. Jacobsen BM, Horwitz KB. Progesterone receptors, their isoforms and progesterone regulated transcription. Mol. Cell. Endocrinol. 2012;357:18–29.

15. Giangrande PH, Kimbrel EA, Edwards DP, McDonnell DP. The opposing transcriptional activities of the two isoforms of the human progesterone receptor are due to differential cofactor binding. Mol Cell Biol 2000;20:3102–15.

16. Wendler A, Albrecht C, Wehling M. Nongenomic actions of aldosterone and progesterone revisited. Steroids 2012;77:1002–6.

17. Abdel-Hafiz H, Takimoto GS, Tung L, Horwitz KB. The inhibitory function in human progesterone receptor N termini binds SUMO-1 protein to regulate autoinhibition and transrepression. J. Biol. Chem. 2002;277:33950–6.

18. Daniel AR, Gaviglio AL, Czaplicki LM, Hillard CJ, Housa D, Lange CA. The progesterone receptor hinge region regulates the kinetics of transcriptional responses through acetylation, phosphorylation, and nuclear retention. Mol. Endocrinol. 2010;24:2126–38.

19. Hagan CR, Daniel AR, Dressing GE, Lange CA. Role of phosphorylation in progesterone receptor signaling and specificity. Mol. Cell. Endocrinol. 2012;357:43–9.

20. Critchley HOD, Kelly RW, Brenner RM, Baird DT. Antiprogestins as a model for progesterone withdrawal. Steroids 2003;68:1061–8.

21. Jabbour HN, Kelly RW, Fraser HM, Critchley HOD. Endocrine regulation of menstruation. Endocr. Rev. 2006;27:17–46.

22. Baulieu EE. Contragestion and other clinical applications of RU 486, an antiprogesterone at the receptor. Science 1989;245:1351–7.

23. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, et al. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. 1995;9:2266–78.

24. Conneely OM, Lydon JP. Progesterone receptors in reproduction: functional impact of the A and B isoforms. Steroids 2000;65:571–7.

25. Conneely OM, Mulac-Jericevic B, Lydon JP. Progesterone-dependent regulation of female reproductive activity by two distinct progesterone receptor isoforms. Steroids 2003;68:771–8.

26. Mulac-Jericevic B, Lydon JP, DeMayo FJ, Conneely OM. Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. Proc. Natl. Acad. Sci. U.S.A. 2003;100:9744–9.

27. Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM. Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. Science 2000;289:1751–4.

28. Mulac-Jericevic B, Conneely OM. Reproductive tissue selective actions of progesterone receptors. Reproduction 2004;128:139–46.

29. Tibbetts TA, DeMayo F, Rich S, Conneely OM, O'Malley BW. Progesterone receptors in the thymus are required for thymic involution during pregnancy and for normal fertility. Proc. Natl. Acad. Sci. U.S.A. 1999;96:12021–6.

30. Tibbetts TA, Conneely OM, O'Malley BW. Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. Biol. Reprod. 1999;60:1158–65.

31. Karas RH, van Eickels M, Lydon JP, Roddy S, Kwoun M, Aronovitz M, et al. A complex role for the progesterone receptor in the response to vascular injury. J. Clin. Invest. 2001;108:611–8.

32. Kastner P, Bocquel MT, Turcotte B, Garnier JM, Horwitz KB, Chambon P, et al. Transient expression of human and chicken progesterone receptors does not support alternative translational initiation from a single mRNA as the mechanism generating two receptor isoforms. J. Biol. Chem. 1990;265:12163–7.

33. Bonéy-Montoya J, Ziegler YS, Curtis CD, Montoya JA, Nardulli AM. Long-range transcriptional control of progesterone receptor gene expression. Mol. Endocrinol. 2010;24:346–58.

34. Ismail PM, Li J, DeMayo FJ, O'Malley BW, Lydon JP. A novel LacZ reporter mouse reveals complex regulation of the progesterone receptor promoter during mammary gland development. Mol. Endocrinol. 2002;16:2475–89.

35. Soyal SM, Mukherjee A, Lee KYS, Li J, Li H, DeMayo FJ, et al. Cre-mediated recombination in cell lineages that express the progesterone receptor. Genesis 2005;41:58–66.

36. Bland R. Steroid hormone receptor expression and action in bone. Clin. Sci. 2000;98:217–40.

37. Mani S. Progestin receptor subtypes in the brain: the known and the unknown. Endocrinology 2008;149:2750–6.

38. Vázquez F, Rodríguez-Manzaneque JC, Lydon JP, Edwards DP, O'Malley BW, Iruela-Arispe ML. Progesterone regulates proliferation of endothelial cells. J. Biol.

Chem. 1999;274:2185–92.

39. Krikun G, Schatz F, Taylor R, Critchley HOD, Rogers PAW, Huang J, et al. Endometrial endothelial cell steroid receptor expression and steroid effects on gene expression. J. Clin. Endocrinol. Metab. 2005;90:1812–8.

40. Brinton RD, Thompson RF, Foy MR, Baudry M, Wang J, Finch CE, et al. Progesterone receptors: form and function in brain. Front Neuroendocrinol 2008;29:313–39.

41. Ozawa H. Steroid Hormones, their receptors and neuroendocrine system. J Nippon Med Sch 2005;72:316–25.

42. Graham JD, Clarke CL. Physiological action of progesterone in target tissues. Endocr. Rev. 1997;18:502–19.

43. Graham JD, Clarke CL. Expression and transcriptional activity of progesterone receptor A and progesterone receptor B in mammalian cells. Breast Cancer Res. 2002;4:187–90.

44. Ingegno MD, Money SR, Thelmo W, Greene GL, Davidian M, Jaffe BM, et al. Progesterone receptors in the human heart and great vessels. Lab. Invest. 1988;59:353–6.

45. Iruela-Arispe ML, Rodriguez-Manzaneque JC, Abu-Jawdeh G. Endometrial endothelial cells express estrogen and progesterone receptors and exhibit a tissue specific response to angiogenic growth factors. Microcirculation 1999;6:127–40.

46. Maybin JA, Duncan WC. The human corpus luteum: which cells have progesterone receptors? Reproduction 2004;128:423–31.

47. Perrot-Applanat M, Cohen-Solal K, Milgrom E, Finet M. Progesterone receptor expression in human saphenous veins. Circulation 1995;92:2975–83.

48. Rodríguez-Manzaneque JC, Graubert M, Iruela-Arispe ML. Endothelial cell dysfunction following prolonged activation of progesterone receptor. Hum. Reprod. 2000;15 Suppl 3:39–47.

49. Otsuki M, Saito H, Xu X, Sumitani S, Kouhara H, Kishimoto T, et al. Progesterone, but not medroxyprogesterone, inhibits vascular cell adhesion molecule-1 expression in human vascular endothelial cells. Arterioscler. Thromb. Vasc. Biol. 2001;21:243–8.

50. Simoncini T. Differential Signal Transduction of Progesterone and Medroxyprogesterone Acetate in Human Endothelial Cells. Endocrinology 2004;145:5745–56.

51. Chennazhi KP, Nayak NR. Regulation of angiogenesis in the primate endometrium: vascular endothelial growth factor. Semin. Reprod. Med. 2009;27:80–9.

52. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin. Reprod. Med. 2007;25:445–53.

53. Lohr PA, Creinin MD. Oral contraceptives and breakthrough bleeding: what patients need to know. J Fam Pract 2006;55:872–80.

54. Lockwood CJ. Mechanisms of normal and abnormal endometrial bleeding. Menopause 2011;18:408–11.

55. Voelker R. Risk of blood clots higher for oral contraceptives with certain progestins. JAMA2011;306:2206.

56. Wu J, Richer J, Horwitz KB, Hyder SM. Progestin-dependent induction of vascular endothelial growth factor in human breast cancer cells: preferential regulation by progesterone receptor B. Cancer Res 2004;64:2238–44.

57. Ancelin M, Buteau-Lozano H, Meduri G, Osborne-Pellegrin M, Sordello S, Plouët J, et al. A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF189 regulates angiogenesis and vascular permeability in human uterus. Proc. Natl. Acad. Sci. U.S.A. 2002;99:6023–8.

58. Shimizu T, Miyamoto A. Progesterone induces the expression of vascular endothelial growth factor (VEGF) 120 and Flk-1, its receptor, in bovine granulosa cells. Anim. Reprod. Sci. 2007;102:228–37.

59. Oertelt-Prigione S. Immunology and the menstrual cycle. Autoimmun Rev 2012;11:A486–92.

60. Gilliver SC. Sex steroids as inflammatory regulators. The Journal of Steroid Biochemistry and Molecular Biology 2010;120:105–15.

61. Hamilton S, Oomomian Y, Stephen G, Shynlova O, Tower CL, Garrod A, et al. Macrophages infiltrate the human and rat decidua during term and preterm labor: evidence that decidual inflammation precedes labor. Biol. Reprod. 2012;86:39.

62. King AE, Critchley HOD. Oestrogen and progesterone regulation of inflammatory processes in the human endometrium. J Steroid Biochem. Mol. Biol. 2010;120:116–26.

63. Bollapragada S, Bollopragada S, Youssef R, Jordan F, Greer I, Norman J, et al. Term labor is associated with a core inflammatory response in human fetal membranes, myometrium, and cervix. Am. J. Obstet. Gynecol. 2009;200:104.e1–

11.

64. Tait AS, Butts CL, Sternberg EM. The role of glucocorticoids and progestins in inflammatory, autoimmune, and infectious disease. J Leukoc. Biol. 2008;84:924–31.

65. Hughes GC. Progesterone and autoimmune disease. Autoimmun Rev 2012;11:A502–14.

66. Martocchia A, Stefanelli M, Cola S, Falaschi P. Sex steroids in autoimmune diseases. Curr Top Med Chem 2011;11:1668–83.

67. Kaushic C, Roth KL, Anipindi V, Xiu F. Increased prevalence of sexually transmitted viral infections in women: the role of female sex hormones in regulating susceptibility and immune responses. J Reprod. Immunol. 2011;88:204–9.

68. McCombe PA, Greer JM, Mackay IR. Sexual dimorphism in autoimmune disease. Curr. Mol. Med. 2009;9:1058–79.

69. Meyer MR, Haas E, Barton M. Gender differences of cardiovascular disease: new perspectives for estrogen receptor signaling. Hypertension 2006;47:1019–26.

70. van Mourik MSM, Macklon NS, Heijnen CJ. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J Leukoc. Biol. 2009;85:4–19.

71. Butts CL, Shukair SA, Duncan KM, Bowers E, Horn C, Belyavskaya E, et al. Progesterone inhibits mature rat dendritic cells in a receptor-mediated fashion. Int. Immunol. 2007;19:287–96.

72. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nat. Rev. Immunol. 2007;7:803–15.

73. Johnson MH. Essential Reproduction. Wiley-Blackwell; 2012.

74. Jones RL. Identification of Chemokines Important for Leukocyte Recruitment to the Human Endometrium at the Times of Embryo Implantation and Menstruation. J Clin. Endocrinol. & Metab. 2004;89:6155–67.

Chapter 2:
Cellular and Molecular Regulation of Vascular Permeability

Abstract

Vascular permeability is a highly coordinated process that integrates vesicular trafficking, complex junctional rearrangements, and refined cytoskeletal dynamics. In response to the extracellular environment, these three cellular activities have been previously assumed to work in parallel to regulate the passage of solutes between the blood and tissues. New developments in the area of vascular permeability however have highlighted the interdependence between trans- and para-cellular pathways, the cross-communication between adherens and tight junctions, and the instructional role of pericytes on endothelial expression of barrier-related genes. Additionally, significant effort has been placed in understanding the molecular underpinings that contribute to barrier restoration following acute permeability events and in clarifying the importance of context-dependent signaling initiated by permeability mediators. Finally, recent findings have uncovered an unpredicted role for transcription factors in the coordination of vascular permeability and clarified how junctional complexes can transmit signals to the nucleus to control barrier function. The goal of this review is to provide a concise and updated view of vascular permeability, discuss the most recent advances in molecular and cellular regulation, and introduce integrated information on the central mechanisms involved in trans-endothelial transport.

Introduction

One of the main roles of endothelial cells is to function as a selective barrier between the blood stream and tissues. As sophisticated gate-keepers, endothelial cells possess a broad number of mechanisms that regulate transport of solutes, large molecules, and cells across the vessel wall. In general terms, the endothelial barrier is controlled by the combined activities of: (1) heterotypic cell associations (inflammatory cells and mural cells); (2) transcellular transport (across the endothelium) and (3) junctional complexes or intercellular junctions (paracellulartranport, ie. between endothelial cells) (Figure 2.1). Together, these extracellular associations and cellular functions maintain and actively regulate transport across the endothelium.

The dynamic and highly responsive control of the endothelial barrier enables macromolecular transport to be reduced or accelerated, facilitates immune surveillance and enables the deposition of matrix proteins immediately outside the vascular wall (provisional matrix) to initiate mechanisms of repair. In recent years, information related to the processes that coordinate transcellular and paracellular transport have been broadly expanded and focus has been placed on integration of these two mechanisms, as well as in understanding tissue-specific control of barrier function. In this review, we will summarize the recent conceptual advancements in both the cellular and molecular control of permeability regulation, and present these findings in the context of previous knowledge in the field.

Mural Cell Control of Vascular Permeability

Historically, the majority of research on vascular permeability has been centered on transcellular regulation and endocytic transport. Nonetheless, in the last four years, a series of publications have focused on the contribution of mural cells as important instructional partners in the control of vascular permeability. These cells appear to convey tissue-specific control to endothelial barrier function.

The term mural cell describes endothelial–associated cells that might not form a permanent sheath, but instead are dynamically associated with capillaries and functional participants of the “vascular unit”. Mural cells include pericytes, smooth muscle cells and macrophages. Depending on the tissue, mural cells also include astrocytes (brain) and podocytes (kidney) as per their tight association with blood vessels.

Recent evidence has brought to light the importance of pericytes in the regulation of permeability. Specifically, mice that lack pericytes showed increased permeability to water and a wide range of low and high molecular mass tracers. The effect was most noticeable in the brain, indicating a stronger relevance of these cells in barrier regulation at this site. Interestingly, in adults, this increase in permeability was mediated by endothelial transcytosis, which was reduced following activation of platelet derived growth factor (PDGFB) signaling [1].

Studies performed in developmental systems have further demonstrated a role for pericytes, and specifically PDGFRB signaling in the development of the blood brain barrier shortly after birth[2]. Pericytes were shown to alter endothelial expression by suppression of molecules that increased vascular permeability [2]. In particular, presence

of pericytes is necessary to regulate the balance between angiopoietin-1 (high) and 2 (low) and thus control pro-permeability signals. Furthermore, through heterotypic cell interactions, pericytes instruct endothelial cell expression to suppress immune surveillance, a central feature of the blood brain barrier. Thus, absence of pericytes in Pdfrb-/- mice yield vessels with robust endothelial expression of IcamI, Alcam and Lgals3 [2]. Combined, these experiments provide strong evidence that pericytes contribute to the stabilization of the endothelial barrier, particularly in the brain, through regulation of endothelial expression and transendothelial transport to enhance barrier stability and suppress inflammation. Although there is much to be uncovered on the molecular cross-talk between endothelial cells and pericytes, the strong in vivo evidence indicating regulation of transcellular transport by pericytes points to a complex signaling circuitry that links heterotypic cell interactions with mechanisms of vesicular transport [3].

Transcellular Permeability

Transcellular permeability is defined as an energy-dependent trafficking of macromolecules from the luminal space to the interstitium by means of vesicular transport. This transport can occur through: (a) caveolae; (b) vesiculo-vacuolar organelles (VVOs) and/or (c) transcellular channels [4,5] (Figure 2.1 and 2.2). In this review, we will highlight the conceptual advancements made in caveolae–mediated transport, and specifically focus on the integrative links between transcellular and paracellular transport.

Caveolae are vesicles with high levels of caveolin-1 (Cav-1). This structural protein is critical for caveolae formation, as deletion of Cav-1 in mice results in the reduction of

albumin transport [6,7]. More recently, it has become evident that along with its structural role, Cav-1 acts as a scaffold protein, recruiting Src kinase and G proteins to caveolae [8]. As such, caveolae are capable of internalizing cell surface macromolecular complexes and participating in cell signaling. Nonetheless, the extent to what this signaling function plays into regulation of vascular transport is yet to be clarified.

Endothelial signaling can affect transcellular trafficking through the phosphorylation of Cav-1 on Y14. Specifically src kinases have been shown to mediate Cav-1 phosphorylation downstream of growth factor signaling or upon generation of reactive oxygen species [9]. While the role of phosphorylation on Cav-1 has been controversial, recent findings have shown that Cav-1 tyrosine phosphorylation is necessary for caveola biogenesis through a direct feedback loop that inactivates Erg-1 (early growth response-1) and thus enables transcription of both Cav-1 and cavin-1 [10].

Studies from Cav-1 null mice have suggested an active cross-talk between transcellular and paracellular pathways. Specifically, complete deletion, as well as, transient siRNA knockdown of Cav-1 result in an increased paracellular transport of albumin in small capillaries and veins [11,12]. This elevation in paracellular permeability, a possible compensatory mechanism for the impaired transcellular transport, was accompanied by abnormal tight junction assembly, detachment of endothelial cells from the basement membrane, and increased NO production [6,11,12]. eNOS inhibition restored junctional integrity in Cav-1 null mice, suggesting that eNOS-dependent redox signaling may indirectly mediate changes in paracellular permeability [11] (Figure 2.2). Recent elegant work has found that NO and peroxynitrile generation in the absence of Cav-1 promotes nitration of p190RhoGAP-A resulting in impaired GAP activity and subsequent

RhoA activation. This increase in active RhoA is responsible for the destabilization of adherens junctions and increase in paracellular transport [13]. These findings provided the missing molecular link to explain the cross-talk between Cav-1 and paracellular junctional complexes. In addition, Cav-1 may affect junctional integrity directly, through its interactions with Src, PKC, claudin-5, and actin-binding proteins, which are all involved in adherens and tight junction assembly and maintenance [8,14]. Cav-1 also binds TRPC1, a calcium transport channel important for the intracellular calcium release underlying actin-myosin remodeling [15,16].

Consistent with the notion that Cav-1 regulates junctional complexes, it is interesting that Cav-1 levels are lower in postcapillary venules [17]. Unlike arteries, venules display increased basal paracellular permeability and exhibit “unstable” junctional complexes (see discussion of constitutive VE-cadherin phosphorylation). These findings might imply that differences in Cav-1 levels may determine whether certain vascular beds are more amenable to increased transcytosis.

Together these findings described above provide molecular basis to the concept that transcellular vesicular trafficking, specifically through the contribution of Cav-1, regulates junctional integrity, and therefore, paracellular permeability in endothelial cells.

Paracellular Permeability

As alluded to previously, maintenance of barrier function also requires the dynamic opening and closing of inter-endothelial junctions, which consist of a complex network of adherent proteins organized into adherens junctions and tight junctions.

Although originally considered structural in function, it is now clear that the anchorage of adherens and tight junctions to the actin cytoskeleton allows the direct transmission of signaling events critical not only for barrier stability but also for the regulation of cell polarity, cellular movement, fluid sensing, and cell-contact inhibition [18]. The distribution and predominance of junctional proteins at inter-endothelial contacts varies between different vascular beds, which suggest that junctional arrangement is unique to the functional needs of specific vascular networks [18,19].

Adherens junctions are mostly formed by the clustering of homophilic calcium-dependent VE-cadherin proteins. The stability of VE-Cadherin complexes between adjacent cells is regulated by phosphorylation. In fact, exposure of endothelial cells to several permeability mediators, such as VEGF, histamine and thrombin, results in tyrosine phosphorylation of VE-cadherin at Y658 and Y731, which correspond to the binding sites for p120 and β-catenin respectively [20]. Phosphorylation of VE-Cadherin results in internalization of the protein and disruption of barrier integrity resulting in vascular permeability. In addition, three other tyrosines (Y645, Y685, and Y733) and one serine (S665) have been reported to be potentially phosphorylated in vitro and participate in the regulation of permeability and leukocyte transmigration [20-23]. Recently, a sophisticated study has demonstrated that phosphorylation of tyrosine residues 658 and 685 of VE-Cadherin is constitutive in veins, but not in arteries. This phosphorylation is mediated by src and can be enhanced in response to bradykinin or histamine. More importantly, point mutations, Y658F and Y685F, prevent internalization of VE-Cadherin and thus block vascular permeability [24]. The endogenous phosphatase for VE-Cadherin, VE-PTP is frequently associated with the protein and prevents VE-cadherin

phosphorylation, promoting an important stabilizing role of endothelial contacts in vivo [25,26].

Intracellularly, VE-cadherin is directly and indirectly bound to a complex network of proteins including catenins, actin binding proteins, RhoGTPases, kinases, and phosphatases, that are important for its tethering and signaling to the actin cytoskeleton [4,18] (Figure 2.2). While β-catenin and plakoglobin prevent VE-cadherin proteolysis, p120-catenin alters retention of VE-cadherin at the cell surface [8,27]. Additionally, β-catenin and p120 are also critical for spatial organization and control of the actin cytoskeleton by way of RhoGTPase activation (p190RhoGAP, Rac1, Cdc42, RhoA), and α-catenin recruitment [18]. Mice engineered to express a VE-cadherin-α-catenin fusion protein developed strong stable junctions, highlighting the relevance of plasticity of cadherin-catenin complexes in the regulation of permeability [28].

Phosphorylation of other adherens junctional components also modulate the affinity of adherens junction complex components for one another, thus affecting junctional stability [8]. Whether these phosphorylation events are important for barrier regulation in vivo is only beginning to be understood. Recently, the generation of a serine phosphodeficient p120 mouse demonstrated the requirement of PKCα mediated p120 phosphorylation for p120/VE-cadherin dissociation following thrombin and lipopolysaccharide (LPS) stimulated permeability [29]. Therefore, generation of phospho-mutant mice will enable dissection of the molecular events that are downstream of individual permeability mediators in vivo.

Major tight junction proteins include claudin-5, occludin, and junctional adhesion molecules (JAM). Similar to adherens junctions, phosphorylation of both tight junction

proteins and their intracellular partners (ZO-1 and MAGUKS) regulate tight junction assembly and mediate changes in vascular permeability [30,31]. Although complete deletion of claudin-5 leads to early lethality shortly after birth due to blood-brain barrier disruption, occludin knock out mice have no apparent defects, making its function in endothelial tight junctions less obvious [32].

While all JAM members are present in endothelial cells, only JAM-C leads to increased permeability when expressed at the cell surface of microvascular cells following stimulation with VEGF or histamine [33]. Although the mechanism of JAM-C mediated permeability is still unclear, recent evidence suggests that JAM-C regulation of αVβ3 integrin localization and activation downstream of Rap1b signaling may account for barrier breakdown [34]. JAM-like molecules have also been implicated in permeability regulation, as mice null for endothelial cell-selective adhesion molecule showed reduced vascular permeability to VEGF [35].

Emerging data has indicated that adherens and tight junctions do not function independently in the regulation of barrier function, and in fact, communication between these complexes is important for permeability regulation. Akt activation downstream of VE-cadherin cell surface clustering results in nuclear expulsion of the transcription factor FoxO1. FoxO1 normally inhibits claudin-5 expression, thus translocation from the nucleus results in enhanced claudin-5 expression at tight junctions [36]. Furthermore, VE-cadherin transmits shear stress signals to stabilize occludin through recruitment of Tiam1/Rac-1 and mediates reduction of occludin phosphorylation [37]. These findings place VE-cadherin as a key sensor and molecular integrator of adherens and tight junctions.

Overall, the data indicates that intracellular signaling cascades and homeostasis of barrier function are more linked than previously appreciated. Furthermore, the interaction between adherens and tight junctions reveals an exquisite level of molecular regulation that is only now starting to be unraveled.

Signaling Mechanisms and Intracellular Regulation of Vascular Permeability

Most mediators of permeability lead to phosphorylation of junctional proteins and reorganization of the acto-myosin apparatus, although these consequences can occur downstream of different signal transduction pathways (Figure 2.3). The kinetics of these changes varies between permeability agents, as some can lead to transient and reversible effects, as in the case of thrombin and histamine, or sustained and prolonged regulation, as seen with VEGF and LPS stimulation. This section will review recent literature on some of the most well studied permeability agents and the signaling pathways that discern them.

1. Inflammatory mediators

Histamine, thrombin, and bradykinin exposure result in a transient increase in vascular permeability followed by barrier stabilization. Thrombin signaling through its receptor, PAR-1, yields a transient increase in vascular permeability which is followed by an equally rapid restoration. PAR-1 activates several downstream G proteins, which promotes intracellular calcium release, RhoA-dependent activation of myosin light chain kinase and cell contraction [38]. Rho activation leads to stress fiber assembly and cell contraction, a mechanism that is responsible for enhancement of permeability.

Histamine also promotes permeability through calcium release and myosin light chain kinase activation, but in addition, mediates src dependent phosphorylation of adherens and tight junction proteins. Conversely, bradykinin, acting through B1 and B2 receptors, results in an eNOS/iNOS dependent increase in permeability, although it is unclear whether nitrosylation of junctional proteins following increased NO production leads to barrier destabilization [4,39].

Long term mediators of permeability such as LPS and TNF-α, result in NF-κB transcriptional expression of cytokines and leukocyte adhesion molecules. ICAM-1 cell surface activation results in RhoA directed stress fiber formation as well as increased NO production, which further potentiates increased permeability [39].

2. Vascular endothelial growth factor

VEGF induces vascular permeability by several mechanisms, including junctional remodeling, induction of fenestrae, and VVOs [40]. VEGF concurrently activates multiple signaling pathways downstream of VEGFR2 that have been implicated in vascular permeability. These include PLC dependent intracellular calcium release, src kinase-mediated phosphorylation/internalization of junctional proteins, RhoGTPase activation, cytoskeletal rearrangement, and eNOS signaling [41]. More recently in vivo data has demonstrated the requirement for VE-PTP/VE-cadherin dissociation [36,42] and FAK dependent β-catenin phosphorylation [43] in VEGF-mediated permeability. Furthermore, the T-cell specific adapter, TSAd was found to be essential for src activation and subsequent phosphorylation of junctional proteins downstream of VEGFR2 [44]. The contribution of eNOS signaling upon VEGFR2 activation has remained elusive, but

recent data suggest that nitrosylation of β-catenin by NO may be an additional mechanism of junctional destabilization by mediating the dissociation of β-catenin from VE-cadherin [45].

Although there have been great strides in understanding the molecular players that coordinate permeability downstream of VEGF, the complexity of the signaling networks has made it difficult to understand how all of these pathways interact to control barrier function. Furthermore, quantitative assessment of each of these signaling pathways in vivo has not been obtained. It is possible that subsets of downstream mediators are activated in distinct vascular beds or under different physiological contexts upon VEGF exposure. With regards to angiogenesis, the in vivo presentation of VEGF isoforms (in the context of matrix or soluble) results in differential signal transduction outputs [46,47]. It is likely that similar nuances part-take in the regulation of vascular permeability by VEGF.

3. Angiopoietin/Tie Receptor Signaling

Tie receptors and their ligands (Ang1-4) are critical regulators of vascular maturation and quiescence [48]. Tie-2 is constitutively phosphorylated upon binding to Ang-1 in mature vessels. In fact, Ang-1 secretion from perivascular cells is important for maintaining vascular stability and endothelial cell adhesion while inhibiting vascular permeability [49,50]. Ang-1/Tie-2 signaling has been shown to inhibit VEGF-mediated vascular permeability via several downstream signaling cascades. These include p190RhoGAP driven cytoskeletal modulation [51], sequestration of Src from VEGFR2 by

the RhoGTPase effector protein mDia [52], inhibition of calcium release [53], and phosphorylation of eNOS by atypical PKC-zeta [54].

One of the main questions in understanding Ang-1 signaling is how it can orchestrate both vascular remodeling and quiescence by signaling through the same receptor. Recent evidence suggests that Ang-1 stimulation leads to differential Tie-2 localization and signaling depending on whether endothelial cells have engaged cell-cell contacts or not. Homotypic cell interactions between endothelial cells trigger recruitment of Tie2 to cell-cell contacts upon Ang-1 exposure leading to enhanced vascular stability following Akt mediated eNOS phosphorylation. In contrast, migrating endothelial cells displayed Dok-R phosphorylation and Tie-2 recruitment to the cell rear [55,56].

Endothelial produced Ang-2, is considered to be the natural antagonist of Ang-1 activity by inhibiting phosphorylation of Tie-2 [48]. Thus, Ang-2 sensitizes the endothelium to both growth factors and inflammatory mediators, which increase vascular destabilization [57]. The mechanism of Ang-2 action is not fully understood, but recent evidence suggests that Ang-2 regulates Tie-2 interaction with αVβ3 integrin, resulting in FAK activation and consequent integrin internalization and degradation [58]. Although both Ang-1 and Ang-2 mediate Tie-2 clustering at cell-cell contacts, their differential signaling may explain their opposing effects on vascular stability. Several groups have also demonstrated that Ang-2 can act as a partial agonist of Tie-2 signaling through Tie-2 phosphorylation [59,60] and can enhance barrier function following endothelial stress [61]. Generation of mice with endothelial specific deletion of Ang-2 will help to address its physiological role in vivo and enable a better understanding of the homeostatic functions of Ang-2 in the endothelium.

Barrier Stabilization

Barrier restoration is critical for maintenance of basal permeability and recovery following exposure to acute inflammatory events, yet our understanding of how this process occurs at the molecular level has remained elusive. Here, we discuss some of the mediators of barrier stability and their known mechanisms of action (Figure 2.4).

Although the mechanisms of barrier breakdown following thrombin have been well studied, the actual process whereby the endothelial barrier is stabilized quickly thereafter has only begun to be understood. Recent evidence has demonstrated that a G protein downstream of thrombin activation Gß1, increases barrier stabilization by redistribution of focal adhesion kinase (FAK) to adherens junctions following Fyn-induced phosphorylation of FAK [62] (Figure 2.4C). How FAK becomes targeted to adherens junctions remains to be clarified.

Signaling via cAMP also contributes to the regulation of barrier function. Increases in cAMP levels downstream of the G protein, Gαs, reduces vascular leakage through activation of protein kinase A (PKA) and the guanine exchange factor, Epac (Figure 2.4C). Epac mediated Rap1 activation results in increased junctional adhesions and reorganization of actin filaments [63]. Emerging evidence on Rap1 suggests it has a cooperative association with VE-Cadherin as they can both modulate each other's responses [64,65]. Interestingly, Rap1 can increase KRIT-1 targeting to endothelial cell-cell junctions, suppressing stress fiber formation and stabilizing junctional integrity. Thus, defects in Rap1 signaling downstream of mutated KRIT-1 protein may explain the loss of vascular integrity seen in cerebral cavernous malformation [66].

More recently, fibroblast growth factor (FGF) has been found to play an important role in adherens junction integrity. Absence of FGF signaling was found to reduce expression of the phosphatase, SHP2, resulting in increased phosphorylation of VE-cadherin, impairing its ability to bind p120 catenin [67,68] (Figure 2.4A). VE-cadherin itself can affect barrier stability by inhibiting growth factor signaling pathways including VEGF, TGFB, and PDGF, which promote permeability following angiogenic responses [19] (Figure 2.4B).

Another emerging and potent barrier stabilizing factor is sphingosine-1-phosphate (S1P). S1P circulates at high levels in the blood and signals through the G-coupled protein receptor S1P1 to mediate cortical actin organization via a number of downstream targets including Rac-1, cortactin, FAK, paxillin, and actinin 1 and 4 [8,38,69] (Figure 2.4D). Two recent studies have unequivocally demonstrated the effect of S1P in barrier stability in vivo. Pharmacological or genetic blockade of the S1P signaling axis results in adherens junctions destabilization, permeability and in some cases angiogenesis [70,71].

Unlike thrombin, S1P signals exclusively through the G protein Gi. Gi activation leads to PLC dependent calcium release, which is necessary for FAK phosphorylation [38]. FAK activation is required for barrier integrity, as impairment of FAK function leads to increased endothelial permeability and subsequently abrogates S1P barrier enhancement [38,69,72]. Although S1P signals through a different G protein cascade, Fyn activation of FAK as seen downstream of thrombin, may also play a role in S1P signaling.

Interestingly, FAK has been shown to both preserve and disrupt the endothelial barrier [43,73,74]. This dual ability has been proposed to be regulated by other events including: differential post-translational modifications, spatial/temporal activation, cellular localization, or association with binding partners [38,75]. Recent evidence in support of this, demonstrated that alternative phosphorylation and cellular localization of FAK contribute to the differential mode of barrier restoration seen following thrombin and S1P stimulation [62,69,76].

Transcriptional Mechanisms of Barrier Regulation

While most events in the regulation of barrier function are non-transcriptional in nature, evidence that transcriptional activation/repression is also required has recently been revealed (Figure 2.5). Both shear stress and Ang-1 signaling to Akt mediates endothelial quiescence by FoxO1 phosphorylation and subsequent exclusion from the nucleus (Figure 2.5A). FoxO1 target genes include Ang-2 and genes important for matrix remodeling and migration. Thus, inhibition of FoxO1 is important for restricting the expression of barrier destabilizing proteins [77,78] (Figure 2.5C). In addition, interaction of FoxO1 with β-catenin and Tcf was found to transcriptionally repress claudin-5 expression. VE-cadherin sequestration of β-catenin from the nucleus inhibits its association with FoxO1, enabling Claudin-5 expression and junctional stability [36] (Figure 2.5B). Conversely, another forkhead member, FoxM1, positively regulates β-catenin expression (Figure 2.5F). Surprisingly basal permeability was not affected following endothelial deletion of FoxM1 in vivo, however barrier stability could not be restored following thrombin treatment [69].

The Krüppel-like family member, KLF4, directly binds the VE-cadherin promoter and upregulates its expression. Basal permeability is increased following KLF4 knockdown in vitro and in mouse lung microvasculature [79] (Figure 2.5D). Analogously, KLF2 also stabilizes barrier function, as heterozygous loss of KLF2 in mice leads to increased basal permeability and exacerbated barrier disruption upon addition of histamine and H_2O_2. How KLF2 mediates barrier function and whether this requires transcriptional activation of KLF2 was not determined [80].

In addition to junctional proteins, elements of the cytoskeleton and regulation of its dynamics are essential to the initiation and restoration of vascular permeability. Along these lines, the contribution of small GTPases, as means of controlling contractility and dynamics of the cytoskeleton, have received significant attention [81-83]. Transcriptional regulation of RhoGTPases was found to be coordinated by factors that regulate multiple aspects of the permeability response. For example, CREB (cAMP response element binding) directly regulates p190RhoGAP, a RhoA inhibitor important for barrier stabilization (Figure 2.5E). In support of this, in vivo expression of endothelial dominant negative CREB enhanced basal permeability and exacerbated the response to thrombin and LPS [84].

Recently, two nuclear hormone receptors, Nur77 and estrogen receptor, have also been implicated in barrier regulation. Nur77 is increased upon exposure to VEGF, histamine, and serotonin resulting in barrier destabilization through the downregulation of several adherent junctional components. Transcriptional activity of Nur77 was found to be required, but whether Nur77 directly binds to the promoters of these adhesion molecules was not addressed [85]. Interestingly, estrogen signaling through the estrogen

receptor directly upregulates claudin-5 expression and thus, possibly is important for barrier stability and restoration [86]. It is apparent from these results that regulation of barrier function cannot simply be explained by transient signaling events. Additional research on transcriptional mediation may reveal further insight into the complexities of permeability regulation.

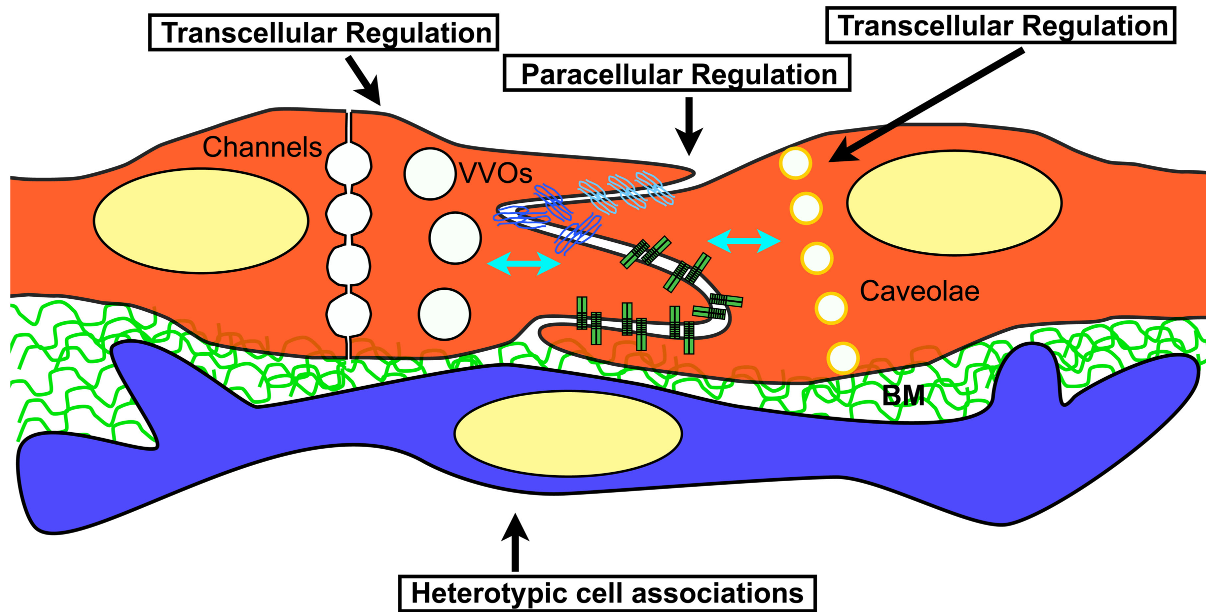


Figure 2.1. Pathways that regulate barrier function in endothelial cells
Scheme shows two endothelial cells and the subendothelial space. Vascular permeability is regulated and maintained through three compartments including: paracellular junctions (adherent and junctional complexes), transcellular pathways (channels, vesiculo-vacuolar organelles (VVOs) and caveolae) and heterotypic cell interactions (usually pericytes). The three pathways are interconnected molecularly (blue arrows), however the details of this cross-talk remain largely unclear. BM: Basement membrane

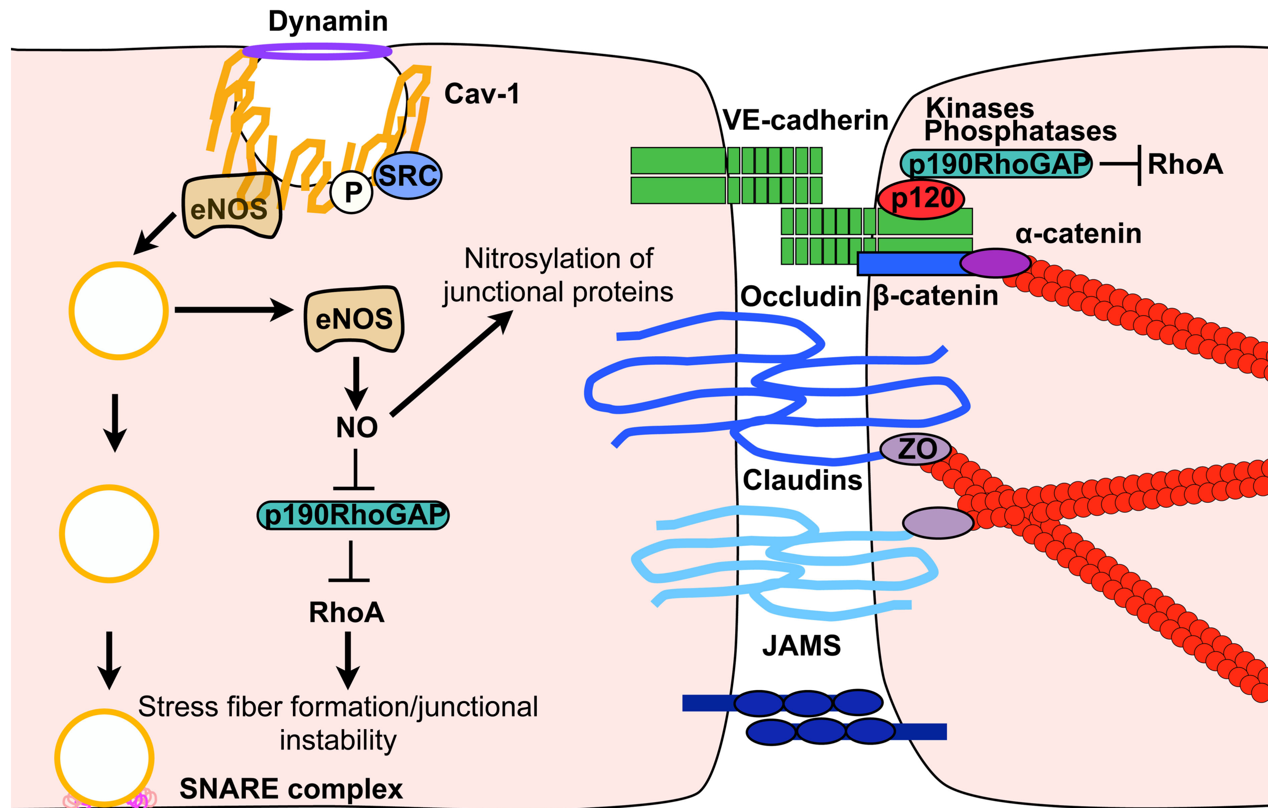


Figure 2.2. Cross talk between transcellular trafficking and paracellular junctional complexes

Caveolae fission and loss of Cav-1 enhance eNOS mediated NO production. Nitrosylation of p190RhoGAP impairs inhibition of RhoA, resulting in stress fiber formation, junctional instability, and increased paracellular permeability. Direct nitrosylation of junctional proteins may also regulate junctional disassembly.

