MOLECULAR MECHANISMS REGULATING IMPLANTATION

BY
SANDEEP PAWAR

DISSEPTION
Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Molecular and Integrative Physiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2014

Urbana, Illinois

Doctoral Committee:
Professor Milan K. Bagchi, Chair
Professor Indrani C. Bagchi
Swanlund Professor Benita S. Katzenellenbogen
Associate Professor Lori T. Raetzman
Assistant Professor Eric C. Bolton
ABSTRACT

Implantation, a critical early event during pregnancy, is a three stage process. It starts with apposition and adhesion of the blastocyst to a receptive uterine epithelium which is then followed by invasion of the blastocyst into a decidualizing stroma. These stages are tightly regulated by steroid hormones Estrogen (E) and Progesterone (P). Genomic profiling in mice has uncovered novel steroid regulated genes in the uterine luminal epithelium and also in the stroma which regulate implantation. Recent evidence also points to the fact that stromal-epithelial interactions are critical for a successful implantation. To understand the mechanisms governing these complex interactions and better address the clinical challenges to infertility, it is important to integrate the information gained from these studies. Chapter I presents a detailed analysis of the knowledge gained from reviewing these studies.

Estrogen acts via its cognate steroid hormone receptor, estrogen receptor alpha (ESR), in order to exert its effects during pregnancy. The studies reported here investigate E’s action via ESR1 and its downstream targets in uterine physiology and is written in three parts. The first part is an investigation of role of epithelial ESR1 in the uterus during stromal cell decidualization. Earlier studies in our lab had indicated an essential role of uterine ESR1 in stromal cell decidualization. This study prompted an investigation into the compartment specific role of ESR1 in uterine stromal cell decidualization. We have used an epithelial specific knockout mouse of Esr1 known as WE<sup>dd</sup> in order to analyse the role of epithelial ESR1 in this process. Our investigation has provided novel insights into a paracrine mechanism of action in which Epithelial ESR1 controls stromal cell decidualization via Leukaemia Inhibitory Factor (LIF) and Indian Hedgehog (IHH).

The second part of this study investigates the role of signal transducer and activator of transcription 3 (STAT3) in the uterine epithelial cells during implantation. STAT3 is activated downstream of LIF, an E target gene, in the uterine epithelial cells during the window of implantation. To identify the molecular pathways regulated by STAT3, we created SW<sup>dd</sup> mice in which Stat3 gene is conditionally inactivated in uterine epithelium. These mutant mice are infertile and exhibit embryo implantation and uterine stromal cell decidualization defect. Further analysis revealed dysregulation of several junctional complexes in the luminal epithelial cells of SW<sup>dd</sup> mice during the window of implantation which adversely affects uterine receptivity and
embryo attachment. Additionally, epithelial STAT3 also controlled certain aspects of stromal cell decidualization by inducing the expression of epidermal growth factor (EGF) production in the luminal epithelium during implantation. A decrease in EGF production adversely affects stromal cell proliferation. Our results uncovered an intricate genetic network operating downstream of STAT3 that regulates uterine epithelial junctional reorganization, stromal proliferation and differentiation, which are critical determinants of successful implantation.

In the fourth chapter of this thesis we describe our ongoing studies investigating the role of an E target gene, endothelial PAS domain – 1 in uterine decidual angiogenesis. Our laboratory has shown that it is local E production acting via ESR1 within the uterine stromal cells that drives and sustains the decidualization response. Furthermore this local E production regulates EPAS-1, also called hypoxia-inducible factor 2 alpha (Hif2α). EPAS-1 is a transcription factor that has been shown to be regulated by hypoxic conditions and regulates molecules essential for an angiogenesis response. In order to study the functional role of EPAS-1, we have created a conditional knockout of the EPAS-1, which has EPAS-1 knocked out in all cells expressing PGR. This conditional knockout mouse, termed Epas-1<sup>d/d</sup>, is infertile. Further analysis revealed that without EPAS-1 in the stromal cells, angiogenesis at the early stages of pregnancy is impaired as marked by a downregulation of an endothelial cell marker, PECAM. Understanding downstream molecules of EPAS-1 will undoubtedly give insight into the molecular pathways essential for uterine decidual angiogenesis.
To my parents Anil Pawar and Annie Pawar
ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Milan Bagchi, for his continued support and encouragement. Dr. Bagchi has helped me tremendously in structuring my research and guiding me in the development of my projects. I would also like to thank Dr. Indrani Bagchi who has fulfilled an advisory role during my Ph.D. studies. Indrani has critiqued my research and challenged my ideas. This continued input has been essential in developing my research projects. Both Milan and Indrani have guided me during my development into a confident research scientist. I will always be grateful for their mentorship and support.

I would also like to thank my other committee members Dr. Benita Katzenellenbogen, Dr. Lori Raetzman and Dr. Eric Bolton whose comments and critiques of my research have been greatly appreciated. I also thank them for their continued advice and support during my search for potential post-doctoral research positions.

I give a very special thank you to Ms Elizabeth Hunt, who has helped me a great deal in performing a variety of mouse surgeries and was always there when I needed help with mouse work. My PhD studies would have taken a significantly longer time if I would have not received help from her. I would like to give special thanks to Dr. Mary Jo Laws who has taught me a lot about how good research is done and many a times has been a soundboard for all my cribbing’s. I would like to thank my current members Dr. Yuechao Zhao, Janelle Mapes and Alison Hantak, as well as past lab members Dr. Elina Starostvetsky, Dr. Cyril Ramathal and Dr. Lavanya Anandan for their helpful critiques of my work.

I would like to express my appreciation to the Reproductive Biology group here at the University of Illinois. I feel fortunate to have worked in an environment that has experts in areas of Reproductive Biology. There are so many great Faculty members in the Reproductive Biology group that are invested in the success of graduate students and post-doctorate fellows. This is one reason why studying Reproductive Biology at UIUC has been so exceptional. Additionally, I would like to give a very large thank you to Karen Doty, the histologist at the Center for Reproduction and Infertility. She has contributed greatly to all my work.

Last but not the least I thank my family and friends. My parents, Anil Pawar and Annie Pawar, their faith in me and my abilities is what keeps me going. My eldest sister, Sulabha, who is my best friend, my mentor and my backup mother. Without her love and guidance I would not be the person I am today. My elder sister Smita, who makes me feel I am the best at everything.
My fiance Niyati, she is truly my rock. She has been with me through thick and thin and her unwavering love and support has helped me immensely in accomplishing my goals. Also, special thanks goes out to my roommates and gym buddies, Vineet Abhishek, Ritoban Basu Thakur, Nachiketa Chakraborty, Vineeth Madhavan, and Amandeep Gargi who helped me adjust to a new life in US and made life interesting at UIUC these past five years. In the end, I would like to thank Ms Lesa Scharnett, a group fitness instructor in CRCE because of whom I found another passion in my life and am as fit as I can be mentally and physically today.
Table of Contents

CHAPTER I: Steroid-Regulated Molecular Pathways Controlling Implantation ..............................1

CHAPTER II: Role of Epithelial Estrogen Receptor Alpha in Stromal Cell Decidualization .....23

CHAPTER III: Role of Signal Transducer And Activator Of Transcription 3 during Implantation .....................................................................................................................................61

CHAPTER IV: Ongoing/Future Studies - Role of Endothelial PAS domain protein-1 (EPAS-1) in Uterine Decidual Angiogenesis ....................................................................................................................................105

CHAPTER V: Conclusion .................................................................................................................................................................................................124
CHAPTER I

Steroid-Regulated Molecular Pathways Controlling Implantation
Introduction

In mammals, the principal function of the uterus is to support the growth and development of the fetus during pregnancy. During establishment of pregnancy, the embryo comes into intimate physical contact with the uterine endometrium and implants into this tissue [1-3]. Successful implantation requires attachment of the embryo to the uterine luminal epithelium and its subsequent invasion into the underlying stromal bed [4]. Embryo invasion triggers a unique transformation of the stromal cells, known as decidualization, which involves sequential proliferation and differentiation of these cells [5,6]. The differentiated stromal tissue supports uterine remodeling and development of an elaborate maternal vasculature that plays a critical role in embryonic growth and survival [7,8]. The events during early pregnancy are profoundly influenced by the ovarian steroid hormones, 17β-estradiol (E) and progesterone (P). The molecular mechanisms via which steroid-regulated pathways control these processes are under intense investigation by many laboratories around the world.

In humans, infertility is one of the most common disturbances of reproductive health. Despite significant advances in assisted reproductive technologies (ART), many couples experience infertility as a result of failed implantation of the fertilized embryos into the uterus and subsequent loss of pregnancy. The implantation rates in ART remain low, even with high-quality embryos, emphasizing the importance of this process as a major cause of pregnancy failure and infertility [9]. Understanding the signaling mechanisms central to implantation has the potential to alleviate many problems associated with infertility and improving the outcome of ART. Because of ethical considerations, research to study the early events during human pregnancy is restricted. However, the mouse has served as an important animal model to investigate the molecular mechanisms underlying female infertility [10]. This review will focus on our emerging understanding of the network of steroid hormone-regulated molecular pathways that regulate endometrial function during implantation in the mouse.

During early pregnancy, E and P act in an opposing fashion to orchestrate changes in the uterine epithelium that render it competent for embryo attachment. In mice, preovulatory ovarian E stimulates uterine epithelial growth and proliferation on days 1 and 2 of pregnancy [11-13]. In the E-dominated proliferative phase, the epithelium displays a distinct columnar phenotype and expresses proteins, such as E-cadherin, which play a key role in the maintenance of the adhesive and polarized phenotype of uterine epithelial cells [14,15]. Starting on day 3 of pregnancy, in response to rising P levels, epithelial cells cease to
proliferate and enter a differentiation program. On day 4 of pregnancy, uterine epithelial cells lose their polarity, as indicated by the downregulation of E-cadherin from junctional complexes, and acquire adhesiveness for the embryo [16]. The attachment of the embryo to the uterine epithelium is followed by the P-driven differentiation of subjacent fibroblastic stromal cells into secretory decidual cells. Thus, critical uterine changes during implantation are orchestrated by an intricate interplay of E and P signaling via their cognate receptors [1,2]. Recent studies have revealed that a series of molecular communications between the epithelial and stromal cells, guided by the steroid hormone receptors present in these tissues, is critical for acquisition of uterine receptivity and progressive functional interactions with the embryo [17-19]. Here we will summarize our emerging understanding of the mechanisms that mediate this cell-cell dialogue.

**ESR1 and PGR control implantation**

The cellular actions of E and P are mediated through their intracellular receptors, estrogen receptor alpha (ESR1) and progesterone receptor (PGR), respectively. These receptors function as ligand-inducible transcription factors [20]. Ligand-occupied ESR1 and PGR bind to specific genomic sites to activate or repress the expression of their target genes. Use of chromatin immunoprecipitation coupled to high throughput sequencing (ChIP-Seq) has identified genome-wide binding sites of ESR1 and PGR [21,22]. These receptors can also control target gene transcription by tethering to other transcription factors bound to the gene regulatory regions. Recent studies indicate that binding of these receptors can occur at distal enhancer regions, which then form complexes with the proximal promoter through a chromosomal looping event [23,24].

A large body of literature indicates that E and P, acting through their receptors, regulate cell proliferation, differentiation and secretory protein production in the uterus [25,26]. Accordingly, a dynamic pattern of expression of ESR1 and PGR proteins is seen in both epithelial and stromal compartments of the uterus during the reproductive cycle and pregnancy. A global knockout of *Esr1* established a critical role of this receptor in the regulation of overall uterine function, particularly in E-induced growth of this tissue [27,28]. Global knockout of *Pgr* established that P signaling via its receptor is essential for decidualization, and is a prerequisite to successful implantation [29]. Although these mouse models underscored the central importance of proper steroid hormone signaling during pregnancy establishment, they exhibit pleiotropic defects. Furthermore, they cannot inform us
of the stromal or epithelial compartment-specific roles of these hormone receptors since the receptors are ablated from all uterine cells.