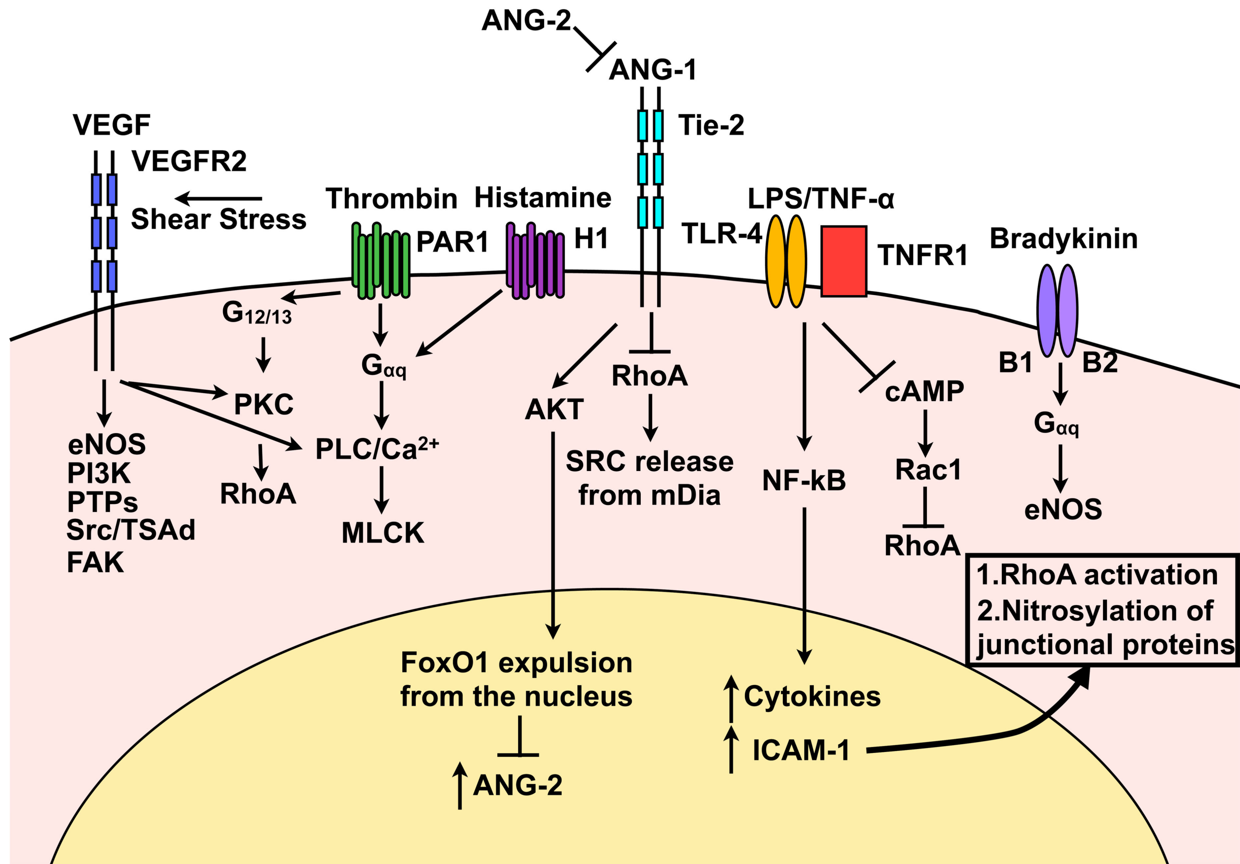


Figure 2.3. Signal transduction pathways that increase paracellular permeability VEGF activation of VEGFR2 initiates several downstream signaling cascades leading to adherens protein internalization, calcium release, and stress fiber formation. Thrombin and histamine, via G-protein coupled receptors, results in RhoA activation, calcium release and the development of stress fibers. Ang-2 inhibits the barrier stabilizing effect of Ang-1 thus making the barrier vulnerable to permeability enhancing agents. LPS and TNF-α signaling result in NF-κB nuclear translocation where increased ICAM-1 expression leads to RhoA activation and NO mediated nitrosylation of junctional proteins. Bradykinin promotes eNOS signaling.

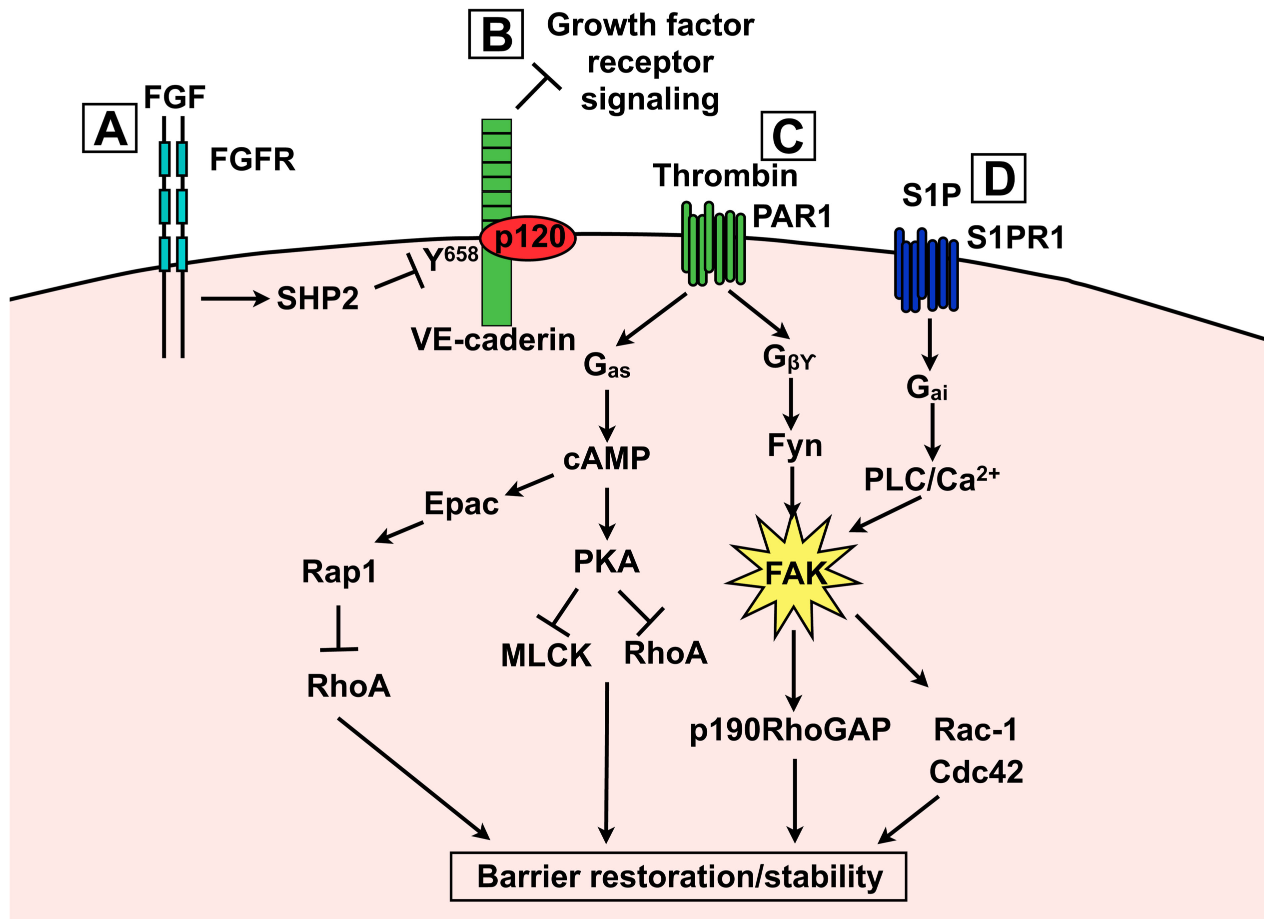


Figure 2.4. Signaling mechanisms leading to enhanced barrier stability and restoration

(A) FGF signaling increases basal barrier function through SHP2 phosphatase mediated p120/VE-cadherin complex stabilization. **(B)** VE-cadherin can inhibit growth factor receptors that normally enhance permeability. **(C)** $G_{\beta\gamma}$ and G_{as} signal transduction results in FAK, Epac/Rap1 and PKA activation. All three of these targets coordinate to increase cortical actin and stabilize junctional complexes. **(D)** S1P signaling leads to FAK phosphorylation via a PLC dependent mechanism. Differential phosphorylation and cellular recruitment of FAK may explain how thrombin and S1P regulate FAK by distinct mechanisms.

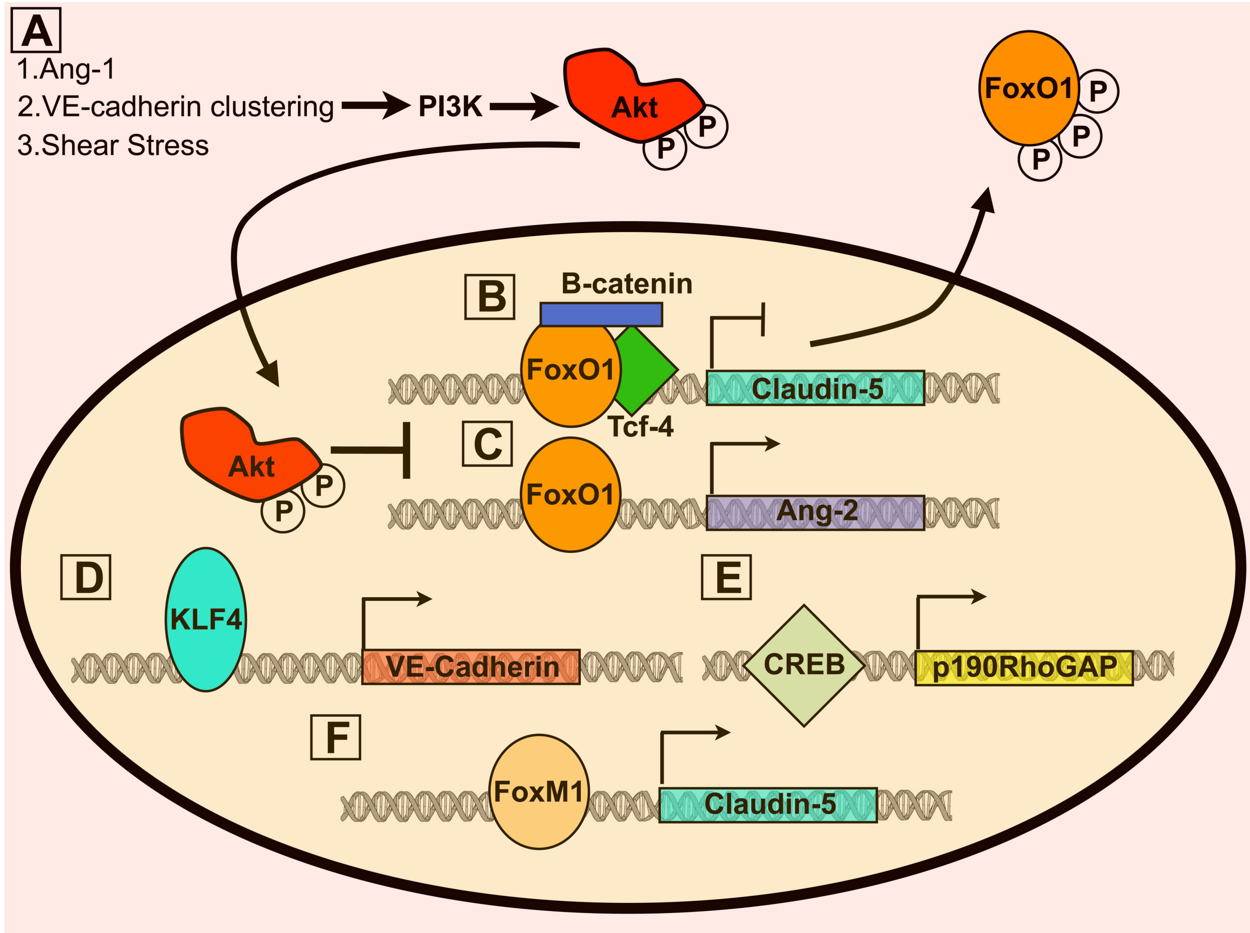


Figure 2.5. Transcriptional regulation of vascular permeability
(A) Akt phosphorylation by several stimuli results in FoxO1 phosphorylation and nuclear translocation. Subsequently, FoxO1 target genes including Claudin-5 **(B)** and Ang-2 **(C)** are expressed and repressed, respectively. Claudin-5 inhibition by FoxO1 requires complex formation between β-catenin and Tcf. Therefore, VE-cadherin/β-catenin complex formation is important for Claudin-5 expression as it prevents β-catenin translocation to the nucleus. **(D)** KLF4 stabilizes the barrier by enhancing expression of VE-cadherin. **(E)** CREB upregulates p190RhoGAP, which is important for inhibiting RhoA activation at adherens junctions. **(F)** Both estrogen receptor (not depicted) and FoxM1 increase Claudin-5 expression, thus promoting barrier stability.

References:

1. Armulik A, Genové G, Mäe M, et al. Pericytes regulate the blood-brain barrier. Nature. 2010; 468: 557–61.

2. Daneman R, Zhou L, Kebede AA, et al. Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature. 2010; 468: 562–6.

3. Armulik A, Genové G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell. 2011; 21: 193-215

4. Mehta D. Signaling Mechanisms Regulating Endothelial Permeability. Physiol Rev. 2006; 86: 279–367.

5. Dvorak AM, Feng D. The vesiculo-vacuolar organelle (VVO). A new endothelial cell permeability organelle. J Histochem Cytochem. 2001; 49: 419–32.

6. Razani B, Engelman JA, Wang XB, et al. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. J Biol Chem. 2001; 276: 38121–38.

7. Schubert W, Frank PG, Razani B, et al. Caveolae-deficient endothelial cells show defects in the uptake and transport of albumin in vivo. J Biol Chem. 2001; 276: 48619–22.

8. Komarova Y, Malik AB. Regulation of endothelial permeability via paracellular and transcellular transport pathways. Annu Rev Physiol. 2010; 72: 463–93.

9. Sun Y, Hu G, Zhang X et al. Phosphorylation of caveolin-1 regulates oxidant-induced pulmonary vascular permeability via paracellular and transcellular pathways. Circ Res. 2009; 105: 676-85

10. Joshi B, Bastiani M, Strgnell SS, et al. Phosphocaveolin-1 is a mechanotransducer that induces caveola biogenesis via Egr1 transcriptional regulation. J Cell Biol. 2012; 199: 425-35

11. Schubert W, Frank PG, Woodman SE, et al. Microvascular hyperpermeability in caveolin-1 (-/-) knock-out mice. Treatment with a specific nitric-oxide synthase inhibitor, L-NAME, restores normal microvascular permeability in Cav-1 null mice. J Biol Chem. 2002; 277: 40091–8.

12. Miyawaki-Shimizu K, Predescu D, Shimizu J, et al. siRNA-induced caveolin-1 knockdown in mice increases lung vascular permeability via the junctional pathway. Am J Physiol Lung Cell Mol Physiol. 2006; 290: L405–13.

13. Siddiqui MR, Komarova YA, Vogel SM, et al. Caveolin-1-eNOS signaling promotes p190RhoGAP-A nitration and endothelial permeability. J Cell Biol. 2011; 30: 841-50

14. Minshall RD, Sessa WC, Stan RV, et al. Caveolin regulation of endothelial function. Am J Physiol Lung Cell Mol Physiol. 2003; 285: L1179–83.

15. Sundivakkam PC, Kwiatek AM, Sharma TT, et al. Caveolin-1 scaffold domain interacts with TRPC1 and IP3R3 to regulate Ca2+ store release-induced Ca2+ entry in endothelial cells. Am J Physiol Cell Physiol. 2009; 296: C403–13.

16. Kwiatek AM, Minshall RD, Cool DR, et al. Caveolin-1 regulates store-operated Ca2+ influx by binding of its scaffolding domain to transient receptor potential channel-1 in endothelial cells. Mol Pharmacol. 2006; 70: 1174–83.

17. Marmon S, Hinchey J, Oh P, et al. Caveolin-1 Expression Determines the Route of Neutrophil Extravasation through Skin Microvasculature. Am J Pathol. 2009; 174: 684–92.

18. Dejana E, Tournier-Lasserve E, Weinstein BM. The Control of Vascular Integrity by Endothelial Cell Junctions: Molecular Basis and Pathological Implications. Dev Cell. 2009; 16: 209–21.

19. Dejana E, Giampietro C. Vascular endothelial-cadherin and vascular stability. Curr Opin Hematol. 2012; 19: 218–23.

20. Potter MD, Barbero S, Cheresh DA. Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenin and maintains the cellular mesenchymal state. J Biol Chem. 2005; 280: 31906–12.

21. Baumeister U, Funke R, Ebnet K, et al. Association of Csk to VE-cadherin and inhibition of cell proliferation. EMBO J. 2005; 24: 1686–95.

22. Gavard J, Gutkind JS. VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat Cell Biol. 2006; 8: 1223–34.

23. Turowski P, Martinelli R, Crawford R, et al. Phosphorylation of vascular endothelial cadherin controls lymphocyte emigration. J Cell Sci. 2008; 121: 29–37.

24. Orsenigo F, Giampietro C, Ferrari A, et al. Phosphorylation of VE-cadherin is modulated by haemodynamic forces and contributes to the regulation of vascular permeability in vivo. Nat Commun. 2012; 3: 1208

25. Vestweber D, Winderlich M, Cagna G, et al. Cell adhesion dynamics at endothelial junctions: VE-cadherin as a major player. Trends Cell Biol. 2009; 19: 8–15.

26. Broermann A, Winderlich M, Block H, et al. Dissociation of VE-PTP from VE-cadherin is required for leukocyte extravasation and for VEGF-induced vascular permeability in vivo. J Exp Med. 2011; 208: 2393–401.

27. Vandenbroucke E, Mehta D, Minshall R, et al. Regulation of Endothelial Junctional Permeability. Ann N Y Acad Sci. 2008; 1123: 134–45.

28. Schulte D, Kuppers V, Dartsch N, et al. Stabilizing the VE-cadherin-catenin complex blocks leukocyte extravasation and vascular permeability. EMBO J. 2011; 30: 4157–70.

29. Vandenbroucke St Amant E, Tauseef M, Vogel SM, et al. PKCα Activation of p120-Catenin Serine 879 Phospho-Switch Disassembles VE-Cadherin Junctions and Disrupts Vascular Integrity. Circ Res. 2012; 111: 739-49.

30. González-Mariscal L, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. Biochim Biophys Acta. 2008; 1778: 729–56.

31. Murakami T, Felinski EA, Antonetti DA. Occludin phosphorylation and ubiquitination regulate tight junction trafficking and vascular endothelial growth factor-induced permeability. J Biol Chem. 2009; 284: 21036–46.

32. Furuse M. Knockout animals and natural mutations as experimental and diagnostic tool for studying tight junction functions in vivo. Biochim Biophys Acta. 2009; 1788: 813–9.

33. Orlova VV, Economopoulou M, Lupu F, et al. Junctional adhesion molecule-C regulates vascular endothelial permeability by modulating VE-cadherin-mediated cell-cell contacts. J Exp Med. 2006; 203: 2703–14.

34. Li X, Stankovic M, Lee BP, et al. JAM-C induces endothelial cell permeability through its association and regulation of {beta}3 integrins. Arterioscler Thromb Vasc Biol. 2009; 29: 1200–6.

35. Wegmann F, Petri B, Khandoga AG, et al. ESAM supports neutrophil extravasation, activation of Rho, and VEGF-induced vascular permeability. J Exp Med. 2006; 203: 1671–7.

36. Taddei A, Giampietro C, Conti A, et al. Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. Nat Cell Biol. 2008; 10: 923–34.

37. Walsh TG, Murphy RP, Fitzpatrick P, et al. Stabilization of brain microvascular endothelial barrier function by shear stress involves VE-cadherin signaling leading to modulation of pTyr-occludin levels. J Cell Physiol. 2011; 226: 3053–63.

38. Thennes T, Mehta D. Heterotrimeric G proteins, focal adhesion kinase, and endothelial barrier function. Microvasc Res. 2012 83: 31-44.

39. Chavez A, Smith M, Mehta D. New insights into the regulation of vascular permeability. Int Rev Cell Mol Biol. 2011; 290: 205–48.

40. Weis SM, Cheresh DA. Pathophysiological consequences of VEGF-induced vascular permeability. Nature. 2005; 437: 497–504.

41. Bates DO. Vascular endothelial growth factors and vascular permeability. Cardiovasc Res. 2010; 87: 262–71.

42. Nottebaum AF, Cagna G, Winderlich M, et al. VE-PTP maintains the endothelial barrier via plakoglobin and becomes dissociated from VE-cadherin by leukocytes and by VEGF. J Exp Med. 2008; 205: 2929–45.

43. Chen XL, Nam JO, Jean C, et al. VEGF-Induced Vascular Permeability Is Mediated by FAK. Dev Cell. 2012; 22: 146–57.

44. Sun Z, Li X, Massena S, et al. VEGFR2 induces c-Src signaling and vascular permeability in vivo via the adaptor protein TSAd. J Exp Med. 2012; 209: 1363-77

45. Thibeault S, Rautureau Y, Oubaha M, et al. S-Nitrosylation of beta-Catenin by eNOS-Derived NO Promotes VEGF-Induced Endothelial Cell Permeability. Mol Cell. 2010; 39: 468–76.

46. Lee SS, Jilani SM, Nikolova GV, et al. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J Cell Biol. 2005; 169: 681–91.

47. Chen TT, Luque A, Lee S, et al. Anchorage of VEGF to the extracellular matrix conveys differential signaling responses to endothelial cells. J Cell Biol. 2010; 188: 595–609.

48. Augustin HG, Koh GY, Thurston G, et al. Control of vascular morphogenesis and homeostasis through the angiopoietin–Tie system. Nat Rev Mol Cell Biol. 2009; 10: 165–77.

49. Wong AL, Haroon ZA, Werner S, et al. Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. Circ Res. 1997; 81: 567-74

50. Thurston G, Rudge JS, Ioffe E, et al. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med. 2000; 6: 460–3.

51. Mammoto T, Parikh SM, Mammoto A, et al. Angiopoietin-1 requires p190 RhoGAP to protect against vascular leakage in vivo. J Biol Chem. 2007; 282: 23910–8.

52. Gavard J, Patel V, Gutkind JS. Angiopoietin-1 prevents VEGF-induced endothelial permeability by sequestering Src through mDia. Dev Cell. 2008; 14: 25–36.

53. Jho D, Mehta D, Ahmmed G, et al.. Angiopoietin-1 Opposes VEGF-Induced Increase in Endothelial Permeability by Inhibiting TRPC1-Dependent Ca2 Influx. Circ Res. 2005; 96: 1282–90.

54. Oubaha M, Gratton JP. Phosphorylation of endothelial nitric oxide synthase by atypical PKC zeta contributes to angiopoietin-1-dependent inhibition of VEGF-induced endothelial permeability in vitro. Blood. 2009; 114: 3343–51.

55. Saharinen P, Eklund L, Miettinen J, et al. Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. Nat Cell Biol. 2008; 10: 527–37.

56. Fukuhara S, Sako K, Minami T, et al. Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1. Nat Cell Biol. 2008; 10: 513–26.

57. Scharpfenecker M, Fiedler U, Reiss Y, et al. The Tie-2 ligand angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. J. Cell Sci. 2005; 118: 771–80.

58. Thomas M, Felcht M, Kruse K, et al. Angiopoietin-2 stimulation of endothelial cells induces alphavbeta3 integrin internalization and degradation. J Biol Chem. 2010; 285: 23842–9.

59. Yuan HT, Khankin EV, Karumanchi SA, et al. Angiopoietin 2 is a partial agonist/antagonist of Tie2 signaling in the endothelium. Mol Cell Biol. 2009; 29: 2011–22.

60. Harfouche R, Hussain SN. Signaling and regulation of endothelial cell survival by angiopoietin-2. Am J Physiol Heart Circ Physiol. 2006; 291: H1635–45.

61. Daly C, Pasnikowski E, Burova E, et al. Angiopoietin-2 functions as an autocrine protective factor in stressed endothelial cells. Proc Natl Acad Sci U S A. 2006; 103: 15491–6.

62. Knezevic N, Tauseef M, Thennes T, et al. The G protein betagamma subunit mediates reannealing of adherens junctions to reverse endothelial permeability increase by thrombin. J Exp Med. 2009; 206: 2761–77.

63. Cullere X, Shaw SK, Andersson L, et al. Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood. 2005; 105: 1950–5.

64. Fukuhara S, Sakurai A, Sano H, et al. Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway. Mol Cell Biol. 2005; 25: 136–46.

65. Kooistra MR, Dube N, Bos JL. Rap1: a key regulator in cell-cell junction formation. J Cell Sci. 2007; 120: 17–22.

66. Glading A, Han J, Stockton RA, et al. KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. J Cell Biol. 2007; 179: 247–54.

67. Murakami M, Nguyen LT, Zhuang ZW, et al. The FGF system has a key role in regulating vascular integrity. J Clin Invest. 2008; 118: 3355–66.

68. Hatanaka K, Lanahan A, Murakami M, et al. Fibroblast Growth Factor Signaling Potentiates VE-Cadherin Stability at Adherens Junctions by Regulating SHP2. PLoS One. 2012; 7: e37600.

69. Sun X, Shikata Y, Wang L, et al. Enhanced interaction between focal adhesion and adherens junction proteins: involvement in sphingosine 1-phosphate-induced endothelial barrier enhancement. Microvasc Res. 2009; 77: 304–13.

70. Jung B, Obinata H, Galvani S et al. Flow-regulated endothelial S1P receptor-1 signaling sustains vascular development. Dev Cell. 2012; 23: 600-10

71. Gaengel K, Niaudet C, Hagikura K et al. The sphingosine-1-phosphate receptor S1PR1 restricts sprouting angiogenesis by regulating the interplay between VE-cadherin and VEGFR2. Dev Cell. 2012; 23: 587-99

72. Shikata Y, Birukov KG, Garcia JG. S1P induces FA remodeling in human pulmonary endothelial cells: role of Rac, GIT1, FAK, and paxillin. J Appl Physiol. 2003; 94: 1193–203.

73. Guo M, Wu MH, Granger HJ, et al. Focal adhesion kinase in neutrophil-induced microvascular hyperpermeability. Microcirculation. 2005; 12: 223–32.

74. Lee YH, Kayyali US, Sousa AM, et al. Transforming growth factor-beta1 effects on endothelial monolayer permeability involve focal adhesion kinase/Src. Am J Respir Cell Mol Biol. 2007; 37: 485–93.

75. Belvitch P, Dudek SM. Role of FAK in S1P-regulated endothelial permeability. Microvasc Res. 2012; 83: 22–30.

76. Shikata Y, Birukov KG, Birukova AA, et al. Involvement of site-specific FAK phosphorylation in sphingosine-1 phosphate- and thrombin-induced focal adhesion remodeling: role of Src and GIT. FASEB J. 2003; 17: 2240–9.

77. Daly C, Wong V, Burova E, et al. Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). Genes Dev. 2004; 18: 1060–71.

78. Dixit M, Bess E, Fisslthaler B, et al. Shear stress-induced activation of the AMP-activated protein kinase regulates FoxO1a and angiopoietin-2 in endothelial cells. Cardiovasc Res. 2008; 77: 160–8.

79. Cowan CE, Kohler EE, Dugan TA, et al. Kruppel-Like Factor-4 Transcriptionally Regulates VE-Cadherin Expression and Endothelial Barrier Function. Circ Res. 2010; 107: 959–66.

80. Lin Z, Natesan V, Shi H, et al. Kruppel-like factor 2 regulates endothelial barrier function. Arterioscler Thromb Vasc Biol. 2010; 30: 1952–9.

81. Haidari M, Zhang W, Chen Z, et al. Myosin light chain phosphorylation facilitates monocyte transendothelial migration by dissociating endothelial adherens junctions. Cardiovasc Res. 2011; 92: 456–65.

82. David S, Ghosh CC, Mukherjee A, et al. Angiopoietin-1 Requires IQ Domain GTPase-Activating Protein 1 to Activate Rac1 and Promote Endothelial Barrier Defense. Arterioscler Thromb Vasc. Biol. 2011; 31: 2643–52.

83. Peng J, He F, Zhang C, et al. Protein kinase Ca signals P115RhoGEF phosphorylation and RhoA activation in TNF-a-induced mouse brain microvascular endothelial cell barrier dysfunction. J Neuroinflammation. 2011; 8: 28

84. Chava KR, Tauseef M, Sharma T, et al. Cyclic AMP response element binding (CREB) protein prevents endothelial permeability increase through transcriptional controlling p190RhoGAP expression. Blood. 2012; 119: 308–19.

85. Zhao D, Qin L, Bourbon PM, et al. Orphan nuclear transcription factor TR3/Nur77 regulates microvessel permeability by targeting endothelial nitric oxide synthase and destabilizing endothelial junctions. Proc Natl Acad Sci U S A. 2011; 108: 12066–71.

86. Burek M, Arias-Loza PA, Roewer N, et al. Claudin-5 as a novel estrogen target in vascular endothelium. Arterioscler Thromb Vasc Biol. 2010; 30: 298–304.

Chapter 3: Physiological Vascular Permeability Requires Induction of Endothelial NR4A1 by Progesterone Receptor

Abstract

Vascular permeability is frequently associated with inflammation and triggered by a cohort of secreted permeability factors such as VEGF. Here we show that the physiological vascular permeability that precedes implantation is directly controlled by progesterone receptor (PR) and is independent of VEGF. Both global and endothelial-specific deletion of PR block physiological vascular permeability in the uterus, while misexpression of PR in the endothelium of other organs results in ectopic vascular leakage. Integration of an endothelial genome-wide transcriptional profile with ChIP-sequencing revealed that PR induces a NR4A1 (Nur77/TR3)-dependent transcriptional program that broadly regulates vascular permeability in response to progesterone. Silencing of NR4A1 blocks PR-mediated permeability responses indicating a direct link between PR and NR4A1. This program triggers concurrent suppression of several junctional proteins and leads to an effective, timely and venous-specific regulation of vascular barrier function.

Introduction

The endothelium constitutes a highly specialized cell population that lines the inner layer of the vascular tree. The particular location of blood vessels imposes functional demands, peculiar to each organ, that exceed its well-accepted role as a barrier and non-thrombogenic surface. To accommodate organ-specific functions, endothelial cells differ in regard to structure, adhesion molecules, metabolic properties, antigenic expression and cell surface determinants [1,2]. However, we are significantly behind in our understanding of how unique vascular functions are developed and maintained to offer specific properties to individual tissues.

In the endometrium, the vascular demands are enhanced by the cyclical requirement for vascular repair and angiogenesis. The repair and re-growth of the endometrium is driven by the sequential and tightly controlled interplay of steroid hormones. In particular, endometrial angiogenesis appears to be regulated by 17-β estradiol (E), likely through the ER-β receptor as per its high expression in the primate endometrial vascular and perivascular cells [3]. Consistent with this prediction, low concentrations of E induce proliferative and migratory responses in endothelial cells [4]. More importantly, ER-β knockout mice acquire abnormal vascular function and hypertension associated with endothelial dysfunction and impaired angiogenesis [5,6]. Furthermore, β-estradiol regulates expression of VEGF and has been shown to promote vascular expansion in the endometrium of primates [7].

A second unique feature of endometrial vessels is cyclic alterations in vascular permeability. These events result in the recurrent formation of a physiological edema during the second half of the endometrial cycle (secretory phase), a time when

progesterone (P) levels peak [8]. Increased permeability alters the functional endometrium and makes it receptive for embryonic implantation. As part of the decidual response, changes in the degree of permeability parallel the ovarian cycle and are extremely pronounced during pregnancy [9]. The leakage of blood-borne proteins to the interstitium is critical to support the highly metabolic trophoblastic cells and to the survival of the blastocyst. Interestingly, animals that lack PR are unable to mount a decidual response [10,11], placing PR as the upstream coordinator of the cellular and molecular changes that regulate decidualization. These changes include alterations in the stroma, matrix and vasculature [12]. However the program that drives the physiological vascular changes in the endometrium is yet to be explored and appears to be distinct from the one that drives pathological vascular permeability.

In this study, we provide evidence that PR is required within the endothelial compartment to mediate physiological vascular permeability. The resulting edema is independent of VEGF and instead triggered by PR-dependent activation of nuclear receptor subfamily, group A, member 1 (NR4A1), a transcription factor that initiates a unique vascular permeability program through concurrent suppression of several endothelial junctional proteins. Ultimately, through this mechanism, PR is able to selectively target the endometrial vasculature in a coordinated and sustained permeability response.

Results

Complete Deletion of PR Leads to Reduced Physiological Vascular Permeability

To dissect the biological function of PR in the endometrial vasculature, we first examined mice with global deletion of PR (PRKO) and wild-type (WT) littermates. Exposure of WT mice to progesterone resulted in uterine hyperplasia (Figure 3.1A) with a concurrent weight increase of 2.3-fold when compared to vehicle-treated mice (Figure 3.1F). In contrast, PRKO uteri failed to mount an equally significant response (Figure 3.1A,F). Histological analysis revealed similar overall structure between WT and PRKO mice regardless of treatment. Uterine sections stained with a collagen IV antibody or perfused intravascularly with *Lycopersicon esculentum* lectin showed similar vascular density between groups whether treated with vehicle or hormones (Figure 3.1B,C,D). Interestingly, even though hormone treatment led to a similar decrease in cell density in both WT and PRKO animals when compared to respective controls, hormone treated PRKO mice showed an increase in cell density compared to similarly treated WT animals (Figure 3.1E).

As a change in uterine hyperplasia could be due to increased interstitial fluid, we assessed whether the changes in uterine weight were due to permeability and accumulation of plasma proteins extravasated from the vascular compartment. Hormone treatment of WT mice resulted in a 4-fold increase in Evans blue content in the uterus compared to vehicle treated WT mice. This was in contrast to PRKO mice in which there were no differences in uterine permeability between hormone and vehicle treated animals (Figure 3.1G). While inhibition of PR by mifepristone (RU486) blocked the effect of progesterone on uterine weight (Figure 3.1H) and Evans blue extravasation

(Figure 3.1I), inhibitors to VEGFR2 (SU11248) and bradykinin (HOE 140) had no effect. These results suggest that progesterone, through PR, regulates uterine vascular permeability independent of classical pathological permeability mediators.

PR Expression in the Vasculature is Restricted to Endothelial Cells of Veins and Lymphatics of the Uterus and Ovary

As the endothelium is responsible for regulation of vascular permeability, we first evaluated whether the effect of progesterone on barrier function was direct, and through PR expression in endothelial cells. Presence of PR in the vasculature has been a point of debate with a number of publications supporting [13-15] and negating expression in endothelial and smooth muscle cells [16-18]. Using PRLacZ mice (Figure 3.2), which report both PRA and PRB promoter activation, we found that indeed endothelial cells were β-gal positive (Figure 3.3A,B). Interestingly, PR positive endothelial cells were restricted to venules and lymphatics of the uterus and ovary, but absent from arterioles (Figure 3.3A, 3.2G). Smooth muscle cells and/or pericytes were also positive, however β-gal reactivity was equivalent in both arterioles and venules (Figure 3.3A). Hormone priming with β-estradiol and progesterone or PMSG/HCG did not significantly alter endothelial β-gal positivity regardless of whether animals were heterozygous or homozygous for the LacZ allele (Figure 3.3A,E,F). Endothelial cell identity was confirmed by co-staining of +/PRLacZ animals with the endothelial marker PECAM-1 (Figure 3.3B). Under physiological conditions, PR promoter activity was not detected in the vascular beds of any other organs (Figure 3.2H) revealing an exclusive organ-specificity for PR to vessels of the uterus and ovary.

Expression of PR in the vasculature was confirmed at the protein level by

immunohistochemistry. Similar to findings from PRLacZ reporter mice, endothelial cells of veins and lymphatic vessels were positive for both PECAM-1 and PR, while arterial endothelial cells lack PR expression (Figure 3.3C). In contrast, smooth muscle cells of both veins and arteries were PR positive. Similar to mouse uteri, expression of PR in human endometrium was also exclusive to the endothelium of veins (Figure 3.3D).

It should be emphasized that PR expression in the endothelium only occurs in a small subset of cells. On average, at any given time PR positive endothelial cells respectively represent 10% and 14% of total uterine lymphatic and venous endothelial cells per histological cross section (Figure 3.3E). Additionally, 20% of uterine lymphatics and 40% of uterine veins express at least one β-gal positive cell per vessel cross section (Figure 3.3F).

PR Signaling in the Endothelium Promotes Vascular Permeability in vivo

To determine whether the effect in vascular permeability was due to PR activity in endothelial cells, we evaluated cell-specific deletion (PR^{ECKO} mice) (Figure 3.4A,B). Cre expression in the uterus and ovary is completely restricted to the endothelium of the vasculature as determined by β-gal positivity using R26R reporter mice (Figure 3.4C).

Absence of PR in the endothelium did not change vascular density, but revealed slight differences in the size of the uteri similar to that seen between hormone treated WT and PRKO mice (Figure 3.4D). Using the Miles assay, control and PR^{ECKO} mice were examined for changes in permeability following hormone treatment. PR^{ECKO} animals had significantly reduced permeability in comparison to control (Figure 3.4E,F). Interestingly, the ovary of PR^{ECKO} animals also showed a slight, but statistically significant decrease in permeability, consistent with the reduced endothelial PR

expression in comparison to the uterus (Figure 3.4G). In contrast, the duodenum, which lacks PR expression, did not exhibit any significant change in permeability in response to progesterone (Figure 3.4H). We further visualized sites of vascular leakage using *Ricinus communis agglutinin I* (RCAI) lectin. Although PR^{ECKO} animals exhibited some areas of RCAI binding, there was an obvious reduction in RCAI positive sites compared to control uteri (Figure 3.4I).

The effect of endothelial PR in vascular permeability in vivo was further scrutinized by ectopic expression using a transgenic mouse model (Figure 3.5A, Table 1). A myc-tag in the transgene enabled distinction between endogenous mouse PR from the human transgenic PR. Comparison of relative levels of transgenic PR protein confirmed that the lung was by far the site of highest expression followed by the intestine, with complete absence from the kidney, uterus, and heart (Figure 3.5B,C). Interestingly, the uterus did not exhibit transgene expression (Figure 3.5D). Consistent with lack of transgene expression in uteri, progesterone treatment resulted in equivalent extravasation of Evans blue (Figure 3.5E). In contrast, vascular permeability in PRTg lungs was 6-fold greater than baseline, while leakage in the duodenum increased by 1.4 fold (Figure 3.5F,G). Immunohistochemical analysis of RCAI-injected mice revealed barrier dysfunction in the lung following hormone treatment and provided additional support to the Miles assay (Figure 3.5H).

PR Activation in Endothelial Cells Results in Inter-endothelial Gaps and Decreased Endothelial Monolayer Resistance

Having established that endothelial PR promoted vascular permeability in vivo, we returned to in vitro settings to gain mechanistic insights. First we examined human

endometrial endothelial cells (HEEC) that express endogenous PR. Similar to the findings in the murine and human endometrial sections, presence of PR was heterogeneous (Figure 3.6A), an important advantage as it allowed for concurrent assessment of PR negative cells in the same culture. To determine the effect of progesterone on junctional complexes, we used β-catenin immunolocalization. Cell-cell integrity was stable in non-treated (Figure 3.6a) and vehicle treated HEECs (Figure 3.6b). However, progesterone treatment induced translocation of β-catenin away from adherens junctions and resulted in the formation of intercellular gaps only in HEECs expressing PR (Figure 3.6c).

A more comprehensive evaluation of the effect of PR on junctional proteins was performed in umbilical vein endothelial cells (HUVECs) infected with a PR lentivirus (Figure 3.6B). Exposure to progesterone resulted in clear loss of both PECAM-1 and VE-cadherin cell surface expression by 24 hours post-treatment. Adapter proteins, β-catenin and ZO-1 relocated from the cell membrane to the cytosol. These effects were absent from HUVECS expressing GFP only and treated with progesterone, confirming the requirement of PR for these events (Figure 3.7A).

To evaluate the progression of junctional breakdown in real-time, we used Electrical Cell-Substrate Impedance Sensing (ECIS) on endothelial monolayers (Figure 3.6C). Following progesterone treatment, human dermal endothelial cells (HDECs) overexpressing PR exhibit a progressive decrease in resistance, with initial barrier destabilization occurring between 4-8h after progesterone addition (Figure 3.6D). At 17 hours, the reduction in barrier resistance was equivalent to that induced by thrombin (at 30 min), a landmark control for these types of experiments. Notably, in contrast to the

short effect mediated by thrombin, progesterone exposure resulted in persistent and continuous barrier breakdown. Endothelial cells that were treated with vehicle, or infected with an adeno-β-gal construct, did not display these alterations whether in the presence or absence of progesterone (Figure 3.6D).

To confirm that the changes in resistance were due to cellular gaps, we visualized β-catenin expression at 24 hours in the same cells measured by ECIS. As expected, those cells exhibiting a decrease in electrical resistance also displayed discontinuous cell-cell adhesion (Figure 3.7B). Furthermore, the effects on barrier integrity were found to be dose-dependent (Figure 3.6E) and ceased after removal of the stimulus only in the presence of low concentrations of progesterone (Figure 3.6F). Surprisingly, inhibition of classical permeability signaling molecules including Src (Figure 3.7C), PI3K (Figure 3.7D), ROCK (Figure 3.7F), and VEGFR2 (Figure 3.7G) did not inhibit progesterone-induced permeability, nor did taxol-mediated microtubule stabilization (Figure 3.7E), suggesting that a novel mechanism may act downstream of PR.

Endothelial PR Signaling Alters Junctional Protein Expression

Using next generation RNA sequencing**,** we explored the notion that PR signaling may transcriptionally alter the expression of endothelial junctional proteins. Following 4 hours of progesterone treatment, we compared the fold change of several genes known to regulate vascular permeability (Figure 3.8A). As expected, many of the genes that encode proteins important for junctional stability such as VE-cadherin (*CDH5*), VE-PTP (*PTPRB*), PECAM-1, and claudin-5 (*CLDN5*) were reduced upon progesterone exposure. qPCR analysis of VE-cadherin and Claudin-5 confirmed the reduction noted

by RNA-seq (Figure 3.8B, Table 2). Western blot analysis demonstrated significant reduction in junctional protein levels starting at 16 hours post-treatment, supporting the kinetics revealed by HUVEC immunofluorescence (Figure 3.8C,D). β-catenin levels remained unchanged both at the RNA and protein level, which correlated with protein translocation rather than reduction.

To determine whether transcriptional activation and subsequent protein synthesis were required for progesterone-mediated permeability, HUVECs were treated with inhibitors of transcription and translation (Figure 3.8E,F). Both inhibitors completely blocked the decrease in monolayer resistance observed upon progesterone treatment, confirming the requirement for transcriptional regulation and de novo protein synthesis downstream of PR signaling.

PR Directly Binds to the NR4A1 Promoter and Regulates NR4A1 Gene Expression

A concrete elucidation of PR's mechanism of action required us to ascertain the cohort of PR-regulated genes in the endothelium and identify within this cohort the intermediate effector(s). We were led to obtain a global read-out of PR binding sites in the HUVEC genome using chromatin immunoprecipitation (ChIP). In the absence of ligand (PR only) we resolved 525 PR binding sites, while activation of the receptor by progesterone (PR+P) resulted in a much higher number (9,906) of identified PR binding sites, 396 of which overlapped with PR only peaks. To identify genes that might be regulated by PR we next associated PR+P binding sites (9,906) with nearby genes within a 50kb range from transcriptional start sites and identified 3,886 predicted bound genes following progesterone treatment (Figure 3.9A).

To identify possible direct target genes, it was necessary to combine the ChIP-

seq and RNA-seq datasets (Figure 3.9B). This analysis would provide a list of genes that not only contained PR binding sites, but would also include genes whose expression was affected in response to progesterone exposure. RNA-seq analysis of HUVECs yielded 406 upregulated and 431 downregulated genes with a p-value less than 0.01 (Figure 3.9B). These genes were then intersected with the list of 3,886 genes predicted as regulated by the PR binding sites obtained from ChIP-seq evaluation. This analysis showed that 93 (23%) of activated and 214 (49%) of repressed genes are likely direct targets of PR. To identify which biological processes PR might regulate, directly activated (Figure 3.9C) and repressed (Table 3) gene lists were subjected to the DAVID Bioinformatics Database for gene ontology (GO) [19] analysis. Interestingly, transcription was the top term associated with directly upregulated genes (Figure 3.9C). As progesterone mediated permeability requires de novo protein synthesis, we further focused on the 28 transcription factors directly upregulated by PR by examining fold upregulation post progesterone treatment (Figure 3.9D).

Notably, only one of these transcription factors, NR4A1, has been previously implicated in vascular permeability [20]. Using the UCSC genome browser, we evaluated the location of PR binding sites in the NR4A1 locus. Two distinct peaks were found between 10-25kb upstream of the NR4A1 start site in PR+P samples, but not in respective controls (Figure 3.9E). qPCR further confirmed significant NR4A1 upregulation as early as 1 hour after progesterone addition (Figure 3.9F). Interestingly, NR4A1 expression continued to increase and sustained elevated levels as long as 24 hours after progesterone stimulation. To further validate direct PR binding at the NR4A1 locus, intervals putatively containing PR binding, along with a negative control region,

were analyzed by ChIP-qPCR (Figure 3.9G, Table 3.2). PR binding was significantly enriched at both regions corresponding to ChIP-seq peaks, as compared to control samples (Figure 3.9G).

NR4A1 is Required for Progesterone Mediated Endothelial Permeability

To determine whether NR4A1 was required for progesterone-mediated permeability, HUVECs were subjected to siRNA against NR4A1. Using qPCR, NR4A1 RNA levels were reduced by ~75% compared to non-targeting siRNA (Figure 3.10A). Interestingly, knockdown of NR4A1 led to a basal increase in HUVEC monolayer resistance compared to non-targeted control cells (Figure 3.10B). While progesterone increased permeability in HUVECS containing non-targeting siRNA, this effect was nearly blocked by the knockdown of NR4A1 (Figure 3.10C).

Immunocytochemistry further supported the concept that NR4A1 acts downstream of PR activation to regulate junctional barrier breakdown in endothelial cells. Although non-targeting HUVECS showed reduced VE-cadherin and PECAM-1 levels as well as β-catenin relocalization, effects were blocked in those with NR4A1 knockdown (Figure 3.10D). Interestingly, knockdown of NR4A1 led to an increase in membrane expression of all three junctional proteins, consistent with the increase in basal resistance seen by ECIS. Protein analysis further demonstrated the reduction of claudin-5, PECAM-1, and VE-cadherin in HUVECs transfected with non-targeting siRNA (Figure 3.10E). Knockdown of NR4A1 also led to a basal increase in junctional protein expression, and treatment with progesterone did not reduce expression levels to the same extent as seen in non-targeting cells. PR levels between non-targeting and knockdown cells were similar, ruling out possible changes in PR expression as the

determinant of this effect.

To further scrutinize these conclusions, HUVECS were infected with an adenovirus containing a dominant negative (Nur DN) construct against all NR4A family members [21]. Similar to NR4A1 knockdown, overexpression of the Nur DN inhibited progesterone mediated permeability (Figure 3.10F). As another member of the NR4A family, NR4A2, was also directly stimulated by PR (Figure 3.9D), we examined the effect of NR4A2 knockdown on permeability (Figure 3.11D). Although three independent siRNAs resulted in a ~70% reduction in NR4A2 RNA expression, none were able to inhibit progesterone-mediated permeability, demonstrating a unique role for NR4A1 in the regulation of barrier function (Figure 3.11E).

The ability of NR4A1 to directly control expression of junctional proteins was also tested in gain-of-function experiments. Overexpression of NR4A1 resulted in a marked reduction in VE-cadherin, claudin-5, and PECAM-1 in the absence of progesterone (Figure 3.10G). Furthermore, expression of NR4A1 alone increased monolayer resistance as determined by ECIS (Figure 3.10H), providing additional functional validation. These results indicate that NR4A1 is required and acts downstream of PR to mediate endothelial specific vascular permeability.

Discussion

The sequential and highly coordinated action of the steroid hormones 17-ß estradiol and progesterone are known to regulate epithelial and stromal functions in the endometrium [22]. Changes imposed by E and P prepare the endometrium for implantation and continue to be essential during the subsequent post-implantation phases to ensure a successful pregnancy [22,23]. Whereas much is known about the molecular and cellular events downstream of epithelial and stromal responses, the unique series of changes imposed to the uterine vasculature prior, during and post-implantation are only known at the level of morphological description. Here we show that PR within the endothelium is responsible for initiating a series of events that lead to physiological edema in the endometrium. Specifically, PR induces expression of the orphan nuclear receptor NR4A1, which, in turn, destabilizes endothelial barrier function within the subpopulation of PR-expressing endothelial cells. The consequence is restricted and sustained vascular permeability directed by circulating progesterone.

The contribution of progesterone as the chief regulator of vascular alterations during the secretory phase was implied from earlier work noting that mice with lack of PR failed to mount a decidual response [10,11]. Because expression of PR in the endothelium was at best sporadic, the effect on vessels was believed to be triggered through the secondary action of permeability modulators. An obvious culprit, VEGF, has been frequently evoked as responsible for the cycle of vascular changes in the uterus. In fact, VEGF is induced by steroid hormones [7,24] and pharmacological blockade of this growth factor in primates impairs endothelial repair and angiogenic growth [25]. Surprisingly, we found that blockade of VEGF does not prevent the physiological edema

that occurs prior to implantation; instead these events appear to be triggered by progesterone-driven mechanisms that are independent of VEGF. These findings pointed to either alternative permeability mediators or a direct role of PR in the endothelium. It should be noted, however, that inactivation of VEGF signaling post-implantation, like progesterone blockade, impacts both permeability and embryo viability. Thus, it appears that the mechanisms that regulate permeability responses pre- and post-implantation are likely distinct.

To continue to test the contribution of PR in the vascular endothelium, we adopted loss and gain-of-function approaches. Mice that lack PR in the endothelium, albeit able to host the typical decidual response by stromal cells, showed impaired ability to mount a physiological edema response. In contrast, transgenic animals that miss-expressed PR on endothelial cells in organs other than the uterus displayed an acute permeability response upon ligand exposure. Together these findings implicated progesterone as the mediator of the permeability responses prior to implantation.

How does progesterone drive vascular permeability? Although the molecular mechanisms of progesterone action via binding to its receptor are well established [26,27], the effects of this hormone on endothelial cells have not been explored at the molecular level. Evaluation of the literature on the effect of PR in epithelial cells was not informative as to how, in endothelial cells, this transcription factor could promote destabilization of barrier function. Furthermore, our in vitro experiments indicated that the effect of PR on endothelial permeability required transcriptional control. Following that lead, we performed global transcriptional profiling (RNA-seq) of endothelial cells treated with progesterone. These data initially failed to provide insights into the process

whereby PR promotes permeability. It was only through the integration of ChIP-sequencing analysis with the transcriptional profile that we were able to identify NR4A1 as the possible link.

The orphan nuclear receptor NR4A1 is a member of the NR4A transcription factor family that is expressed by a broad number of cell types. The effects mediated by NR4A1 are pleotropic, cell-type dependent and impact metabolism, homeostasis and inflammation [28]. Recently NR4A1 has been also shown to be expressed by endothelial cells and to induce pathological permeability responses [20]. The reports implicating NR4A1 in permeability opened the possibility for a role of NR4A1 downstream of PR signaling.

A hallmark of vascular leakage is the formation of intercellular gaps via disruption of cell-cell contacts resulting in a loss of barrier integrity [29,30]. Along these lines, endothelial cells expressing PR showed disruption of cell-cell interactions upon exposure to the ligand. Interestingly, silencing of NR4A1 in cells expressing PR and treated with the ligand blocked the effect of progesterone on permeability. These findings clearly indicated that PR was upstream of NR4A1 in the control of endothelial barrier function. Furthermore, we found that NR4A1 coordinates an effective program of transcriptional repression of junctional proteins, including VE-Cadherin, Claudin-5 and PECAM1.

Our results indicate that under homeostatic conditions, PR is highly restricted to uterine blood vessels, at the exclusion of vessels from other organs. Interestingly, expression of PR is selective to veins and lymphatic vessels. Endothelium from arteries is conspicuously absent of PR, while expression is highly noted in the smooth muscle

layer of these vessels. This exquisite specificity enables local and controlled functions triggered by a systemically distributed ligand.

As for progesterone effects in other vascular beds, we and others have noted that in human specimens, PR is expressed by patches of endothelial cells within the human coronary vasculature and other large vessels [13,31,32]. Interestingly, evaluation of progesterone effects on the progression of atherosclerosis using the mouse carotid injury model suggested a negative effect of this hormone on the resolution of injury. In fact, treatment of wild-type animals with progesterone worsened the response to injury while PRKO mice were unaffected, supporting the notion that long-term therapeutic use of progesterone and its derivatives may be deleterious to vascular function [33]. Although under non-pathological conditions PR cannot be detected within the coronary vasculature in the mouse, expression in human atherosclerotic vessels has been shown [13]. Therefore, it is possible that PR expression follows injury and subsequently mediates these effects.

The findings presented here are in accordance with, and further explain, the uterine vascular fragility experienced by users of long-term progestin-only contraceptives [34-36]. In fact, prolonged exposure to progestins results in abnormal endometrial bleeding despite increased levels of tissue factor expression [37].

Structural and molecular differences in the endothelium of distinct tissues reflect its role in meeting the diverse requirements of individual organ sites. The recurrent cycles of physiological permeability in the endometrium are unique to this tissue and must be timely regulated. Here we showed that this physiological permeability requires a molecular toolkit distinct from that of pathological permeability. Combined the findings

highlight the process by which endothelial cells detect and respond to systemic hormones to trigger local, timely and effective changes in barrier function.

Materials and Methods

Mouse Models

PRLacZ mice were generated by insertion of the LacZ gene into exon 1 by homologous recombination directly downstream of the PR-B initiation site on chromosome 9. To facilitate insertion, the PRLacZ reporter construct was flanked by a 1.25 kb (short arm) segment and 6.6 kb segment (long arm) of the endogenous PR locus. The construct also included a floxed neomycin gene for selection. The neomycin gene was later removed by Cre excision through mating PRLacZ mice to EIIaCre transgenic mice [38]. Incorporation of the construct by homologous recombination was verified using PCR. For generation of tie1-progesterone receptor (PRTg) mouse, the transgenic construct consisted of a 950bp tie1 minimal promoter fragment linked to human PR DNA. To distinguish between mouse endogenous PR and the human transgenic PR, a myc-tag was inserted preceding the stop codon of the PR construct. Mice were genotyped by Southern via digestion of total genomic DNA with *Ava*II and probing with an eGFP fragment of tie1-PR construct that distinguishes it from the endogenous PR locus. Additional mouse lines and their respective genotyping including: VE-cadherin Cre [39], floxed PR [40], PRKO [40] and R26R LacZ [41] have previously been described. All animals were housed in a pathogen-free environment in an AAALAC-approved vivarium at UCLA, and experiments were performed in accordance with the guidelines of the Committee for Animal Research at the same institution.

Hormone Treatment

8-12 week old female tie1-PRTg, PRLacZ, PR^{ECKO}, PRKO and littermate controls were treated with hormones as previously described [10]. Days 1-3 mice were injected

subcutaneously each day with 100ng 17 β–estradiol (Sigma, St. Louis, MO) in 0.1ml sesame oil; Days 4 and 5 no treatment; Day 6-8, 1μg progesterone (Sigma, St. Louis, MO) and 6.7ng 17 β–estradiol. Inhibitors were concurrently administered with hormonal or vehicle treatment at the following concentrations: icabant (Sigma, St. Louis, MO) was injected ip at 500ug/kg, Sunitinib (Sigma, St. Louis, MO) was given orally at 40mg/kg, and mifepristone 100mg/kg subcutaneously (RU486; Sigma, St. Louis, MO).

Morphometric analysis of uteri following hormone treatment

Microscopic images from the Zeiss LSM 510 META multiphoton microscope were imported into Image Pro (Media Cybernetics, Bethesda, MD) image analysis software. Cell density and vessel number were calculated by measuring boxed areas of endometrium and counting the number of nuclei and vessels, respectively, in the area of interest.

Vascular Permeability Assays

Following hormone treatment, mice were injected i.v. with either Evans blue dye (1 ml/kg of 3% Evans blue) or select lectins and allowed to circulate for 20 minutes before perfusion fixation (1% paraformaldehyde). Evans blue was allowed to circulate for 20 minutes, and the vasculature was perfusion-fixed (1% paraformaldehyde in 50 mM citrate buffer, pH 3.5). Uteri, ovary, lung, and intestine were removed, blotted dry, and weighed (wet weight). Evans blue was extracted from tissues with formamide overnight at 55°C and measured in duplicate by a spectrometer at 620 nm. Alternatively, mice were injected i.v. with *Lycopersicon esculentum* (Tomato) or *Ricinus communis agglutinin I* (RCA I) (Vector Laboratories, Burlingame, CA) to label the entire vasculature uniformly or mark sites of vascular permeability, respectively. Tissues were

fixed by vascular perfusion of 1% paraformaldehyde, sectioned (300μm) on a Vibratome, mounted using 90% glycerol in PBS, and imaged using a Zeiss LSM 510 META multiphoton microscope. Z stack images were analyzed using Zen software (Zeiss,Germany).

Immunohistochemistry

Tissue sections were immunostained with antibodies against PR (1:400; clone SP2, Lab vision, Kalamazoo, MI) and PECAM-1 (1:100 MEC 13.1, BD Biosciences, Franklin Lakes, NJ). Antigen retrieval using Tris-EDTA (pH 9.0) was required for PR staining of formalin embedded tissues. 488 and 564 Alexa Fluor secondary antibodies (1:300) were used to recognize primary antibodies (Molecular Probes, Life Technologies, Grand Island, NY). Sections were analyzed using a Zeiss LSM 510 META multiphoton microscope with built in Axiocam and acquired using Zen software (Zeiss, Germany). For β-galactosidase staining, vibratomed sections (300-400μm) were permeabilized with detergent, rinsed, and incubated with X-gal overnight. 5μm sections were stained with nuclear fast red for nuclei visualization and mounted on slides using Permount (Fischer Scientific, NJ). Bright field images were obtained using an Olympus BX40 microscope (Olympus, PA) with an Olympus F1H033971 camera (Olympus, PA). Images were taken at room temperature and objectives included: 4x UplanFl 0.13, 10xUPlanF1 0.3, 20x UPlanApo 0.8 oil, 40x UPlanApo 1.0 oil, and 100xUPlan Apo 1.35 oil iris Ph3. Images were analyzed using Magnafire software.

Immunoblotting and Immunoprecipitation

Both organs and cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1%

NP-40, 0.25% sodium deoxycolate, 1 mM EDTA, 1 mM sodium vanadate, 10 mM β-glycerophosphate, and protease inhibitors [1 mM phenylmethanesulfonylfluoride (PMSF), 20 μg/ml leupeptin, and 20 μg/ml aprotinin]). Immunoprecipitation of human PR from PRTg mice was performed using equal amounts of whole tissue extracts, as determined by the DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA), followed by incubation with anti-human monoclonal PR antibody (clone PgR 1294, DAKO; Carpenteria, CA) and proteinA-sepharose beads. Proteins were resolved by SDS-PAGE, transferred to reinforced nitrocellulose (Optitran BA-S 83; Dassel, Germany), and incubated overnight with the following antibodies: anti-PR (1:2000; clone SP2, Lab Vision, Kalamazoo, MI), anti-VE-cadherin (1:1000; Cell Signaling, Danvers, MA), anti-PECAM-1 (1:1000; Cell Signaling, Danvers, MA), anti-Claudin-5 (Invitrogen; Grand Island, NY), β-catenin (1:2000, Sigma, St. Louis, MO), β-actin (1:10,000, Sigma, St. Louis, MO), and anti-myc (1:1000; Cell Signaling, Danvers, MA). Blots were incubated with HRP-conjugated secondary (1:5000; Bio-Rad Laboratories, Hercules, CA), developed with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Kalamazoo, MI) and imaged by a Bio-Rad ChemiDoc XRS+ and accompanying Image Lab software (Bio-Rad Laboratories, Hercules, CA).

Electrical Cell-Substrate Impedance Sensing

Human umbilical vein endothelial cells, passages 4-6, were cultured in MCDB-131 (VEC Technologies, Rensselaer, NY) with the addition of 10% fetal bovine serum (Omega Scientific, Tarzana, CA) that was stripped using 0.25% dextran coated charcoal (Sigma, St. Louis, MO). PR infected HUVECS were seeded onto 8W10E+ arrays and treated with 100nM progesterone 48h after cells reached confluence (8 wells per array, 40

electrodes per well; Applied Biophysics, Troy, NY). Data was acquired and analyzed using ECIS software (Applied Biophysics, Troy, NY). Inhibitors included PI-103 (100nM; EMD Millipore, Billerica, MA), paclitaxel (100nM; Sigma, St. Louis, MO), Y27632 (10uM; Sigma, St. Louis, MO), SU6656 (10uM; EMD Millipore, Billerica, MA), and SU4312 (10uM; Sigma, St. Louis, MO). HUVECs were pretreated with inhibitors for 2h before progesterone treatment. For Nur77/TR3 functional experiments, a Nur family dominant negative adenovirus was a generous gifts from Dr. Peter Tontonoz and has been described previously [21]. The NR4A1 overexpression was done using a construct designed by the Tontonoz lab and viral amplification done by ViraQuest (North Liberty, IA). Cells were incubated in adenovirus for 1h in the absence of serum and examined by ECIS 48 h after infection.

Immunocytochemistry of cultured endothelial cells

For immunocytochemistry, HUVECS were seeded onto Lab-Tek II 8-well slides (Thermo Scientific, Rochester, NY) and stimulated with 100 nM progesterone. Cells were fixed for 20 minutes with 4% PFA, permeabilized with 0.1% Triton-X100, and blocked for 1h with 10% donkey serum. Primary antibodies were incubated overnight in 1% serum and included β-catenin (1:350, Sigma, St. Louis, MO), PECAM-1 (1:400, M-20; Santa Cruz Biotechnology, Santa Cruz, CA), VE-cadherin (1:200, C-20; Santa Cruz Biotechnology, Santa Cruz, CA), and ZO-1 (1:500, Clone 1A12; Invitrogen; Grand Island, NY). Alexa Fluor secondary antibodies were incubated for 1h at RT (1:300, Invitrogen, Grand Island, NY). Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI, 1:1000; Invitrogen, Grand Island, NY). Images were acquired using a Zeiss LSM 520 multiphoton microscope (Zeiss, Germany).

siRNA transfection of HUVECs

Confluent HUVEC monolayers were transfected with a Stealth single siRNA (Invitrogen, Grand Island, NY) to NR4A1 and NR4A2 using siPORT reagent (Ambion, Life Technologies, Grand Island, NY). Briefly, siPORT was incubated for 30 min. at RT with Opti-MEM before addition and 20 min. RT incubation with the siRNA. Cells were washed and incubated in antibiotic free DMEM with 1% FBS followed by the addition of the siRNA mixture. Cells were incubated for 4 hours with the siRNA then washed and replaced with MCDB-131 with 10% charcoal stripped FBS. The procedure was repeated again 48 hours later and cells were used for experiments 48-72 hours after the final transfection. Knockdown efficiency was assessed using qPCR. Negative Control Hi GC siRNA was used as a control (Invitrogen, Grand Island, NY). The NR4A1 siRNA sequence is CACAUGUGCGGACACCAUAAUGCUG.

Chromatin immunoprecipitation and library preparation Chromatin Immunoprecipitation

For each condition (non-infected-negative control, PR+P, PR only, and IgG control) $10x10^6$ and $2x10^6$ cultured HUVECs were used per IP for ChIP-seq and ChIP-qPCR, respectively. HUVECs were infected with a PR lentivirus, grown to confluence, and then treated with progesterone for 1h. Cells were then crosslinked with 1% formaldehyde, resuspended in 400 µl of lysis buffer (1% SDS, 20 mM EDTA and 50 mM Tris-HCl (pH 8.0)) containing protease inhibitors (Roche, Indianapolis, IN), and sonicated using Misonix cup-horn sonicator to achieve, on average, 200bp fragments for ChIP-seq and 500bp fragments for ChIP-qPCR. The lysate was diluted with ChIP

dilution buffer containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA and 16.7 mM Tris-HCl (pH 8.1) and immunoprecipitated with 3 ug of anti-PR or IgG antibody overnight at 4 degrees.

The complexes were captured using protein A Dynabeads (Invitrogen, Grand Island, NY) and washed twice with the following buffers: low-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1); high-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 500 mM NaCl); LiCl wash buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris-HCl (pH 8.1)) and TE (10 mM Tris-HCl and 1 mM EDTA (pH 8.0)). After elution with 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1% SDS, crosslinks were reversed by overnight incubation at 65°C. Samples were then treated with RNase A for 30 min at 37°C and proteinase K for 2 h at 56°C. DNA was subsequently purified using Qiagen MinElute Columns according to manufacturers instructions. DNA concentration was measured using a Qubit (Invitrogen, Grand Island, NY). The library for sequencing was constructed using Ovation Ultralow IL Multiplex System 1-8 according to manufacturer's instructions (Nugen, San Carlos, CA). Libraries were sequenced using HIseq-2000 (Illumina, San Diego, CA) to obtain 50 bp long reads. ChIP-seq data sets have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE43789.

ChIP-seq analysis

Debarcoding of the multiplex runs was performed using Fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit). Tags were mapped to the human genome (hg19) using bowtie v0.12.7 [42] excluding non-unique mappings (-m 1). 12-22 million

uniquely mapped reads were obtained for each sample. Wig files were created using Homer [43] and visualized on UCSC (Kent et al., 2002) genome browser as custom tracks. Peak identification was performed with MACS v1.3.7.1 [44]. Peaks for PR and PR+P conditions were called using either input, negative control (non-infected cells) or IgG control as a reference and only peaks that were present in all three comparisons were included in the final list of PR binding sites. To identify genes that are potentially regulated by PR, peaks were mapped to nearby genes within 50kb range from the transcriptional start site using Genomic Regions Enrichment of Annotations Tool (GREAT) [45]. Peak intersections and overlaps with differentially expressed genes were performed using Galaxy [46] and in house shell scripts.

RNA-seq analysis

Debarcoding of the multiplex runs was performed using in house shell script. Reads were then processed and aligned to the human genome (hg19) using TopHat v2.0.4 [47] with default parameters. Approximately 50 million and 42 million mapped reads where obtained for PR and PR+P samples, respectively. The aligned read files were further processed with Cufflinks v2.0.1 [48]. Assemblies for PR and PR+P endothelial cells were merged using CuffMerge and differential expression was determined using Cuffdiff. Genes with a p-value smaller than 0.01 where considered as differentially expressed. For the generation of heatmaps for each gene log2 ratio of a given sample rpkm was divided with the average of the two samples (PR and PR+P) rpkm's and visualized using treeview [49].

RNA isolation, qPCR, and library preparation

Total RNA was extracted using RNeasy Kit (Qiagen, Valencia, CA), cDNA generated

using SuperScript First-strand Synthesis System (Invitrogen, Grand Island, NY) and quantitative real-time PCR was performed using SYBR Green reagent (Qiagen, Valencia, CA) and detected using an Opticon2 PCR machine (MJ Research; BioRad, Hercules, CA). The library for sequencing was constructed using an Illumina Multiplex System according to manufacturer's instructions (Illumina, San Diego, CA). Libraries were sequenced using HIseq-2000 (Illumina, San Diego, CA) to obtain 50 bp long reads. RNA-seq data sets have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE43788. For details on how differentially expressed genes were identified and analyzed see Extended Experimental Procedures.

Statistical Analysis

For statistical analysis, Student's unpaired two-tailed t-test was used for all comparisons.

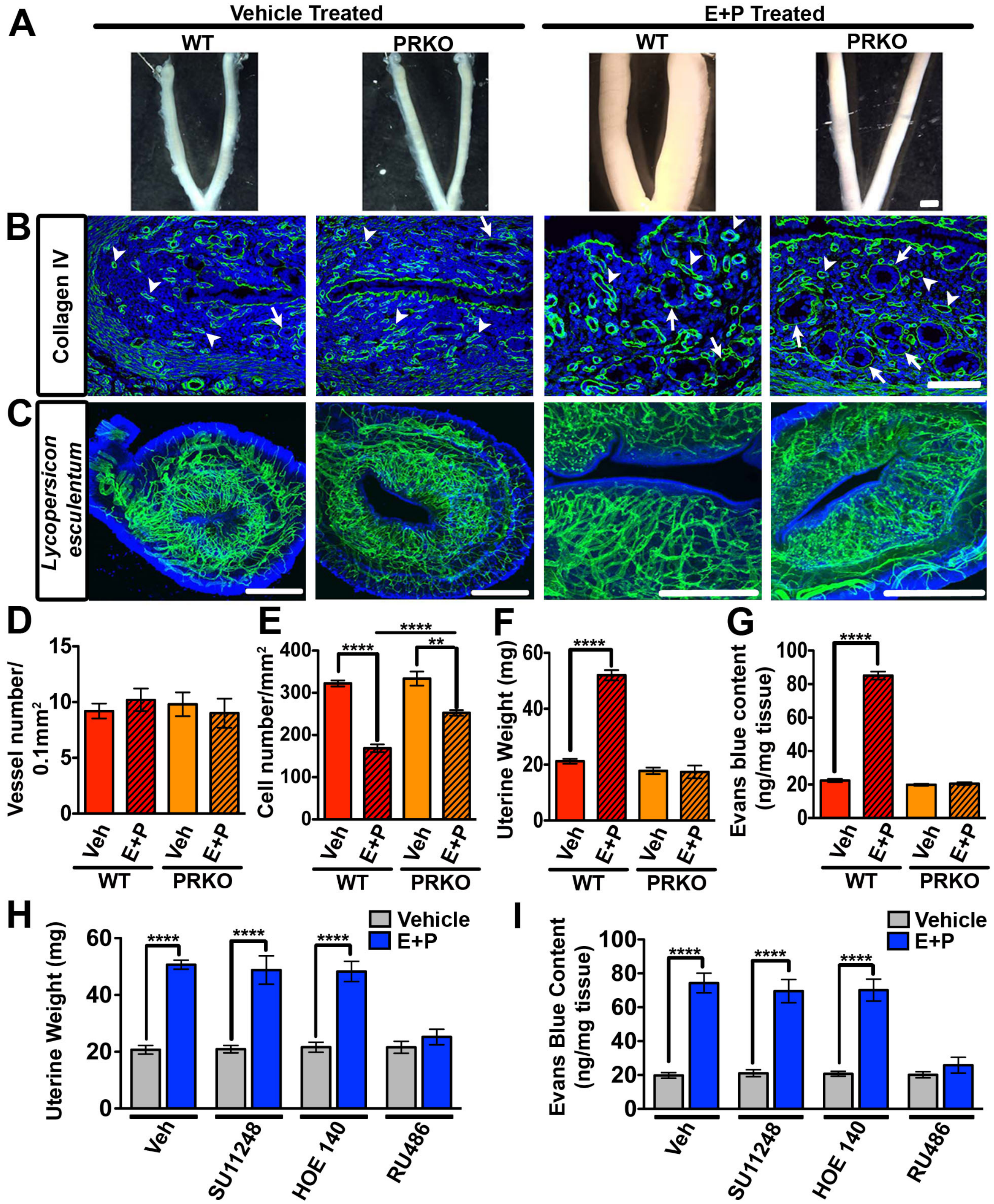

A
Vehicle Treated
E+P Treated
WT
PRKO
WT
PRKO
B
Collagen IV
C
Lycopersicon esculentum
D
Vessel number/ 0.1mm²
E
Cell number/mm²
F
Uterine Weight (mg)
G
Evans blue content (ng/mg tissue)
Veh
E+P
WT
PRKO
H
Uterine Weight (mg)
Vehicle
E+P
Veh
SU11248
HOE 140
RU486
I
Evans Blue Content (ng/mg tissue)

Figure 3.1. Reduced physiological permeability in the uterus in PRKO mice
(A) WT but not PRKO uteri undergo physiological hyperplasia after hormonal stimulation. Scale bar = 3mm. **(B)** Collagen IV immunostaining (green) detects basement membrane of glands (arrows) and blood vessels (arrowheads). Scale bar = 100 µm. **(C)** Intravascular perfusion with FITC conjugated *Lycopersicum esculentum* (tomato lectin, green) reveals well vascularized PRKO uteri despite poor response to hormonal treatment. Scale bar = 1 mm. **(D)** Quantification of vessel number/0.1mm^2 in WT and PRKO mice. n=5/group. **(E)** Quantification of uterine cell density/mm^2 in WT and PRKO mice. n=5/group. **(F)** Uterine wet weight in WT vehicle-treated and matching hormonal-treated group. n=5/group. **(G)** Vascular permeability, as measured by the Miles assay, is attenuated in hormonal-treated PRKO uteri compared to similarly treated WT mice. n=5/group. **(H,I)** Quantification of uterine weight and Miles blue content following hormonal stimulation and concurrent inhibition of VEGFR2 (n=5), bradykinin (n=3), and PR (n=5). **p < 0.01, ****p < 0.0001. In all panels, error bars = +/- SEM and data was analyzed using an unpaired two-tailed Student-T test.