**Tissue recombination studies: initial evidence for epithelial-stromal crosstalk**

In a series of elegant studies, Cunha *et al* used uterine tissue recombinants obtained from *Esr1*-null and *Pgr*-null mice to dissect individual roles of epithelial and stromal steroid receptors in regulating uterine proliferation and differentiation [30]. To examine the role of ESR1 in E-induced epithelial proliferation, tissue recombinants were prepared, using epithelium and stroma from wild-type (WT) and *Esr1*-null mice [31]. This study reported that the mitogenic effect of E in uterine epithelium is independent of epithelial ESR1, but is dependent on stromal ESR1. This surprising result suggested that uterine epithelial proliferation is a paracrine event dictated by E signaling originating in the stromal compartment. With a similar approach using *Pgr*-null mice, Cunha’s group went on to demonstrate that stromal PGR is both necessary and sufficient to mediate the anti-proliferative effects of P on E-induced epithelial cell proliferation [32]. Collectively, Cunha’s experiments suggested that uterine epithelial cell proliferation is controlled by stromal ESR1 and its antagonism is mediated by stromal PGR, indicating that E or P acting on uterine stromal cells initiate paracrine mechanisms that modulate epithelial proliferation. However, since these studies used neonatal mouse tissue, questions were raised whether these results truly reflect adult uterine physiology. Nevertheless, these studies underscored, for the first time, the importance of stromal-epithelial communications in regulating key uterine functions.

**Conditional deletion of *Esr1* and *Pgr* in the uterus**

The advent of the Cre-Lox strategy for generating tissue-specific gene deletion has allowed researchers to make great advances in understanding the cell-type-specific roles of ESR1 and PGR in the adult mouse uterus. Recently, Korach’s group has addressed the epithelial cell-specific role of ESR1 by using the WNT7A-Cre mouse model, which allows ablation of the *Esr1* gene specifically in luminal and glandular epithelia [33]. They reported that the epithelial ESR1 does not control E-mediated epithelial cell proliferation, raising the possibility that stromal ESR1 actually regulates epithelial growth via paracrine mechanisms. This observation is in excellent agreement with Cunha’s tissue recombination studies [31]. Korach’s group showed further that ESR1 induces the expression of insulin-like growth factor-1 (IGF-1) in the stromal cells [34]. Since IGF-1 receptor is abundantly expressed on
the luminal and glandular epithelium, this finding is consistent with the view that IGF-1 is a paracrine factor of stromal origin mediating E-driven uterine growth [35]. It is pertinent to mention here that our recent findings support a role of stroma-derived growth factors, although distinct from IGF-1, in mediating E-induced epithelial proliferation [36]. So, it is conceivable that multiple E-regulated signals of stromal origin might be involved in uterine epithelial growth.

Compartment-specific conditional knockout mice have also proved valuable in delineating the role of PGR in epithelial-stromal crosstalk. DeMayo’s group generated and characterized a uterine epithelial-specific knockout of Pgr [37]. These knockout mice are infertile due to defects in embryo attachment, stromal cell decidualization, and the inability to suppress E-induced epithelial cell proliferation. The findings of this study suggested that epithelial PGR is a critical regulator of epithelial receptivity. It also mediates P regulation of E-induced epithelial proliferation, although no mechanism for this has been proposed. Importantly, previous studies by this group showed that P regulates the production of the morphogen Indian Hedgehog (IHH) by the luminal epithelium, which then acts on the stromal cells to promote decidualization. Taken together, these studies highlight the critical role of epithelial PGR in regulating stromal cell decidualization via a paracrine mechanism.

**Downstream Mediators of E and P Function during Implantation**

Extensive use of gene expression profiling has uncovered many genes that are regulated by E and P during implantation [38-40]. During the past several years, with the advent and continued refinement of gene knockout and knock-in strategies, transgenic mice have become powerful tools for determining the functional roles of several of these E- and P-regulated pathways in various aspects of uterine physiology [41,42]. Studies employing these animal models have established that these factors critically regulate uterine growth and differentiation, which in turn control embryo-endometrial interactions during early pregnancy. Here we provide a brief description of the physiological relevance of these steroid-regulated genes in the context of epithelial-stromal interactions during implantation and decidualization.

**Steroid-induced epithelial-stromal crosstalk involving glandular epithelium, luminal epithelium and stroma: role of LIF, STAT3 and the EGF family**

The leukemia inhibitory factor (LIF), produced by the endometrial glands during implantation, plays a central role in a crosstalk involving three different uterine cell-types. It
was discovered as an obligatory factor for implantation by Stewart’s group in 1992 [43]. LIF, a member of the interleukin-6 (IL-6) family, functions through the LIF receptor, which is associated with the signal transducer GP130 [44]. In the uterus, its expression is induced specifically in the glandular epithelium in response to a transient surge of E immediately prior to implantation [45]. LIF receptors are primarily located in the luminal epithelium at the time of implantation. Our recent studies indicated that the conditional ablation of epithelial Esr1 leads to a loss of LIF production by the glands, confirming the E regulation of this factor (Sandeep Pawar, Indrani C. Bagchi, and Milan K. Bagchi, unpublished results). However, it is unclear whether ESR1 directly controls LIF expression in this tissue. In female mice lacking LIF, the embryos fail to attach to the luminal epithelium due to a defect in uterine receptivity [43]. It was reported that, during implantation, LIF, secreted from the glands, binds to its receptors in luminal epithelial cells and activates the JAK-STAT3 pathway, as described in Figure 1. To understand the molecular mechanisms via which the LIF-STAT3 signaling pathway controls uterine functions during implantation, Pawar et al created a mutant mouse model in which Stat3 is specifically deleted in the uterine epithelium, but is retained in the stroma [46]. Using this mouse model, it was revealed that STAT3, upon activation following LIF signaling, alters the molecular organization of epithelial junctional complexes at the time of implantation. STAT3 suppresses the expression of the tight junction proteins claudin-1, -3 and -4 within the implantation window. It also down regulates the expression of α and β-catenin, which likely contributes to the redistribution of E-cadherin away from the lateral adherent junctional complexes during implantation. The concerted downregulation of the tight and adherent junction complexes in the luminal epithelium, driven by STAT3 signaling, critically alters the uterine epithelial phenotype by disrupting cell-cell linkages and triggering a loss of epithelial polarization. This presumably leads to proper organization and presentation of cell surface adhesion molecules that allow interaction with the blastocyst trophoectoderm initiating the process of implantation. Pawar et al also reported that the conditional loss of uterine epithelial STAT3 results in impaired decidualization [46]. Specifically, it was noted that, in the absence of uterine epithelial STAT3, the stromal cells exhibit a significantly reduced capacity to undergo proliferation in response to a decidual stimulus. These results suggested a role of epithelial STAT3 in promoting production and/or secretion of a paracrine factor(s) from the uterine epithelium, which in turn induces stromal cell proliferation, as depicted in Figure 1.1. Indeed, a marked reduction in the levels of a subset of EGF family growth factors was observed in the Stat3-null uterine epithelium in the period following implantation, indicating that these
growth factors are likely downstream paracrine effectors generated by epithelial STAT3 signaling. Consistent with this hypothesis, a marked reduction in the level of active, phosphorylated EGFR was observed in the uterine stroma of these mutant mice. Furthermore, administration of exogenous EGF family factors was effective in rescuing the uterine stromal proliferation defect in mice lacking uterine epithelial STAT3.

Recent studies by two other groups have also indicated a role for uterine stromal STAT3 in controlling the decidualization process [47,48]. They used conditional mutant mice, developed by crossing floxed Stat3 mice with Pgr-Cre mice, which resulted in Stat3 gene deletion in both epithelial and stromal compartments of the uterus. Loss of uterine STAT3 led to alterations in pathways in the stromal cells with consequent dysregulation of E and P signaling in the uterus as reported by these investigators. Collectively, these studies provided valuable insights into the mechanisms by which LIF-STAT3 signaling allows transition of uterine epithelium and stroma to proper functional states that permit embryo attachment, invasion and the subsequent decidualization of the underlying stroma.

**Steroid-induced epithelial-stromal crosstalk originating in the epithelium: role of the IHH-COUP-TFII pathway**

Indian Hedgehog (Ihh) represents a prototype hormone-regulated paracrine factor that links epithelial and stromal functions in the uterus. It is a member of the Hedgehog family of morphogens that is induced by P in the uterus [49]. Recent studies by the DeMayo group, using ChIP-Seq, showed that PGR binds directly to the Ihh promoter [22]. In the endometrium, Ihh is expressed in the luminal and glandular epithelium prior to implantation and its expression decreases thereafter [49]. Its receptor Patched-1 (PTCH-1) is also upregulated in a similar temporal fashion in the luminal epithelium and stroma. To study IHH-mediated signaling in the uterus, Lee et al created a conditionally deleted mutant of Ihh using Pgr-Cre mice [50]. The resulting mutant females are infertile, exhibiting implantation defects and failure to respond to an artificial decidual stimulus. Defects in implantation were attributed to sustained ESR1 activity in mutant mice [51]. Loss of IHH also resulted in reduced expression of PTCH-1 and the transcription factor COUP-TFII (Chicken Ovalbumin Upstream Promoter-Transcription Factor II, also known as NR2F2) in the subepithelial stroma [50]. Taken together, these observations suggested that IHH is a major effector of PGR signaling in the uterus. When secreted from the luminal epithelium, it influences stromal function by regulating COUP-TFII, as shown in Figure 1.2, and thereby mediates the communication between the uterine epithelium and stroma required for embryo implantation.
COUP-TFII, which is expressed almost exclusively in the uterine stroma, acts downstream of IHH [50]. To assess its uterine function, a conditional COUP-TFII knockout mouse was generated using Pgr-Cre mice. Similar to Ihh-null mice, COUP-TFII-null females are infertile due to an implantation failure [52,53]. It was demonstrated that COUP-TFII-null mice exhibit enhanced E activity in the epithelium during the implantation window, which inhibits epithelial maturation and thus leads to failure of embryo attachment. It was also reported that COUP-TFII regulates PGR expression in the uterine stroma [52]. Stromal PGR, acting through paracrine mechanisms, down regulates ESR1 activity in the luminal epithelium to promote a receptive uterus. So, suppression of stromal PGR in COUP-TFII-null mice may result in dysregulated E signaling in the epithelium. Interestingly, COUP-TFII-null mice also failed to undergo experimentally induced decidualization, a defect linked to decreased expression of bone morphogenetic protein 2 (BMP2), a factor induced in the uterine stroma in response to P stimulation [54]. Previous studies in our laboratory and the DeMayo laboratory have shown that WNT4 acts downstream of PGR and BMP2 to regulate stromal decidualization [55,56]. Taken together, the Ihh-null and COUP-TFII-null mice demonstrated that the IHH-COUP-TFII signaling pathway functionally link the epithelial and stromal compartments, as indicated in Figure 2, and is essential for successful implantation.

Steroid-induced epithelial-stromal crosstalk originating in the stroma: regulation by HAND2 and FGFs

Although the tissue recombination studies of Cunha’s group indicated that stromal PGR plays a pivotal role in mediating the antiproliferative effects of P on the uterine epithelium, the molecular mechanisms by which P achieves this regulation remained unclear until recently. Microarray-based profiling of P-responsive transcripts during the implantation window in the mouse identified the basic helix-loop-helix transcription factor heart and neural crest derivatives-expressed protein 2 (Hand2) as a P-regulated gene in the uterine stroma [36,57]. HAND2 is robustly expressed in the uterine stroma by day 3 of gestation but is notably absent in the epithelium. Its stromal expression persists through the peri-implantation phase and is detectable until day 8.5 of pregnancy [58]. Recent genome-wide PGR binding studies indicated that Hand2 is a direct target of regulation by this receptor [22].

A study by Li et al revealed that HAND2 plays a central role in controlling the paracrine mechanisms that mediate the antiproliferative effects of P in the endometrium [36,59]. Deletion of Hand2 in the mouse uterus, using Pgr-Cre, resulted in infertility due to failed implantation. Hand2-null mice showed persistent luminal epithelial proliferation on
day 4 of pregnancy, indicating that, in the absence of HAND2, pregnant uteri fail to achieve a receptive state. HAND2 was shown to regulate the expression of a subset of stromal fibroblast growth factors (FGFs). Unopposed, stromal FGFs, presumably induced by E via stromal ESR1, act in a paracrine fashion to target receptors on the luminal epithelium and mediate epithelial proliferation through ERK1/2 activation. HAND2 mediates the anti-proliferative effects of P by inhibiting the production of FGFs, and thus terminating E-driven epithelial proliferation, as illustrated in Figure 1.3. Additionally, phosphorylation and activation of epithelial ESR1 was also shown to be persistent in Hand2-null mice. Together, these findings show that loss of HAND2 removes the brake by which the uterus shifts from a proliferative state to a differentiated state and creates a tissue unreceptive to the blastocyst.

This study provides a plausible mechanism by which epithelial proliferation is controlled in a paracrine manner by signaling molecules produced downstream of steroid action in the stroma.

**Dysfunction of stromal-epithelial crosstalk leads to reproductive diseases**

During the past several years, the use of conditional knockout mice has allowed us to make great strides in identifying key molecular players important for the implantation process. Many of these factors, of both stromal and epithelial origin, have been well characterized in vitro and in vivo. An emerging concept is that dysregulation of these factors not only affects fertility, but is also linked to other reproductive pathologies, such as endometriosis and endometrial cancer. Endometriosis, a chronic disease characterized by endometrial epithelium and stroma implanting outside of the uterus, is often associated with inflammation, abdominal pain, and infertility [60,61]. Because endometriotic tissue exhibits deregulation in differentiation gene networks associated with PGR signaling, endometriosis is often considered a P resistant disease, although the mechanism is not fully understood [62]. In addition, several lines of evidence link endometriosis with elevated expression of P450 aromatase and consequent increase in 17β-estradiol (E) signaling in the endometriotic tissue [61].

Bulun’s group has shown that specific steroid hormone receptor isoforms are differentially regulated in stromal cells isolated from normal and endometriotic tissue [63]. For example, the PGR-B isoform, but not PGR-A, is downregulated in endometriotic stroma [64]. It was proposed that the stromal dysregulation of PGR contributes to endometriosis through the downregulation of 17β-hydroxysteroid dehydrogenase type 2 (HSD17β2), an enzyme that metabolizes E into estrone, a weakly estrogenic metabolite [65]. This impaired E
metabolism, coupled with the abnormal high expression of P450 aromatase that is seen in endometriotic tissue, leads to excessive local E signaling, which presumably drives the growth of endometriotic lesions. Additional studies have linked impaired expression of other key P-regulated pathways in the stroma to the pathogenesis of endometriosis [66-68]. Bulun’s group has also reported that the level of ESR2 expression is significantly greater in endometriotic stroma versus normal [69]. They postulated that the increased ESR2 levels observed in endometriosis are a consequence of hypomethylation at a CpG island within the Esr2 promoter, and that ESR2 overexpression leads to the observed suppression of ESR1 and PGR. Taken together, these reports support the concept that aberrant steroid receptor expression in endometriosis, likely driven by epigenetic alterations, results in dysregulation of paracrine signaling between the stroma and the epithelium, leading to abnormal epithelial proliferation and stromal differentiation observed in this disease.

Recent studies revealed that impaired stromal-epithelial interactions may also contribute to endometrial hyperplasia, a precursor to endometrial cancer, which is associated with unregulated E signaling [68]. Treatment regimens for this condition often include progestin therapy to inhibit E mediated proliferation. Jones et al. identified HAND2 as the most commonly hypermethylated and silenced gene in endometrial hyperplasia and cancer [70]. The degree of HAND2 methylation was positively correlated with the severity of hyperplasia. It was further shown that uterine samples from patients that did not respond to progestin treatment exhibited greater HAND2 methylation than positive responders. Morphological changes similar to complex atypical hyperplasia were also observed in uteri of aged Hand2-null mice as seen in Figure 1.4, including increased gland to stroma ratio and irregularities in glandular shape, compared to age matched control mice. Loss of Hand2 also results in down-regulation of the tumor suppressor Pten, an event that often occurs early during endometrial carcinogenesis [68]. Collectively, these results indicated that stromal epigenetic modifications of key target genes, such as HAND2, have a profound effect on the protective effects of P on the epithelium and can now be considered as predictors of progestin responsiveness in endometrial cancer treatment regimens.

**Conclusion**

Steroid hormones, acting through their cognate nuclear receptors, regulate various aspects of uterine physiology and pregnancy. In preparation for implantation, these hormones orchestrate the cellular events in different uterine compartments to achieve blastocyst attachment to the luminal epithelium and subsequent invasion into the stroma. A
defect in any of these steps may cause infertility. This review has highlighted several key steroid-regulated pathways that play an important role in epithelial-stromal crosstalk during early pregnancy. Tissue-specific gene knockout mouse models combined with powerful bioinformatic approaches have helped us understand how these pathways act in different uterine compartments to synchronize the critical events to achieve successful implantation. We have also described briefly the emerging studies that suggest that epigenetic alterations at specific gene loci impact on uterine functions, leading to reproductive disorders and cancer. These novel and important insights, along with future studies, will help develop new therapeutic approaches in treating infertility and other reproductive disorders arising from aberrant steroid hormone signaling in the uterus.
Figure 1.1 Steroid-mediated glandular epithelium-luminal epithelium-stromal crosstalk in the uterus. E induces LIF in the glandular epithelium (blue), which acts in a paracrine manner on the luminal epithelium (purple) to activate the JAK/STAT pathway to mediate a shift in junctional integrity required for implantation. Activation of JAK/STAT also promotes proliferation of the stroma (pink) via expression and secretion of EGF family proteins from the luminal epithelium. Dashed lines represent indirect events. E, estrogen; ESR1, E receptor; LIF, leukemia inhibitory factor; LIFR, LIF receptor; JAK/STAT3, Janus kinase/signal transducers and activators of transcription 3; EGF, epidermal growth factor; EGFR, EGF receptor.
Figure 1.2 Steroid-mediated luminal epithelium-stromal crosstalk in the uterus. P drives IHH expression in the luminal epithelium (purple). IHH acts on the stroma to promote decidualization through activation of COUP-TFII signaling. Decidualization results in morphologically distinct stroma (green) compared to undifferentiated stroma (pale pink). Dashed lines represent indirect events. P, progesterone; PR, P receptor; IHH, Indian hedgehog; PTCH1, patched 1; COUP-TFII, chicken ovalbumin upstream promoter-transcription factor II; BMP2, bone morphogenetic protein; WNT4, wingless-type MMTV integration site family, member 4.
Figure 1.3 Steroid-mediated stromal-epithelial crosstalk in the uterus. Prior to implantation, E, acting through ESR1 in the stroma (pink), drives epithelial (purple) proliferation through the secretion of paracrine acting FGFs. During the periimplantation period, P, acting on PGR in the stroma, promotes the expression of HAND2. HAND2 inhibits expression of FGFs and blocks E-induced epithelial proliferation. Dashed lines represent indirect events. E, estrogen; ESR1, E receptor; FGF, fibroblast growth factor; FGFR, FGF receptor; ERK1/2, extracellular signal regulated kinase1/2; P, progesterone; PGR, P receptor; HAND2, heart and neural crest derivatives expressed 2.
Figure 1.4 Epigenetic silencing of HAND2 through hormonal dysregulation or environmental insults leads to signs of complex atypical uterine hyperplasia.


CHAPTER II

Role of Epithelial Estrogen Receptor Alpha in Stromal Cell Decidualization
ABSTRACT

Differentiation of uterine stromal cells, known as decidualization, is critical for embryo implantation. The role of estrogen receptor alpha (ESR1) during this process is unclear. Development of a conditional Esr1-null mouse model in our laboratory showed that deletion of this gene in both epithelial and stromal compartments of the uterus leads to a complete blockade of decidualization, indicating a critical role of uterine ESR1 during this process. To further address the compartment-specific uterine function of ESR1, we created WE<sup>dd</sup> mice in which Esr1 is specifically ablated in the uterine luminal and glandular epithelia, but is retained in the stroma. Interestingly, the uteri of WE<sup>dd</sup> mice also failed to undergo decidualization. The loss of epithelial ESR1 did not affect stromal progesterone receptor (PGR) expression, but markedly reduced the production of leukemia inhibitory factor (LIF) in the glandular epithelium. Strikingly, administration of exogenous LIF restored decidualization in WE<sup>dd</sup> mice. We showed that LIF induces the expression of Indian hedgehog (Ihh) in the luminal epithelium. IHH then acts on the stromal cells to induce the expression of Nr2f2 a transcription factor that promotes decidualization. Further analysis also revealed that LIF and P act synergistically to induce Ihh expression in the uterine epithelium by activating the mitogen activated protein kinase (MAPK) pathway. Collectively, these results functionally link uterine epithelial ESR1 to stromal differentiation via a paracrine mechanism involving LIF and IHH.
INTRODUCTION

Steroid hormones estrogen (E) and progesterone (P) play crucial roles during early pregnancy. These hormones function via their cognate receptors to coordinate a complex series of interactions between the embryo and the receptive uterus to initiate the process of implantation (1-3). In mouse and human, one of the prerequisites for successful implantation is the differentiation of endometrial stromal cells into a unique secretory tissue, known as the decidua. This remarkable transformation event, known as decidualization, comprises morphogenetic and biochemical changes driven by the estrogen and progesterone receptors leading to successful establishment of pregnancy (4, 5). Decidualization is also accompanied by the creation of an extensive vascular network within the stromal bed that supports embryonic growth until placentation ensues (6, 7).

The development of mutant mouse models lacking receptors for progesterone (Pgr) and estrogen (Esr) has firmly established the necessity of steroid signaling for implantation. The Pgr-null mice display a refractory uterus that fails to respond to an experimentally induced deciduogenic stimulus, establishing a central role played by P in regulating the decidualization phase of pregnancy (8, 9). In contrast, the estrogen receptor alpha (Esr1)-null mice, although impaired in its preparedness for blastocyst attachment, exhibited decidual response challenging the essentiality of Esr1 in stromal differentiation during early pregnancy (10). It was later revealed that the uteri of these mutant mice expressed a truncated ESR1, raising the possibility that it may mediate the decidual response seen in these mice (11, 12). Thus, the role of Esr1 in the regulation of decidualization to support establishment of pregnancy remained unresolved.

In the present investigation, we have employed genetic and molecular approaches to establish for the first time the critical role of Esr1 signaling in the regulation of endometrial stromal cell differentiation. We initially developed conditional knockout mice in which Esr1 is deleted from both epithelial and stromal compartments of the uterus. Our results revealed that these mice displayed a complete blockade of decidualization. To analyze further the compartment-specific role of ESR1, we created mice in which Esr1 is specifically ablated in the uterine luminal and glandular epithelia, but is retained in the stroma. Interestingly this deletion also resulted in a complete blockade of decidualization, indicating that epithelial ESR1 controls critical aspects of endometrial stromal cell differentiation. Our studies further uncovered a novel paracrine mechanism in which epithelial ESR1 controls stromal cell decidualization via LIF and IHH signaling cascades. These findings are of high clinical relevance because proper E signaling is critical for implantation and establishment of
pregnancy. A deeper understanding of the E mediated signaling in the uterus would help in improving *in vitro* fertilization techniques.

**RESULTS**

To investigate the function of ESR1 during decidualization, we created a conditional knockout of the *Esr1* gene in the uterus of adult mice by employing the Cre-LoxP strategy. Transgenic mice expressing Cre under the control of *Pgr* promoter were used previously to ablate “flooed” genes in the uterus (13). We crossed these *Pgr*-Cre mice with those harboring the “flooed” *Esr1* gene (*Esr1*<sup>fl/fl</sup>) to create the *Esr1*<sup>dd</sup> mice in which the *Esr1* gene is deleted in uterine cells expressing PGR. The ablation of *Esr1* in various compartments of *Esr1*<sup>dd</sup> uteri was confirmed by immunohistochemistry (IHC). Uterine sections obtained from *Esr1*<sup>dd</sup> mice failed to show any ESR1 protein expression in the luminal epithelium, glandular epithelium, and stroma (Fig. 2.1A).