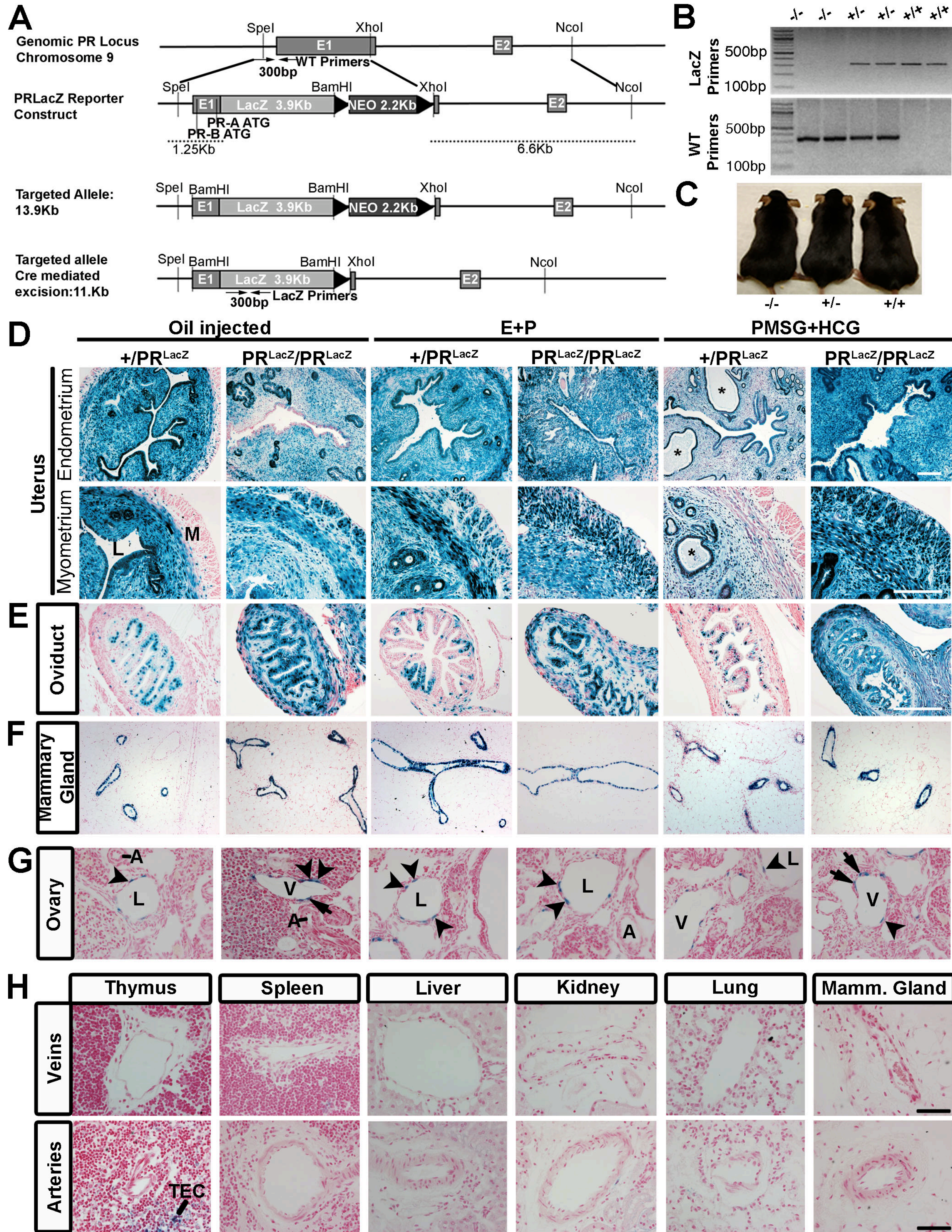

A
Genomic PR Locus
Chromosome 9
SpeI
XhoI
NcoI
E1
E2
300bp
WT Primers
PRLacZ Reporter
Construct
BamHI
LacZ 3.9Kb
NEO 2.2Kb
PR-A ATG
PR-B ATG
1.25Kb
6.6Kb
Targeted Allele:
13.9Kb
Targeted allele
Cre mediated
excision:11.Kb
LacZ Primers
B
-/- -/- +/- +/- +/+ +/+
LacZ
Primers
WT
Primers
500bp
100bp
C
-/-
+/-
+/+
D
Oil injected
E+P
PMSG+HCG
+/PR^LacZ
PR^LacZ/PR^LacZ
Uterus
Endometrium
Myometrium
L
M
E
Oviduct
F
Mammary
Gland
G
Ovary
A
L
V
H
Thymus
Spleen
Liver
Kidney
Lung
Mamm. Gland
Veins
Arteries
TEC

Figure 3.2. PR LacZ knock-in mice report expression of both PR isoforms
(A) The LacZ reporter gene encoding β-gal was inserted by homologous recombination into exon 1 (E1) of the endogenous murine PR locus directly downstream of the ATG start sites for both PR isoforms, PR-B and PR-A. Dotted lines at the 5' and 3' flanks indicate areas of homology between the genomic locus and the LacZ reporter construct. A floxed neo cassette was used as a selection marker and excised through Cre-mediated recombination. Black arrows delineate the two distinct sets of forward and reverse primers (WT and LacZ) used for genotyping. **(B)** Both WT primers and LacZ primers reveal a 300bp band by PCR. **(C)** Similar to WT mice (-/-), heterozygous (+/-) and homozygous (+/+) mice are viable and survived into adulthood in a frequency identical to wild-type mice. Unlike -/- and +/- animals, +/+ mice are infertile. Animals depicted are 3 months of age. Transverse uterine **(D)** and oviduct **(E)** sections (5 μm) from both +/PR^{LacZ} and PR^{LacZ}/PR^{LacZ} exhibit β-gal positive cells (blue) in smooth muscle, stromal, and epithelial cells**.** PR^{LacZ}/PR^{LacZ} animals exhibit increased numbers of β-gal positive cells compared to +/PR^{LacZ} animals even without exogenous hormones. Exposure of +/PR^{LacZ} to PMSG and HCG did not drastically alter PR expression in the uterus, but did lead to changes in glandular size. L = lumen; M = myometrium; Scale bar = 150 μm; * indicate areas of enlarged glands after PMSG/HCG injection. **(E)** Transverse sections through the oviduct of +/PR^{LacZ} and PR^{LacZ}/PR^{LacZ} animals. **(F)** Histological sections of the mammary gland demonstrate PR promoter activity localization in the glandular epithelium. **(G)** Histological sections from the ovary following X-gal staining. Arrowheads and arrows represent β-gal positive endothelial and smooth muscle cells respectively. A = artery, V = vein, L = lymphatic, TEC= thymic epithelial cell. Scale bar = 50μm. **(H)** Histological sections of veins and arteries from X-gal stained non-reproductive tissues. Nuclei are stained with nuclear fast red. Scale bar = 50 μm.

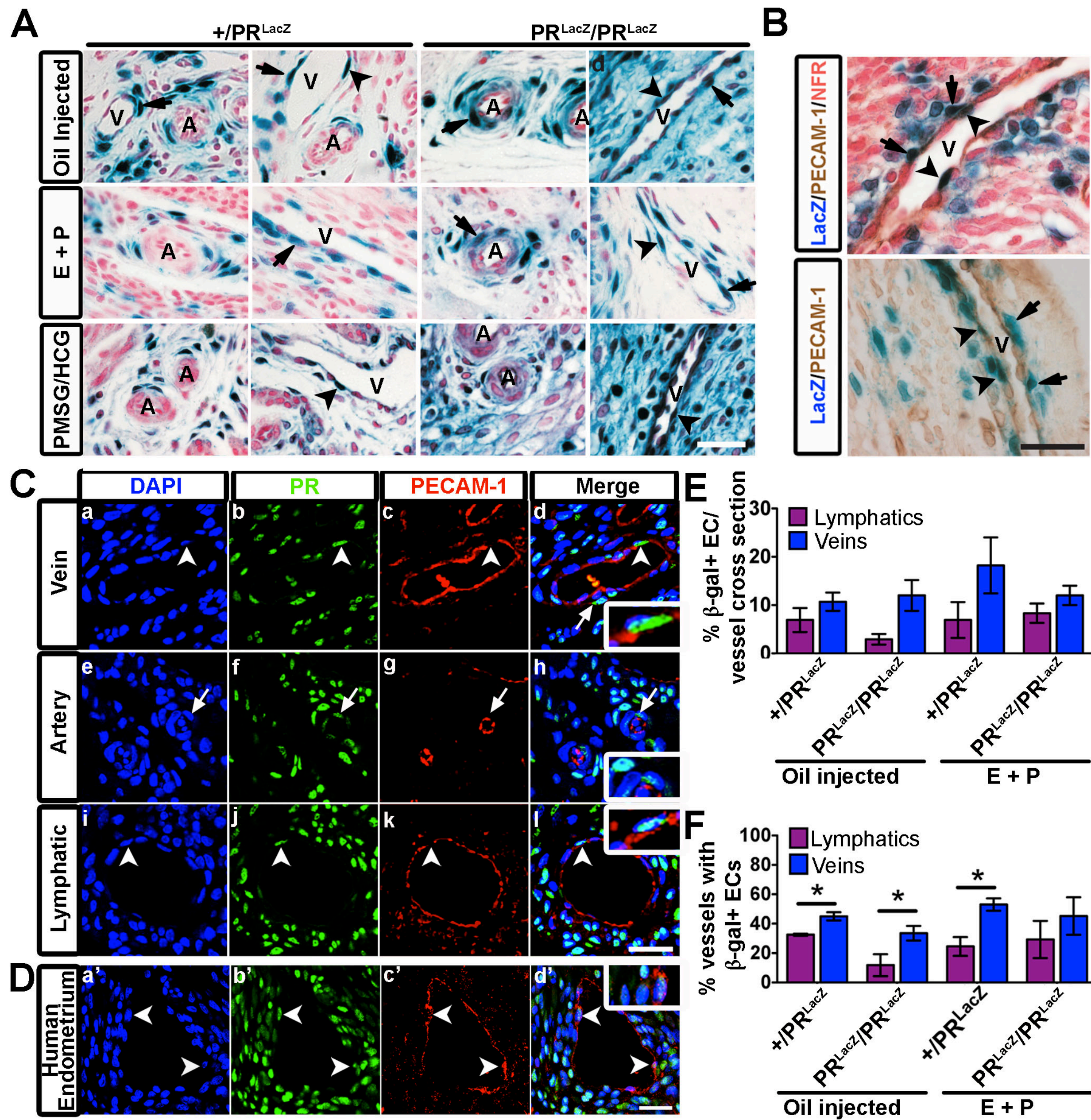

A
+/PR^LacZ
PR^LacZ/PR^LacZ
Oil Injected
E + P
PMSG/HCG
B
LacZ/PECAM-1/NFR
LacZ/PECAM-1
C
DAPI
PR
PECAM-1
Merge
Vein
Artery
Lymphatic
D
Human Endometrium
E
% β-gal+ EC/ vessel cross section
Lymphatics
Veins
Oil injected
E + P
F
% vessels with β-gal+ ECs
Lymphatics
Veins
Oil injected
E + P

Figure 3.3. PR expression in the murine vasculature
(A) Transverse uterine sections from +/PR^{LacZ} and PR^{LacZ}/PR^{LacZ} mice treated with oil, β-estradiol and progesterone (E+P), or PMSG/HCG demonstrate β-gal positivity in the vasculature. Endothelial cells (arrowheads) from veins (V) but not arteries (A) were positive for β-gal. PR promoter activity in smooth muscle cells (arrows) was detected in both arteries and veins. Nuclear Fast Red (NFR) was used as a counterstain. Scale bar = 25 μm. **(B)** PR promoter activity (β-gal) in venous endothelial cells was verified by co-staining with PECAM-1 (brown). V = vein; Scale bar = 25 μm. **(C)** Immunofluorescence of murine uterine sections stained for PECAM-1 (red) and PR (green). Nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI, blue). PR is expressed in endothelial cells (arrowheads) of veins and lymphatic vessels, and smooth muscle cells (arrows) of veins and arteries. Scale bar = 25 μm. Insets are higher magnification images of a PR positive endothelial cell. **(D)** Venous endothelial expression of PR (green) in human endometrial sections as confirmed by colocalization with PECAM-1 (red). Nuclei are stained with DAPI (blue). Scale bar = 25 μm. Inset is a higher magnification image of a PR positive endothelial cell. **(E)** Percentage of β-gal+ endothelial cells per total number of endothelial cells in each vessel cross-section. n = 2-5 and 300-500 endothelial cells/condition. **(F)** Percentage of lymphatics and veins in the uterus that contain at least one β-gal+ endothelial cell per cross-section. n = 2-5 and 300-500 endothelial cells/counted; $^*p < 0.05$. In all panels, error bars = +/- SEM and data was analyzed using an unpaired two-tailed Student-T test.

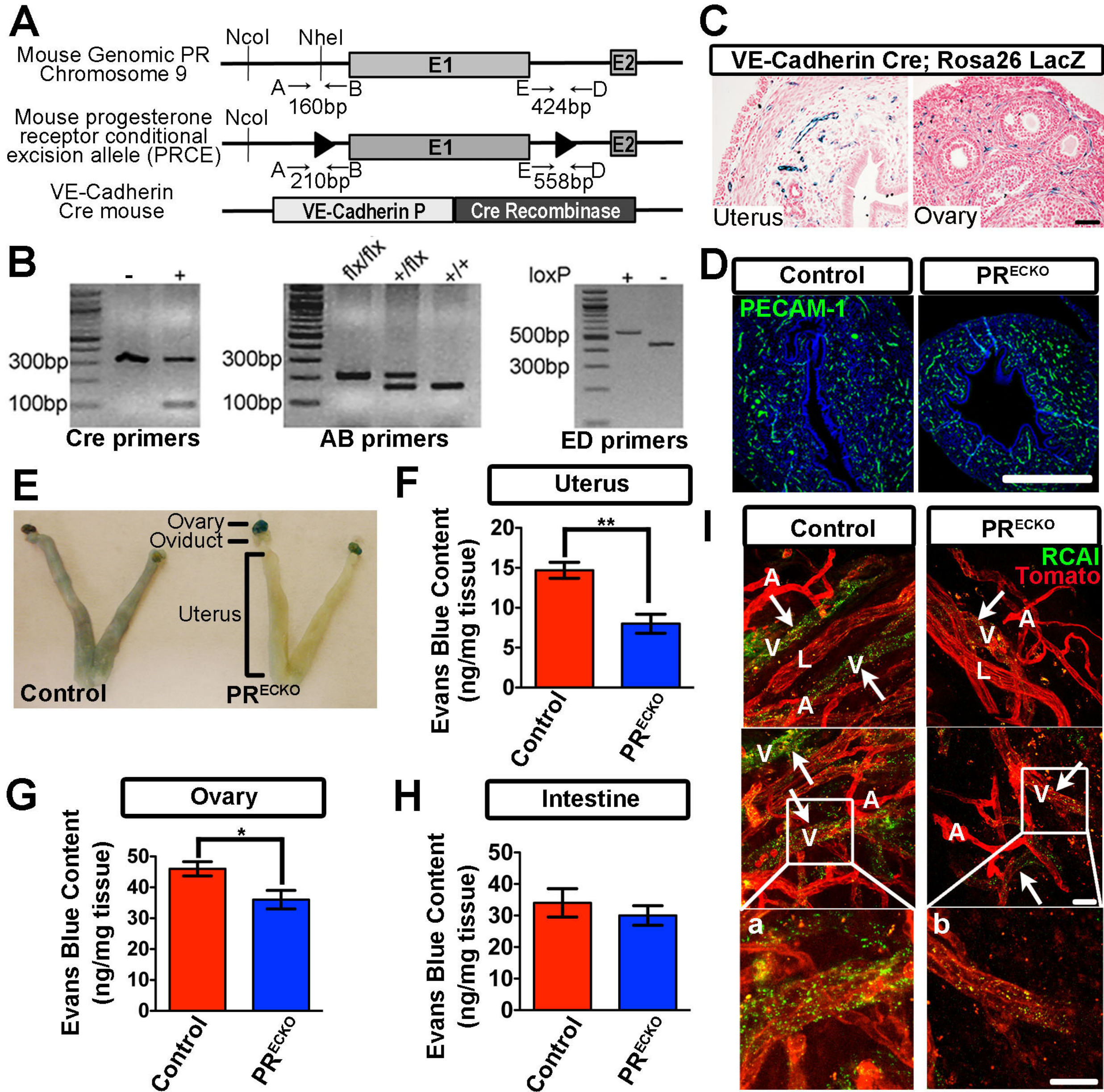

A
Mouse Genomic PR
Chromosome 9
NcoI
NheI
E1
E2
160bp
424bp
Mouse progesterone
receptor conditional
excision allele (PRCE)
210bp
558bp
VE-Cadherin
Cre mouse
VE-Cadherin P
Cre Recombinase
B
Cre primers
AB primers
ED primers
flx/flx
+/flx
+/+
loxP
300bp
100bp
500bp
C
VE-Cadherin Cre; Rosa26 LacZ
Uterus
Ovary
D
Control
PRECKO
PECAM-1
E
Ovary
Oviduct
Uterus
Control
PRECKO
F
Uterus
Evans Blue Content
(ng/mg tissue)
G
Ovary
H
Intestine
I
RCAI
Tomato
a
b

Figure 3.4. Reduced vascular permeability in PR^{ECKO} mice
(A) Generation of PR^{ECKO} mice was accomplished by mating PRCE mice to constitutive VE-Cadherin Cre mice. **(B)** Presence of VE-Cadherin Cre was determined by a 100bp band using general EIIa Cre primers. A 300bp control band confirmed PCR efficiency. **(C)** VE-cadherin Cre recombination efficiency (β-gal, blue) in the uterus and ovary are restricted to the endothelium. Scale bar = 50 μm. **(D)** Tiled images comparing control and PR^{ECKO} vascular density in the uterus. Vessels were stained for PECAM-1 (green). DAPI (blue) visualized nuclei. Scale bar = 0.5 mm. **(E)** PR^{ECKO} animals (n=8) have reduced Evans Blue extravasation compared to control (n=7) following hormone treatment**.** **(F-H)** Quantification of Evans blue content from the uterus, ovary and intestine. **p < 0.01; *p < 0.05. **(I)** *Ricinus communis agglutinin* I (green) and *Lycopersicon esculentum* (red) staining from uteri of control and PR^{ECKO} animals following hormone treatment. Arrows indicate sites of permeability (green). A= artery, V=vein, L=lymphatic. **(a,b)** Enlarged images of the area contained within the white boxes. Scale bar = 50 μm. In all panels, error bars = +/- SEM and data was analyzed using an unpaired two-tailed Student-T test.

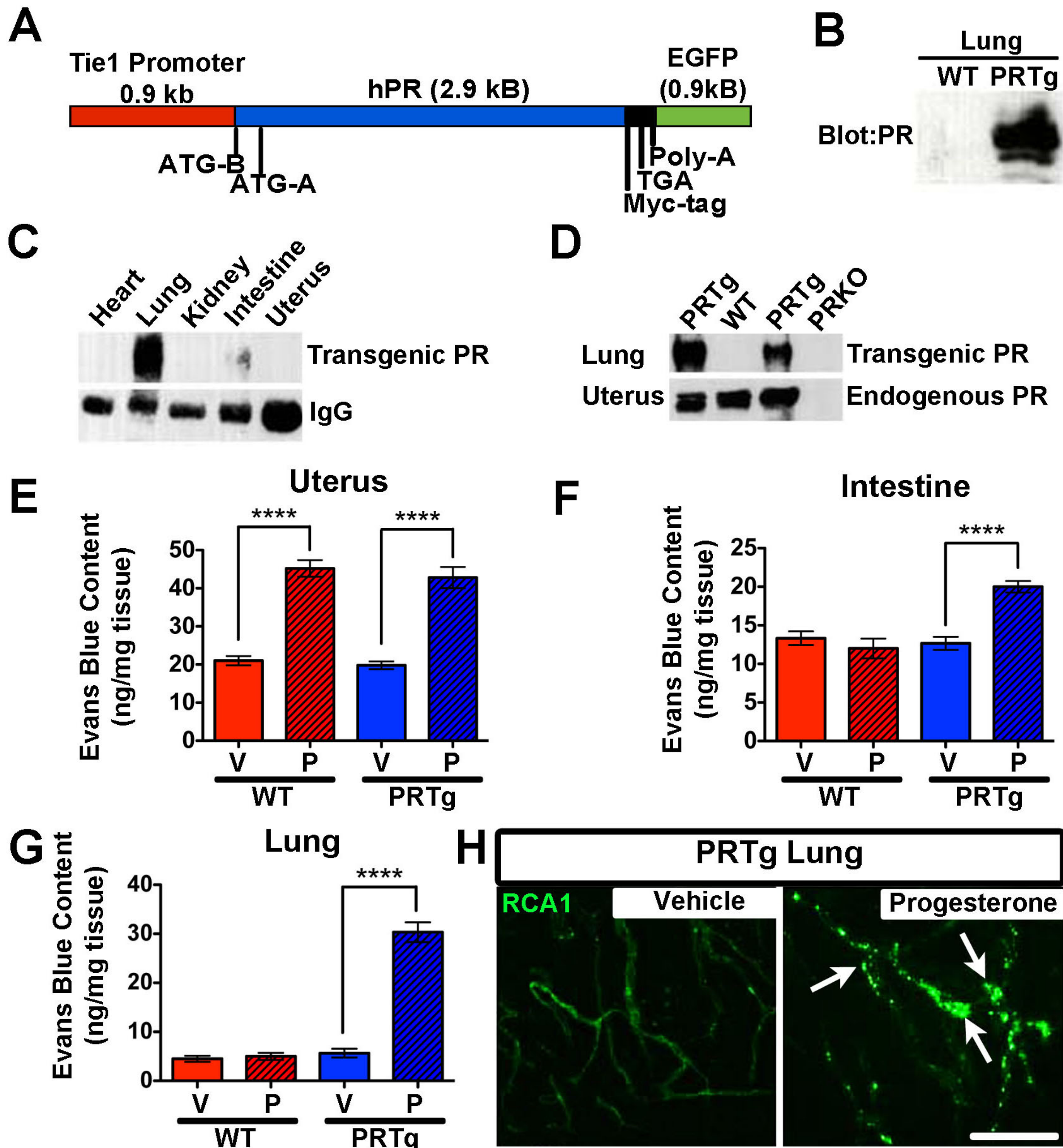

A
Tie1 Promoter
0.9 kb
hPR (2.9 kB)
EGFP
(0.9kB)
ATG-B
ATG-A
Poly-A
TGA
Myc-tag
B
Lung
WT
PRTg
Blot:PR
C
Heart
Lung
Kidney
Intestine
Uterus
Transgenic PR
IgG
D
PRTg
WT
PRTg
PRKO
Lung
Uterus
Transgenic PR
Endogenous PR
E
Uterus
Evans Blue Content
(ng/mg tissue)
****
****
50
40
30
20
10
0
V
P
V
P
WT
PRTg
F
Intestine
Evans Blue Content
(ng/mg tissue)
****
25
20
15
10
5
0
V
P
V
P
WT
PRTg
G
Lung
Evans Blue Content
(ng/mg tissue)
****
40
30
20
10
0
V
P
V
P
WT
PRTg
H
PRTg Lung
RCA1
Vehicle
Progesterone

Figure 3.5. Ectopic expression of PR in lung endothelial cells leads to enhanced permeability
(A) Transgenic mice containing the 950bp tie1 minimal promoter fragment directs PR expression to the endothelium. **(B)** Transgenic PR was detected in PRTg total lung lysates by Western blot analysis. **(C)** Equal levels of total protein lysate from indicated organs were evaluated for the presence of the human PR transgene. IgG indicates the level of antibody used for immunoprecipitation. **(D)** Comparison of transgenic (human) and endogenous (murine) PR in lung and uterus respectively. **(E-G)** Quantification of vascular permeability in wild-type (WT) and PRTg mice normalized to respective organ weight. V = vehicle treated; P = progesterone treated. n = 6; ****p < 0.001. (**H**) *Ricinus communis agglutinin I* staining (arrows) in wild-type and PRTg vessels of the lungs. Scale bar=150um. In all panels, error bars represent SEM and data was analyzed using an unpaired two-tailed Student-T test.

Table 3.1. Mouse models used in this study.

Transgenic lines, genotypes and selected references are indicated.

Mouse Model	Genotype	Reference
PRCE	$PR^{loxP/loxP}$	Hashimoto-Partyka et al. 2006
PR^{ECKO}	VE-cadherin Cre; $PR^{loxP/loxP}$	This Work
PRKO	EIIa Cre; $PR^{loxP/loxP}$	Hashimoto-Partyka et al. 2006
PR^{LacZ}	PR-LacZ	This Work
Tie1-PR	Tg (Tie1-PR)	This Work
VE-cad/ROSA	VE-Cadherin Cre; R26R	Alva et al. 2006; Soriano, 1999

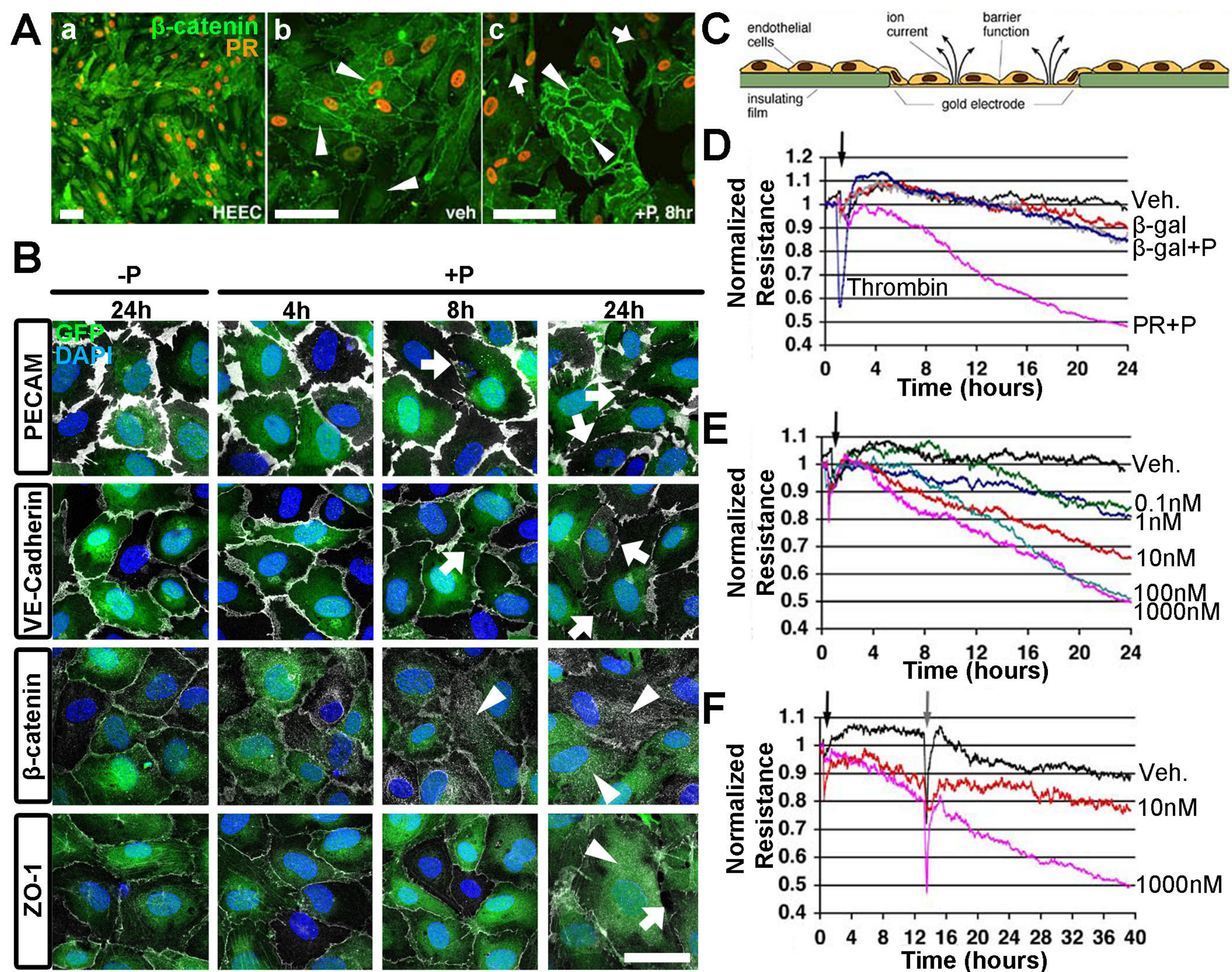


Figure 3.6. PR activation in endothelial cells results in decreased monolayer resistance

(A) Human endometrial EC (HEEC) express endogenous PR (orange). Progesterone treatment results in junctional breakdown in PR-expressing HEEC, but not PR-negative cell islands as determined by β-catenin staining (green). Arrowheads demonstrate areas of intact junctions. Arrows indicate loss of β-catenin at the junctions of PR positive cells. Scale bar = 100 μm. **(B)** HUVECs infected with a PR lentivirus (green) were treated for 4, 8, and 24 hours with progesterone (100nM). PECAM-1, VE-cadherin, β-catenin, and ZO-1 (white) were used to visualize junctions. DAPI (blue) shows nuclei. Activation of PR leads to intercellular gaps and reduced junctional protein expression (arrows). Arrowheads show translocation of β-catenin from the cell membrane to cytosol. Scale bar = 50 μm. **(C)** Diagram depicting electrical cell-substrate impedance sensing (ECIS). Resistance values are calculated based on fluctuation in current movement through the endothelial monolayer. **(D)** Progesterone decreases HDEC monolayer resistance in the presence of PR, but not in cells infected with a β-gal control construct. Thrombin = positive control. **(E)** Effect of progesterone on the barrier is dose-dependent. **(F)** Barrier function stabilizes when 10nM but not 1μM P is removed from the media. Black arrows = stimulus addition, Grey arrow =stimulus removal.

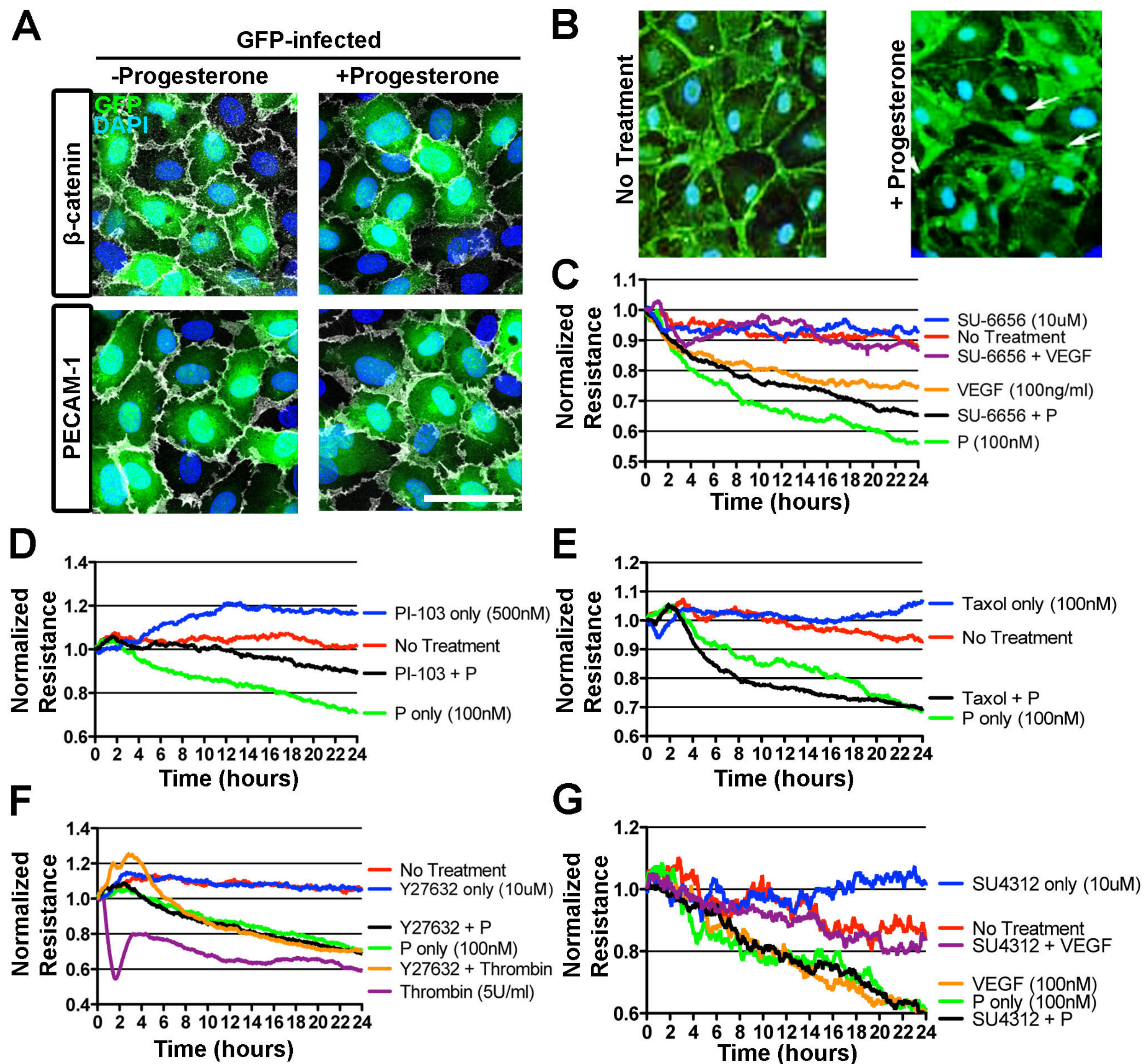


Figure 3.7. Inhibition of classical permeability mediators does not prevent progesterone mediated permeability

(A) Progesterone treatment for 24 hours does not lead to changes in β-catenin or PECAM-1 (white) in HUVECS infected with a GFP control construct (green). Nuclei are stained with DAPI (blue). Scale bar = 50 µm. **(B)** HUVECS grown on ECIS electrodes visualized in the presence or absence of progesterone for 24 hours. Arrows denote areas of junctional breakdown represented by β-catenin staining (green). PR positive nuclei are stained in blue. **(D-G)** Inhibition of permeability mediators including: Src kinase (SU-6656, 10uM) **(C)**, PI3K (PI-103, 500nM) **(D)**, ROCK (Y27632, 10uM) **(F)**, and VEGFR2 (SU4312, 10uM) **(G)** and inhibition of microtubule reorganization (taxol, 100nM) **(E)** did not affect progesterone mediated permeability as measured by ECIS.

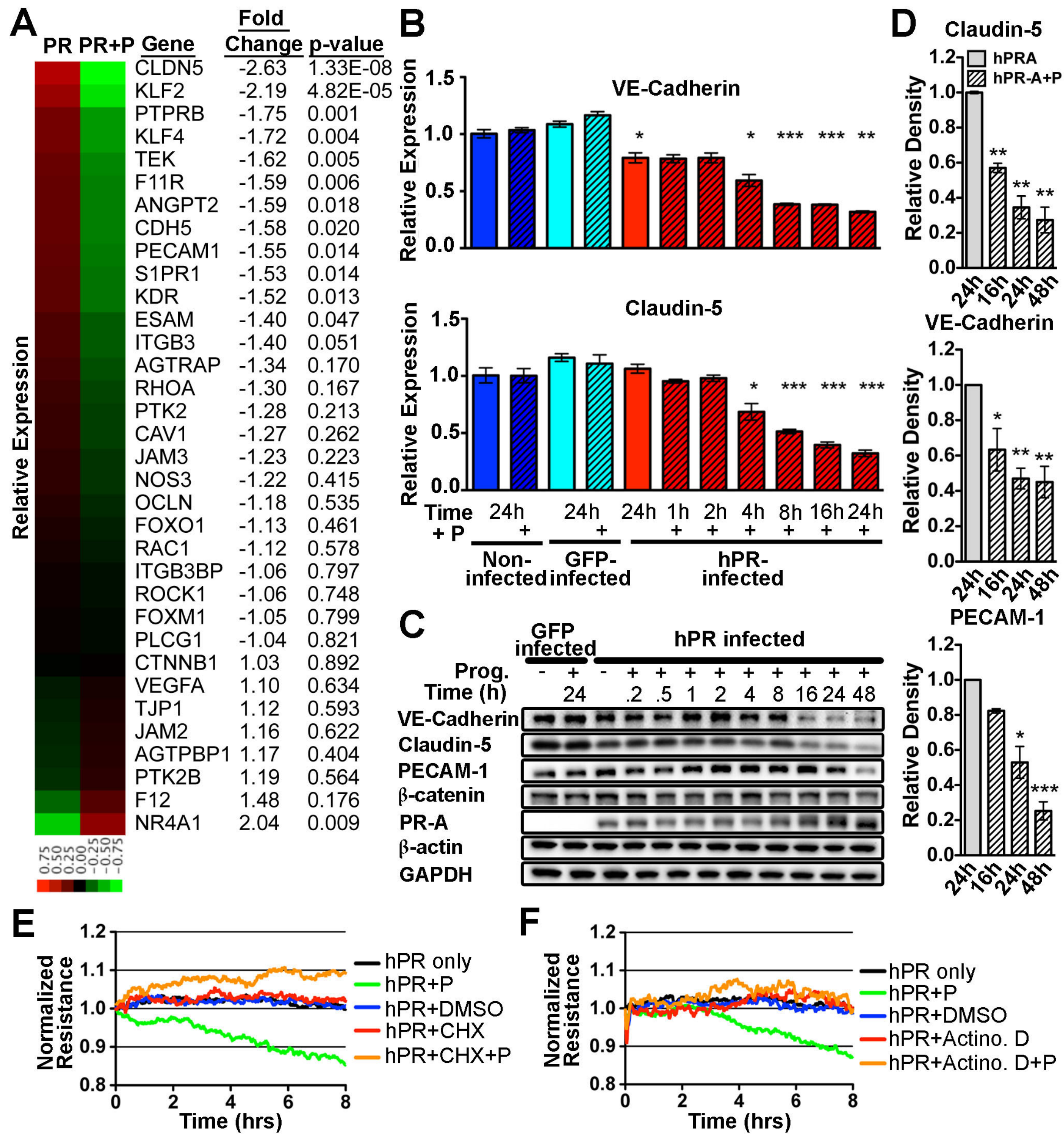

A
PR PR+P Gene Fold Change p-value
CLDN5 -2.63 1.33E-08
KLF2 -2.19 4.82E-05
PTPRB -1.75 0.001
KLF4 -1.72 0.004
TEK -1.62 0.005
F11R -1.59 0.006
ANGPT2 -1.59 0.018
CDH5 -1.58 0.020
PECAM1 -1.55 0.014
S1PR1 -1.53 0.014
KDR -1.52 0.013
ESAM -1.40 0.047
ITGB3 -1.40 0.051
AGTRAP -1.34 0.170
RHOA -1.30 0.167
PTK2 -1.28 0.213
CAV1 -1.27 0.262
JAM3 -1.23 0.223
NOS3 -1.22 0.415
OCLN -1.18 0.535
FOXO1 -1.13 0.461
RAC1 -1.12 0.578
ITGB3BP -1.06 0.797
ROCK1 -1.06 0.748
FOXM1 -1.05 0.799
PLCG1 -1.04 0.821
CTNNB1 1.03 0.892
VEGFA 1.10 0.634
TJP1 1.12 0.593
JAM2 1.16 0.622
AGTPBP1 1.17 0.404
PTK2B 1.19 0.564
F12 1.48 0.176
NR4A1 2.04 0.009
Relative Expression
B
VE-Cadherin
Claudin-5
Relative Expression
Time 24h 24h 24h 1h 2h 4h 8h 16h 24h
+ P
Non-infected
GFP-infected
hPR-infected
C
GFP infected
hPR infected
Prog.
Time (h) 24 .2 .5 1 2 4 8 16 24 48
VE-Cadherin
Claudin-5
PECAM-1
β-catenin
PR-A
β-actin
GAPDH
D
Claudin-5
hPRA
hPR-A+P
Relative Density
VE-Cadherin
PECAM-1
24h 16h 24h 48h
E
Normalized Resistance
Time (hrs)
hPR only
hPR+P
hPR+DMSO
hPR+CHX
hPR+CHX+P
F
Normalized Resistance
Time (hrs)
hPR only
hPR+P
hPR+DMSO
hPR+Actino. D
hPR+Actino. D+P

Figure 3.8. PR activation leads to changes in expression of junctional proteins
(A) Heat map representing the relative expression and fold change of genes known to regulate vascular permeability in PR vs. PR+P HUVECS at 4h. **(B)** qPCR confirmation of VE-cadherin (*CDH5*) and claudin-5 (*CLDN5*) expression following progesterone treatment (hatched bars). Graphs display an average of three biological replicates. Ct values were normalized to those from GAPDH and made relative to non-infected cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. **(C)** Western blot analysis of total protein levels from GFP control or PR infected total HUVEC lysate following progesterone treatment. GAPDH/β-actin = loading controls. Blots are representative of three independent experiments. **(D)** Densitometry analysis of VE-cadherin, claudin-5, and PECAM-1 levels following progesterone treatment (hatched bars). Bands were normalized to GAPDH and made relative to PR infected HUVECS (solid gray bars). Graphs display an average of three biological replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **(E,F)** Inhibition of progesterone mediated permeability by cycloheximide (10ug/ml) and actinomycin D (10ug/ml), respectively. Error bars = +/- SEM and data was analyzed using an unpaired two-tailed Student-T test.

Table 3.2. Primers used for qPCR and ChIP-seq analysis.
Primer sets were generated using Primer3 Input (version 0.4.0) and product sizes were validated using gel electrophoresis.