*Esr1*<sup>fl/fl</sup> and *Esr1*<sup>dd</sup> mice were then subjected to experimentally induced decidualization in which a mechanical stimulation of the steroid-primed uteri triggers a decidual response in the absence of an implanting embryo (14). This artificial stimulus mimics the embryonic signal during implantation and sets in motion the decidualization program. Examination of the gross anatomy of the stimulated and unstimulated uterine horns of *Esr1*<sup>fl/fl</sup> and *Esr1*<sup>dd</sup> mice indicated that as expected, the uterine horns of *Esr1*<sup>fl/fl</sup> mice exhibited a robust decidual response within 72 h after receiving the artificial stimulation (Fig. 2.1B, upper left panel). In contrast, the *Esr1*-deficient uteri under identical conditions failed to show any decidualization (Fig. 2.1B, upper right panel). When the decidual response was assessed by measurement of uterine wet weight gain, the *Esr1*-deficient uteri exhibited a markedly reduced weight gain relative to that seen in the *Esr1*<sup>fl/fl</sup> uteri (Fig. 2.1C).

We further analyzed the decidualization response of *Esr1*<sup>dd</sup> uteri by monitoring the expression of alkaline phosphatase (ALP), a classical biomarker of decidualization. As shown in Fig. 2.1D panel a, the expression of ALP was prominent in the antimesometrial decidual cells of the control *Esr1*-intact uteri at 72 h following decidual stimulation. In contrast, no expression of this decidual marker was seen in the uteri of *Esr1*<sup>dd</sup> mice at a similar time point, indicating a complete blockade of stromal cell differentiation in these mice (Fig. 2.1D, panel b). While these studies with *Esr1*<sup>dd</sup> mice established the critical role played by ESR1 in decidualization, we next performed compartment-specific deletion of *Esr1* to gain mechanistic insight into the role of ESR1 and its downstream signaling pathway in the regulation of stromal differentiation.
Uterine epithelial ESR1 is indispensable for stromal cell decidualization

To ablate ESR1 from uterine epithelium, we crossed \textit{Esr1}^{fl/fl} mice with transgenic mice expressing Cre under the control of an epithelium specific Wnt-7a promoter (13, 14). This created the \textit{WE}^{dd} mice in which the \textit{Esr1} gene is deleted exclusively in the uterine glandular and luminal epithelial cells expressing Wnt-7a. The ablation of \textit{Esr1} gene in the uterine epithelium of \textit{WE}^{dd} mice was confirmed when uterine sections obtained from these mice failed to show any \textit{ESR1} mRNA (Fig. 2.7A) or protein expression in the luminal and glandular epithelium, but the expression in the stroma remained unaltered (Fig. 2.7B compare panel a and b).

A six-month breeding study demonstrated that \textit{WE}^{dd} mutant mice are infertile (Table 1). While \textit{Esr1}^{fl/fl} mice exhibited normal litter size and pregnancy rates, the \textit{WE}^{dd} females failed to become pregnant when mated with wild-type males. Further analysis revealed a defect in oviductal function and embryo transport in \textit{WE}^{dd} females (data not shown). We then subjected \textit{Esr1}^{fl/fl} and \textit{WE}^{dd} mice to experimentally induced decidualization as described previously. As expected, the decidual response was pronounced in the uterine horns of \textit{Esr1}^{fl/fl} mice (Fig. 2.2A, upper left panel). However, \textit{WE}^{dd} uteri under identical conditions failed to show any decidualization (Fig. 2.2A, upper right panel). The \textit{WE}^{dd} uteri exhibited a markedly reduced wet weight gain relative to that seen in the \textit{Esr1}^{fl/fl} uteri (Fig. 2.2B). Taken together, these data indicate that \textit{Esr1} in the uterine epithelium is indispensable for stromal cell decidualization.

During decidualization, the uterine stromal cells initially undergo proliferation for 24 to 48 h followed by differentiation (15, 16). To further investigate the decidualization defect in mice lacking \textit{Esr1} in uterine epithelium, we monitored proliferation and differentiation of steroid hormone-primed stromal cells in response to a decidual stimulation by using known markers of these events. Uteri of \textit{Esr1}^{fl/fl} mice exhibited widespread staining for Ki67 in the stromal cells within 20 h of receiving the decidual stimulus, clearly indicative of extensive stromal cell proliferation (Fig. 2.2C, panels a and c). In contrast, the uterine sections from \textit{WE}^{dd} mice exhibited significantly reduced Ki67 staining under identical conditions (Fig.2.2C, panels b and d). Quantitation of the Ki67 signal revealed a significant reduction in the number of Ki67-positive cells in the stromal compartment of the \textit{WE}^{dd} uteri (Fig. 2.2D). We further investigated the proliferation defect in \textit{WE}^{dd} uterine stroma by analyzing the expression of factors that control various cell cycle checkpoints, including cyclins, and cyclin-dependent kinases (Cdks) (17). Our results revealed a marked down regulation of cyclin E1 (\textit{Ccne1}) and E2 (\textit{Ccne2}), their corresponding Cdk, Cdk2, and cyclin D1 (\textit{Ccnd1}) in
stromal cells of $WE^{dd}$ uteri within 20 h of receiving the decidual stimulus (Fig. 2.2E), indicating a defect in stromal cell proliferation in uteri lacking epithelial ESR1.

We next investigated whether stromal differentiation is impaired as a consequence of a defect in stromal proliferation. We observed prominent expression of ALP in the decidual cells of the $Esr1^{ff}$ uteri at 72 h after decidual stimulation. However, no expression of this decidual marker was seen in the uteri of $WE^{dd}$ mice at a similar time point, indicating a complete blockade of stromal cell differentiation in these mice (Fig. 2.2F, compare panel a and b). Collectively, these results indicate that ESR1 in the uterine epithelium is a critical regulator of stromal cell proliferation and differentiation during early pregnancy.

**Epithelial ESR1 controls decidualization of stromal cells via LIF**

To gain insights into the underlying mechanism by which epithelial ESR1 controls differentiation of stromal cells, we first monitored the expression of ESR1 at the onset of decidualization. We noted that the expression of ESR1 in $Esr1^{ff}$ uteri is strongest in the glandular epithelium, lower in the stroma, and negligible in the luminal epithelium at 20h post administration of decidual stimulation (Fig. 2.3A, panel a). In comparison in the $WE^{dd}$ uteri, ESR1 is still expressed in the stroma but is absent from both luminal and glandular epithelium (Fig. 2.3A, panel b). The high expression of glandular ESR1 in $Esr1^{ff}$ uteri and its absence in the $WE^{dd}$ uteri led us to hypothesize that ESR1 in the glands might control the secretion of a factor which promotes stromal decidualization. One such factor that has been shown previously to be regulated by ESR1 and is of critical importance in implantation is LIF (18-20). Therefore, we examined the expression of Lif in $Esr1^{ff}$ and $WE^{dd}$ animals during decidualization. As shown in Fig. 2.3B, the expression of Lif was dramatically reduced in uteri lacking epithelial ESR1, while the levels of PGR and its target gene HAND2 remained unaltered between $Esr1^{ff}$ and $WE^{dd}$ uteri (Figs. 2.3B and 2.9). These results indicated that the loss of epithelial ESR1 does not affect the PGR-mediated signaling in uterus during decidualization. These results also suggested that LIF is a potential downstream target of ESR1 and might be involved in the regulation of stromal decidual response.

To address the role of LIF in decidualization, we next investigated whether LIF is able to rescue the decidualization defect exhibited by the $WE^{dd}$ uteri. Recombinant mouse LIF was administered intraperitoneally to $WE^{dd}$ mice and subjected to experimentally-induced decidualization. Uteri were dissected 72 h later, and the decidual response was measured. Remarkably we observed that LIF treatment restored the decidual response in the mutant uterine horns, as measured by morphological examination (Fig. 2.3C, compare panels...
a, b, and c), alkaline phosphatase activity (Fig. 2.3C, compare panels d, e, and f) and wet weight gain (Fig. 2.3D). These results strongly support our hypothesis that LIF is a downstream effector of epithelial ESR1 and regulates stromal cell decidualization.

**LIF regulates stromal cell decidualization via paracrine-mediated signaling involving Ihh pathway**

To investigate the mechanism by which LIF promotes the differentiation of stromal cells, we first considered the possibility that LIF secreted from the glands act directly on stromal cells to promote decidualization. Stromal cells isolated from ESR1^{f/f} uteri were cultured in the presence and absence of LIF. We observed that LIF by itself did not promote stromal differentiation, and when added along with P, LIF failed to exhibit any additive effect on stromal decidualization (Fig. 2.8A and 2.8B). These results indicated that LIF does not act directly on the stromal cells to induce decidualization. We next considered the possibility that LIF induces the expression of a factor in the luminal epithelium, which in turn acts on stromal cells to promote decidualization. A potential paracrine factor is IHH which has been shown previously to be secreted from the luminal epithelium, binds to its receptor PTCH1 in the stroma and induces the expression of Nr2f2, a transcription factor that promotes stromal cell decidualization (24, 25, 26, 27). Interestingly, LIF has been shown to regulate Ihh expression during pregnancy raising the possibility that LIF regulates stromal decidualization by inducing IHH (21). In order to test this possibility we monitored the expression of genes involved in the Ihh signaling cascade during decidualization. As shown in Fig. 2.3E, we observed a significant downregulation of the factors involved in Ihh signaling cascade in WE^{dd} uteri during decidualization, indicating decidual impairment due to defective Ihh signaling. Importantly, administration of LIF to WE^{dd} uteri was able to induce the expression of the genes involved in Ihh signaling and rescue the decidualization defect in the mutant uterus (Fig. 2.3F). Collectively, these results strongly indicate that LIF regulates differentiation of endometrial stromal cells via Ihh pathway.

**LIF and P act synergistically to induce Ihh via the MAPK pathway**

Previous studies have shown induction of Ihh by both P and LIF in the uterus during early pregnancy (21, 22). However, the mechanism by which either P or LIF induces IHH remains unknown (23). To gain an insight into the regulation of Ihh by P and LIF, we administered P and LIF to ovariectomized animals and monitored Ihh expression. As expected, Ihh was induced in uterine epithelial cells within 6 h of P injection. We observed a
similar induction of \textit{Ihh} with LIF. However upon co-administration of P and LIF, there was a dramatic induction of \textit{Ihh} indicating a synergistic action of P and LIF on \textit{Ihh} expression (Fig. 2.4A).

We next investigated the signaling pathway that is functioning downstream of LIF and is involved in \textit{Ihh} expression. Previous studies have shown that in the uterus LIF acts through Signal Transducer and Activator of Transcription (STAT3) to exert its effects on the luminal epithelium to promote receptivity during implantation (24, 25). In order to test this signaling mechanism we examined \textit{Ihh} induction by P and LIF in the uteri of control (\textit{Stat3}\textsuperscript{fl/fl}) and epithelial knockout of \textit{Stat3} (\textit{SW}\textsuperscript{dd/d}) animals. In \textit{SW}\textsuperscript{dd/d} mutants, \textit{Stat3} has been excised from the luminal and glandular epithelial cells of the uterus, thus blocking the signaling of LIF involving the JAK/STAT3 pathway. Our results show that LIF and P still retained the ability to act synergistically to induce \textit{Ihh} in the \textit{SW}\textsuperscript{dd/d} uteri (Fig. 2.4B), indicating that LIF does not act via the STAT3 pathway to induce \textit{Ihh} during pregnancy. LIF is also known to act via the MAPK signaling pathway to exert its effects on target cells (26-28). In order to examine whether the MAPK signaling pathway was activated downstream of LIF, we compared the expression levels of phosphorylated-ERK1/2 (p-ERK1/2) in uteri of animals treated with LIF, P or P along with LIF. An increase in the level of p-ERK1/2 as well as total ERK was observed in uterine epithelium of LIF treated samples (Fig. 2.4C, Fig. 2.10, compare panels a and b). This expression was further enhanced in response to P along with LIF (Fig. 2.4C, Fig. 2.10, compare panels b and d). P by itself also activated MAPK signaling pathway in the uterine epithelium but to a lower extent than LIF or P along with LIF (Fig. 2.4C, compare panels b, c and d). Similarly, phospho-SHP2 an adaptor molecule in the MAPK signaling pathway was also activated in the same manner as p-ERK1/2 (Fig. 2.4D, compare panels a through d). Taken together, these results indicate that P and LIF synergize to induce \textit{Ihh} by activation of MAPK pathway. Consistent with these results, we observed that administration of PD184352, an inhibitor of the ERK1/2 (29), to uteri led to downregulation of the MAPK pathway and concomitant suppression of \textit{Ihh} production (Fig. 2.4E compare panels a and b), while the levels of PGR remained unaltered in response to the treatment (Fig. 2.4F).

We further investigated the regulation of \textit{Ihh} expression in the uterus by monitoring the expression profiles of \textit{Lif}, \textit{Ihh} and p-ERK1/2 on different days of pregnancy. \textit{Lif} is expressed in the uterus in a biphasic manner; its expression levels are high on day 1 and 2 of pregnancy but then decline to minimal levels by day 3. The levels increase again by day 4 with a transient surge in E levels (Fig. 2.5A). The levels of \textit{Ihh} are low on day 1 of
pregnancy. The levels increase on day 2 reaching maximum levels by day 3, followed by a reduction on day 4 of gestation (Fig. 2.5B). Levels of pERK1/2 are prominent in the uterine luminal epithelium from day 1 to 3 of pregnancy, reaching maximum levels on day 3, following which it is excluded from the luminal epithelium and is expressed in the stroma (Fig. 2.5C, panels a through d). These overlapping expression patterns of Lif, Ihh and pERK1/2 suggest a regulatory network wherein LIF and P via ERK1/2 signaling pathway regulate Ihh production.

To establish this regulatory network, we administered PD184352, an inhibitor of the ERK1/2 pathway, to uterine horn on day 2 of pregnancy. When we looked at the expression of Ihh on day 3 of gestation, at a time when its expression is supposed to be highest, we observed a significant reduction in Ihh levels in comparison to untreated control uterine horns; however Pgr levels remain unaffected by this treatment indicating a specific effect of ERK inhibitor on Ihh induction (Fig. 2.5D). Collectively, these data establish an intricate regulation of Ihh expression by LIF and P via the MAPK signaling pathway during early pregnancy.

DISCUSSION

This study, for the first time, provides genetic evidence that uterine epithelial Esr1 signaling is indispensable for stromal cell decidualization, a key event for the onset and establishment of pregnancy. Earlier studies using global knockout of Esr1 have shown that Esr1 does not play a role in decidualization function (10, 30). The uteri of these mutant mice created by Lubahn et al, however, still expressed a truncated Esr1 that retained DNA and hormone binding activities raising the possibility that this residual Esr1 activity mediated the decidual response seen in these mutant mice (11, 31, 32). Thus, the role of Esr1 in the establishment of a differentiated uterus to support implantation remained unclear until now. The decidualization defect displayed in WE<sup>ddd</sup> mice unequivocally supports a critical compartmental specific role of Esr1 in the regulation of stromal differentiation.

Impaired decidual response in uteri lacking epithelial ESR1 pointed to a paracrine regulation of stromal differentiation by epithelial ESR1. A possible candidate of this paracrine mediator emerged when we noticed the distinct expression pattern of ESR1 in the uteri of Esr1<sup>ff</sup> and WE<sup>ddd</sup> mice during early stages of decidualization. In Esr1<sup>ff</sup> uteri ESR1 was expressed strongly in the glands while this expression was absent in WE<sup>ddd</sup> uteri (Fig. 2.2A). The difference in ESR1 expression pattern in decidual uteri of the two genotypes raised the possibility that ESR1 might be regulating the secretion of a critical factor from the
glands, which controls stromal cell decidualization. In WE<sup>dd</sup> uteri the lack of glandular ESR1 prevents secretion of this factor leading to impaired decidual response. Indeed our studies revealed that LIF synthesized in the glandular epithelium is the downstream mediator of epithelial ESR1 and is involved in the control of stromal function.

Although previous studies have shown that LIF plays a critical role in implantation and decidualization (25, 33), the molecular mechanism by which LIF controls decidualization of stromal cells has not been addressed to this date. A recent report indicated a direct effect of LIF’s action on the stromal cells during decidualization (34). Contrary to this report, our studies have shown that LIF does not exhibit a direct effect on stromal differentiation. Instead, LIF regulates decidualization in an indirect manner by controlling IHH signaling in the endometrial epithelium. It has been shown previously that P induces IHH in the endometrial epithelium, which then acts in the stroma to regulate expression of Nr2f2, an essential factor for stromal cell decidualization (35, 36). Our studies indicate that LIF acting in concert with P promotes maximal induction of Ihh during early pregnancy.

Signaling by LIF is initiated when it binds to its receptors on the target cell. LIF is known to activate three distinct pathways, JAK-STAT3, Ras/ERK/MAP kinase and/or PI3kinase/AKT (37, 38). Previous studies have shown that in the uterus during implantation, LIF functions by activating JAK-STAT3 pathway (24, 39). In this study we show for the first time activation of MAPK pathway in response to LIF signaling in the uterus. We observed that induction of Ihh in response to LIF signaling remains unaffected in uteri lacking epithelial Stat3. Instead administration of PD184352, which inhibits ERK1/2 to uteri, resulted in a marked suppression of Ihh indicating activation of MAPK rather than JAK-STAT3 pathway in response to LIF signaling. Consistent with these results we observed that Lif, Ihh, and pERK exhibit similar expression patterns in the uterus during early pregnancy. We noted that between days 1 to 4 of gestation, LIF is secreted from endometrial glands in a biphasic manner. On day 2 of gestation, LIF acts synergistically with P to induce Ihh via the Ras/ERK/MAPK pathway. The level of LIF in the uterus declines on day 3 and rises again on day 4 prior to implantation. The surge of LIF on day 4 activates JAK-STAT3 pathway in the luminal epithelial cells and regulates a distinct set of genes that are known to play critical roles in uterine receptivity and embryo implantation.

Although research over the past decade has identified several factors that regulate uterine function during implantation (13, 19, 35, 36), there is only limited insight into the molecular mechanisms and signaling pathways that interconnect the various factors and steroid hormone signaling during early pregnancy.
Our studies for the first time reveal that a complex interplay of E and P signaling via their target molecules LIF and IHH in the glandular and luminal epithelium respectively is pivotal for successful differentiation of endometrial stroma during decidualization (Fig. 2.6). Based on the findings described herein, we propose that E acting via epithelial ESR1 induces the expression of LIF in the glands between days 1 to 2 of pregnancy. Glandular LIF in concert with P then act on the luminal epithelial cells to activate MAPK pathway and induce \( Ihh \) expression between days 2 to 3 of gestation. The activation of the hedgehog pathway involving IHH-PTCH1-NR2F2 then promotes differentiation of stromal cells during decidualization. In summary, we have uncovered a novel mechanism by which epithelial \( Esr1 \) in a paracrine manner controls decidualization of endometrial stromal cells.

**MATERIALS AND METHODS**

**Mice, hormone treatments, and tissue collection**

All experiments involving mice were conducted in accordance with National Institutes of Health standards for the use and care of mice. The animal protocols were approved by the University of Illinois Institutional Animal Care and Use Committee. For mating studies, \( Esr1^{flo} \), \( WE^{dd} \) female mice were housed with wild-type C57BL/6 male mice (Charles Rivers). The presence of a vaginal plug after mating was designated as day 1 of pregnancy.

Female mice were subjected to bilateral ovariectomy. Two weeks later, mice were injected subcutaneously with vehicle (sesame oil) or P (1 mg) for 3 consecutive days. The animals were killed and uteri were collected after 24 h. In certain experiments mice were pretreated with P (1 mg) for 2 days before E (100 ng) administration. Uteri were collected 24 h after E treatment and epithelial proliferation was assessed.

Decidualization was experimentally induced in non-pregnant mice as described previously (14). Briefly, mice were first ovariectomized. Two weeks following ovariectomy, mice were injected with 100 ng of E in 0.1 ml of sesame oil for 3 consecutive days. This was followed by daily injections of 1 mg of P and 10 ng of E for 3 consecutive days. Decidualization was then initiated in one horn by injection of 50ul oil. The other horn was left unstimulated. The mice were treated with E+P additionally post stimulation, and then killed to collect the uterine tissue either 20h post stimulus or 72h post stimulus. For the rescue of decidualization, 100ul of Vehicle (PBS) or LIF (1ug in 100ul PBS) was added intraperitoneally every 2h for 8h pre-stimulus. Uteri were collected 20h or 72h post stimulus.
Uteri from different days of pregnancy were collected for RNA isolation and IHC. One horn of the uterus was fixed in 10% formalin prior to IHC, and the other horn was flash frozen in Liquid N2 prior to RNA isolation.

**Intraluminal administration of inhibitor**

MEK inhibitor PD184352, was initially dissolved in DMSO, and then diluted in HBSS. Ten μl of inhibitor (50 μM) was injected into one horn intraluminally 12h prestimulus, while the other horn served as vehicle control. LIF was administered as described above and uterine horns were collected 20h post stimulus. Similarly, 10 μl of inhibitor (50 μM) was injected into one horn intraluminally on day 2 of pregnancy, while the other horn served as vehicle control. Uterine horns were collected on day 3 of pregnancy.

**Isolation of uterine epithelial and stromal cells**

Uteri were removed at 0h, 20h or 72h post stimulus. With the aid of a dissecting microscope, each uterine horn was cut transversely into two equal segments. The tissues were then incubated in 10 ml of a solution of 0.5% bovine pancreatic trypsin (Cooper Biomedical, Malvern, PA) in Ca and Mg-free PBS, pH 7.4. Tissues were first incubated at 4ºC for 1 h and then at 37ºC for 40 min. After incubation, the uterine tissue was squeezed with forceps dissociating the epithelium from the uterine pieces. The epithelial fragments in the supernatant were collected with a pipette; this procedure was repeated three or four times. The epithelial fragments were collected by centrifugation at 100 X g for 5 min. The resulting cell pellet was subjected to RNA isolation protocol.

The remaining uterine tissue was incubated with 0.5g/L collagenase at 37ºC for 40 min. After incubation, the tube was shaken vigorously and the supernatant was passed through a 70um mesh to remove tissue debris. Stromal cells were collected by centrifugation at 1700 X g for 5 min. For primary stromal cell culture, cells were grown in DMEM/F-12 medium containing 5% charcoal-stripped FBS. To induce in vitro decidualization, the cells were treated with or without a hormone cocktail containing 1 nM E, 1 mM P for 24-72h.

**Culture of uterine stromal cells**

The uterine stromal cells, isolated as described above, were diluted in Dulbecco’s modified Eagle’s Medium-F12 medium (DMEM-F12; with 100 unit/L penicillin, 0.1 g/L streptomycin, 1.25 mg/L Fungizone) with 2% heat-inactivated fetal calf serum. The live cells were counted by trypan blue staining using a hemocytometer. Cells were then seeded in 6-well cell culture plates. The unattached cells were removed by washing several times with
HBSS after 2 h, and cell culture was continued after addition of fresh medium supplemented with P (1 mM) and E (10 nM).