Primers for qPCR			
Gene			**Product Size**
VE-cadherin	Forward	CACCACCAGCTACGATGTGT	184 bp
	Reverse	TCGTAGCCGTAGATGTGCAG	
Claudin-5	Forward	GAGGCGTGCTCTACCTGTTT	239 bp
	Reverse	GTACTTCACGGGGAAGCTGA	
NR4A1	Forward	CTTCTCAAGGTCCCTGCACA	249 bp
	Reverse	TCTTGTCAATGATGGGTGGA	
NR4A2	Forward	GGCGAACCCTGACTATCAAA	209 bp
	Reverse	CTGGGTTGGACCTGTATGCT	

Primers for ChIP-PCR			
Region			**Product Size**
NR4A1 Peak 1	Forward	CAGACTTTCCCCATCTCAGC	116 bp
	Reverse	AGGAGGCGACAATGTAGCAG	
NR4A1 Peak 2	Forward	TTGTTCTGTGCTGTGCTGTG	112 bp
	Reverse	AGAGCAGGGGAAGGAAGAAA	
Control	Forward	TCCCACTTCCAGAGAACCTG	124 bp
	Reverse	ACAGACGCGGAGAACTCCTA	

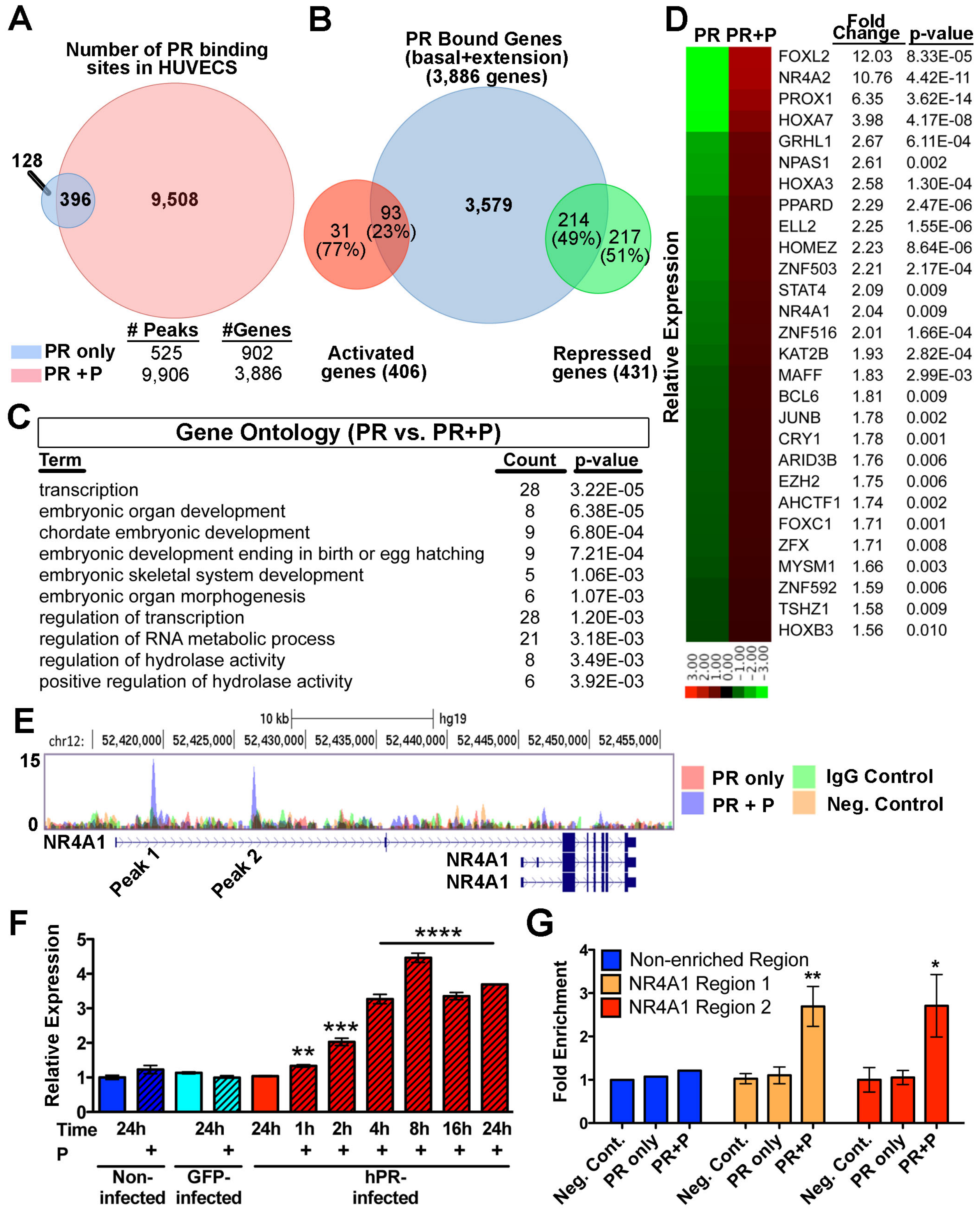
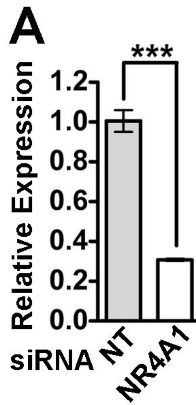

A
Number of PR binding sites in HUVECS
128
396
9,508
Peaks
#Genes
PR only 525 902
PR +P 9,906 3,886
B
PR Bound Genes (basal+extension) (3,886 genes)
3,579
31 (77%)
93 (23%)
214 (49%)
217 (51%)
Activated genes (406)
Repressed genes (431)
C
Gene Ontology (PR vs. PR+P)
Term Count p-value
transcription 28 3.22E-05
embryonic organ development 8 6.38E-05
chordate embryonic development 9 6.80E-04
embryonic development ending in birth or egg hatching 9 7.21E-04
embryonic skeletal system development 5 1.06E-03
embryonic organ morphogenesis 6 1.07E-03
regulation of transcription 28 1.20E-03
regulation of RNA metabolic process 21 3.18E-03
regulation of hydrolase activity 8 3.49E-03
positive regulation of hydrolase activity 6 3.92E-03
D
PR PR+P
Fold Change p-value
FOXL2 12.03 8.33E-05
NR4A2 10.76 4.42E-11
PROX1 6.35 3.62E-14
HOXA7 3.98 4.17E-08
GRHL1 2.67 6.11E-04
NPAS1 2.61 0.002
HOXA3 2.58 1.30E-04
PPARD 2.29 2.47E-06
ELL2 2.25 1.55E-06
HOMEZ 2.23 8.64E-06
ZNF503 2.21 2.17E-04
STAT4 2.09 0.009
NR4A1 2.04 0.009
ZNF516 2.01 1.66E-04
KAT2B 1.93 2.82E-04
MAFF 1.83 2.99E-03
BCL6 1.81 0.009
JUNB 1.78 0.002
CRY1 1.78 0.001
ARID3B 1.76 0.006
EZH2 1.75 0.006
AHCTF1 1.74 0.002
FOXC1 1.71 0.001
ZFX 1.71 0.008
MYSM1 1.66 0.003
ZNF592 1.59 0.006
TSHZ1 1.58 0.009
HOXB3 1.56 0.010
Relative Expression
3.00 2.00 1.00 0.00 -1.00 -2.00 -3.00
E
10 kb
hg19
chr12: 52,420,000 52,425,000 52,430,000 52,435,000 52,440,000 52,445,000 52,450,000 52,455,000
15
0
NR4A1
Peak 1
Peak 2
NR4A1
NR4A1
PR only
PR + P
IgG Control
Neg. Control
F
Relative Expression
5
4
3
2
1
0
**
***
****
Time 24h 24h 24h 1h 2h 4h 8h 16h 24h
P + + + + + + + +
Non-infected
GFP-infected
hPR-infected
G
Fold Enrichment
4
3
2
1
0
Non-enriched Region
NR4A1 Region 1
NR4A1 Region 2
**
*
Neg. Cont.
PR only
PR+P
Neg. Cont.
PR only
PR+P
Neg. Cont.
PR only
PR+P

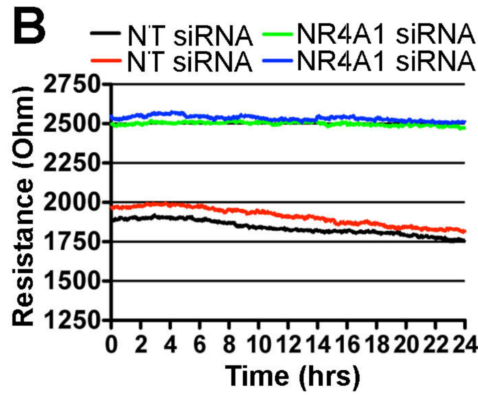
Figure 3.9. NR4A1 is a direct target of PR

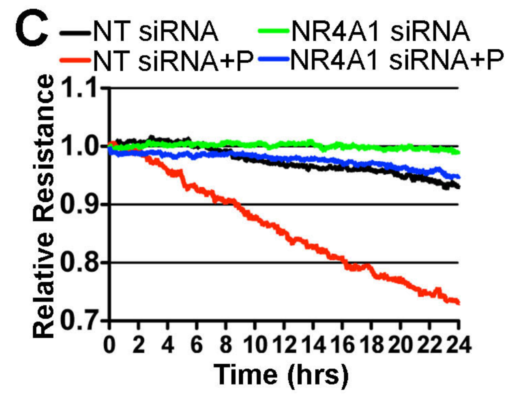
(A) Venn diagram of PR binding peaks between HUVECS infected with PR (blue) and those expressing PR and treated with progesterone for 1 hour (red) as determined by MACS. Gene numbers were predicted based on GREAT annotation analysis of binding peaks within 50kb of the transcriptional start site. **(B)** Venn Diagram representing the overlap between genes predicted to be regulated by PR from ChIP-seq data and genes with a p value less than 0.01 as determined by RNA-seq. **(C)** Top gene ontology terms from activated genes bound by PR as predicted by DAVID. **(D)** Heat map depicting expression and fold change of the 28 transcription factors that were in the top gene ontology pathway. **(D)** Depiction of two binding peaks upstream of the NR4A1 gene in the presence of progesterone. **(E)** qPCR analysis demonstrates a significant increase in expression of NR4A1 following progesterone treatment. Graph is an average of three biological replicates. Ct values were normalized to GAPDH and made relative to non-infected HUVECS. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. **(F)** ChIP-PCR analysis of both NR4A1 binding peaks indicates enrichment following progesterone. Ct values were normalized to those from GAPDH and made relative to negative control samples from each corresponding group. *$p < 0.05$, **$p < 0.01$. In all panels, error bars = +/- SEM and data was analyzed using an unpaired two-tailed Student-T test.

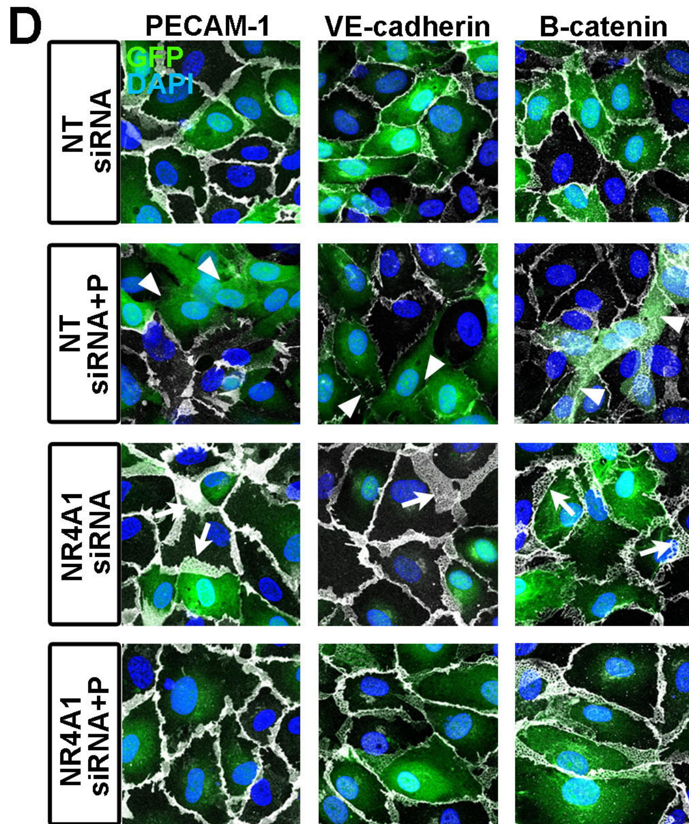
Table 3.3. Gene Ontology terms from an analysis of genes directly repressed by PR.

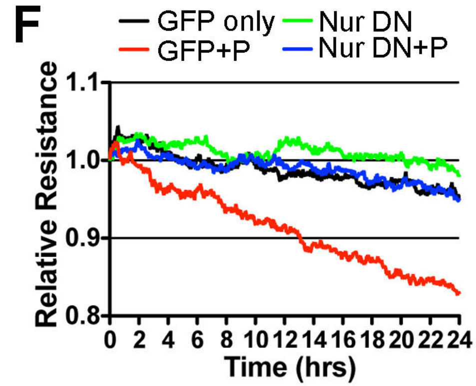
Gene Ontology-Repressed Genes		
Term	**Count**	**p-value**
regulation of cell proliferation	33	2.15E-09
response to organic substance	30	1.75E-08
positive regulation of smooth muscle cell proliferation	8	8.22E-08
regulation of smooth muscle cell proliferation	9	8.36E-08
positive regulation of cell proliferation	21	2.08E-07
blood vessel development	16	3.96E-07
vasculature development	16	5.39E-07
regulation of locomotion	14	7.84E-07
enzyme linked receptor protein signaling pathway	18	1.22E-06
angiogenesis	12	2.30E-06

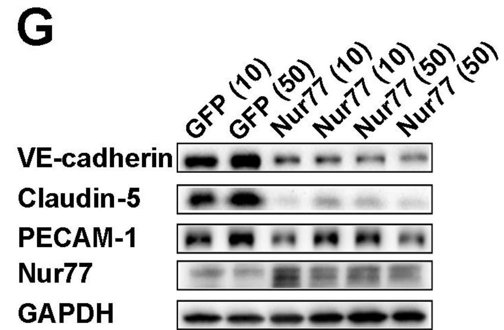

A
***
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
siRNA
NT
NR4A1

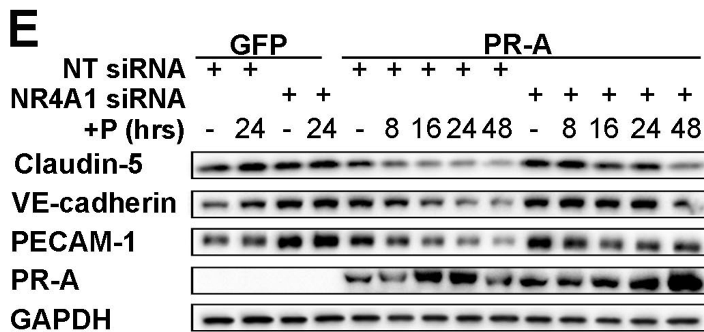

B
NT siRNA
NR4A1 siRNA
NT siRNA
NR4A1 siRNA
Resistance (Ohm)
2750
2500
2250
2000
1750
1500
1250
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hrs)


C
NT siRNA
NR4A1 siRNA
NT siRNA+P
NR4A1 siRNA+P
Relative Resistance
1.1
1.0
0.9
0.8
0.7
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hrs)


D
PECAM-1
VE-cadherin
B-catenin
GFP
DAPI
NT siRNA
NT siRNA+P
NR4A1 siRNA
NR4A1 siRNA+P


F
GFP only
Nur DN
GFP+P
Nur DN+P
Relative Resistance
1.1
1.0
0.9
0.8
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hrs)


G
GFP (10)
GFP (50)
Nur77 (10)
Nur77 (10)
Nur77 (50)
Nur77 (50)
VE-cadherin
Claudin-5
PECAM-1
Nur77
GAPDH


E
GFP
PR-A
NT siRNA
NR4A1 siRNA
+P (hrs)
- 24 - 24 - 8 16 24 48 - 8 16 24 48
Claudin-5
VE-cadherin
PECAM-1
PR-A
GAPDH

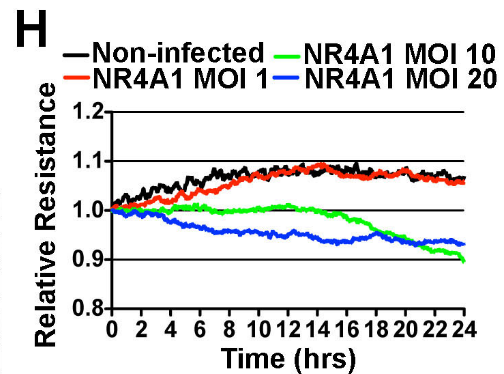

H
Non-infected
NR4A1 MOI 10
NR4A1 MOI 1
NR4A1 MOI 20
Relative Resistance
1.2
1.1
1.0
0.9
0.8
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hrs)

Figure 3.10. Knockdown of NR4A1 inhibits progesterone-mediated permeability
(A) qPCR analysis of NR4A1 following transfection of HUVECS with either non-targeting (NT; grey bar) or NR4A1 siRNA (white bar). Ct values were normalized to GAPDH and made relative to HUVECS expressing non-targeting siRNA. Graph represents an average of three independent experiments. ***$p < 0.001$. **(B)** Increased basal resistance of the HUVEC monolayer following transfection with NR4A1 siRNA. **(C)** Inhibition of progesterone mediated permeability following expression of NR4A1 siRNA. **(D)** HUVECs expressing PR (green; GFP) and transfected with either non-targeting or NR4A1 siRNA were treated with progesterone (100nM) for 24h. PECAM, VE-cadherin, and β–catenin (white) were used to visualize junctions. DAPI (blue) denotes nuclei. Reduction of junctional proteins is seen in cells expressing non-targeting siRNA (arrowheads), while those expressing NR4A1 siRNA have increased junctional expression (arrows). Scale bar = 50 μm. **(E)** Junctional proteins from PR expressing HUVEC lysates following transfection with either non-targeting or NR4A1 siRNA. GAPDH = loading control. **(F)** Inhibition of progesterone mediated permeability following expression of a NR4A family dominant negative (Nur DN). **(G)** Decreased expression of junctional proteins from HUVECs 48h after infection with a NR4A1 adenovirus (MOI 10 and 50). GAPDH = loading control. **(H)** Overexpression of NR4A1 in HUVECS leads to a decrease in monolayer resistance. In all panels error bars = +/- SEM. Data was analyzed using an unpaired two-tailed Student-T test.

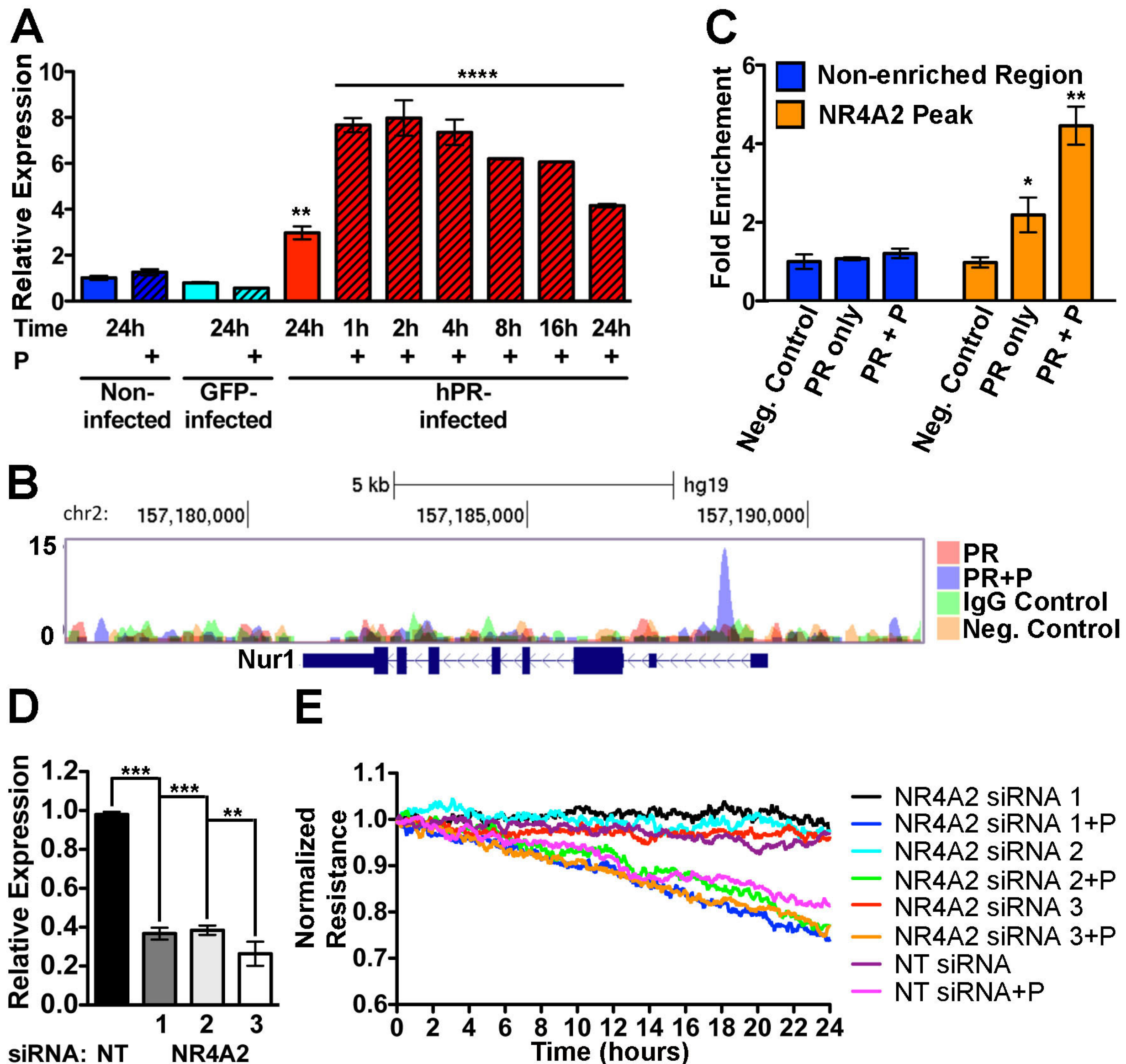


Figure 3.11. NR4A2 is not involved in progesterone mediated permeability
(A) qPCR demonstrates the significant increase in NR4A2 expression following progesterone treatment. Graph represents the average of three independent experiments. **p > 0.01, ****p > 0.0001. **(B)** ChIP-seq analysis demonstrates a clear PR binding peak upstream of the NR4A2 gene only in the presense of progesterone. **(C)** ChIP-PCR of PR expressing HUVECS confirms binding of PR at the DNA correlating to PR binding peaks by ChIP-seq. *p > 0.05, **p > 0.01. **(D)** Knockdown of NR4A2 was accomplished using three independent siRNA constructs. **p > 0.01, ***p > 0.001. **(E)** Knockdown of NR4A2 by three independent siRNAs had no effect on progesterone-mediated permeability. In all panels, error bars represent SEM and data was analyzed using an unpaired two-tailed Student-T test.

References

1. Atkins GB, Jain MK, Hamik A. Endothelial differentiation: molecular mechanisms of specification and heterogeneity. Arterioscler. Thromb. Vasc. Biol. 2011;31:1476–84.

2. Regan ER, Aird WC. Dynamical systems approach to endothelial heterogeneity. Circ Res 2012;111:110–30.

3. Arnal J-F, Fontaine C, Billon-Galés A, Favre J, Laurell H, Lenfant F, et al. Estrogen receptors and endothelium. Arterioscler. Thromb. Vasc. Biol. 2010;30:1506–12.

4. Bernelot Moens SJ, Schnitzler GR, Nickerson M, Guo H, Ueda K, Lu Q, et al. Rapid estrogen receptor signaling is essential for the protective effects of estrogen against vascular injury. Circulation 2012;126:1993–2004.

5. Iafrati MD, Karas RH, Aronovitz M, Kim S, Sullivan TR, Lubahn DB, et al. Estrogen inhibits the vascular injury response in estrogen receptor alpha-deficient mice. Nat. Med. 1997;3:545–8.

6. Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, et al. Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. Science 2002;295:505–8.

7. Sugino N, Kashida S, Karube-Harada A, Takiguchi S, Kato H. Expression of vascular endothelial growth factor (VEGF) and its receptors in human endometrium throughout the menstrual cycle and in early pregnancy. Reproduction 2002;123:379–87.

8. Jerome F Strauss I, Robert L Barbieri MD. Yen and Jaffe's Reproductive Endocrinology. Elsevier Health Sciences; 2009.

9. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin. Reprod. Med. 2007;25:445–53.

10. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, et al. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. 1995;9:2266–78.

11. Lydon JP, DeMayo FJ, Conneely OM, O'Malley BW. Reproductive phenotpes of the progesterone receptor null mutant mouse. J Steroid Biochem. Mol. Biol. 1996;56:67–77.

12. Large MJ, DeMayo FJ. The regulation of embryo implantation and endometrial decidualization by progesterone receptor signaling. Mol. Cell. Endocrinol. 2012;358:155–65.

13. Vázquez F, Rodríguez-Manzaneque JC, Lydon JP, Edwards DP, O'Malley BW, Iruela-Arispe ML. Progesterone regulates proliferation of endothelial cells. J. Biol. Chem. 1999;274:2185–92.

14. Maybin JA, Duncan WC. The human corpus luteum: which cells have progesterone receptors? Reproduction 2004;128:423–31.

15. Krikun G, Schatz F, Taylor R, Critchley HOD, Rogers PAW, Huang J, et al. Endometrial endothelial cell steroid receptor expression and steroid effects on gene expression. J Clin. Endocrinol. Metab. 2005;90:1812–8.

16. Press MF, Udove JA, Greene GL. Progesterone receptor distribution in the human endometrium. Analysis using monoclonal antibodies to the human progesterone receptor. Am. J. Pathol. 1988;131:112–24.

17. Perrot-Applanat M, Cohen-Solal K, Milgrom E, Finet M. Progesterone receptor expression in human saphenous veins. Circulation 1995;92:2975–83.

18. Ismail PM, Li J, DeMayo FJ, O'Malley BW, Lydon JP. A novel LacZ reporter mouse reveals complex regulation of the progesterone receptor promoter during mammary gland development. Mol. Endocrinol. 2002;16:2475–89.

19. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37:1–13.

20. Zhao D, Qin L, Bourbon P-M, James L, Dvorak HF, Zeng H. Orphan nuclear transcription factor TR3/Nur77 regulates microvessel permeability by targeting endothelial nitric oxide synthase and destabilizing endothelial junctions. Proc. Natl. Acad. Sci. U.S.A. 2011;108:12066–71.

21. Pei L, Castrillo A, Tontonoz P. Regulation of macrophage inflammatory gene expression by the orphan nuclear receptor Nur77. Mol. Endocrinol. 2006;20:786–94.

22. Wetendorf M, DeMayo FJ. The progesterone receptor regulates implantation, decidualization, and glandular development via a complex paracrine signaling network. Mol. Cell. Endocrinol. 2012;357:108–18.

23. Franco HL, Rubel CA, Large MJ, Wetendorf M, Fernandez-Valdivia R, Jeong J-W, et al. Epithelial progesterone receptor exhibits pleiotropic roles in uterine development and function. FASEB J 2012;26:1218–27.

24. Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, et al. Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. J Clin. Endocrinol. Metab. 1996;81:3112–8.

25. Fan X, Krieg S, Kuo CJ, Wiegand SJ, Rabinovitch M, Druzin ML, et al. VEGF blockade inhibits angiogenesis and reepithelialization of endometrium. FASEB 2008;22:3571–80.

26. McKenna NJ, O'Malley BW. From ligand to response: generating diversity in nuclear receptor coregulator function. J Steroid Biochem. Mol. Biol. 2000;74:351–6.

27. Rubel CA, Lanz RB, Kommagani R, Franco HL, Lydon JP, DeMayo FJ. Research Resource: Genome-Wide Profiling of Progesterone Receptor Binding in the Mouse Uterus. Mol. Endocrinol. 2012;

28. Zhao Y, Bruemmer D. NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology. Arterioscler. Thromb. Vasc. Biol. 2010;30:1535–41.

29. Dejana E, Orsenigo F, Lampugnani MG. The role of adherens junctions and VE-cadherin in the control of vascular permeability. J. Cell. Sci. 2008;121:2115–22.

30. Komarova Y, Malik AB. Regulation of endothelial permeability via paracellular and transcellular transport pathways. Annu. Rev. Physiol. 2010;72:463–93.

31. Hegele-Hartung C, Fritzemeier KH, Diel P. Effects of a pure antiestrogen and progesterone on estrogen-mediated alterations of blood flow and progesterone receptor expression in the aorta of ovariectomized rabbits. J Steroid Biochem. Mol. Biol. 1997;63:237–49.

32. Winkler M, Kemp B, Classen-Linke I, Fischer DC, Zlatinsi S, Neulen J, et al. Estrogen receptor alpha and progesterone receptor A and B concentration and localization in the lower uterine segment in term parturition. J. Soc. Gynecol. Investig. 2002;9:226–32.

33. Karas RH, van Eickels M, Lydon JP, Roddy S, Kwoun M, Aronovitz M, et al. A complex role for the progesterone receptor in the response to vascular injury. J. Clin. Invest. 2001;108:611–8.

34. Shoupe D, Mishell DR, Bopp BL, Fielding M. The significance of bleeding patterns in Norplant implant users. Obstet Gynecol 1991;77:256–60.

35. Kovacs G. Progestogen-only pills and bleeding disturbances. Hum Reprod 1996;11 Suppl 2:20–3.

36. Krikun G, Critchley H, Schatz F, Wan L, Caze R, Baergen RN, et al. Abnormal uterine bleeding during progestin-only contraception may result from free radical-induced alterations in angiopoietin expression. Am. J. Pathol. 2002;161:979–86.

37. Runic R, Schatz F, Wan L, Demopoulos R, Krikun G, Lockwood CJ. Effects of norplant on endometrial tissue factor expression and blood vessel structure. J

Clin. Endocrinol. Metab. 2000;85:3853–9.

38. Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee E, et al. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc. Natl. Acad. Sci. U.S.A. 1996;93:5860–5.

39. Alva JA, Zovein AC, Monvoisin A, Murphy T, Salazar A, Harvey NL, et al. VE-Cadherin-Cre-recombinase transgenic mouse: a tool for lineage analysis and gene deletion in endothelial cells. Dev. Dyn. 2006;235:759–67.

40. Hashimoto-Partyka MK, Lydon JP, Iruela-Arispe ML. Generation of a mouse for conditional excision of progesterone receptor. Genesis 2006;44:391–5.

41. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 1999;21:70–1.

42. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10:R25.

43. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell 2010;38:576–89.

44. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 2008;9:R137.

45. McLean CY, Bristor D, Hiller M, Clarke SL, Schaar BT, Lowe CB, et al. GREAT improves functional interpretation of cis-regulatory regions. Nat. Biotechnol. 2010;28:495–501.

46. Blankenberg D, Gordon A, Kuster Von G, Coraor N, Taylor J, Nekrutenko A, et al. Manipulation of FASTQ data with Galaxy. Bioinformatics 2010;26:1783–5.

47. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 2009;25:1105–11.

48. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 2010;28:511–5.

49. de Hoon MJL, Imoto S, Nolan J, Miyano S. Open source clustering software. Bioinformatics 2004;20:1453–4.

Chapter 4:
Endothelial PR Mediates Leukocyte Trafficking by Selective Regulation of Endothelial-leukocyte Adhesion Molecules

Abstract

Female sex hormones play an important role in the regulation of the immune system and are believed to underlie gender differences in immune mediated diseases. Although there is evidence to implicate progesterone as an anti-inflammatory agent, little is known about the specific targets and molecular mechanisms. Here we demonstrate that global deletion of progesterone receptor (PR) results in a significant increase in the number of CD3e+ T cells, Mac-1+ macrophages and Mac-1+, Gr-1+ polymorphonuclear cells (PMNs) exclusively in the uterus. Furthermore, using cell specific deletion of PR, we determined that PR signaling in the endothelium preferentially mediates macrophage and PMN transmigration. Integration of next generation RNA- and ChIP-sequencing revealed that PR directly suppresses VCAM-1 and E-selectin expression in the endothelium under both physiological and pathological conditions. These events lead to a reduction in the ability of the endothelium to bind to CD45+ cells under flow. Together, these findings demonstrate a direct role for PR in the endothelium in discriminating immune trafficking of leukocyte subsets by altering the relative expression of specific leukocyte-adhesive proteins by the endothelium.

Introduction

It is well accepted that gender differences underlie the susceptibility and progression of several diseases with immune etiologies [1-3]. While various factors may contribute to this sexual dimorphism, female sex hormones are believed to play an essential role in the regulation of the immune system. This assumption is supported by correlative findings that hormonally governed processes, such as menstruation and pregnancy, parallel fluctuations in immune trafficking [4-7]. Furthermore, pregnancy, a time when progesterone levels are high, results in alterations in the incidence and severity of many immune-mediated diseases, demonstrating a link between female sex hormones and the systemic immune response [8-10]. Currently, the exact mechanism by which progesterone regulates physiological immune trafficking, as well as its cellular and molecular targets, remains to be clarified.

While much is known in relation to immune trafficking following an acute inflammatory response, the process of leukocyte extravasation during physiological conditions is incompletely understood. Many cells of the immune system frequently transmigrate across the endothelium of specific organs and lymph nodes to survey for foreign antigens in the tissue. Leukocyte diapedesis into specific sites requires locally produced chemokines/cytokines, which not only activate leukocytes, but are important for endothelial expression of endothelial-leukocyte adhesion molecules such as VCAM-a and ICAMs [11]. The expression of these molecules seems to be regulated by a mechanism distinct from the process mediated by inflammation.

The endothelium acts as an important gatekeeper in the trafficking of leukocytes from the blood into the interstitial space [12]. In the absence of an inflammatory stimulus,

endothelial cells reduce the levels of leukocyte-interactive proteins such as P- and E-selectin at the cell surface and transcriptionally downregulate VCAM-1, ICAM-1/2, and E-selectin. Thereby interactions with circulating leukocytes are suppressed [13]. Upon exposure to an acute stimulus, endothelial cells secrete cytokine/chemokines and express endothelial-leukocyte adhesion molecules either through the release from Weibel-palade bodies (E-selectin/P-selectin) or by transcriptional upregulation (VCAM-1, ICAM-1/2, PECAM-1, JAMs, E-selectin) [13]. The expression of these molecules results in a series of spatio-temporal events that facilitate the rolling, adhesion, and transmigration of leukocytes across the endothelium.

The uterus imposes unique functional demands on the vasculature, as it requires coordinated fluctuations in immune trafficking during many physiological reproductive processes. Moreover, specific subsets of leukocytes that traffic through the uterus vary according to the cyclical stage as well as reproductive context, yet it is unclear what signals drive this differential recruitment [14,15]. Thus, it is possible that slight alterations on endothelial adhesive molecules underlie the selective trafficking of immune cells into the uterus. Therefore an understanding of the hormonal regulation of these molecules may yield insight into transmigration of particular leukocyte subsets in the absence of an inflammatory stimulus.

Progesterone is generally considered anti-inflammatory, as increased influx of immune cells into the human endometrium is seen during periods of progesterone withdrawal [14,16,17]. Moreover, mice with complete deletion of PR show increased leukocyte infiltrate (in the uterus, suggesting that progesterone controls immune cell trafficking [18]. While several reports have demonstrated expression of PR within different

human vascular beds, its biological role in this tissue is only beginning to be uncovered [19-25]. Furthermore, in vitro studies have demonstrated repression of VCAM-1 by progesterone implicating the hormone in the regulation of leukocyte adhesion molecules, although the biological significance of these findings is yet to be explored in vivo [26,27]. Therefore, we were interested in examining the biological consequences of PR inactivation within the endothelial cell compartment in vivo, and to explore whether progesterone signaling transcriptionally modulates endothelial cell activation and expression of endothelial-leukocyte adhesion molecules.

Here we provide evidence that PR signaling in the endothelium regulates the physiological trafficking of select leukocyte subsets into the uterus. Under both physiological and pathological conditions, PR is able to directly repress the endothelial-leukocyte adhesion molecules VCAM-1 and E-selectin, yet does not significantly alter the expression of JAM-A, JAM-C, or ICAM-2. Concurrent treatment of endothelial cells with progesterone and lipopolysaccharide resulted in reduction in leukocyte binding under flow. These findings expand our understanding of the cell specific function of progesterone in the endothelium and its potential role in immune regulation through selective leukocyte trafficking.

Results

Global deletion of PR results in increased trafficking of leukocytes into the uterus

As reported previously, mice with global deletion of PR **(**PRKO) have increased recruitment of leukocytes into the uterus following estrogen stimulation, suggesting that PR is required for physiological immune trafficking [18]. We confirmed these findings, but were also interested in assessing what individual leukocyte subsets were present in the uterus in the absence of PR. Using flow cytometry, we first examined the number of CD45+ cells in the uterus as a percent of total cell number. As expected, PRKO mice had a 6-fold increase in total CD45+ cells (Figure 4.1A). Using a set of pan markers we determined that the majority of CD45+ cells present in the uterus consisted of T cells (CD3e+), macrophages (Mac-1+), and PMNs (Mac-1+, Gr-1+) and these populations were significantly increased in PRKO mice (Figure 4.1B).

Next, we examined whether this change in leukocyte number altered the relative proportion of these subpopulations as a percent of total CD45+ cells. Interestingly, while all three populations increased in total numbers, PMNs and T cells made up a higher proportion of the CD45+ population, while macrophages represented a smaller proportion compared to controls (Figure 4.1C,D,E). PR mediated leukocyte recruitment was uterine specific, as there were no change in the number of CD45+ cells (Figure 4.1F) or individual leukocyte subsets in the lung, spleen, and peripheral blood (Figure 4.1G).

Conditional deletion of PR from the endothelium leads to selective recruitment of PMNs into the uterus

As the endothelium plays an important role in mediating leukocyte transmigration, we evaluated whether the effect of progesterone on leukocyte trafficking was direct, and through PR signaling in endothelial cells. To do this, we examined leukocyte number in the uteri of mice with cell-specific deletion of PR (PR^{ECKO}). On average, PR^{ECKO} mice exhibited a two-fold increase in the number of CD45+ cells in the uterus compared to controls (Figure 4.2A). As opposed to PRKO animals, PR^{ECKO} mice had a significant increase in the total number of uterine PMNs and macrophages, but not T cells (Figure 4.2B). This selectivity was further evidenced by the fact that only the relative proportion of PMNs increased in PR^{ECKO} mice compared to controls (Figure 4.2D,E,F). Using cell sorting and subsequent cytospin, we determined that the PMN population is a morphologically distinct subset with multi-segmented nuclei, in contrast to Mac-1+ and Gr-1+ single positive cell populations (Figure 4.2C). Similar to PRKO mice, this effect was predominantly uterine specific, as the lung and spleen of PR^{ECKO} mice did not display any difference in total CD45 numbers (Figure 4.2H) or in the number of individual leukocyte subsets (Figure 4.2G). In contrast to findings in PRKO mice, the peripheral blood of PR^{ECKO} mice displayed a slight, yet significant increase in total number of CD45+ cells, which seem to be due to increased number of macrophages and PMNs (Figure 4.2G,H).

Progesterone regulates the expression of multiple endothelial-leukocyte adhesion molecules

Using next generation RNA sequencing, we explored whether PR signaling may transcriptionally alter the expression of endothelial-leukocyte adhesion molecules by the endothelium. To do this, we employed a cell culture based system in which human umbilical vein endothelial cells (HUVECs) expressed human PR following infection with a lentivirus (Figure 5.1). We first assessed whether progesterone stimulation alone could alter the expression of adhesion molecules. We then compared fold change in expression of cell adhesion molecules between PR infected HUVECs in the presence or absence of progesterone for 4h. Out of the 11 endothelial-leukocyte adhesion molecules examined, progesterone negatively regulated VCAM-1, E-selectin (SELE), JAM-C (JAM3), JAM-A (F11R), ICAM-2 and PECAM-1 (Figure 4.3A). However, only the expression of VCAM-1 and E-selectin reached significance ($p<0.01$).