**Real-time PCR analysis**

Uterine tissue was either homogenized or uterine epithelium was isolated and total RNA was extracted by using TRIZOL reagent, according to the manufacturer’s protocol. cDNA was prepared by standard protocols. The cDNA was amplified to quantify gene expression by real-time PCR, using gene-specific primers and SYBR Green (Applied Biosystems, Warrington, UK). The expression level of Rplp0 (36B4) or Cytokeratin 18 (Ck18) was used as the internal control. For each treatment, the mean Ct and standard deviation were calculated from individual Ct values obtained from three replicates of a sample. The normalized ΔCt in each sample was calculated as mean Ct of target gene subtracted by the mean Ct of internal control gene. ΔΔCt was then calculated as the difference between the ΔCt values of the control and treatment sample. The fold change of gene expression in each sample relative to a control was computed as \(2^{-\Delta\Delta Ct}\). The mean fold induction and standard errors were calculated from three or more independent experiments.

**Immunohistochemistry**

Formalin fixed uterine pieces were processed for paraffin embedding. Cross sections (5um thickness) were mounted onto microscope slides (Fisher Scientific). For immunostaining, uterine sections were deparaffinized in xylene (three times for 5 min each), rehydrated through a graded series of treatment with ethanol (100%, 95%, 85%, and 70% for 5 min each), and rinsed in tap water. For all samples, antigen retrieval was performed by boiling the sections in 0.01 M sodium citrate buffer (pH 6.0) for 20 min, followed by incubation at RT for 30 min. A 5% solution of normal donkey serum (Jackson Immunoresearch) in PBS was used as a blocking buffer. Sections were incubated with the following primary antibodies diluted in blocking solution (0.25% bovine serum albumin [BSA], 0.3% Triton X-100, sterile PBS) overnight at 4°C: PH3 (BD Pharmingen), pSTAT3-TY 705 (ABCAM), pERK1/2 (Santa Cruz), ERK1/2( Santa Cruz), pShp2 (ABCAM), ESR1 (Novus Biologicals). The sections were washed and incubated with either biotinylated secondary antibodies (Jackson Immunoresearch Laboratories Inc.) secondary antibodies for 60 min. This was followed by incubation with streptavidin-conjugated horseradish peroxidase (Histostain kit; Zymed Laboratories Inc.) for 45 min. Sections were stained with 3-amino-9-ethyl carbazole (AEC) solution (Zymed Laboratories Inc.) and counterstained with Mayer’s
hematoxylin (Sigma). Stained sections were mounted in diazabicyclo (2, 2, 2) octane (DABCO), Tris-Cl (pH 8.5), and sterile double-distilled water (ddH2O). Negative controls included incubation with donkey serum and omission of the primary antibody for all samples.

**Image capture and quantitation of immunostaining**

The images of immunohistochemical staining were captured by using a Leica (Nussloch, Germany) DM2500 light microscope fitted with a Qimaging Retiga 2000R camera (Qimaging, British Columbia, Canada). For counting, at least 5 to 6 individual 20x fields from each sample were captured. The numbers of positively stained uterine LE nuclei in each field were averaged and expressed as a percentage of the total number of these cells. Images were processed on ADOBE Photoshop version 8. The standard deviation was determined for each averaged total. ANOVA single-factor analysis was conducted on the grouped means to determine statistical significance at a significance of a $P$ value of $< 0.01$.

**Statistical analysis**

Statistical analysis was performed by t-test or ANOVA. The values were expressed as mean ± SEM and considered significant if $p<0.01$. 
Figure 2.1. Ablation of Esr1 in the uterus epithelium leads to a defect in stromal cell decidualization. A. Uterine cross sections from Esr1^{ff} and Esr1^{dd} mice were subjected to immunohistochemistry using anti-ESR1. Esr1^{dd} mice display efficient deletion of ESR1 (b) when compared to Esr1^{ff} control mice (a). B. Esr1^{ff} and Esr1^{dd} mice were subjected to an artificial decidual stimulus. Uteri were collected 72 hours after administration of oil stimulus. Esr1^{dd} uteri fail to give a decidual response.
Figure 2.1 (cont.) C. Ratio of right to left horn in weight. $Esr1^{d/d}$ uterine horns fail to decidualize. D. Alkaline phosphatase activity was detected in the decidualized stroma. (a) sections of $Esr1^{ff}$ uteri (b) sections of $Esr1^{d/d}$ uteri. Purple color indicates alkaline phosphatase activity.
Figure 2.2 Uterine epithelial ESR1 is indispensable for stromal cell decidualization. A. \( WE^{dd} \) mutants have decidualization failure. \( Esr1^{ff} \), 72 h after stimuli. The right horn (R) was stimulated, and the left horn (L) was unstimulated. Only right horn is decidualized. \( WE^{dd} \), 72 h after stimuli. Neither right (stimulated) nor left (unstimulated) horn is decidualized. B. Ratio of right to left horn in weight. \( WE^{dd} \) uterine horns fail to decidualize. *\( p \leq 0.001 \) (t-test, \( n=5 \)).
Figure 2.2 (cont.) C. Cell proliferation was measured by Ki67 immunostaining in uterine sections of $Esr1^{+/+}$ (upper panel) and $WE^{+/+}$ (lower panel) mice, 20h after decidual stimulation. Magnification: a and c: 10x, b and d: 20x. D. Percentage of Ki67-positive cells in the stromal compartment of $Esr1^{+/+}$ and $WE^{+/+}$ mice, 20 h after decidual stimulation. The data represents average number of cells from multiple fields of multiple uterine sections. *$p \leq 0.01$ (t-test, n=4).
Figure 2.2 (cont.) E. Stromal cells were isolated from uteri 20 h after decidual stimulation and total RNA was prepared. Real-time PCR was performed to monitor the expression of mRNAs corresponding to the cell cycle genes Ccne1, Ccne2, Ccnd1, Cdk2, Cdk4 and Cdk6. Rplp0 encoding a ribosomal subunit protein was used as internal control to normalize gene expression. The data are represented as the mean fold induction ± SEM, *p ≤ 0.01. F. Alkaline phosphatase activity was detected in the decidualized stroma. (a) sections of Esr1f/f uteri (b) sections of WE d/d uteri. Purple color indicates alkaline phosphatase activity.
Figure 2.3. LIF regulates stromal cell decidualization via IHH. A. ESR1 is differentially expressed in the uterus during decidualization. (a) $Esr1^{f/f}$, ESR1 20 h after stimuli, black arrows indicate glandular expression (b) $WE^{d/d}$, ESR1 20 h after stimuli, black arrows indicate lack of glandular expression. B. Real-time PCR was performed to monitor the expression of $Lif$ and $Pgr$ in the uterus of $Esr1^{f/f}$ and $WE^{d/d}$ mice at 20h post stimuli. Ck18, encoding an epithelial specific protein was used as internal control to normalize gene expression. The data are represented as the mean fold induction ± SEM, *p ≤ 0.01.
Figure 2.3 (cont.) C. LIF rescues stromal cell decidualization in \( WE^{dd} \) mice. \( Esr1^{ff} \) and \( WE^{dd} \) mice were subjected to experimentally induced decidualization with vehicle (Saline) or LIF. Uteri were assessed for gross anatomy and ALP activity 72h after stimuli. (a, d) \( Esr1^{ff} \) mice treated with vehicle; (b, e) \( WE^{dd} \) mice treated with vehicle; (c, f) \( WE^{dd} \) mice treated with LIF (a) \( Esr1^{ff} \) with vehicle. The right horn (R) was stimulated, and the left horn (L) was unstimulated, R decidualizes, L does not decidualize. (b) \( WE^{dd} \) without LIF, neither R (stimulated) or L (unstimulated) horn is decidualized. (c) \( WE^{dd} \) with LIF, R decidualizes, L does not decidualize. ALP activity is strong in (d), absent in (e), and restored in (f). D. Ratio of right to left horn in wet weight. LIF rescues wet weight gain defect in \( WE^{dd} \) mice. *\( p \leq 0.001 \) (t-test, n=7).
Figure 2.3 (cont.) E. Ihh signaling is downregulated in WE<sup>d/d</sup> mice uteri. Real-time PCR was performed to monitor the expression of Ihh, Ptc1 and Nr2f2 in the uterus of Esr1<sup>f/f</sup> and WE<sup>d/d</sup> mice at 20h post stimuli. The level of Rplp0 or Ck18 was used as internal control to normalize gene expression. F. Ihh signaling is restored in WE<sup>d/d</sup> mice during the rescue of decidualization by LIF. Real-time PCR was performed to monitor the expression of Ihh, Ptc1 and Nr2f2 in the uterus of WE<sup>d/d</sup> treated with Saline or LIF at 20h post stimuli. The level of Rplp0 or Ck18 was used as internal control to normalize gene expression. The data are represented as the mean fold induction ± SEM, *p ≤ 0.01, **p ≤ 0.001.
Figure 2.4. LIF and P act synergistically to induce expression of Ihh. A. Expression level of Ihh mRNA in the LE of WT ovariectomized mice injected with Oil, P (1mg), LIF (1ug, 3 injections at 2h intervals) or P+LIF. LE was isolated at 6 h post injection. The data are represented as the mean fold induction ± SEM, *p ≤ 0.01, **p ≤ 0.001. B. Expression level of Ihh mRNA in the LE of Stat3\(^{fl/fl}\) and SW\(^{d/d}\) ovariectomized mice injected with Oil, P (1mg), LIF (1ug, 3 injections at 2h intervals) or P+LIF. LE was isolated at 6 h post injection. The data are represented as the mean fold induction ± SEM.
Figure 2.4 (cont.) C. Immunohistochemical localization of pERK1/2 in WT overectomized mice uteri injected with (a) Oil, (b) LIF (1ug, 3 injections at 2h intervals), (c) P (1mg) or (d) P along with LIF for 6h. D. Immunohistochemical localization of pSHP2 of WT overectomized mice uteri injected with (a) Oil, (b) LIF (1ug, 3 injections at 2h intervals), (c) P (1mg) or (d) P along with LIF for 6h.
Figure 2.4 (cont.) E. Downregulation of ERK1/2 in the uterus inhibits Ihh induction by LIF. Immunohistochemical localization of pERK1/2 in WT overectomized mice uteri pretreated with (a) Saline and P along with LIF (b) PD184352 and P along with LIF, for 6h. Note reduced expression of pERK1/2 in LE of uteri treated with PD184352. F. Real-time PCR was performed to monitor the expression of Ihh in the uterus of WT mice treated with DMSO and (P along with LIF in Saline) or PD184352 and (P along with LIF in Saline). Ck18 was used as internal control to normalize gene expression.
Figure 2.5. LIF in conjunction with P regulates Ihh expression during pregnancy. A. Real-time PCR was performed to monitor the expression of mRNAs corresponding to Lif in uterus on days 1 to 4 of gestation. The relative levels of gene expression on different days of pregnancy were determined by setting the expression level of Lif mRNA on day 1 of pregnancy at 1.0. Ck18, an epithelium specific protein, was used as internal control to normalize gene expression. B. Real-time PCR was performed to monitor the expression of mRNAs corresponding to Ihh in uterus on days 1 to 4 of gestation. The relative levels of gene expression on different days of pregnancy were determined by setting the expression level of Ihh mRNA on day 1 of pregnancy at 1.0. Ck18, encoding an epithelium specific protein, was used as internal control to normalize gene expression.
Figure 2.5 (cont.) C. Uterine sections from day 1 to day 4 (a–d) of pregnancy were subjected to immunohistochemical analysis using anti-pERK1/2 antibody. L, G and S indicate luminal epithelium, glandular epithelium and stroma, respectively.
Figure 2.5 (cont.) D. Real-time PCR was performed to monitor the expression of Ihh, Pgr in uterine horns of WT day 3 pregnant mice treated with either DMSO or PD184352. Ck18 was used as internal control to normalize gene expression. The data are represented as the mean fold induction ± SEM, *p ≤ 0.01, **p ≤ 0.001
Figure 2.6 Conceptual model of epithelial ESR1 action in the mouse uterus during implantation. In the preimplantation uterus around day 1-2 of pregnancy, E acting through its receptor ESR1 induces *Lif* production in the glands. LIF secreted from the glands, acts via the LIF receptors on the luminal epithelium and activates ERK signaling synergistically with P which acts through its receptor PGR. The JAK-STAT3 pathway is not activated by LIF in the luminal epithelial cells at this time. Activated MAPK then functions as a downstream effector of LIF and P to induce the expression of *Ihh* in the luminal epithelium. IHH secreted from the luminal epithelium activates the IHH-PTCH1-NR2F2 signaling cascade in the uterine stromal cells. These chain of signaling events help promote stromal proliferation and differentiation, which are key events during early pregnancy.
Figure 2.7 Deletion of Esr1 expression in the uterus of WE\textsuperscript{dd} mice. A. Uterine epithelial RNA was extracted from Esr1\textsuperscript{ff} and WE\textsuperscript{dd} mice on day 4 of pregnancy (n= 3) and analyzed by Real-time PCR. Relative levels of Esr1 mRNA expression in uterine epithelium WE\textsuperscript{dd} mice are compared to those in Esr1\textsuperscript{ff} control mice. Ck18 was used as internal control to normalize gene expression. The data are represented as the mean fold induction ± SEM, **p < 0.001. B. Immunohistochemical localization of ESR1 in the uterus of Esr1\textsuperscript{ff} and WE\textsuperscript{dd} 18h after E treatment. Note the absence of glandular and epithelial expression of ESR1 in WE\textsuperscript{dd} uteri, whereas stromal expression is intact.
Figure 2.8 LIF does not have a direct on stromal cell decidualization. Real-time PCR was performed to monitor the expression of mRNAs corresponding to *Prp* (A) and *Alp* (B) in isolated stromal cells from hormone primed WT mice uteri treated with vehicle, LIF (100ng/ml), P (1μm) or P along with LIF for 72h. *Rplp0*, was used as internal control to normalize gene expression.
Figure 2.9 PGR expression and its downstream target Hand2 in the stroma, is not affected in WE<sup>dd/d</sup> mice. Immunohistochemical localization of PGR (upper panel) and HAND2 (lower panel) in the uterus of Esr1<sup>fl/fl</sup> and WE<sup>dd/d</sup> 20h after decidualization.
Figure 2.10 Expression of ERK in the uterus is altered by hormone and LIF treatment
Immunohistochemical localization of ERK1/2 of WT overectomized mice uteri injected with (a) Oil, (b) LIF (1ug, 3 injections at 2h intervals), (c) P (1mg) or (d) P along with LIF for 6h.
Table 2.1. Ablation of uterine epithelial *Esr1* leads to infertility

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Number of litters</th>
<th>Number of litters per animal (Mean ± SEM)</th>
<th>Number of pups</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Esr1</em>^{ff}</td>
<td>6</td>
<td>33</td>
<td>5.5 ± 0.4</td>
<td>233</td>
</tr>
<tr>
<td><em>WE</em>^{did}</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Result of six month breeding study are shown
Bibliography


CHAPTER III

Role of Signal Transducer And Activator Of Transcription 3 during Implantation
ABSTRACT

Embryo implantation is regulated by a variety of endometrial factors, including cytokines, growth factors and transcription factors. Earlier studies identified the leukaemia inhibitory factor (LIF), a cytokine produced by uterine glands, as an essential regulator of implantation. LIF, acting via its cell surface receptor, activates the signal transducer and activator of transcription 3 (STAT3) in the uterine epithelial cells. However, the precise mechanism via which activated STAT3 promotes uterine function during implantation remains unknown. To identify the molecular pathways regulated by STAT3, we created $SW^{d/d}$ mice in which $Stat3$ gene is conditionally inactivated in uterine epithelium. The $SW^{d/d}$ mice are infertile due to a lack of embryo attachment to the uterine luminal epithelium and consequent implantation failure. Gene expression profiling of uterine epithelial cells of $SW^{d/d}$ mice revealed dysregulated expression of specific components of junctional complexes, including E-cadherin, $\alpha$- and $\beta$-catenin, and several claudins, which critically regulate epithelial junctional integrity and embryo attachment. Additionally, uteri of $SW^{d/d}$ mice exhibited markedly reduced stromal proliferation and differentiation, indicating that epithelial STAT3 controls stromal function via a paracrine mechanism. The stromal defect arose from a drastic reduction in the production of several members of the epidermal growth factor (EGF) family in luminal epithelium of $SW^{d/d}$ uteri and the resulting lack of activation of EGF receptor signaling and mitotic activity in the stromal cells. Collectively, our results uncovered an intricate molecular network operating downstream of STAT3 that regulates uterine epithelial junctional reorganization, and stromal proliferation and differentiation, which are critical determinants of successful implantation.
INTRODUCTION

In mammals, a hormonally-primed receptive uterus is a pre-requisite for attachment of the embryo to the uterine epithelium to initiate the process of implantation (1,2). Failure of implantation due to a non receptive uterus is a major cause of infertility in humans (3,4). In rodents and primates, the attached embryo breaches the uterine luminal epithelium during implantation and invades the underlying stromal compartment to trigger a remarkable transformation of the stromal tissue (5-7). During this process, known as decidualization, the fibroblastic stromal cells proliferate and subsequently differentiate into distinct decidual cells, which support the growth and development of the implanted embryo until placentation ensues (7).

Implantation is regulated by a timely interplay of various transcription factors, cytokines and growth factors (1,8,9). Spatio-temporal expression analyses have revealed that these factors are expressed in various uterine compartments, such as luminal epithelium, glandular epithelium, and stroma, overlapping the window of implantation (8,10-12). Extensive research over the past decade, using genetically altered mutant mouse models, has identified a number of factors that critically regulate uterine function in the preimplantation and post implantation phases of pregnancy (5,13-16). Colin Stewart and co-workers were the first to describe an obligatory role of the leukaemia inhibitory factor (LIF) in implantation (17). In female mice lacking LIF, the embryos fail to attach to the luminal epithelium due to a defect in uterine receptivity (8,17). While it is clear that during implantation, LIF, secreted from the glands, binds to its receptors in luminal epithelial cells and activates the JAK-STAT pathway, there is only limited insight into the molecular mechanisms that function downstream of STAT signaling to promote uterine receptivity for embryo implantation (18-20).

It was previously reported that STAT3, a member of the STAT family of transcription factors, is activated in uterine luminal epithelium in response to LIF signaling, raising the possibility that downstream pathways regulated by STAT3 help create the receptive state of the uterus during implantation (18,21). Global deletion of Stat3 gene is embryonic lethal, necessitating the development of conditional deletion of this gene to study its function during implantation (22). We, therefore, performed a conditional knockout of the Stat3 gene in the uterus of adult mice by employing the Cre-LoxP strategy (23). This resulted in the creation of mutant mice in which Stat3 is specifically ablated in the uterine epithelium, but is retained in the stroma. Using this unique mouse model, we uncovered the mechanisms by which LIF regulates uterine epithelial and stromal functions during the receptive phase. Our studies
revealed that LIF, by activating STAT3, regulates uterine epithelial junctional integrity and cell-cell communication, and influences paracrine signaling between epithelial and stromal compartments to control uterine proliferation and differentiation. Collectively, these studies provide novel insights into the mechanisms by which LIF-STAT3 signaling allows transition of uterine epithelium and stroma to proper functional states that permit embryo attachment and invasion.

RESULTS

Biphasic expression and activation of STAT3 in the preimplantation uterus

The spatio-temporal profiles of Stat3 mRNA and the active phosphorylated form of STAT3 protein were examined in the mouse uterus during early pregnancy by real-time PCR and immunohistochemistry (IHC), respectively. As shown in Figure 3.1A, the expression of Stat3 mRNA, which was high on day 1 of pregnancy, decreased to low levels by day 3. This was followed by a second rise in mRNA levels on day 4 at the time of embryo implantation. Consistent with the mRNA profile, we observed prominent expression of active STAT3 protein, phosphorylated at Y705, in uterine luminal epithelium on day 1 of pregnancy (Figure 3.1B, panel a). The level of phospho STAT3 declined on days 2 and 3 of gestation (Figure 3.1B, panel b and c), but reappeared in uterine luminal and glandular epithelium on day 4 at the time of implantation (Figure 3.1B panel d). The level of phospho STAT3 increased further on day 5 of gestation. Interestingly, this later expression was localized to both luminal epithelium and sub-luminal stroma (Figure 3.1B, panel e). The biphasic expression and activation of STAT3 in the periimplantation uterus showed a striking overlap with previously reported biphasic LIF expression in the uterus during early pregnancy (25,26). This observation supports the view that LIF is a modulator of STAT3 activation in the uterus during this critical period.

Conditional ablation of Stat3 in the uterine epithelium leads to infertility

Earlier studies also localized the LIF receptor predominantly in the luminal epithelium [18], indicating that an analysis of STAT3 activation and function in this tissue would provide a critical understanding of the molecular events controlled by LIF during early pregnancy. To investigate the function of STAT3 in the uterine epithelium, we created a conditional knockout of Stat3 in the uteri of adult mice. Transgenic mice expressing Cre under the control of Wnt-7a promoter was previously used to ablate “floxed” genes selectively in uterine epithelial cells (27,28) We, therefore, crossed the Wnt7a-Cre mice with
mice harbouring “floxed” Stat3 (Stat3<sup>f/f</sup>) (29-31) to create SW<sup>dd</sup> mice. In uterine epithelial cells of SW<sup>dd</sup> mice, Cre recombinase deletes exon 22 of Stat3, which encodes the tyrosine residue (Y705) essential for STAT activation.

We employed real-time PCR and immunohistochemistry (IHC) to confirm the deletion of Stat3 in the uterine epithelium of these mutant mice. As shown in Figure 3.8A, Stat3 mRNA was markedly downregulated in uterine epithelial cells, but not in the stromal cells, of SW<sup>dd</sup> mice on day 4 of pregnancy. Similarly, STAT3 protein was robustly expressed throughout Stat3<sup>f/f</sup> uterine epithelium on day 4 of pregnancy, whereas SW<sup>dd</sup> mice display STAT3 expression solely in the stromal tissue (Fig 3.8B) confirming successful abrogation of Stat3 gene specifically in uterine epithelium of SW<sup>dd</sup> mice.

A six-month breeding study demonstrated that SW<sup>dd</sup> mutant mice are infertile (Table 1). While Stat3<sup>f/f</sup> mice exhibited normal litter size and pregnancy rates, the SW<sup>dd</sup> females failed to become pregnant when mated with wild-type males. However copulatory plugs were observed upon mating, indicating normal mating behavior. To investigate the cause of infertility in SW<sup>dd</sup> females, we examined their ovarian functions by inducing superovulation. Prepubertal Stat3<sup>f/f</sup> and SW<sup>dd</sup> mice were treated with a regimen of gonadotropins as described in Materials and Methods. We observed that, upon gonadotropin stimulation, the number of eggs produced by the Stat3<sup>f/f</sup> females was comparable to that produced by the SW<sup>dd</sup> females (Figure 3.9A), indicating that ovulation is not affected in these mutant mice. To further examine the ovulation and fertilization in these mice under normal physiological conditions, blastocysts were recovered from uteri of Stat3<sup>f/f</sup> and SW<sup>dd</sup> mice on day 4 of pregnancy prior to implantation. Once again, no significant difference was found in either the number or the morphology of the embryos recovered from Stat3<sup>f/f</sup> and SW<sup>dd</sup> uteri (Figure 3.9B and data not shown). In further support of normal ovarian activity, the serum levels of progesterone (P) and estrogen (E) were found to be comparable in Stat3<sup>f/f</sup> and SW<sup>dd</sup> females on day 4 of pregnancy (Figures 3.9C and 3.9D). Collectively, these results suggested that the infertility of SW<sup>dd</sup> females is not due to impairment in the hypothalamic-pituitary-ovarian axis or lack of fertilization, but is likely due to defective implantation or pregnancy failure following implantation.