We next assessed whether progesterone stimulation altered the expression of these molecules in the presence of, lipopolysaccharide (LPS) as an acute inflammatory stimulus. Specifically, we evaluated fold change expression in HUVECs when in the presence of LPS alone and treated concurrently with LPS and progesterone at 4 and 8h. Progesterone negatively regulated the expression of MADCAM1, VCAM-1, E-selectin, JAM-A (F11R), JAM-C (JAM3), ICAM-2, and PECAM-1 (Figure 4.3B). Similar to addition of progesterone alone, only VCAM-1 and E-selectin were determined to be significant. Interestingly, ICAM-1 expression increased following progesterone treatment, but this was not apparent until 8h after treatment, and was also not considered significant.

PR binding to specific promoter regions indicates direct transcriptional regulation of endothelial-leukocyte adhesion molecules

To further explore whether PR could directly regulate endothelial-leukocyte adhesion molecule expression, we obtained a global read-out of PR binding sites in the HUVEC genome using ChIP-sequencing. To identify whether the differentially expressed adhesion molecules were direct targets of PR, it was necessary to combine the ChIP-seq and RNA-seq datasets. These genes were then intersected with the list of 3,886 genes predicted as regulated by the PR binding sites obtained from ChIP-seq evaluation. The analysis revealed 214 and 94 genes that were likely directly repressed or activated by PR respectively. Of the 11 adhesion molecules examined, seven of these were predicted to be direct targets of PR. These included MADCAM1, VCAM-1, ICAM-2, E-selectin (SELE), JAM-A (F11R), and JAM-C (JAM3), and ICAM-1 (Figure 4.3A).

Confirmation of leukocyte adhesion molecule expression

In order to confirm the findings from the global transcriptome analysis we more closely examined protein and transcript levels in HUVECs following stimulation by progesterone alone or in combination with LPS (Figure 4.4). As predicted, the RNA levels of VCAM-1 and E-selectin were significantly reduced upon addition of progesterone. This effect was also noted following LPS stimulation as early as 1 hour after progesterone treatment (Figure 4.5 A,C). The changes in transcript levels of VCAM-1 and E-selectin also correlated with a significant decrease in protein levels (Figure 4.4D,E). Interestingly, transcripts for ICAM-1 were significantly increased at 4h post progesterone treatment, although this change did not correlate with a significant change in ICAM-1 protein levels (Figure 4.4B,D).

As the transcription factor NF-kB controls the expression of many of these endothelial-leukocyte adhesion molecules following LPS stimulation, we further assessed whether progesterone affected NF-kB levels and activation. Total levels of NF-kB p65 subunit remained constant following progesterone treatment. Interestingly, phosphorylation of p65 at serine 536 was enhanced in the presence of progesterone (Figure 4.4F).

PR activation results in the reduction of leukocyte binding under flow

To understand whether the regulation of endothelial-leukocyte adhesion molecules hold a functional significance, we assessed whether leukocyte binding to an endothelial monolayer was altered following concurrent treatment with progesterone and LPS. Using a Bioflux flow apparatus we first confirmed the validity and efficiency of the assay by examining human leukocyte binding to a HUVEC monolayer in the presence of LPS under a shear rate of 1dyn/cm^2. As expected, LPS stimulation significantly increased the number of CD45+ cells that bound to the endothelium (Figure 4.5A). HUVECs expressing PR were then treated with progesterone and LPS for 4 and 8 hours and exposed to human peripheral blood leukocytes. Progesterone treatment reduced the number of bound CD45+ cells at both time points, although this was more pronounced at 4h (Figure 4.5C,D). Quantification of bound CD45+ cells per channel confirmed the reduction in binding (Figure 4.5B,E).

Discussion

Steroid hormones are well known regulators of the immune system, however, their cell- and tissue-specific effects in the regulation of inflammation are far less understood [5]. Progesterone, in particular, appears to regulate trafficking or retention of inflammatory cells in the uterus, as loss of progesterone signaling preceding menstruation and prior to labor, parallels a marked influx of immune cells into the human endometrium [4-7,28,29]. Moreover, PRKO mice were found to have increased immune cell infiltration into the uterus, demonstrating that these effects are PR-dependent [18]. While these studies used immunohistochemistry to identify immune populations, we further quantified leukocyte subsets in PRKO mice using flow cytometry. We demonstrate here that complete loss of PR results in an increase in multiple leukocyte subsets including, T cells, macrophages, and PMNs.

As demonstrated in Chapter 3, vascular PR expression is restricted to the murine uterus and detected only in the endothelium of veins and lymphatics. As veins and lymphatics are the primary mode of leukocyte entrance and exit from tissues, it seemed likely that PR signaling in the endothelium held functional significance with regard to immune regulation. As the endothelium functions as a barrier in the regulation of leukocyte trafficking between the blood and tissue, we hypothesized that PR in the endothelium may act to inhibit physiological immune influx into uterine tissue. Using mice with conditional deletion of PR from the endothelium, we demonstrated a 2-fold increase in CD45+ cells in the uterus. This was in contrast to the 6-fold increase we noted in PRKO mice, indicating that the endothelium may be one of many cell types downstream of progesterone signaling that regulates influx of inflammatory cells. This is

in congruence with data demonstrating that uterine smooth muscle and stromal cells secrete a myriad of cytokines during several reproductive processes [17,30-33]. Unlike PRKO animals, loss of PR in the endothelium resulted in an increase in only macrophage and PMN numbers. These findings highlighted a role for PR in the endothelium, but more importantly they demonstrated the ability of the endothelium to discern and recruit different leukocyte subsets under the regulation of PR.

For this reason, we were interested in examining whether progesterone differentially controlled the expression of endothelial-leukocyte adhesion molecules by the endothelium. To do this, we utilized an in vitro culture system in which we could study the direct effects of progesterone on isolated endothelial cells. Using both RNA- and ChIP-sequencing analysis we demonstrated that PR directly suppresses the expression of the adhesive proteins VCAM-1 and E-selectin, yet does not significantly alter the expression of other adhesion molecules including JAM-A, ICAM-1, ICAM-2, PECAM-1, and JAM-C.

E-selectin is critical for the slowing and rolling of leukocytes on the endothelium while VCAM-1 controls the firm adhesion and arrest of leukocytes by binding to leukocytes expressing the integrin complex VLA4 [12]. Thus reduction in the levels of both of these proteins suggests that progesterone affects the distinct steps in the leukocyte adhesion cascade. Interestingly, ICAM-1 levels, although not significant, were increased following progesterone addition. Similar to VCAM-1, ICAM-1 is involved in the firm adhesion of leukocytes to the apical surface of endothelial cells through interactions with leukocytes that express LFA-1 or Mac-1 [34-36]. As VCAM-1 levels are altered, maintenance of ICAM-1 expression may preferentially mediate the trafficking of distinct

leukocyte subsets that express corresponding ligands. As chemokine/cytokine signaling dictate the conformational state of integrins on leukocyte surfaces [37], cytokines in combination with adhesion molecules may further define the leukocyte subset recruited to the uterus. Therefore, we cannot rule out that differential cytokine production is also responsible for the transmigration of immune subpopulations in a context and stimulus dependent manner.

Physiologically, changes in the ovarian cycle parallel fluctuations in immune cell trafficking [5,6,38]. During the secretory phase (progesterone - high), T cells, macrophages, and natural killer cells are the predominant leukocyte subsets in the uterus. Following the withdrawal of progesterone that precedes menstruation, there is a large influx of macrophages, neutrophils, and eosinophils most likely important for the destruction and repair of the endometrium [39]. Our results indicate that progesterone signaling in the endothelium may be one mechanism by which the uterus inhibits trafficking of macrophages and neutrophil subsets, yet allows T cell and NK cell infiltration preceding menstruation through differential expression of VCAM-1 and ICAM-1.

To address whether altered endothelial-leukocyte expression resulted in a functional effect, we examined leukocyte binding to endothelial monolayers under flow. Addition of progesterone following LPS stimulation resulted in the reduction of the number of CD45+ leukocytes bound to the endothelium by 50%. Although progesterone did not completely abrogate leukocyte binding, it is unclear whether this is the reflection of inefficient PR expression, or to the increased levels of ICAM-1. Further experiments addressing the identity of the leukocyte subsets capable of binding to the endothelium following progesterone stimulation will be needed to clarify this issue. In summary, the

results of this study provide novel insight into the functional role of endothelial PR in physiological leukocyte trafficking. PR differentially regulates the expression of a particular subset of endothelial-leukocyte adhesion molecules that potential confer selectivity of leukocyte recruitment.

Materials and Methods

Mouse Models

Mouse lines and their respective genotyping including: VE-cadherin Cre [40], floxed PR [41], and PRKO mice [41] have previously been described. All animals were housed in a pathogen-free environment in an AAALAC-approved vivarium at UCLA, and experiments were performed in accordance with the guidelines of the Committee for Animal Research at the same institution.

Flow Cytometry

Uterus and lung were dissociated by chemical digestion with 1% collagenase (Sigma, St. Louis, MO) and 0.25% DNaseA (Sigma, St. Louis, MO) for 20 min at 37°C. Subsequently cells from these organs as well as the spleen were passed through both a 70-and 40-μm strainer. Blood was obtained by cardiac puncture and stored in EDTA-coated tubes. All samples were collected in Dulbecco's HBSS (Gibco, Invitrogen; Grand Island, NY) and were stored on ice during staining. Red blood cells were lysed in RBC Lysis Buffer. Fcγ receptors were blocked for 10 min and surface antigens were stained for 30 min at 4 °C with the following monoclonal antibodies: Gr-1-PE, CD45-APC, CD3e-PE, Mac-1-FITC, CD19-FITC and 7-AAD (7-amino-actinomycin D) for viability (BD Pharmingen, Franklin Lakes, NJ). Cells (1×10^6) were resuspended in 100 μl flow staining buffer (1% FBS plus, 0.1% Pen/Strep, 0.1% HEPES). Cells were run on an LSRII and analysis was done using FloJo Software and forward- and side-scatter parameters were used for exclusion of doublets.

Cell Culture

Human umbilical vein endothelial cells, passages 4-6, were cultured in MCDB-131 (VEC Technologies, Rensselaer, NY) with the addition of 10% fetal bovine serum (Omega Scientific, Tarzana, CA) that was stripped using 0.25% dextran coated charcoal (Sigma, St. Louis, MO). For immunocytochemistry, HUVECS were seeded onto Lab-Tek II 8-well slides (Thermo Scientific, Rochester, NY). Cells were fixed for 20 minutes with 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and incubated overnight with anti-CD45 (1:400, Vector Labs, Burlingame, CA). Alexa Fluor secondary antibodies were incubated for 1h at RT (1:300, Invitrogen, Grand Island, NY). Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI, 1:1000; Invitrogen, Grand Island, NY). Images were acquired using a Zeiss LSM 520 multiphoton microscope (Zeiss, Germany).

Leukocyte Adhesion Assay

300,000 HUVECS were seeded onto 48 well custom made plates designed for use in by a Fluxion BioFlux 200 (Fluxion Biosciences, San Francisco, CA). Human blood samples were collected from the UCLA Virus core and peripheral blood mononuclear cells were collected by Ficoll gradient and resuspended in RPMI. To examine leukocyte binding, HUVECS were exposed to $2x10^6$ leukocytes/ml at a shear rate of $1dyn/cm^2$ for 10 minutes. Cells were immediately fixed by exposure to 4% paraformaldehyde for 25 min. and subsequent staining was preformed as described in cell culture methods.

Immunoblotting

Proteins were resolved by SDS-PAGE, transferred to reinforced nitrocellulose (Optitran BA-S 83; Dassel, Germany), and incubated overnight with the following antibodies: anti-

PR (1:2000; clone SP2, Lab Vision, Kalamazoo, MI), anti-VCAM-1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p65 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-ICAM-1 (1:1000, Cell Signaling, Danvers, MA), anti-phospho-p65 (S536-1:000, Cell Signaling, Danvers, MA), and anti-GAPDH (1:1000, Millipore, Billerica, MA). Blots were incubated with HRP-conjugated secondary (1:5000; Bio-Rad Laboratories, Hercules, CA), developed with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Kalamazoo, MI) and imaged by a Bio-Rad ChemiDoc XRS+ and accompanying Image Lab software (Bio-Rad Laboratories, Hercules, CA).

Chromatin Immunoprecipitation

Cells were then crosslinked with 1% formaldehyde, resuspended in 400 μl of lysis buffer (1% SDS, 20 mM EDTA and 50 mM Tris-HCl (pH 8.0)) containing protease inhibitors (Roche, Indianapolis, IN), and sonicated using Misonix cup-horn sonicator to achieve 200bp fragments. The lysate was diluted with ChIP dilution buffer containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA and 16.7 mM Tris-HCl (pH 8.1) and immunoprecipitated with 3 ug of anti-PR or IgG antibody overnight at 4 degrees. The complexes were captured using protein A Dynabeads (Invitrogen, Grand Island, NY) and washed twice with the following buffers: low-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1); high-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 500 mM NaCl); LiCl wash buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris-HCl (pH 8.1)) and TE (10 mM Tris-HCl and 1 mM EDTA (pH 8.0)). After elution with 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1% SDS, crosslinks were reversed by overnight incubation at 65°C. Samples were then treated with RNase

A for 30 min at 37°C and proteinase K for 2 h at 56°C. DNA was subsequently purified using Qiagen MinElute Columns according to manufacturers instructions. DNA concentration was measured using a Qubit (Invitrogen, Grand Island, NY).

ChIP-seq analysis

Debarcoding of the multiplex runs was performed using Fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit). Tags were mapped to the human genome (hg19) using bowtie v0.12.7 [42] excluding non-unique mappings (-m 1). 12-22 million uniquely mapped reads were obtained for each sample. Wig files were created using Homer [43] and visualized on UCSC [44]genome browser as custom tracks. Peak identification was performed with MACS v1.3.7.1 [45]. Peaks for PR and PR+P conditions were called using either input, negative control (non-infected cells) or IgG control as a reference and only peaks that were present in all three comparisons were included in the final list of PR binding sites. To identify genes that are potentially regulated by PR, peaks were mapped to nearby genes within 50kb range from the transcriptional start site using Genomic Regions Enrichment of Annotations Tool (GREAT) [46]. Peak intersections and overlaps with differentially expressed genes were performed using Galaxy [47] and in house shell scripts.

RNA isolation, qPCR, and library preparation

Total RNA was extracted using RNeasy Kit (Qiagen, Valencia, CA), cDNA generated using SuperScript First-strand Synthesis System (Invitrogen, Grand Island, NY) and quantitative real-time PCR was performed using SYBR Green reagent (Qiagen,

Valencia, CA) and detected using an Opticon2 PCR machine (MJ Research; BioRad, Hercules, CA). The library for sequencing was constructed using an Illumina Multiplex System according to manufacturer's instructions (Illumina, San Diego, CA). Libraries were sequenced using HIseq-2000 (Illumina, San Diego, CA) to obtain 50 bp long reads. RNA-seq data sets have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE43788.

RNA-seq analysis

Debarcoding of the multiplex runs was performed using in house shell script. Reads were then processed and aligned to the human genome (hg19) using TopHat v2.0.4 [48] with default parameters. Approximately 50 million and 42 million mapped reads where obtained for PR and PR+P samples, respectively. The aligned read files were further processed with Cufflinks v2.0.1[49]. Assemblies for PR and PR+P endothelial cells were merged using CuffMerge and differential expression was determined using Cuffdiff. Genes with a p-value smaller than 0.01 where considered as differentially expressed. For the generation of heatmaps for each gene log2 ratio of a given sample rpkm was divided with the average of the two samples (PR and PR+P) rpkm’s and visualized using treeview [50].

Statistical Analysis

For statistical analysis, Student’s unpaired two-tailed t-test was used for all comparisons.

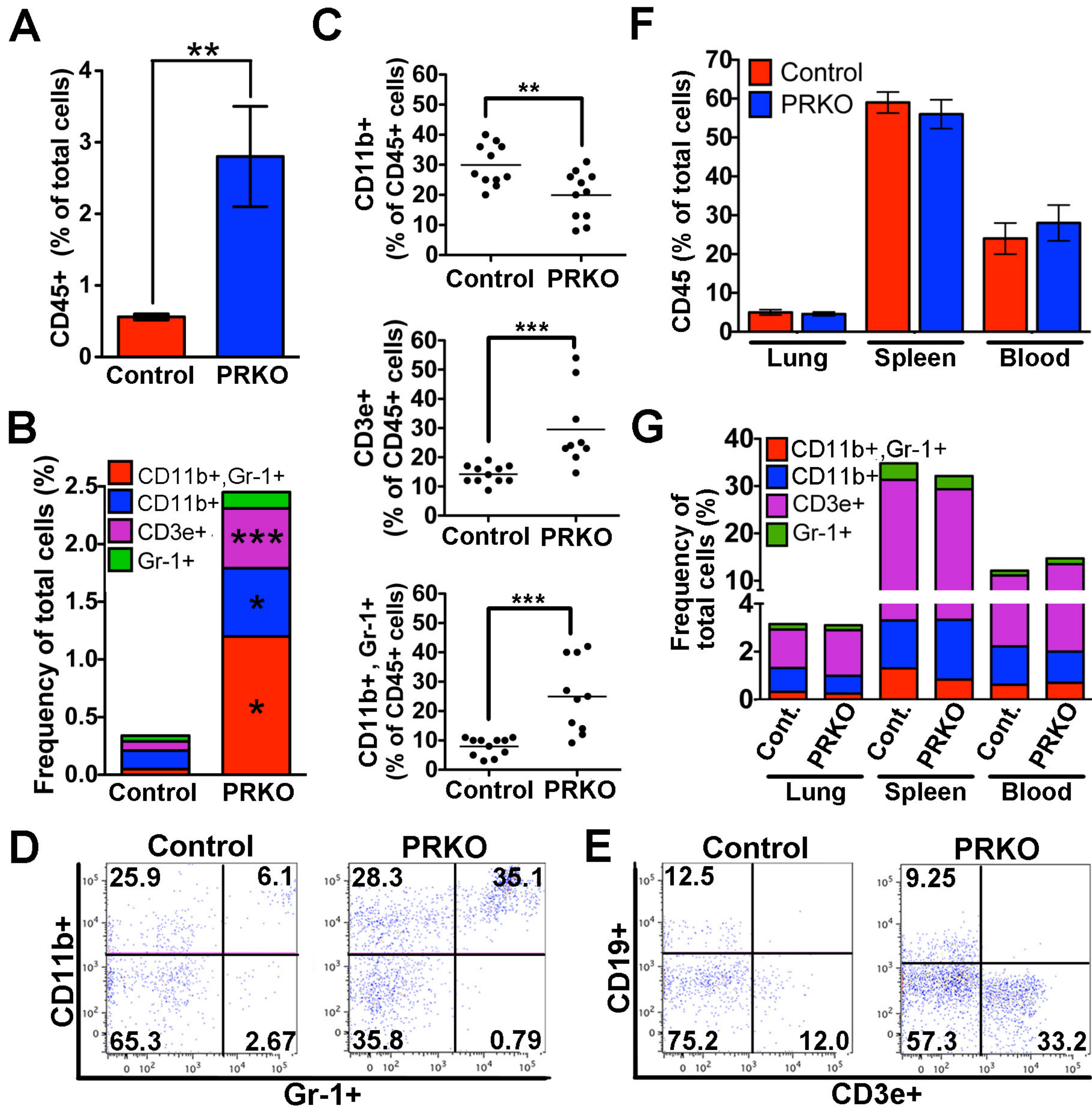


Figure 4.1: Increased leukocyte trafficking into the uterus of PRKO mice
(A) CD45+ cells as a percent of total cells in the uterus of PRKO mice compared to controls. **(B)** Quantification of individual leukocyte subsets (as a percent of total cells) from PRKO and control uteri. CD3e+ = T cells, Mac-1+=macrophages, and Gr-1+, Mac-1+=PMNs **(C)** Quantification of leukocyte subpopulations as percentage of CD45+ cells in the uterus. **(D, E)** Dot plots depict leukocyte cell populations as percentage of the number of CD45+ cells. **(F)** CD45+ cells (as a percent of total cells) between control and PRKO lung, spleen, and peripheral blood. **(G)** The number of CD3e+, Gr-1+, Gr-1+,Mac-1+, and Mac-1+ cells (as a percent of total cells) between control and PRKO lung, spleen, and peripheral blood. In all panels, error bars represent +/- SEM, n=11 for controls and 9-11 for PRKO mice. Data was analyzed using an unpaired two-tailed Student-T test. . *p < 0.05, **p < 0.01, ***p < 0.001

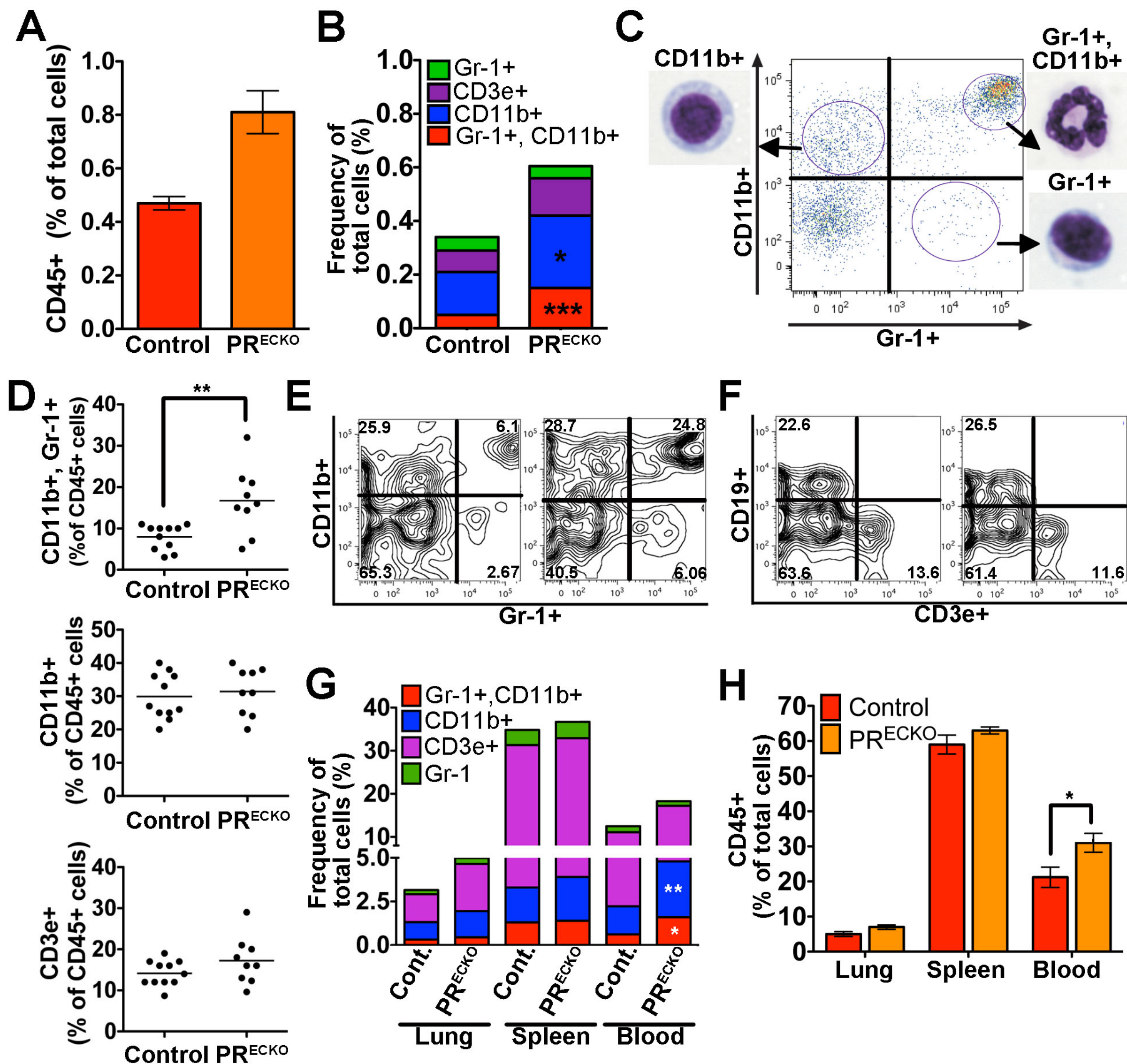
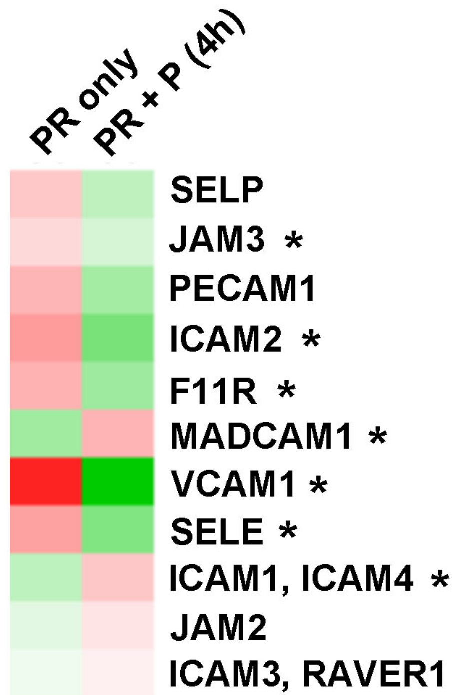


Figure 4.2: Selective increase in PMNs and macrophages in PR^{ECKO} uteri
(A) 2-fold increase in CD45+ cells in the uterus of PR^{ECKO} mice compared to controls. **(B)** Quantification of individual leukocyte subsets (as a percent of total cells) from PR^{ECKO} and control uteri. CD3e+ = T cells, Mac-1+=macrophages, and Gr-1+, Mac-1+=PMNs. **(C)** Morphological analysis to confirm leukocyte subpopulations. **(D)** Quantification of leukocyte subpopulations as percentage of CD45+ cells in the uterus shows. **(E, F)** Contour plots depict leukocyte cell populations as percentage of the number of CD45+ cells. **(G)** The number of CD3e+, Gr-1+, Gr-1+/Mac-1+, and Mac-1+ cells (as a percent of CD45+cells) does not differ between control and PR^{ECKO} mice lung, spleen, and peripheral blood. **(H)** Quantification of the number of CD45+ cells (as a percent of total cells) between control and PR^{ECKO} mice in the lung, spleen, and peripheral blood. In all panels, error bars represent +/- SEM and data was analyzed using an unpaired two-tailed Student-T test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$



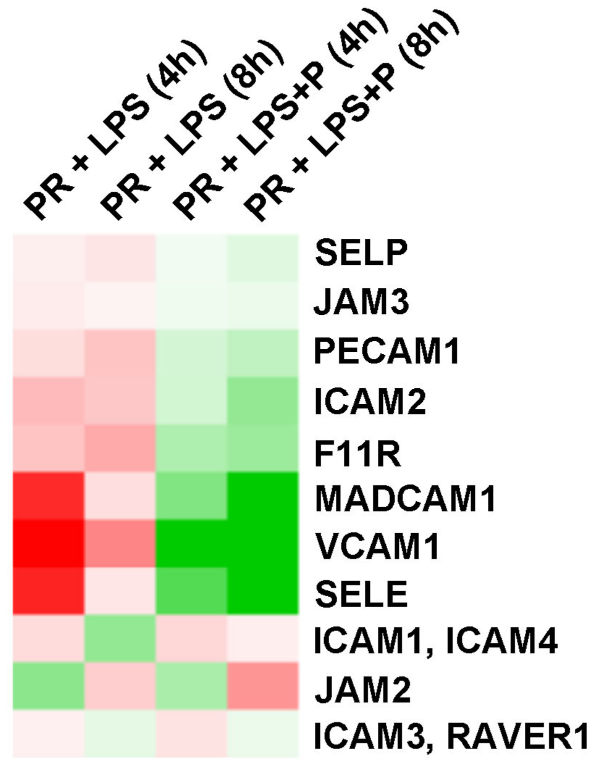


Figure 4.3: Transcriptional profile of PR target genes
(A) Heat map depicting differential expression of endothelial-leukocyte adhesion molecules by HUVECS in the presence and absence of progesterone. * symbolize genes that were predicted to be directly bound by PR (peaks within 200kb of transcriptional start site) by ChIP-seq analysis. **(B)** Heat map depicting differential expression of endothelial-leukocyte adhesion molecules by HUVECS in the presence of LPS alone or in combination with progesterone.

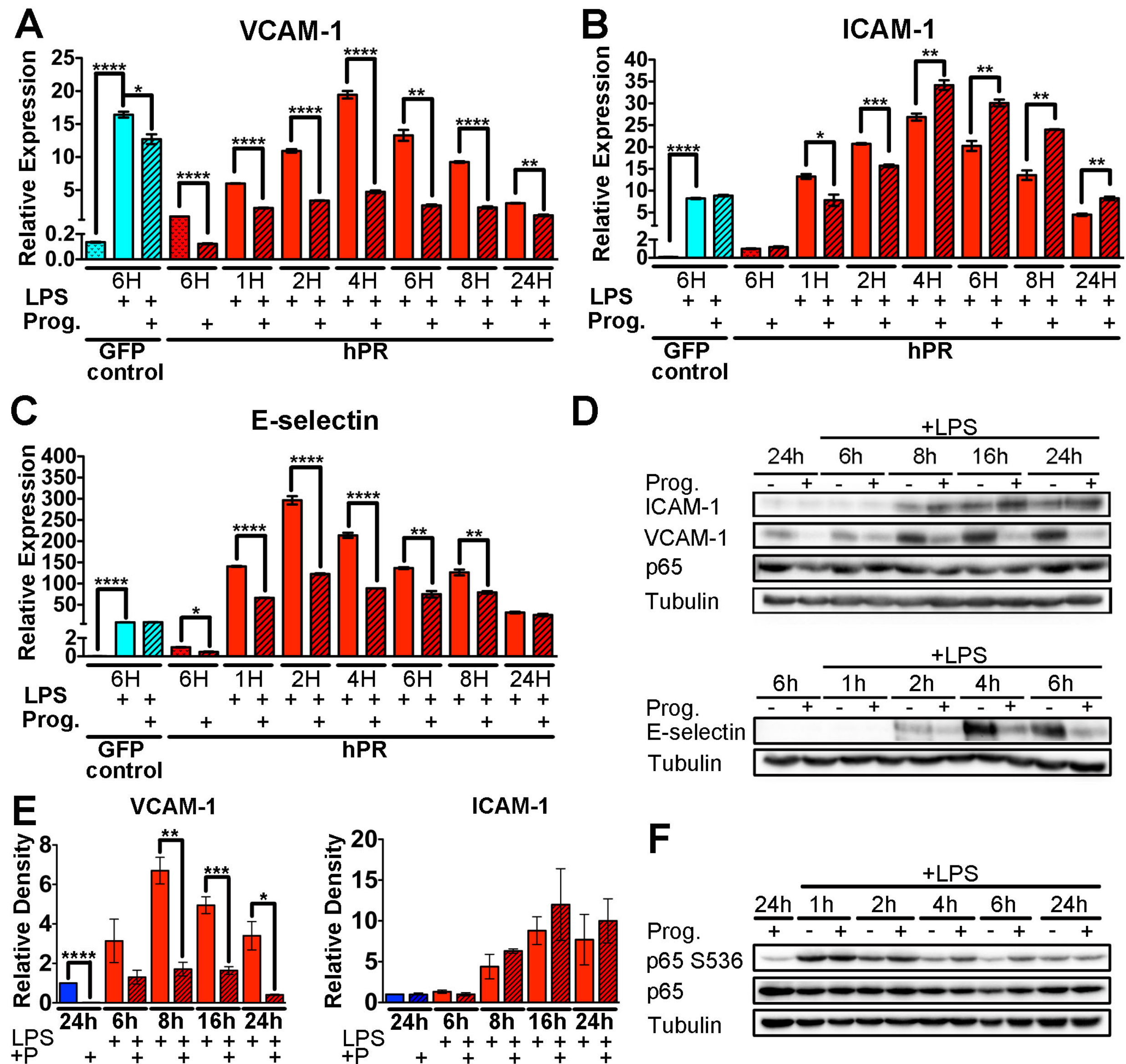


Figure 4.4: PR regulation of endothelial leukocyte adhesion molecules
(A-C) qPCR analysis of VCAM-1 **(A)**, ICAM-1 **(B)** and E-selectin **(C)** expression following progesterone treatment (red hatched bars). Graph is an average of three biological replicates run in triplicate. Ct values were normalized to GAPDH and made relative to PR infected HUVECS in the absence of LPS or progesterone (red dotted bar). GFP infected cells were used as a control and did not respond to progesterone (light blue bars). **(D)** Western blot demonstrating a decrease in LPS induced VCAM-1 expression following progesterone treatment. **(E)** Densitometry of VCAM-1 and ICAM-1 protein levels following progesterone treatment (hatched bars). Bands were normalized to tubulin and made relative to PR infected HUVECs in the absence of treatment (solid blue bars). Graphs display an average of three biological replicates. **(F)** Western blot of phospho- p65 (S536) and total p65 levels. In all panels, error bars represent +/- SEM. Data was analyzed using an unpaired two-tailed Student-T test. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$.

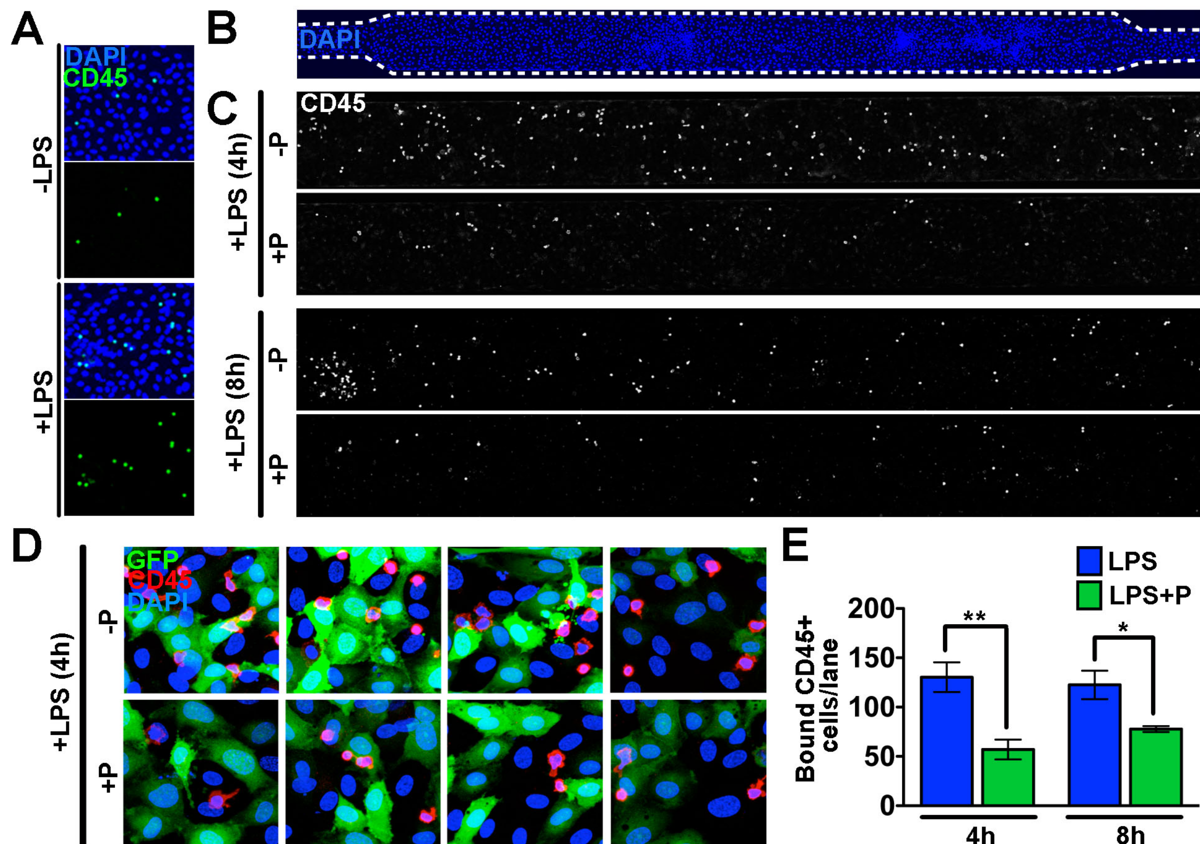


Figure 4.5: PR activation reduces leukocyte binding under flow
(A) Validation of increased leukocyte binding (CD45+; green) under flow (1dyn/cm^2) following LPS stimulation of HUVEC monolayers. DAPI (blue) denotes nuclei. **(B)** Tile scan of a flow channel. DAPI (blue) denotes HUVEC nuclei, while dotted line demarks the outline of the channel. **(C)** Progesterone treatment (4h and 8h) of PR expressing HUVECs reduces the amount of leukocyte binding (CD45+; white) to the endothelium in the presence of LPS. Images are representative of three independent experiments and depict an enlarged area from tile scans of an entire flow channel. **(D)** Higher magnification images of leukocyte binding (CD45; red) to HUVECs overexpressing PR (green; GFP). DAPI (blue) denotes nuclei. **(E)** Quantification of leukocyte binding to the endothelium. Graph depicts the average of three independent experiments. Error bars represent +/- SEM. Data was analyzed using an unpaired two-tailed Student-T test. *$p < 0.05$, **$p < 0.01$.

References:

1. Kaushic C, Roth KL, Anipindi V, Xiu F. Increased prevalence of sexually transmitted viral infections in women: the role of female sex hormones in regulating susceptibility and immune responses. J Reprod Immunol 2011;88:204–9.

2. McCombe PA, Greer JM, Mackay IR. Sexual dimorphism in autoimmune disease. Curr. Mol. Med. 2009;9:1058–79.

3. Meyer MR, Haas E, Barton M. Gender differences of cardiovascular disease: new perspectives for estrogen receptor signaling. Hypertension 2006;47:1019–26.

4. Challis JR, Lockwood CJ, Myatt L, Norman JE, Strauss JF, Petraglia F. Inflammation and pregnancy. Reprod Sci 2009;16:206–15.

5. Gilliver SC. Sex steroids as inflammatory regulators. The Journal of Steroid Biochemistry and Molecular Biology 2010;120:105–15.

6. King AE, Critchley HOD. Oestrogen and progesterone regulation of inflammatory processes in the human endometrium. The Journal of Steroid Biochemistry and Molecular Biology 2010;120:116–26.

7. van Mourik MSM, Macklon NS, Heijnen CJ. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. Journal of Leukocyte Biology 2009;85:4–19.

8. Adams Waldorf KM, Nelson JL. Autoimmune disease during pregnancy and the microchimerism legacy of pregnancy. Immunol. Invest. 2008;37:631–44.

9. Hughes GC. Progesterone and autoimmune disease. Autoimmun Rev 2012;11:A502–14.

10. Martocchia A, Stefanelli M, Cola S, Falaschi P. Sex steroids in autoimmune diseases. Curr Top Med Chem 2011;11:1668–83.

11. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol 2005;

12. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 2007;7:678–89.

13. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nat Rev Immunol 2007;7:803–15.

14. Hamilton SA, Tower CL, Jones RL. Identification of chemokines associated with the recruitment of decidual leukocytes in human labour: potential novel targets for

preterm labour. PLoS One 2013;8:e56946.

15. Oertelt-Prigione S. Immunology and the menstrual cycle. Autoimmun Rev 2012;11:A486–92.

16. Hamilton S, Oomomian Y, Stephen G, Shynlova O, Tower CL, Garrod A, et al. Macrophages infiltrate the human and rat decidua during term and preterm labor: evidence that decidual inflammation precedes labor. Biol. Reprod. 2012;86:39.

17. Shynlova O, Tsui P, Dorogin A, Lye SJ. Monocyte chemoattractant protein-1 (CCL-2) integrates mechanical and endocrine signals that mediate term and preterm labor. The Journal of Immunology 2008;181:1470–9.

18. Tibbetts TA, Conneely OM, O'Malley BW. Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. Biol. Reprod. 1999;60:1158–65.

19. Ingegno MD, Money SR, Thelmo W, Greene GL, Davidian M, Jaffe BM, et al. Progesterone receptors in the human heart and great vessels. Lab. Invest. 1988;59:353–6.

20. Iruela-Arispe ML, Rodriguez-Manzaneque JC, Abu-Jawdeh G. Endometrial endothelial cells express estrogen and progesterone receptors and exhibit a tissue specific response to angiogenic growth factors. UMIC [Internet] 1999;6:127–40. Available from: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=10466115&retmode=ref&cmd=prlinks

21. Rodríguez-Manzaneque JC, Graubert M, Iruela-Arispe ML. Endothelial cell dysfunction following prolonged activation of progesterone receptor. Hum Reprod 2000;15 Suppl 3:39–47.

22. Krikun G, Schatz F, Taylor R, Critchley HOD, Rogers PAW, Huang J, et al. Endometrial endothelial cell steroid receptor expression and steroid effects on gene expression. Journal of Clinical Endocrinology & Metabolism 2005;90:1812–8.

23. Maybin JA, Duncan WC. The human corpus luteum: which cells have progesterone receptors? Reproduction 2004;128:423–31.

24. Perrot-Applanat M, Cohen-Solal K, Milgrom E, Finet M. Progesterone receptor expression in human saphenous veins. Circulation 1995;92:2975–83.

25. Vázquez F, Rodríguez-Manzaneque JC, Lydon JP, Edwards DP, O'Malley BW, Iruela-Arispe ML. Progesterone regulates proliferation of endothelial cells. J. Biol. Chem. 1999;274:2185–92.

26. Otsuki M, Saito H, Xu X, Sumitani S, Kouhara H, Kishimoto T, et al.

Progesterone, but not medroxyprogesterone, inhibits vascular cell adhesion molecule-1 expression in human vascular endothelial cells. Arterioscler. Thromb. Vasc. Biol. 2001;21:243–8.

27. Simoncini T. Differential Signal Transduction of Progesterone and Medroxyprogesterone Acetate in Human Endothelial Cells. Endocrinology 2004;145:5745–56.

28. Jones RL. Identification of Chemokines Important for Leukocyte Recruitment to the Human Endometrium at the Times of Embryo Implantation and Menstruation. Journal of Clinical Endocrinology & Metabolism 2004;89:6155–67.

29. Red-Horse K, Drake PM. Human pregnancy: the role of chemokine networks at the fetalmaternal interface. Expert Rev Mol Med 2004; 6:1-14

30. Gotkin JL, Celver J, McNutt P, Shields AD, Howard BC, Paonessa DJ, et al. Progesterone reduces lipopolysaccharide induced interleukin-6 secretion in fetoplacental chorionic arteries, fractionated cord blood, and maternal mononuclear cells. Am. J. Obstet. Gynecol. 2006;195:1015–9.

31. Hardy DB, Janowski BA, Corey DR, Mendelson CR. Progesterone receptor plays a major antiinflammatory role in human myometrial cells by antagonism of nuclear factor-kappaB activation of cyclooxygenase 2 expression. Molecular Endocrinology 2006;20:2724–33.

32. Luk J, Seval Y, Ulukus M, Ulukus EC, Arici A, Kayisli UA. Regulation of monocyte chemotactic protein-1 expression in human endometrial endothelial cells by sex steroids: a potential mechanism for leukocyte recruitment in endometriosis. Reprod Sci 2010;17:278–87.

33. Shields AD, Wright J, Paonessa DJ, Gotkin J, Howard BC, Hoeldtke NJ, et al. Progesterone modulation of inflammatory cytokine production in a fetoplacental artery explant model. Am. J. Obstet. Gynecol. 2005;193:1144–8.

34. Martin-Padura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, et al. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol 1998;142:117–27.

35. Woodfin A, Voisin M-B, Imhof BA, Dejana E, Engelhardt B, Nourshargh S. Endothelial cell activation leads to neutrophil transmigration as supported by the sequential roles of ICAM-2, JAM-A, and PECAM-1. Blood 2009;113:6246–57.

36. Johnson-Léger CA, Aurrand-Lions M, Beltraminelli N, Fasel N, Imhof BA. Junctional adhesion molecule-2 (JAM-2) promotes lymphocyte transendothelial migration. Blood 2002;100:2479–86.

37. Lawson C, Wolf S. ICAM-1 signaling in endothelial cells. Pharmacol Rep

2009;61:22–32.

38. Oertelt-Prigione S. The influence of sex and gender on the immune response. Autoimmun Rev 2012;11:A479–85.

39. Henriet P, Gaide Chevronnay HP, Marbaix E. The endocrine and paracrine control of menstruation. Mol. Cell. Endocrinol. 2012;358:197–207.

40. Alva JA, Zovein AC, Monvoisin A, Murphy T, Salazar A, Harvey NL, et al. VE-Cadherin-Cre-recombinase transgenic mouse: a tool for lineage analysis and gene deletion in endothelial cells. Dev. Dyn. 2006;235:759–67.

41. Hashimoto-Partyka MK, Lydon JP, Iruela-Arispe ML. Generation of a mouse for conditional excision of progesterone receptor. Genesis 2006;44:391–5.

42. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10:R25.

43. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell 2010;38:576–89.

44. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al. The human genome browser at UCSC. Genome Res. 2002;12:996–1006.

45. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 2008;9:R137.

46. McLean CY, Bristor D, Hiller M, Clarke SL, Schaar BT, Lowe CB, et al. GREAT improves functional interpretation of cis-regulatory regions. Nat. Biotechnol. 2010;28:495–501.

47. Blankenberg D, Gordon A, Kuster Von G, Coraor N, Taylor J, Nekrutenko A, et al. Manipulation of FASTQ data with Galaxy. Bioinformatics 2010;26:1783–5.

48. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 2009;25:1105–11.

49. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 2010;28:511–5.

50. de Hoon MJL, Imoto S, Nolan J, Miyano S. Open source clustering software. Bioinformatics 2004;20:1453–4.

Chapter 5:
Selective Suppression of Endothelial Cytokine Production by Progesterone Receptor

Abstract

Steroid hormones are well-recognized suppressors of the inflammatory response, however, their cell- and tissue-specific effects in the regulation of inflammation are far less understood, particularly for the sex-related steroids. To determine the contribution of progesterone in the endothelium, we have characterized and validated an in vitro culture system in which human umbilical vein endothelial cells constitutively express human progesterone receptor (PR). Using next generation RNA-sequencing, we identified a selective group of cytokines that are suppressed by progesterone both under physiological conditions and during pathological activation by lipopolysaccharide. In particular, IL-6, IL-8, CXCL2/3, and CXCL1 were found to be direct targets of PR, as determined by ChIP-sequencing. Regulation of these cytokines by progesterone was also confirmed by bead-based multiplex cytokine assays and quantitative PCR. These findings provide a novel role for PR in the direct regulation of cytokine levels secreted by the endothelium. They also suggest that progesterone-PR signaling in the endothelium directly impacts leukocyte trafficking in PR-expressing tissues.

Introduction

Inflammation contributes to the susceptibility and progression of many diseases that exhibit gender based differences in prevalence. These include, but are not limited to, autoimmune disease, cardiovascular disease and sexually transmitted infections [1-3]. The prevailing hypothesis is that endocrine-immune interactions drive this sexual dimorphism by affecting the sensitivity to various inflammatory stimuli. Evidence for this emanates from studies demonstrating the requirement for the immune system in hormonally controlled processes including implantation, cycling, and pregnancy [4-9]. For example, symptoms of rheumatoid arthritis and multiple sclerosis are reduced during pregnancy, suggesting that hormones not only modulate local inflammatory reactions, but can affect systemic immune responses as well [10-12]. While much is known of the cellular and molecular control of the immune system by estrogen, glucocorticoids, and androgen signaling, the action of progesterone and its downstream targets are far less understood.

Progesterone has been generally assumed to play an anti-inflammatory role in immune regulation. In fact, the physiological reduction of progesterone prior to menstruation and preceding labor results in a marked influx of inflammatory cells (macrophages, neutrophils, and T cells) into the decidua resembling a local inflammatory response [6,13-15]. Moreover, mice with complete deletion of PR (PRKO) were found to have increased immune cell infiltration into the uterus and impaired thymic function [16,17]. At the cellular level, PR expression has been demonstrated in a variety of immune cell types indicative of a direct regulation by progesterone [5,11,18]. However, these findings do not explain progesterone control of other leukocyte

populations that do not express PR in vivo, such as natural killer cells and granulocytes. Therefore, it is likely that paracrine factors such as cytokines and chemokines act as effectors of steroid hormones, thus enabling systemic immune modulation in the absence of leukocyte steroid receptors. In fact, there is ample evidence in the literature for regulation of immune function by progesterone through its affect on smooth muscle, stromal, and perivascular cells [15,19-22]. Due to its multiple cellular targets, a comprehensive dissection of cell specific signaling, as well as direct downstream targets of PR, is necessary to understand the multiple immune-modulatory functions of progesterone.

The endothelium is an active participant in immune cell trafficking and is an important barrier in the regulation of leukocyte extravasation into tissues [23,24]. Upon activation by an inflammatory stimulus, endothelial cells acquire new capabilities including cytokines/chemokine secretion and the expression of endothelial-leukocyte adhesion molecules [24]. Several reports have demonstrated expression of PR within different human vascular beds [25-30], including endothelial cells of human atherosclerotic vessels [31]. Functionally, progesterone has been found to mediate endothelial cell proliferation, transcriptional repression of endothelial-leukocyte adhesion molecules, as well as MMP secretion [30-32] implicating a direct function of progesterone in the endothelium. Therefore, we hypothesized that progesterone signaling may modulate the immune system by transcriptionally altering endothelial cell activation and expression of immunomodulatory factors.

Here we provide evidence that PR signaling in the endothelium directly regulates cytokine expression both under physiological conditions as well as following an acute

inflammatory stimulus. PR is able to selectively and directly target a cohort of endothelial cytokines resulting in transcriptional repression and reduction in protein levels by the endothelium. These findings expand our understanding of the cell specific function of progesterone in the endothelium and its potential role in immune regulation through direct mediation of cytokine production.

Results

Generation and validation of a lentivirus for expression of human PR

A detailed, comprehensive histological examination of PR expression in the mouse confirmed the presence of PR in the endothelium (Figure 5.1A). Interestingly, PR positive endothelial cells were restricted to veins, but absent from arteries. Although expressed by different vascular beds in humans, endothelial PR expression in the mouse was restricted to the vasculature of the uterus, suggestive of its unique importance in this organ.

In order to gain a better molecular understanding of progesterone function in the endothelium, we overexpressed human PR in human umbilical vein endothelial cells (HUVECs) using lentiviral infection. Full-length human PR cDNA (hPR) was cloned downstream of a CMV promoter and preceding sequences for eGFP and puromycin resistance (Figure 5.1B). A lentivirus expressing eGFP under the control of a CMV promoter was used as a control. HUVECs overexpressing the GFP or hPR construct looked morphologically normal when compared to uninfected HUVECs (Figure 5.1C). PR protein colocalized with GFP positive cells, confirming GFP as an indicator of PR expressing cells (Figure 5.1D). Western blot analysis demonstrated the expression of both PR isoforms, PR-A and PR-B, in HUVECs expressing hPR, but not of the cells expressing GFP alone (Figure 5.1E). PR protein levels were most optimally expressed at viral concentrations between 0.13 and 0.16 μg/mL, while higher concentrations led to cell death and reduction in PR protein expression.

HUVECs expressing hPR were treated with progesterone to evaluate ability of the transduced receptor to respond to progesterone (Figure 5.2A). PR was mainly

localized to the nucleus in both the presence and absence of progesterone, but was almost exclusively localized to the nucleus following progesterone treatment (Figure 5.2A). To determine the optimal infection efficiency of the hPR lentivirus, cells were infected with progressively lower viral concentrations. Titration of virus demonstrated that concentrations less than 0.16 μg/mL led to a reduction of PR expressing cells upon quantification (Figure 5.2B). Therefore, all subsequent experiments were performed at an hPR concentration of 0.16 μg/mL.

PR negatively regulates endothelial cytokine production

Using next generation RNA sequencing**,** we explored whether PR signaling may transcriptionally alter the expression of cytokines by the endothelium. First, we assessed which cytokines might be specifically altered when lipopolysaccharide (LPS) was applied to endothelial cultures for 4 and 8 hours. A total of 70 cytokines were included in the initial analysis. Of these 70, only 27 showed transcript expression by the endothelium (Figure 5.3A), and only 15 of these were significantly ($p<0.01$) altered in the presence of LPS at both 4 and 8 hours of treatment (Figure 5.3B). To determine if progesterone altered the expression of these 15 genes, we examined fold change expression between HUVECs in the presence of LPS alone and those treated concurrently with LPS and progesterone (Figure 5.3B). Although majority of these cytokines were downregulated by progesterone, only five: including CCL2, IL-6, IL-8, CXCL1 and CXCL2, were considered to be statistically significantly ($p<0.01$). To assess if progesterone alone, in the absence of LPS, was able to reduce the expression of these same five genes, we compared fold change expression between hPR infected

cells in the presence or absence of progesterone for 4h (Figure 5.3C). Even in the absence of LPS, progesterone still negatively regulated the expression of IL-8, IL-6, CXCL1, and CXCL1/2, suggesting that progesterone may modulate cytokine production even in the absence of an acute inflammatory stimulus.

PR binding peaks reveal direct transcriptional regulation of cytokine production

To further explore whether PR could directly regulate cytokine expression, we obtained a global read-out of PR binding sites in the HUVEC genome using ChIP-sequencing. Activation of the receptor by progesterone resulted in 9,906 PR binding sites. To identify whether the 5 cytokines significantly regulated by progesterone might be direct targets of PR, it was necessary to combine the ChIP-seq and RNA-seq datasets. RNA-seq analysis of HUVECs yielded 431 downregulated genes with a *p*-value less than 0.01. These genes were then intersected with the list of 3,886 genes predicted as regulated by the PR binding sites obtained from ChIP-seq evaluation. The analysis revealed 214 genes that were likely directly repressed by PR. Of this list, 4 out of the 5 cytokines found to be downregulated by progesterone were also predicted to be direct targets of PR (Figure 5.3C).

PR negatively regulates cytokine production in the endothelium

To confirm whether treatment of the endothelium with progesterone leads to a reduction in cytokine secretion, we performed a 42-multiplex bead-based cytokine array. This approach was set up to determine whether reduced RNA transcript levels correlated with cytokine protein production. Of 42 cytokines analyzed, only 24 were at sufficiently high levels to be detected by the array (Table 5.1). Analysis of these 24

cytokines uncovered selective regulation of 8 by progesterone, including fractalkine, GRO, IL-6, IL-8, IP-10, MCP-1, PDGF-AA, and PDGFAB/BB. Cytokine regulation was PR-dependent as per evaluation of HUVECs transduced with a GFP control construct. These findings were very similar to the RNA- and ChIP-seq analysis, as IL-6, IL-8, CCL2/MCP-1, and CXCL1/GRO were all found to be targets of PR (Figure 5.3C).

Confirmation of cytokine transcript levels following progesterone treatment

In order to confirm the findings from the global transcriptome analysis and the multiplex cytokine array, we more closely examined protein and transcript levels following concurrent exposure of HUVECs to progesterone and LPS (Figure 5.4-5.6). As predicted, the protein levels for IL-6 were significantly reduced in the presence of progesterone (Figure 5.4A). This correlated with a significant decrease in transcript levels as early as 1 hour after progesterone treatment (Figure 5.4B).

IL-8/CXCL8 showed a similar expression pattern to that of IL-6, but regulation by progesterone was not as pronounced (Figure 5.5A,D). CXCL10/IP-10 RNA levels were negatively regulated by progesterone at early time points, yet protein levels were not significantly reduced until 24 hours after treatment (Figure 5.5B,E). Interestingly, transcript levels of CXCL1/GRO were significantly downregulated by progesterone at very early time points of 1 and 2 hours (Figure 5.5F). This correlated with a reduction in protein at 4 and 8 hours, yet this phenomenon did not extend to 24 hours (Figure 5.5C).

Progesterone significantly reduced RNA levels of the CCL family member, MCP-1/CCL2, yet decreased protein levels did not reach significance (Figure 5.6A,C). Alternatively, the CX3C family member, CX3CL1/fractalkine, although not found to be

significantly reduced by RNA-seq analysis, did show significant reduction in both RNA and protein expression at all times of progesterone treatment (Figure 5.6B,D).

Discussion

Hormones are believed to play an important role in the sexual dimorphism underlying diseases with immune etiologies [5,11,12]. Clearly interdependence exists between different hormonal signaling pathways in immune regulation, yet this complexity makes it difficult to assess the contributions of individual steroid hormones, particularly in vivo. Although several studies have demonstrated the anti-inflammatory properties of progesterone within the context of its reproductive functions [4-9], little is understood as to its direct cellular and molecular targets with respect to immune regulation. As the vascular endothelium is known to mediate leukocyte homing and selective extravasation, we hypothesized that progesterone signaling might transcriptionally modulate the activation state of the endothelium in response to an acute inflammatory stimulus. Using unbiased global expression analysis we demonstrated that progesterone signaling, via PR, directly suppresses a select group of cytokine and chemokines expressed by the endothelium. This reduction was seen both under physiological and pathological activation by LPS, indicating that endothelial cells are also susceptible to anti-inflammatory regulation by progesterone.

Our results indicate that under homeostatic conditions, endothelial expression of PR is selective to veins and conspicuously absent from arteries. This exquisite specificity is consistent with the fact that immune trafficking occurs predominantly in venules and lymphatics [24]. Thus, restricted expression allows spatial regulation of PR function in response to a systemically distributed ligand. Although PR is not constitutively expressed in the endothelium, the focal and sporadic expression may be indicative of a tightly regulated and precisely localized function. While not much is

known of the role of PR in the endothelium, a select group of in vitro studies have shown that PR can inhibit the expression of the endothelial-leukocyte adhesion molecule, VCAM-1, and the cytokines IL-8 and MCP-1 [32,43,44], yet no study has assessed global expression of endothelial genes upon progesterone stimulation.

To examine the contribution of PR in the vascular endothelium at the molecular level, we employed a cell culture based system using HUVECs. Using global transcriptome analysis we examined cytokine production from the endothelium in the presence of LPS. Of 70 cytokines examined, 29 of these displayed altered expression in the presence of LPS, yet only 15 were considered statistically significant. From these, 5 were significantly downregulated by progesterone (IL-8, IL-6, CCL2, CXCL1 and CXCL2/3) while 4 (IL-6, IL-8, CXCL1, and CXCL2) were directly bound by PR. These findings are intriguing as not only does the endothelium itself preferentially produce a unique subset of cytokines in response to LPS, but only a small proportion of these are presumably controlled by PR. Therefore, progesterone may modulate specific leukocyte subsets in response to an acute inflammatory event. Indeed, the majority of the direct cytokine/chemokine targets of progesterone noted in this study were found to be neutrophil/monocyte attractants [14,45].

Biologically, the local tissue response to withdrawal of progesterone shows many features characteristic of an inflammatory response [5,7,46]. Following progesterone decline in the circulation that precedes menstruation, there is a significant influx of neutrophils, eosinophils, and macrophages into the uterus, which are likely critical for focal inflammatory mediated endometrial repair [47]. Analysis of whole decidual tissue has implicated MCP-1, IL-8, IL-6, MDC, fractalkine, eotaxin, and MCP-3 following the

decline in progesterone levels that initiate menstruation [6,13,48-52]. Moreover, IL-8 and MCP-1 levels as well as monocyte numbers are increased in human decidua from women taking the PR inhibitor, mifepristone [53]. Furthermore, the expression of these two cytokines (both in vitro and in vivo) was inhibited by progesterone [21,51,54,55].

Similar to menstruation, proinflammatory cytokines also play a central role in the mechanisms of term and inflammation/infection-induced preterm parturition [13,56,57]. Cytokines associated with this process also include MCP-1, IL-8 and IL-6, in addition to RANTES, and MIP-B1 [13,14,58]. As progesterone is capable of inhibiting the expression of MCP-1, IL-8, and IL-6 in the endothelium, it is possible the vasculature plays a critical role in maintaining an immunosuppressive environment in the uterus prior to these immune-mediated events. Naturally, other cell types, including stromal and epithelial cells also play key roles in immune regulation [13].

Interestingly, we determined that GRO/CXCL1/2/3 is a direct target of progesterone in the endothelium, yet a role for GRO has not been revealed with regard to reproductive immune infiltration. Recently, progesterone has been found to inhibit expression of GRO in ovarian and endometrial cancer cells as well as dendritic cells [59,60]. As GRO is a potent chemoattractant for neutrophils, more so than IL-8, suppression of GRO by progesterone may play an even stronger role in the inhibition of neutrophil trafficking.

To confirm that progesterone mediated changes in RNA expression correlated with differential protein production, we performed an unbiased bead-based multiplex cytokine array. While all of the cytokines determined to be transcriptionally modulated following RNA-seq analysis were regulated at the protein level, two additional cytokines,

fractalkine/CX3CL1 and CXCL10/IP-10, were also found to be downregulated following analysis of the multiplex array. Subsequent qPCR analysis confirmed this reduction at the RNA level.

Based on our analysis of the RNA-seq data, CXCL10 did not meet the criteria as for being regulated by LPS, and thus was excluded from further evaluation. In addition, although LPS significantly modulated CX3CL1 expression, it was not significantly altered by progesterone. It is likely that these two cytokines are targets of progesterone, but due to the stringent statistical analysis used for our RNA-seq datasets these cytokines were not found to be significant.

Conclusion

The results of this study provide detailed insight into the endothelial cell specific role of progesterone signaling in the regulation of cytokine production. PR directly suppresses the expression of a small subset of cytokines both under physiological conditions and following stimulation by LPS. These results confirm PR as an anti-inflammatory agent in the endothelium, with potential for the negative regulation of immune cell trafficking into tissues. Understanding the factors and cell populations that control immune cells will further clarify gender differences in disease as well as dysregulated immune-mediated reproductive processes such as preterm labor.

Materials and Methods

Virus Production and Transduction

Human PR cDNA was PCR amplified and cloned into a lentiviral vector using the following primers with attached restriction site sequences: 5'-PR-XbaI (GCTATCTAGAATGACTGAGCTGAAGGCA) and 3'-PR-STOP-EcoRI (GCTAGAATTCCTACTTTTTATGAAAGAGAAG). Lentivirus-based vectors encoding PR cDNA were generated by transient cotransfection of 293T cells with a three-plasmid combination, as described previously, with slight modifications [33]. The construct pMD.G was used for the production of the VSV-G viral envelope in combination with the packaging constructs pMDLg/pRRE and pRSV–REV, whereas the pRRL constructions correspond to the different transfer vectors. Briefly, 100 mm dishes of nonconfluent 293T cells were co-transfected with 6.5 µg of pMDLg/pRRE, 3.5 µg of pMDG (encoding the VSV-G envelope), 2.5 µg of pRSV–REV and 10 µg of pRRL-hPR, by the CaPi-DNA coprecipitation method [34,35]. The plasmid vectors were provided by Dr Luigi Naldini (University of Torino, Italy). Next day, the medium was adjusted to make a final concentration of 10 mM sodium butyrate and the cells were incubated for 8 h to obtain high-titer virus production as previously described [36]. Conditioned medium was harvested 16 h later and passed through 0.45 mm filters. Viral titer was determined by assessing viral p24 antigen concentration by ELISA (the Alliance® HIV-I p24 ELISA Kit, Perkin Elmer) and hereafter expressed as µg of p24 equivalent units per milliliter.

Cell Culture

Human umbilical vein endothelial cells were cultured in MCDB-131 media (VEC Technologies, Rensselaer, NY) supplemented with charcoal stripped fetal bovine serum (Omega Scientific, Tarzana, CA). For bead-based multiplex cytokine arrays, HUVECs were grown to confluence in 48-well plates and treated with LPS (1μM; 0111:B4; Sigma, St. Louis, MO) and/or progesterone (100nM; Sigma, St. Louis, MO) for 4, 8, and 24 hours. Media without serum was collected, and run in triplicate on a 42-plex array analyzed by Eve Technologies. For immunocytochemistry, HUVECs were seeded onto Lab-Tek II 8-well chamber slides (Thermo Scientific, Rochester, NY) and fixed with 4% paraformaldehyde. Cells were probed with an antibody against PR (1:400; clone SP2; Lab Vision, Kalamazoo, MI) followed by an Alexa Fluor secondary (1:300, Invitrogen, Grand Island, NY). Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI, 1:1000; Invitrogen, Grand Island, NY). Images were acquired using a Zeiss LSM 520 multiphoton microscope (Zeiss, Germany).

Immunoblotting

Total HUVEC lysate were resolved by SDS-PAGE, and nitrocellulose membranes (Optitran BA-S 83; Dassel, Germany) were incubated overnight with an anti-PR antibody (1:2000; clone SP2; Lab Vision, Kalamazoo, MI) and anti-GAPDH antibody (1:1000; Millipore, Billerica, MA). Blots were incubated with HRP-conjugated secondary (1:5000; Bio-Rad Laboratories, Hercules, CA) and developed using Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Kalamazoo,

MI). A Bio-Rad ChemiDoc XRS+ and accompanying Image Lab software was used for detection (Bio-Rad Laboratories, Hercules, CA).

RNA Isolation, qPCR, and Library Preparation

Total RNA was extracted using RNeasy Kit (Qiagen, Valencia, CA). RNA was reverse transcribed using SuperScript First-strand Synthesis System (Invitrogen, Grand Island, NY). qPCR was performed using SYBR Green reagent (Qiagen, Valencia, CA) and PCR products were run on an Opticon2 PCR machine (MJ Research; BioRad, Hercules, CA). Libraries for RNA-sequencing were generated using an Illumina Multiplex System (Illumina, San Diego, CA) and sequenced using HIseq-2000 (Illumina, San Diego, CA). RNA-seq datasets have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE43788.

RNA-seq Analysis

Multiplex runs were debarcoded by in house UNIX shell script. Reads were aligned to the human genome (hg19) using TopHat v2.0.4 [37] and processed with Cufflinks v2.0.1 [38]. Assemblies for all samples were merged using CuffMerge and pairwise differential expression was assessed using Cuffdiff. Genes with a *p*-value smaller than 0.01 where considered as significant. Heatmaps with relative expression were generated by visualizing log2 value of each gene rpkm divided with the average rpkm of all samples using Java treeview [39].

ChIP-sequencing and Analysis

HUVECs were infected with hPR lentivirus, grown to confluence, and treated with progesterone for 1h. For each condition (non-infected negative control, PR+P, PR only, and IgG control) 10×10^6 cultured HUVECs were used per IP. Cells were crosslinked with 1% formaldehyde, resuspended in 400 µl of lysis buffer (1% SDS, 20 mM EDTA and 50 mM Tris-HCl (pH 8.0)) containing protease inhibitors (Roche, Indianapolis, IN), and sonicated to achieve 200bp fragments. Samples were immunoprecipitated with 3 µg of anti-PR or IgG antibody. Protein A Dynabeads (Invitrogen, Grand Island, NY) were used to isolate antibody-PR complexes and eluted using 50 mM Tris-HCl, pH 8.0. Crosslinks were reversed by incubation at 65°C and DNA was purified using Qiagen MinElute Columns. Libraries were generated using Ovation Ultralow IL Multiplex System 1-8 (Nugen, San Carlos, CA) and sequenced using HIseq-2000 (Illumina, San Diego, CA). ChIP-seq data sets have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE43789.

Multiplex runs were debarcoded using Fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit) and reads were mapped to the human genome (hg19) using bowtie v0.12.7 [40]. 12-22 million uniquely mapped reads were obtained for each sample. Peak identification was performed with MACS v1.3.7.1 [41]. Peaks were called by comparing peaks in PR and PR+P conditions to that of the input, negative control (non-infected cells) or IgG control. Only peaks that appeared in all three comparisons were determined to be noteworthy. Genes potentially regulated by PR were determined by mapping peaks to nearby genes within 200kb of the transcriptional start site using the Genomic Regions Enrichment of Annotations Tool [42]. Intersection of

PR binding associated genes with differentially expressed genes was performed using Unix shell scripts.

Statistical Analysis

All data was analyzed using a Student unpaired two-tailed t-test. *p*-values less than 0.05 were considered to be statistically significant.

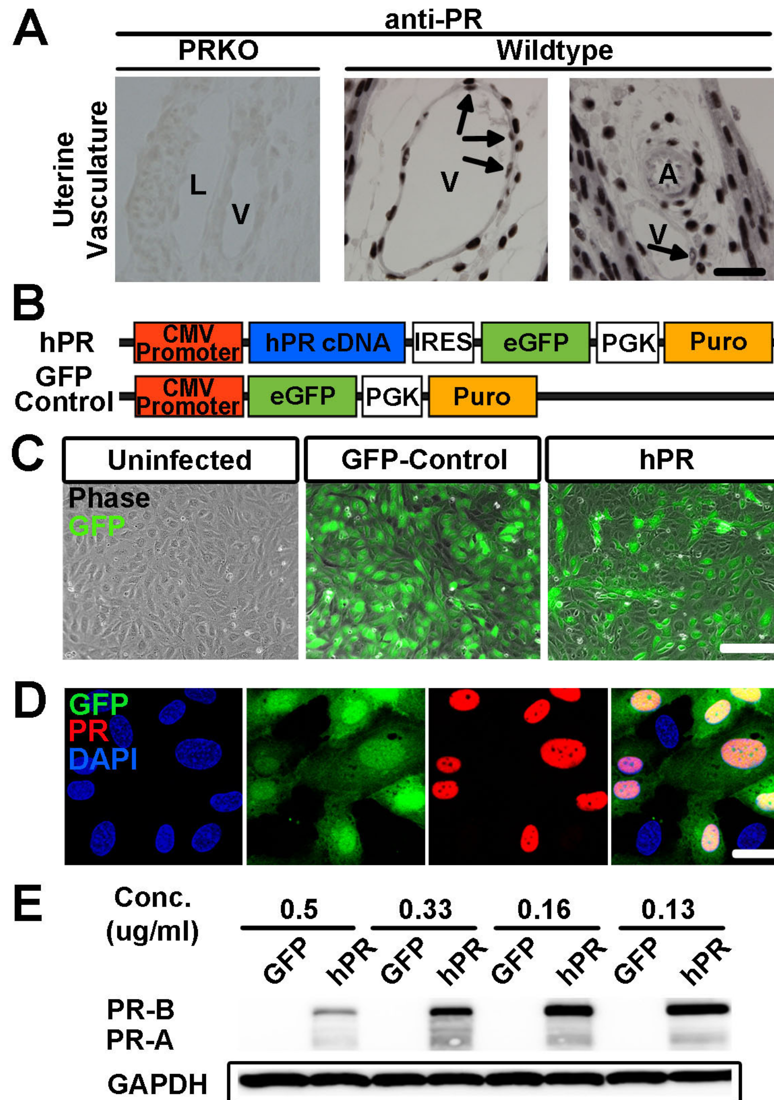


Figure 5.1 Generation of a lentivirus for human PR expression
(A) Histological sections demonstrate PR expression in the endothelium of veins (V) and lymphatics (L), but not arteries (A) in murine uterine vasculature. PRKO tissue was used as a negative control. Arrows indicate PR positive endothelial cells. Scale = 25 µm. **(B)** Scheme depicting human PR (hPR) and GFP control lentiviral constructs. PR cDNA was cloned directly following a CMV promoter. eGFP and puromycin resistance were used to determine infection efficiency and confer selection, respectively. **(C)** Human umbilical vein endothelial cells infected with a GFP-control or hPR lentivirus. GFP (green) and phase images were superimposed to determine infection efficiency. Scale bar = 50 µm. **(D)** Confirmation that GFP expression (green) correlates with PR expression (red). DAPI (blue) marks cell nuclei. Scale bar = 20 µm. **(E)** Western blot analysis of total protein levels from GFP control or hPR infected HUVECs at various viral concentrations. GAPDH was used as a loading control. In all panels, results are representative of 3-5 independent experiments.

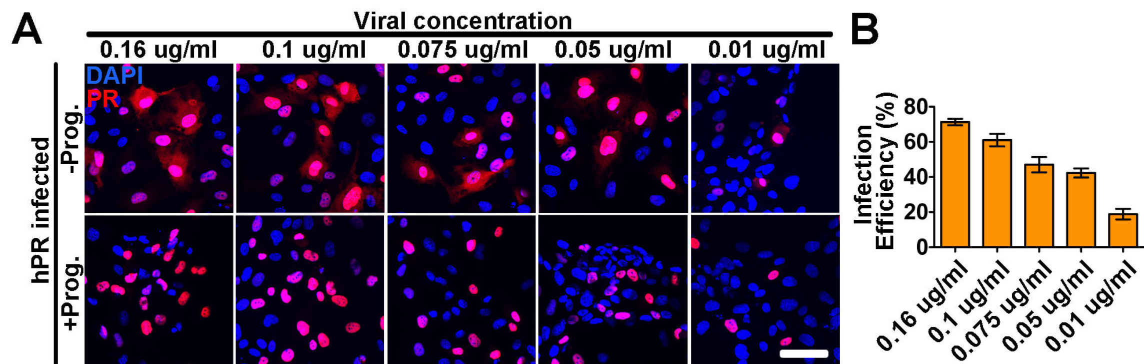


Figure 5.2. Validation of hPR responsiveness to progesterone
(A) HUVECs overexpressing hPR were treated with or without progesterone (100 nM) for 1 hour. PR (red) localization was exclusively confined to the nucleus in the presence of progesterone. DAPI (blue) marks cell nuclei. Scale bar = 50 µm. **(B)** Infection efficiency of the hPR lentivirus at several different viral concentrations. Efficiency was determined by dividing PR positive cells (red) by total number of nuclei (DAPI, blue) in 10 independent fields from three biological replicates.

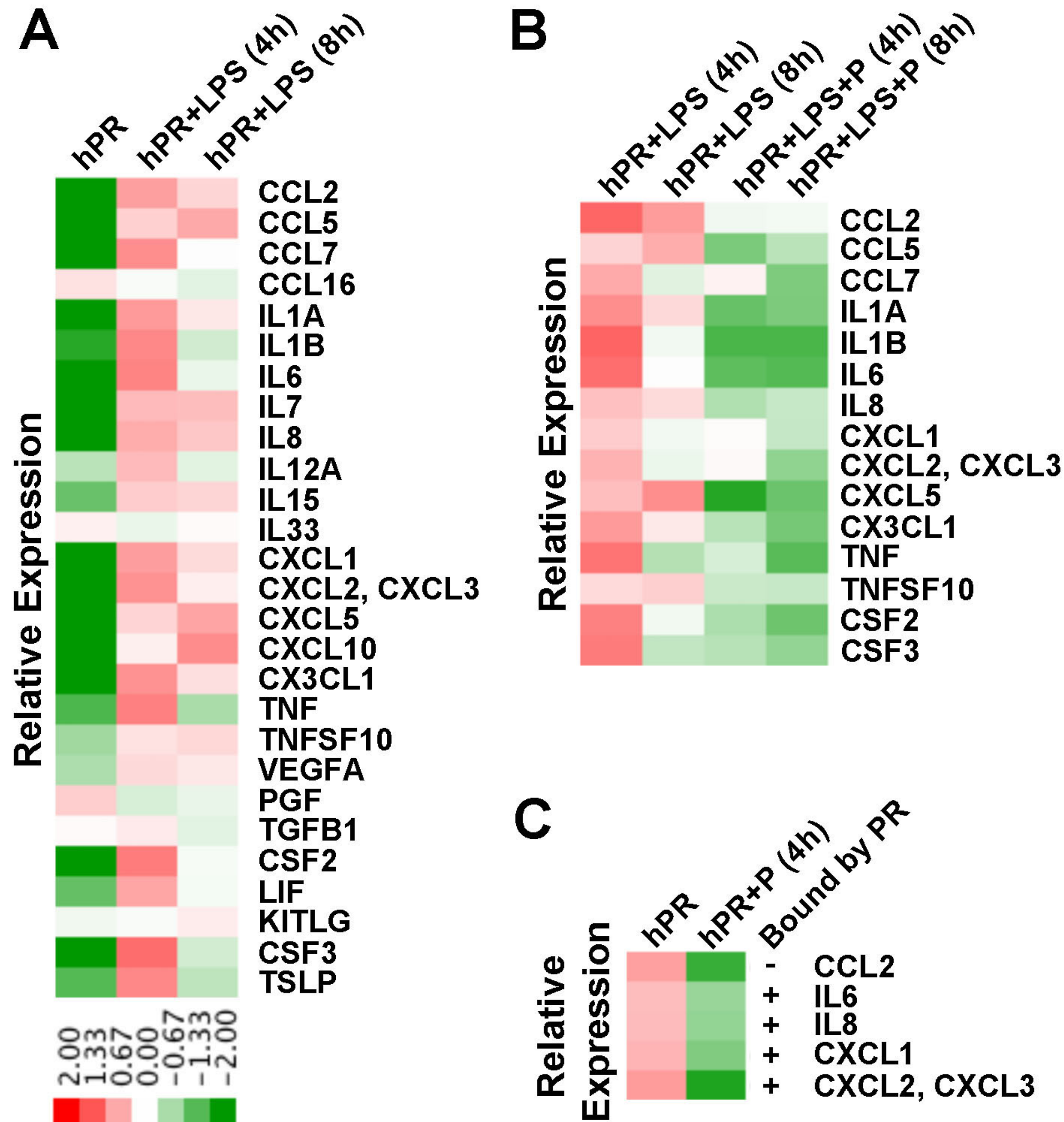


Figure 5.3. PR regulation of cytokine and chemokine expression
(A) Heat map depicting differential expression of cytokine and chemokines strongly regulated by LPS stimulation of HUVECs for 4 and 8 hours compared to nontreated cells. **(B)** Heat map comparing differential gene expression between LPS treatment in the presence of absence of progesterone. Genes included those found to be significantly ($p < 0.01$) upregulated by LPS treatment as determined from the heat map in panel A. **(C)** Heat map comparing differential gene expression between HUVECs treated with or without progesterone. Genes analyzed were those that were significantly ($p < 0.01$) downregulated by progesterone in the heat map from panel B. + symbolize genes that were directly bound by PR by ChIP-seq analysis.

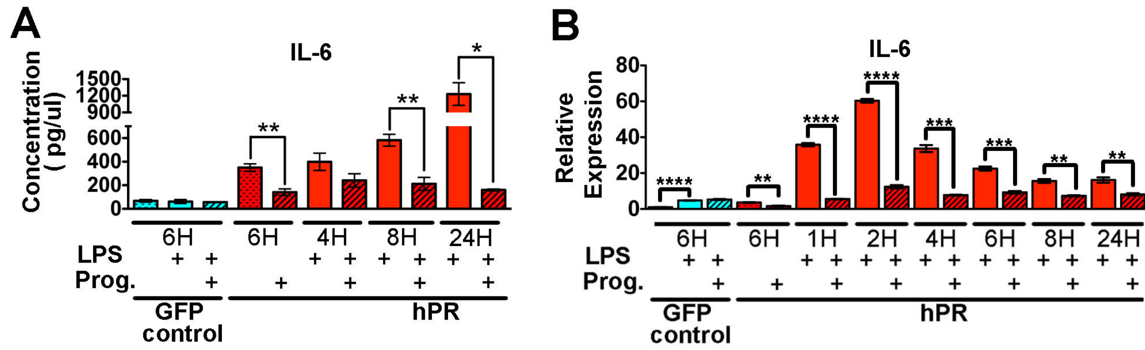


Figure 5.4. IL-6 repression by progesterone stimulation
(A) Protein expression (pg/μL) of IL-6 determined by cytokine array. HUVECs were treated with LPS (solid bars) or in combination with progesterone (hatched bars) for the indicated times. Graphs depict an average of three biological replicates. *p < 0.01, **p < 0.001. **(B)** qPCR confirmation of IL-6 expression. HUVECs were treated with LPS (solid bars) or in combination with progesterone (hatched bars) for the indicated times. Ct values were normalized to GAPDH and made relative to PR infected HUVECS in the absence of both LPS and progesterone (red dotted bar). GFP infected cells were used as a control and did not respond to progesterone (light blue bars). Graphs depict an average of three biological replicates run in triplicate. *p < 0.01, **p < 0.001, ***p < 0.0001, ****p < 0.00001.

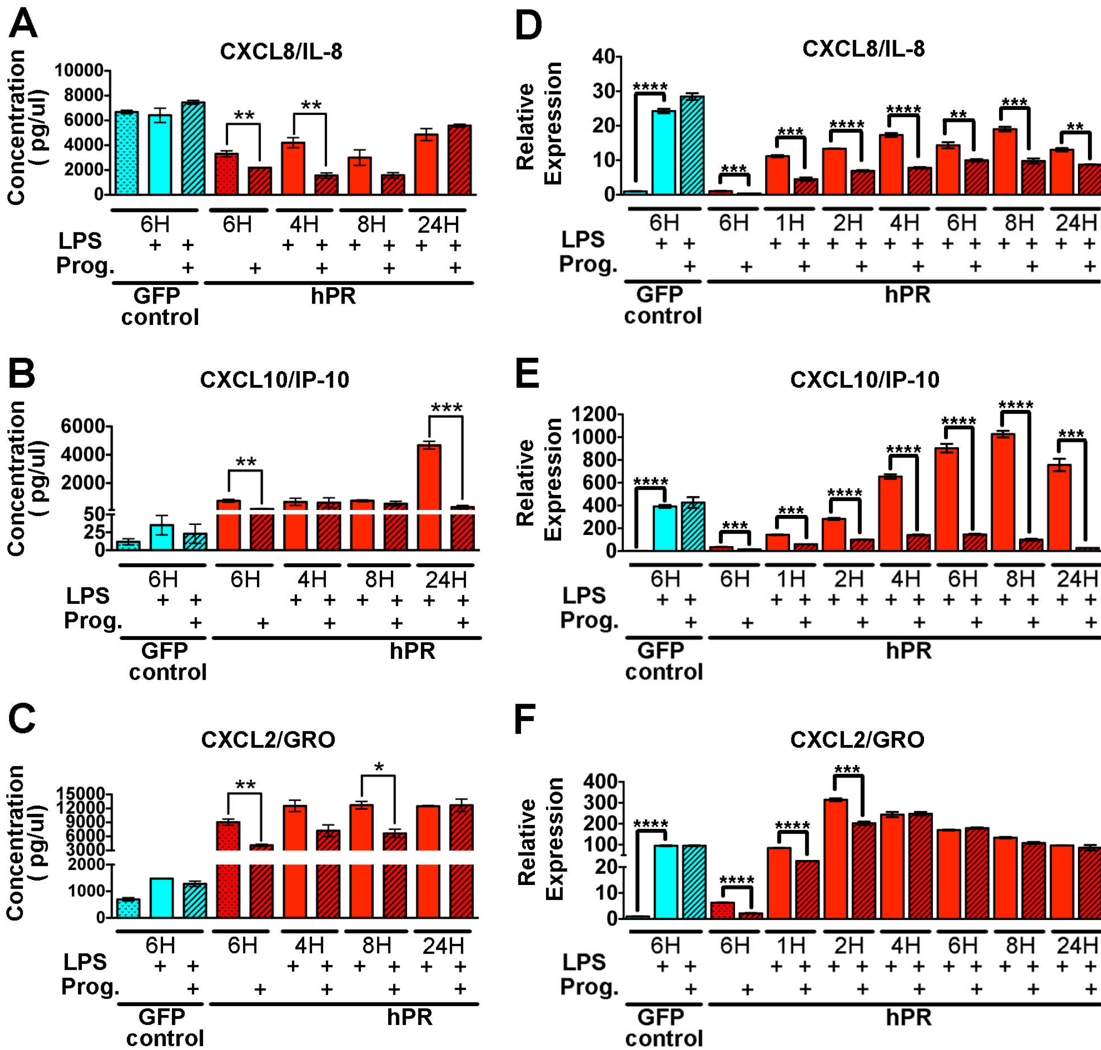


Figure 5.5. Progesterone regulation of CXC chemokine family members
(A-C) Protein expression (pg/μL) of CXCL8/IL-8 (A), CXCL10/IP-10 (B), and CXCL2/GRO (C) as determined by cytokine array. HUVECs were treated with LPS (solid bars) or in combination with progesterone (hatched bars) for the indicated times. Graphs depict an average of three biological replicates. *p < 0.01, **p < 0.001, ***p < 0.0001. **(D-F)** qPCR confirmation of CXCL8/IL-8 (D), CXCL10/IP-10 (E), and CXCL2/GRO (F). HUVECs were treated with LPS (solid bars) or in combination with progesterone (hatched bars) for the indicated times. Ct values were normalized to GAPDH and made relative to PR infected HUVECs in the absence of both LPS and progesterone (red dotted bar). GFP infected cells were used as a control and did not respond to progesterone (light blue bars). Graphs depict an average of three biological replicates run in triplicate. ***p < 0.0001, ****p < 0.00001.

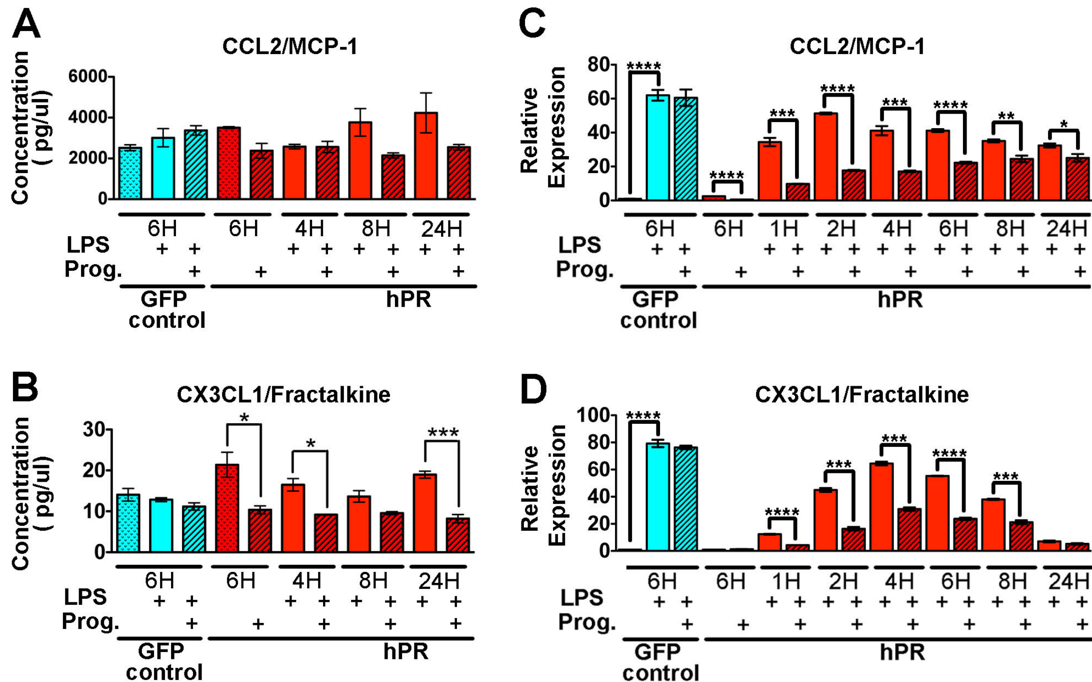


Figure 5.6. Progesterone regulation of the CC and CX_3C family members
(A,B) Protein expression (pg/µL) of CCL2 (A) and CX3CL1/fractalkine (B) as determined by cytokine array. HUVECs were treated with LPS (solid bars) or in combination with progesterone (hatched bars) for the indicated times. Graphs depict an average of three biological replicates. **(C,D)** qPCR confirmation of CCL2 (C) and CX3CL1 (D). HUVECs were treated with LPS (solid bars) or in combination with progesterone (hatched bars) for the indicated times. Ct values were normalized to GAPDH and made relative to PR infected HUVECs in the absence of both LPS and progesterone (red dotted bar). GFP infected cells were used as a control and did not respond to progesterone (light blue bars). Graphs depict an average of three biological replicates run in triplicate. *p < 0.01, **p < 0.001, ***p < 0.0001, ****p < 0.00001.

Table 5.1
Effect of Progesterone on Cytokine/Chemokine Secretion by Endothelial Cells[a]

Cytokine	+LPS/P	Cytokine	+LPS/P
EGF	-	IL-12 (p70)	-
Eotaxin	-	IL-13	-
FGF-2	-	IL-15	-
Flt-3	ND	IL-17	ND
Fractalkine	↓	IP-10	↓
G-CSF	-	MCP-1	↓
GM-CSF	-	MCP-3	-
GRO(Pan)	↓	MDC	-
INF-alpha2	-	MIP-1alpha	ND
IL-1beta	ND	MIP-1beta	ND
IL-1ra	-	PDGF-AA	↓
IL-2	ND	PDGFAB/BB	↓
IL-3	ND	RANTES	-
IL-4	ND	CD40L	ND
IL-5	ND	siL-2Ralpha	ND
IL-6	↓	TGFalpha	ND
IL-7	ND	TNFbeta	ND
IL-8	↓	VEGFA	-
IL-9	ND	IFN-gamma	ND
IL-10	ND	IL-1alpha	-
IL-12 (p40)	-	TNFalpha (78)	ND
		TNFalpha (80)	ND

[a] Samples were run using Multiplexing LASER Bead Technology based on uniquely colored bead sets able to recognize up to 100 analytes per well. Human 42-plex 96-well plates were used to simultaneously detect 42 different cytokine/chemokines per sample. Cell culture supernatant from LPS and LPS/P treated HUVECs were run in duplicate and average concentrations were calculated by comparing the fluorescent intensity of each analyte to a cytokine/chemokine specific standard curve (0.64 pg/mL-10,000 pg/mL). Analyte sensitivities were 0.1 pg/mL-30 pg/mL with most being in the 0.1 pg/mL-1 pg/mL range. ND = non detectable; - = no change; ↓ = decreased analyte concentration.

References

1. Kaushic C, Roth KL, Anipindi V, Xiu F. Increased prevalence of sexually transmitted viral infections in women: the role of female sex hormones in regulating susceptibility and immune responses. J Reprod. Immunol. 2011;88:204–9.

2. McCombe PA, Greer JM, Mackay IR. Sexual dimorphism in autoimmune disease. Curr. Mol. Med. 2009;9:1058–79.

3. Meyer MR, Haas E, Barton M. Gender differences of cardiovascular disease: new perspectives for estrogen receptor signaling. Hypertension 2006;47:1019–26.

4. Challis JR, Lockwood CJ, Myatt L, Norman JE, Strauss JF, Petraglia F. Inflammation and pregnancy. Reprod Sci 2009;16:206–15.

5. Gilliver SC. Sex steroids as inflammatory regulators. J Steroid Biochem. Mol. Biol. 2010;120:105–15.

6. Jones RL. Identification of Chemokines Important for Leukocyte Recruitment to the Human Endometrium at the Times of Embryo Implantation and Menstruation. J Clin. Endocrinol. Metab. 2004;89:6155–67.

7. King AE, Critchley HOD. Oestrogen and progesterone regulation of inflammatory processes in the human endometrium. J Steroid Biochem. Mol. Biol. 2010;120:116–26.

8. Red-Horse K, Drake PM. Human pregnancy: the role of chemokine networks at the fetalmaternal interface. Expert Rev Mol Med 2004; 6:1-14

9. van Mourik MSM, Macklon NS, Heijnen CJ. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J Leukoc. Biol. 2009;85:4–19.

10. Adams Waldorf KM, Nelson JL. Autoimmune disease during pregnancy and the microchimerism legacy of pregnancy. Immunol. Invest. 2008;37:631–44.

11. Hughes GC. Progesterone and autoimmune disease. Autoimmun Rev 2012;11:A502–14.

12. Martocchia A, Stefanelli M, Cola S, Falaschi P. Sex steroids in autoimmune diseases. Curr Top Med Chem 2011;11:1668–83.

13. Hamilton SA, Tower CL, Jones RL. Identification of chemokines associated with the recruitment of decidual leukocytes in human labour: potential novel targets for preterm labour. PLoS One 2013;8:e56946.

14. Hamilton S, Oomomian Y, Stephen G, Shynlova O, Tower CL, Garrod A, et al.

Macrophages infiltrate the human and rat decidua during term and preterm labor: evidence that decidual inflammation precedes labor. Biol. Reprod. 2012;86:39.

15. Shynlova O, Tsui P, Dorogin A, Lye SJ. Monocyte chemoattractant protein-1 (CCL-2) integrates mechanical and endocrine signals that mediate term and preterm labor. J Immunol 2008;181:1470–9.

16. Tibbetts TA, DeMayo F, Rich S, Conneely OM, O'Malley BW. Progesterone receptors in the thymus are required for thymic involution during pregnancy and for normal fertility. Proc. Natl. Acad. Sci. U.S.A. 1999;96:12021–6.

17. Tibbetts TA, Conneely OM, O'Malley BW. Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. Biol. Reprod. 1999;60:1158–65.

18. Butts CL, Shukair SA, Duncan KM, Bowers E, Horn C, Belyavskaya E, et al. Progesterone inhibits mature rat dendritic cells in a receptor-mediated fashion. Int Immunol 2007;19:287–96.

19. Gotkin JL, Celver J, McNutt P, Shields AD, Howard BC, Paonessa DJ, et al. Progesterone reduces lipopolysaccharide induced interleukin-6 secretion in fetoplacental chorionic arteries, fractionated cord blood, and maternal mononuclear cells. Am. J. Obstet. Gynecol. 2006;195:1015–9.

20. Hardy DB, Janowski BA, Corey DR, Mendelson CR. Progesterone receptor plays a major antiinflammatory role in human myometrial cells by antagonism of nuclear factor-kappaB activation of cyclooxygenase 2 expression. Mol Endocrinol 2006;20:2724–33.

21. Luk J, Seval Y, Ulukus M, Ulukus EC, Arici A, Kayisli UA. Regulation of monocyte chemotactic protein-1 expression in human endometrial endothelial cells by sex steroids: a potential mechanism for leukocyte recruitment in endometriosis. Reprod Sci 2010;17:278–87.

22. Shields AD, Wright J, Paonessa DJ, Gotkin J, Howard BC, Hoeldtke NJ, et al. Progesterone modulation of inflammatory cytokine production in a fetoplacental artery explant model. Am. J. Obstet. Gynecol. 2005;193:1144–8.

23. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 2007;7:678–89.

24. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nat Rev Immunol 2007;7:803–15.

25. Ingegno MD, Money SR, Thelmo W, Greene GL, Davidian M, Jaffe BM, et al. Progesterone receptors in the human heart and great vessels. Lab. Invest. 1988;59:353–6.

26. Iruela-Arispe ML, Rodriguez-Manzaneque JC, Abu-Jawdeh G. Endometrial endothelial cells express estrogen and progesterone receptors and exhibit a tissue specific response to angiogenic growth factors. Microcirculation 1999;6:127–40.

27. Krikun G, Schatz F, Taylor R, Critchley HOD, Rogers PAW, Huang J, et al. Endometrial endothelial cell steroid receptor expression and steroid effects on gene expression. J Steroid Biochem. Mol. Biol.2005;90:1812–8.

28. Maybin JA, Duncan WC. The human corpus luteum: which cells have progesterone receptors? Reproduction 2004;128:423–31.

29. Perrot-Applanat M, Cohen-Solal K, Milgrom E, Finet M. Progesterone receptor expression in human saphenous veins. Circulation 1995;92:2975–83.

30. Rodríguez-Manzaneque JC, Graubert M, Iruela-Arispe ML. Endothelial cell dysfunction following prolonged activation of progesterone receptor. Hum Reprod 2000;15 Suppl 3:39–47.

31. Vázquez F, Rodríguez-Manzaneque JC, Lydon JP, Edwards DP, O'Malley BW, Iruela-Arispe ML. Progesterone regulates proliferation of endothelial cells. J. Biol. Chem. 1999;274:2185–92.

32. Otsuki M, Saito H, Xu X, Sumitani S, Kouhara H, Kishimoto T, et al. Progesterone, but not medroxyprogesterone, inhibits vascular cell adhesion molecule-1 expression in human vascular endothelial cells. Arterioscler. Thromb. Vasc. Biol. 2001;21:243–8.

33. Naldini L, Blömer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc. Natl. Acad. Sci. U.S.A. 1996;93:11382–8.

34. Chen C, Okayama H. High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 1987;7:2745–52.

35. Sakoda T, Kaibuchi K, Kishi K, Kishida S, Doi K, Hoshino M, et al. smg/rap1/Krev-1 p21s inhibit the signal pathway to the c-fos promoter/enhancer from c-Ki-ras p21 but not from c-raf-1 kinase in NIH3T3 cells. Oncogene 1992;7:1705–11.

36. Sakoda T, Kasahara N, Hamamori Y, Kedes L. A high-titer lentiviral production system mediates efficient transduction of differentiated cells including beating cardiac myocytes. J. Mol. Cell. Cardiol. 1999;31:2037–47.

37. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 2009;25:1105–11.

38. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated

transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 2010;28:511–5.

39. de Hoon MJL, Imoto S, Nolan J, Miyano S. Open source clustering software. Bioinformatics 2004;20:1453–4.

40. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10:R25.

41. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 2008;9:R137.

42. McLean CY, Bristor D, Hiller M, Clarke SL, Schaar BT, Lowe CB, et al. GREAT improves functional interpretation of cis-regulatory regions. Nat. Biotechnol. 2010;28:495–501.

43. Okada M, Matsumori A, Ono K, Furukawa Y, Shioi T, Iwasaki A, et al. Cyclic stretch upregulates production of interleukin-8 and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in human endothelial cells. Arterioscler. Thromb. Vasc. Biol. 1998;18:894–901.

44. Simoncini T. Differential Signal Transduction of Progesterone and Medroxyprogesterone Acetate in Human Endothelial Cells. Endocrinology 2004;145:5745–56.

45. Romano M, Sironi M, Toniatti C, Polentarutti N, Fruscella P, Ghezzi P, et al. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. Immunity 1997;6:315–25.

46. Oertelt-Prigione S. Immunology and the menstrual cycle. Autoimmun Rev 2012;11:A486–92.

47. Henriet P, Gaide Chevronnay HP, Marbaix E. The endocrine and paracrine control of menstruation. Mol. Cell. Endocrinol. 2012;358:197–207.

48. Angstwurm MW, Gärtner R, Ziegler-Heitbrock HW. Cyclic plasma IL-6 levels during normal menstrual cycle. Cytokine 1997;9:370–4.

49. Critchley HO, Jones RL, Lea RG, Drudy TA, Kelly RW, Williams AR, et al. Role of inflammatory mediators in human endometrium during progesterone withdrawal and early pregnancy. J Clin Endocrinol Metab. 1999;84:240–8.

50. Hannan NJ, Jones RL, Critchley HOD, Kovacs GJ, Rogers PAW, Affandi B, et al. Coexpression of fractalkine and its receptor in normal human endometrium and in endometrium from users of progestin-only contraception supports a role for fractalkine in leukocyte recruitment and endometrial remodeling. J Clin Endocrinol Metab. 2004;89:6119–29.

51. Jones RL, Kelly RW, Critchley HO. Chemokine and cyclooxygenase-2 expression in human endometrium coincides with leukocyte accumulation. Hum Reprod 1997;12:1300–6.

52. Zhang J, Lathbury LJ, Salamonsen LA. Expression of the chemokine eotaxin and its receptor, CCR3, in human endometrium. Biol. Reprod. 2000;62:404–11.

53. Critchley HO, Kelly RW, Lea RG, Drudy TA, Jones RL, Baird DT. Sex steroid regulation of leukocyte traffic in human decidua. Hum Reprod 1996;11:2257–62.

54. Kelly RW, Illingworth P, Baldie G, Leask R, Brouwer S, Calder AA. Progesterone control of interleukin-8 production in endometrium and chorio-decidual cells underlines the role of the neutrophil in menstruation and parturition. Hum Reprod 1994;9:253–8.

55. Loudon JAZ, Elliott CL, Hills F, Bennett PR. Progesterone represses interleukin-8 and cyclo-oxygenase-2 in human lower segment fibroblast cells and amnion epithelial cells. Biol. Reprod. 2003;69:331–7.

56. MacIntyre DA, Sykes L, Teoh TG, Bennett PR. Prevention of preterm labour via the modulation of inflammatory pathways. J. Matern. Fetal. Neonatal. Med. 2012;25 Suppl 1:17–20.

57. Norman JE, Bollapragada S, Yuan M, Nelson SM. Inflammatory pathways in the mechanism of parturition. BMC Pregnancy Childbirth 2007;7 Suppl 1:S7.

58. Robertson SA, Christiaens I, Dorian CL, Zaragoza DB, Care AS, Banks AM, et al. Interleukin-6 is an essential determinant of on-time parturition in the mouse. Endocrinology 2010;151:3996–4006.

59. Kavandi L, Collier MA, Nguyen H, Syed V. Progesterone and calcitriol attenuate inflammatory cytokines CXCL1 and CXCL2 in ovarian and endometrial cancer cells. J Cell Biochem 2012;113:3143–52.

60. Zhao Y, Koga K, Osuga Y, Izumi G, Takamura M, Harada M, et al. Cyclic Stretch Augments Production of Neutrophil Chemokines and Matrix Metalloproteinases-1 (MMP-1) from Human Decidual Cells, and the Production was Reduced by Progesterone. Am J Reprod Immunol 2013;69:454–62.

Chapter 6: Conclusions

Progesterone is critical for all reproductive processes including cycling, implantation, and maintenance of pregnancy [1]. Two biologically important and cyclically controlled alterations in the decidua during the progesterone-predominant secretory phase are vascular permeability and immune cell trafficking. While these changes are assumed to be under progesterone control, the exact cellular and molecular regulation remains largely unknown. Using loss-of-function studies in combination with whole transcriptome analysis we were able to dissociate the contribution of endothelial PR from other PR positive cells, thus establishing an endothelial-specific role for PR in the control of physiological vascular permeability and immune regulation. In this work, we demonstrate that:

1. Restricted expression of PR in veins and lymphatics ensures vessel-selectivity to progesterone signaling

2. Physiological cycles of permeability in the uterus are cell autonomous, independent of VEGF, and require the activation of NR4A1 (Nur77/TR3) for barrier instability

3. Endothelial PR contributes to the select regulation of PMN/macrophage trafficking into the uterus

4. PR negatively regulates the expression of a select group of cytokines/chemokines by the endothelium

Endothelial PR Mediates Vascular Permeability

In Chapter 3, we set out to understand the biological significance of PR expression in the endothelium, with a particular focus on vascular permeability. Using a PR reporter

mouse, we first demonstrated that PR is highly restricted to uterine blood vessels, at the exclusion of vessels from other organs. This exquisite specificity enables local and unique functions from a systemically distributed ligand. Endothelial PR expression was seen only in venous and lymphatic endothelium, which is consistent with the function of these vessels in maintaining fluid homeostasis in the tissue. While PR is only present in a small subset of total endothelial cells, these findings suggest the need for a very controlled mechanism of PR action in the vasculature.

In terms of progesterone effects in other vascular beds, we and others have noted that in human specimens, PR is expressed by patches of endothelial cells within the human coronary vasculature and other large vessels [2-5]. Although under non-pathological conditions we do not detect PR expression within the coronary vasculature in the mouse, expression in human atherosclerotic vessels has been shown [3]. Therefore it is possible that PR upregulation occurs following injury and subsequently alters vascular function.

Uterine vascular permeability is temporally regulated during menstruation and implantation, both times when physiological levels of circulating progesterone are high [6,7]. While these effects were assumed to be under progesterone control, definitive evidence for PR requirement in these processes has not been determined. Using both global and conditional PR knockout mice, we were able to demonstrate that PR in the endothelium mediates the vascular permeability response preceding the time of implantation. While the function of pathological permeability mediators such as VEGF and histamine are well documented in the literature, not much is known of the mechanisms that drive physiological permeability. We show here, that in the uterus,

physiological permeability is controlled by a mechanism distinct from that of pathological permeability and does not require VEGF signaling. Thus our findings provide novel insights into the molecular regulation of physiological permeability.

In contrast to the large cohort of downstream targets of PR in the epithelium and stroma [8], the cellular and molecular mechanism of progesterone action on the endothelium are poorly understood. We found that PR activation in the endothelium alters cell-cell junctions, resulting in gap formation, and decreased monolayer resistance. Combining global expression analysis with PR binding by ChIP-seq we were able to determine relevant downstream effectors of PR. Interestingly, PR was found to upregulate the orphan nuclear receptor NR4A1, shown recently to mediate permeability downstream of VEGF [9] . Following this lead, evaluation of NR4A1 loss of function models confirmed the requirement for NR4A1 in PR mediated permeability. Although we have not addressed the mechanism of NR4A1 action, we believe that NR4A1 transcriptionally downregulates endothelial cell-cell adhesion molecules. Future studies will be needed to address the relationship between PR and NR4A1, as well as NR4A1 targets in the endothelium.

Progesterone regulates the trafficking of a subpopulation of leukocytes into the uterus

In addition to vascular permeability, hormone fluctuations have been found to parallel alterations in the trafficking of leukocytes into the uterus and are required for a variety of reproductive processes, including implantation, menstruation, and labor [10,11]. While progesterone is generally considered anti-inflammatory, it is still unclear whether

progesterone affects immune cells directly or through paracrine signaling in other cell types. The endothelium acts as the initial barrier during leukocyte extravasation into tissues, and thus it is an interesting target for PR control of immune trafficking. Therefore, we evaluated the contribution of endothelial PR in physiological immune regulation by examining PR control of endothelial-leukocyte adhesion molecules (Chapter 4) and cytokine production by the endothelium (Chapter 5).

In Chapter 4 we demonstrate that both global and conditional deletion of PR from the endothelial compartment leads to defects in leukocyte trafficking under homeostatic conditions. While PRKO uteri display increased numbers of T cells, macrophages, and PMNS, loss of PR from the endothelium resulted in only an increase in the PMN and macrophage subpopulations. These findings confirm the anti-inflammatory role of progesterone, however they highlight the unique function of the endothelium in selective regulation of particular leukocyte subtypes. Therefore, PR signaling in other cell types, most likely cells of the decidua, also function in immune recruitment through paracrine regulation. To further understand this unique selectivity in leukocyte control by the endothelium, we assessed differences in the expression of endothelial-leukocyte adhesion molecules using RNA-seq. We found that PR directly downregulates the expression of a select group of these proteins, potentially explaining endothelial leukocyte selectivity. Future work should focus on how preferential selection of macrophage/PMNs occurs, as well as address whether differences in endothelial-leukocyte adhesion have functional significance in vivo. Furthermore, it will also be important to understand if loss of PR in the endothelium has biological consequences on physiological reproductive processes such as implantation.

Progesterone controls the secretion of a select set of cytokine/chemokines from the endothelium

In Chapter 5 we explored whether PR activation in the endothelium altered expression of cytokines. In order to study cytokine production by the endothelium alone, we generated and validated in vitro culture system. Using next generation RNA-sequencing, in combination with bead-based multiplex cytokine arrays, we identified a selective group of cytokines that are suppressed by progesterone both under physiological conditions and during an acute inflammatory response. In particular, IL-6, IL-8, CXCL2/3, and CXCL1 were found to be direct targets of PR, as determined by ChIP-sequencing. These findings confirm the increase in cytokine levels seen during times of progesterone withdrawal such as labor and menstruation [12-18]. The findings from both Chapter 4 and 5 suggest that progesterone-PR signaling in the endothelium directly impacts leukocyte trafficking in PR-expressing tissues.

Progesterone signaling in the vasculature: a clinical perspective

Cardiovascular disease (CVD) remains the major cause of mortality in the Western world. Interestingly, men are 3-5 times more likely to develop cardiovascular disease than women of the same age, but in women this risk increases with age and rises sharply after menopause [19]. The apparent gender-related protection has been attributed to circulating sex hormones and, consequently, hormone replacement therapy (HRT) has been used clinically to prevent/ameliorate heart disease during post-menopause. Nonetheless, results from several epidemiological studies and clinical trials on the cardiovascular effects of HRT have cast serious concerns on those earlier assumptions.

The Women's Health Initiative (WHI), Heart and Estrogen/Progestin Replacement Study, and the Women International Study of Long Duration Oestrogen after Menopause, have all reported that combined estrogen and progestin HRT had no benefit to cardiovascular function. [20-24]. Interestingly, while the estrogen-only arm of the WHI showed an increase in stroke and venous thromboembolism, this risk was significantly lower when compared to that seen with women taking both estrogen and progestin [25]. In addition to these side effects, women also showed a marked increase in the incidence of myocardial infarction and stroke [22]. Therefore the implication is that progestins are likely the stronger inducers of these negative cardiovascular events. In further agreement, women taking progestin-only contraceptives exhibit symptoms similar to those seen with HRT, including vessel fragility, breakthrough bleeding and thromboembolism [26-29]. Unfortunately, the paucity of information on the function of progesterone in the vasculature has impaired a more detailed and mechanistic analysis of the current epidemiological and clinical data.

Currently, it remains unclear whether the side effects of HRT and progestin-only contraceptives are due to the binding of progestins to receptors located in non-reproductive tissues, effects on lipid metabolism, or through direct signaling within cells of the vasculature. While PR is expressed by a subset of endothelial cells in women, [3,30-33] an understanding of the biological significance of this expression has not been fully clarified.

Here we demonstrate a novel link between progesterone signaling and vascular function. While physiological permeability is tightly regulated and short-lived, the prolonged exposure to progestins may lead to a more sustained and dysregulated

permeability response. Prolonged permeability can lead to physiological consequences such as platelet activation and thrombus formation [34] Furthermore, platelet aggregation has been recognized to be an essential contributing factor in the development of atherosclerotic plaques and has been implicated in other patho-physiological events such as occlusive stroke, deep vein thrombosis, and pulmonary embolism [35,36]. Thus, constant, dysregulated permeability resultant from exposure to progestins may lead to more severe cardiovascular consequences that resemble those in women taking HRT and contraceptives.

In addition to cardiovascular disease, hormones are believed to play an important role in the susceptibility and progression of other immune based diseases that exhibit gender-based differences in prevalence [11,37,38]. Several lines of evidence have demonstrated marked changes in the severity of various diseases during different stages of the menstrual cycle and during pregnancy, linking hormonal control to systemic immune regulation [37]. Currently there are still significant gaps in our understanding of the mechanisms that mediate sex-biased immune responses. We determined that PR in the endothelium alters immune trafficking through the regulation of cytokines and endothelial-leukocyte adhesion molecules. These results confirm PR as an anti-inflammatory agent in the endothelium, with potential for the negative regulation of immune cell trafficking into tissues. Therefore, we believe these findings clarify cell specific hormonal regulation of sex differences in disease.

Summary

In summary, we have shown a unique requirement for endothelial progesterone signaling in physiological vascular permeability and immune regulation. In vivo loss of

function models and a systems approach combining global transcriptome analysis and transcription factor binding by ChIP-seq was valuable in the advancement of our understanding of PR biological function and target gene regulation. Elucidating the physiological role of progesterone in the endometrial vasculature is not only vital to advancements in reproductive physiology but will also aid in our understanding of pathological vascular dysfunction that can result in implantation failure, cycling defects and preterm labor. We hope that the determination of downstream targets of PR in the vasculature will reveal insights into the function of PR in other vascular beds outside of the endometrium and thus may explain some of the current epidemiological/clinical findings on women taking exogenous progestins.

References:

1. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, et al. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. 1995;9:2266–78.

2. Hegele-Hartung C, Fritzemeier KH, Diel P. Effects of a pure antiestrogen and progesterone on estrogen-mediated alterations of blood flow and progesterone receptor expression in the aorta of ovariectomized rabbits. J Steroid Biochem Mol Biol 1997;63:237–49.

3. Vázquez F, Rodríguez-Manzaneque JC, Lydon JP, Edwards DP, O'Malley BW, Iruela-Arispe ML. Progesterone regulates proliferation of endothelial cells. J. Biol. Chem. 1999;274:2185–92.

4. Otsuki M, Saito H, Xu X, Sumitani S, Kouhara H, Kishimoto T, et al. Progesterone, but not medroxyprogesterone, inhibits vascular cell adhesion molecule-1 expression in human vascular endothelial cells. Arterioscler. Thromb. Vasc. Biol. 2001;21:243–8.

5. Winkler M, Kemp B, Classen-Linke I, Fischer DC, Zlatinsi S, Neulen J, et al. Estrogen receptor alpha and progesterone receptor A and B concentration and localization in the lower uterine segment in term parturition. J. Soc. Gynecol. Investig. 2002;9:226–32.

6. Jerome F Strauss I, Robert L Barbieri MD. Yen and Jaffe's Reproductive Endocrinology. Elsevier Health Sciences; 2009.

7. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin. Reprod. Med. 2007;25:445–53.

8. Wetendorf M, DeMayo FJ. The progesterone receptor regulates implantation, decidualization, and glandular development via a complex paracrine signaling network. Mol. Cell. Endocrinol. 2012;357:108–18.

9. Zhao D, Qin L, Bourbon P-M, James L, Dvorak HF, Zeng H. Orphan nuclear transcription factor TR3/Nur77 regulates microvessel permeability by targeting endothelial nitric oxide synthase and destabilizing endothelial junctions. Proc. Natl. Acad. Sci. U.S.A. 2011;108:12066–71.

10. Oertelt-Prigione S. Immunology and the menstrual cycle. Autoimmun Rev 2012;11:A486–92.

11. Gilliver SC. Sex steroids as inflammatory regulators. J Steroid Biochem Mol Biol 2010;120:105–15.

12. Angstwurm MW, Gärtner R, Ziegler-Heitbrock HW. Cyclic plasma IL-6 levels

during normal menstrual cycle. Cytokine 1997;9:370–4.

13. Critchley HO, Jones RL, Lea RG, Drudy TA, Kelly RW, Williams AR, et al. Role of inflammatory mediators in human endometrium during progesterone withdrawal and early pregnancy. J Clin Endocrinol Metab 1999;84:240–8.

14. Hamilton SA, Tower CL, Jones RL. Identification of chemokines associated with the recruitment of decidual leukocytes in human labour: potential novel targets for preterm labour. PLoS One 2013;8:e56946.

15. Hannan NJ, Jones RL, Critchley HOD, Kovacs GJ, Rogers PAW, Affandi B, et al. Coexpression of fractalkine and its receptor in normal human endometrium and in endometrium from users of progestin-only contraception supports a role for fractalkine in leukocyte recruitment and endometrial remodeling. J Clin Endocrinol Metab 2004;89:6119–29.

16. Jones RL. Identification of Chemokines Important for Leukocyte Recruitment to the Human Endometrium at the Times of Embryo Implantation and Menstruation. J Clin Endocrinol Metab 2004;89:6155–67.

17. Jones RL, Kelly RW, Critchley HO. Chemokine and cyclooxygenase-2 expression in human endometrium coincides with leukocyte accumulation. Hum Reprod 1997;12:1300–6.

18. Zhang J, Lathbury LJ, Salamonsen LA. Expression of the chemokine eotaxin and its receptor, CCR3, in human endometrium. Biol. Reprod. 2000;62:404–11.

19. Turgeon JL, McDonnell DP, Martin KA, Wise PM. Hormone therapy: physiological complexity belies therapeutic simplicity. Science 2004;304:1269–73.

20. Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, et al. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. JAMA 1998;280:605–13.

21. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 2002;288:321–33.

22. Manson JE, Hsia J, Johnson KC, Rossouw JE, Assaf AR, Lasser NL, et al. Estrogen plus progestin and the risk of coronary heart disease. N. Engl. J. Med. 2003;349:523–34.

23. Cushman M, Kuller LH, Prentice R, Rodabough RJ, Psaty BM, Stafford RS, et al. Estrogen plus progestin and risk of venous thrombosis. JAMA 2004;292:1573–80.

24. Vickers MR, MacLennan AH, Lawton B, Ford D, Martin J, Meredith SK, et al. Main

morbidities recorded in the women's international study of long duration oestrogen after menopause (WISDOM): a randomised controlled trial of hormone replacement therapy in postmenopausal women. BMJ 2007;335:239.

25. Curb JD, Prentice RL, Bray PF, Langer RD, Van Horn L, Barnabei VM, et al. Venous thrombosis and conjugated equine estrogen in women without a uterus. Arch. Intern. Med. 2006;166:772–80.

26. Kovacs G. Progestogen-only pills and bleeding disturbances. Hum Reprod 1996;11 Suppl 2:20–3.

27. Hickey M, Fraser IS. Surface vascularization and endometrial appearance in women with menorrhagia or using levonorgestrel contraceptive implants. Implications for the mechanisms of breakthrough bleeding. Hum Reprod 2002;17:2428–34.

28. Krikun G, Critchley H, Schatz F, Wan L, Caze R, Baergen RN, et al. Abnormal uterine bleeding during progestin-only contraception may result from free radical-induced alterations in angiopoietin expression. Am. J. Pathol. 2002;161:979–86.

29. Voelker R. Risk of blood clots higher for oral contraceptives with certain progestins. JAMA 2011;306:2206.

30. Perrot-Applanat M, Cohen-Solal K, Milgrom E, Finet M. Progesterone receptor expression in human saphenous veins. Circulation 1995;92:2975–83.

31. Iruela-Arispe ML, Rodriguez-Manzaneque JC, Abu-Jawdeh G. Endometrial endothelial cells express estrogen and progesterone receptors and exhibit a tissue specific response to angiogenic growth factors. Microcirculation 1999;6:127–40.

32. Ingegno MD, Money SR, Thelmo W, Greene GL, Davidian M, Jaffe BM, et al. Progesterone receptors in the human heart and great vessels. Lab. Invest. 1988;59:353–6.

33. Maybin JA, Duncan WC. The human corpus luteum: which cells have progesterone receptors? Reproduction 2004;128:423–31.

34. Davì G, Patrono C. Platelet activation and atherothrombosis. N. Engl. J. Med. 2007;357:2482–94.

35. Huo Y, Ley KF. Role of platelets in the development of atherosclerosis. Trends Cardiovasc. Med. 2004;14:18–22.

36. Nieswandt B, Pleines I, Bender M. Platelet adhesion and activation mechanisms in arterial thrombosis and ischaemic stroke. J. Thromb. Haemost. 2011;9 Suppl 1:92–104.

37. Hughes GC. Progesterone and autoimmune disease. Autoimmun Rev 2012;11:A502–14.

38. Martocchia A, Stefanelli M, Cola S, Falaschi P. Sex steroids in autoimmune diseases. Curr Top Med Chem 2011;11:1668–83.