**Deletion of Stat3 from uterine epithelium prevents embryo attachment**

In mice, attachment of the embryos to the uterine wall is accompanied by increased vascular permeability at the implantation sites, which can be scored visually as distinct blue bands following an intravenous injection of Chicago blue dye (32). As shown in Figure 3.2A,
Stat3<sup>f/f</sup> mice displayed distinct implantation sites on day 5 of pregnancy. In contrast, the SW<sup>d/d</sup> females did not show any sign of implantation. Histological analysis of uterine sections from Stat3<sup>f/f</sup> females on day 5 of pregnancy showed, as expected, a close contact of embryonic trophectoderm with uterine luminal epithelium (Figure 3.2B, panel a). In contrast, in SW<sup>d/d</sup> uteri, embryos did not attach to luminal epithelium. Instead, blastocysts remained free-floating in the lumen (Figure 3.2B, panel b). Taken together, these results indicated that the loss of Stat3 expression in the uterine epithelium resulted in the inability of the luminal epithelium to attach to the embryos.

**STAT3 regulates junctional remodelling in uterine luminal epithelium**

To gain insights into the mechanisms underlying the implantation defect observed in SW<sup>d/d</sup> mice, we isolated luminal epithelial cells from Stat3<sup>f/f</sup> and SW<sup>d/d</sup> uteri on day 4 of pregnancy and performed gene expression profiling, using Affymetrix Mouse GeneChip arrays. Interestingly, our study revealed an up-regulation of transcripts corresponding to two distinct classes of junctional molecules, catenins and claudins, in SW<sup>d/d</sup> uterine epithelium compared to Stat3<sup>f/f</sup> control uteri. The microarray data (GEO accession # GSE44087) was validated by real-time PCR analysis. As shown in Figure 3.3A, we confirmed a marked up-regulation of mRNAs corresponding to claudins 1, 3, and 4 and alpha (α) and beta (β) catenin in the luminal epithelium of SW<sup>d/d</sup> uteri.

An important feature of the receptive uterus is the reorganization of junctional proteins in uterine epithelial cell membrane at the time of implantation. In the nonreceptive state, E-cadherin, a Ca<sup>2+</sup>-dependent transmembrane adhesion molecule, connects adjacent epithelial cells by linking their cytoskeletons via catenins to help maintain cell-cell adhesion and apical-basal polarity (31,33). Similarly, claudins, which are components of tight junctions, help maintain a paracellular barrier between the cells of an epithelium (34,35). These junctional proteins associate with various peripheral membrane proteins, which are in turn connected to the actin cytoskeleton. As the uterus attains receptive status, the epithelial cell junctions are reorganized as evidenced by the redistribution of membrane proteins at junctional complexes, resulting in the loss of cell polarity that allows acquisition of adhesiveness for the embryo (36,37).

We next investigated whether the non-receptive state of the uterine epithelium of SW<sup>d/d</sup> mice is associated with an altered expression of specific junctional proteins, such as E-cadherin, β-catenin and claudin-1, in the epithelial junctions. As shown in Fig. 3.3B (panel a), low levels of E-cadherin expression were seen only in the apical tips of Stat3<sup>f/f</sup> uterine
epithelium during the receptive phase. The lateral junctions were devoid of any E-cadherin expression. Low levels of β-catenin were present in lateral junctions of Stat3f/f uterine epithelium (Fig. 3.3B, panel b). In contrast, in SWd/d uterine epithelium, prominent expression of E-cadherin was observed in both apical and lateral junctions (panel d) and markedly elevated levels of β-catenin were seen in the lateral junctions (panel e). Interestingly, E-cadherin and β-catenin were co-localized specifically in the lateral junctions of SWd/d uterine epithelium, consistent with the presence of intact intercellular cadherin-catenin adherent junctional complexes (Figure 3.3B, panel f).

We also examined the status of the tight junctions in Stat3f/f and SWd/d uterine epithelia. As shown in Figure 3.3C, the claudin-1 (CLDN1) protein, which was barely detectable in Stat3f/f luminal epithelium (panel b), was present in the apical and basolateral junctions of SWd/d uterine epithelium (panel e). Collectively, these results suggested that, in the absence of epithelial Stat3, proper reprogramming of luminal epithelial membrane proteins fails to occur with consequent dysregulated distribution of adherent and tight junctional complexes and lack of epithelial function required for embryo attachment.

**LIF-induced STAT3 activation triggers uterine epithelial reorganization**

We next examined whether LIF-induced activation of STAT3 during the transition of uterine luminal epithelium from non-receptive to receptive phase is accompanied by altered expression and redistribution of the catenins, claudins and E-cadherin in uterine epithelial junctional complexes. In this experiment, we used the delayed implantation model in which pregnant mice are ovariectomized to remove the source of ovarian hormones and then treated with exogenous LIF to initiate embryo attachment to uterine luminal epithelium. Our results revealed that uterine epithelial STAT3 remains unphosphorylated (at Y705), cytosolic, and inactive in the absence of exogenous LIF (Figure 3.3D, panels a and b). Both apical and lateral junctions of luminal epithelium contained E-cadherin (green, panel c), and β-catenin (red) was present mainly in the lateral junctions. Prominent expression of claudin-1 was observed in both apical and basolateral surfaces of the uterine luminal epithelium (panel d). Interestingly, administration of LIF, which initiated implantation in delayed mice, also led to phosphorylation (at Y705) and nuclear localization of phosphorylated STAT3 in uterine epithelial cells (panels e and f), however total STAT3 protein levels remain unaltered (compare panel a and e). We noted concomitant downregulation of E-cadherin at epithelial apical and lateral adherent junctions (panel g). In the presence of LIF, β-catenin staining, although reduced, was still detectable in the lateral surfaces. We observed marked
downregulation of CLDN1 in the uterine luminal epithelial apical and basolateral tight junctions in response to exogenous LIF (Figure 3.3D, compare panels d and h). Taken together, our results are consistent with the hypothesis that LIF-mediated activation of STAT3 critically regulates the dynamics of uterine luminal epithelial adherent and tight junctional complexes containing E-cadherin/β-catenin and claudin-1, respectively. Hence, we propose that a major cause of implantation failure in mice lacking uterine epithelial Stat3 is the inability of LIF to downregulate the expression and distribution of critical components of epithelial junctional complexes. The resulting epithelium fails to present a proper surface for attachment to the embryonic trophectoderm and remains non-receptive and refractive to implantation.

**Ablation of Stat3 in the uterine epithelium leads to a defect in decidualization**

During implantation, embryo attachment to the uterine epithelium is followed by decidualization of underlying stromal cells (7,38). Since embryos fail to attach to epithelium of SWd/d uteri, we assessed the decidual response in these mutant mice by performing experimentally-induced decidualization, which occurs independent of embryo attachment, as described previously (24). Ovariectomized Stat3f/f and SWd/d mice were treated with a well-established regimen of steroid hormones and then decidualization reaction was initiated in one uterine horn by intra luminal injection of oil while the other horn was left unstimulated. We then examined the gross anatomy of the stimulated and unstimulated uterine horns of Stat3f/f and SWd/d mice. As expected, the uterine horns of Stat3f/f mice exhibited a robust decidual response at 72 h after receiving the artificial stimulation (Figure 3.4A, panel a). In contrast, the SWd/d uteri under identical conditions showed a drastically reduced decidual response (Figure 3.4A, panel b). When the decidual response was assessed by measurement of uterine wet weight gain, the SWd/d uteri exhibited a markedly reduced weight gain relative to that seen in the Stat3f/f uteri (Figure 3.4B).

**Uterine epithelial Stat3 regulates stromal proliferation during decidualization**

During decidualization, the uterine stromal cells initially undergo proliferation for 24–48 h followed by differentiation (39). To further investigate the decidualization defect in mice lacking Stat3 in uterine epithelium, we monitored the proliferation and differentiation of steroid hormone-primed stromal cells in response to a decidual stimulation, using known markers of these events. As shown in Figure 3.4C panel a, uteri of Stat3f/f mice exhibited an intense staining for Ki67 in the stromal cells within 20 h of receiving the decidual stimulus,
clearly indicative of extensive stromal cell proliferation, which is typically seen during normal pregnancy in response to embryo attachment (Figure 3.4C, panel c). In contrast, the uterine sections from SW<sup>dd</sup> mice showed a markedly altered pattern of Ki67 staining under identical conditions (Figure 3.4C, panels b and d). A generally reduced proliferative activity was observed in the stromal compartment of the mutant uteri. However, a few bands of proliferative cells persisted in the sub-epithelial stroma, indicating that proliferation of these cells is independent of epithelial STAT3. Quantitation of the Ki67 signal in areas of reduced proliferation revealed a significant reduction in the number of Ki67-positive cells in the stromal compartment of the SW<sup>dd</sup> uteri (Figure 3.4D), indicating a defect in stromal cell proliferation in these mutant mice.

We further investigated the proliferation defect in SW<sup>dd</sup> uterine stroma by analyzing the expression of factors that control various cell cycle checkpoints, including cyclins, cyclin-dependent kinases (Cdks), and Cdk inhibitors (40). Our results revealed a marked down regulation of cyclin E1 (Ccne1) and E2 (Ccne2) in stromal cells of SW<sup>dd</sup> uteri on day 5 of pregnancy. In contrast, the level of p21, an inhibitor of cyclin E was upregulated in stromal cells of SW<sup>dd</sup> uteri during decidualization (Figure 3.4E).

We next investigated whether uterine stromal differentiation is also impaired in the absence of epithelial Stat3. Our results revealed an intense expression of alkaline phosphatase, a classical marker of decidualization, in the decidual cells of Stat3<sup>f/f</sup> uteri at 72 h following decidual stimulation. In contrast, no expression of this decidual marker was seen in the uteri of SW<sup>dd</sup> mice at a similar time point, indicating a complete blockade of stromal cell differentiation in these mice (Figure 3.4F, compare panels a and b). Taken together, our results indicated that Stat3 is a critical regulator of stromal cell proliferation as well as differentiation during decidualization.

**Uterine epithelial STAT3 controls EGF receptor signaling in the stroma**

A defect in stromal proliferation resulting from ablation of uterine epithelial Stat3 in SW<sup>dd</sup> mice raised the interesting possibility that action of this transcription factor in the epithelium controls stromal proliferation by a paracrine mechanism. Previous studies reported that several members of the EGF family growth factors are expressed in the uterine epithelium and the EGF receptors (ERBB) are expressed in the uterine stroma during pregnancy (10,11). It was also reported that the expression of EGF-like factors in uterine luminal epithelium is drastically reduced in the Lif-null mice during implantation (41,42). However, the mechanisms that regulate the production of EGF-like growth factors
downstream of LIF in the preimplantation uterus, and most importantly, the precise uterine functions controlled by these factors remained unclear. We, therefore, considered the possibility that EGF ligands are potential paracrine mediators that function downstream of epithelial STAT3 to control stromal proliferation. Indeed, our studies revealed a dramatic reduction in the expression of several EGF family ligands in the epithelial cells of \(Sw^{d/d}\) uteri compared to \(Stat3^{f/f}\) controls on day 5 of pregnancy (Figure 3.5A).

The EGF family ligands exert their responses through the cell surface ERBB receptors. Stimulation of ERBBs by these growth factors leads to phosphorylation of specific tyrosine residues on the receptors, which in turn activate downstream signaling pathways (43). While ERBB1 is the major EGF receptor expressed in the uterine stroma, ERBB2 and ERBB3 are exclusively expressed in the luminal epithelium during the implantation window and ERBB4 is expressed in the sub-myometrial stroma and the myometrium (10,44). The signaling by EGF, HB-EGF, and AREG is mediated by either an ERBB1 homodimer or a heterodimer of ERBB1 and ERBB2-4 (43) We, therefore, determined the expression of ERBB1 in uteri on day 5 of pregnancy. We found it to be widely expressed in the stroma and the levels were comparable in uteri of \(Stat3^{f/f}\) and \(Sw^{d/d}\) mice (Figure 3.5B, compare panels a and b). We also monitored the expression of the active, phosphorylated form of ERBB1 in uteri of \(Stat3^{f/f}\) and \(Sw^{d/d}\) mice on day 5 of pregnancy. While a widespread expression of the phosphorylated ERBB1 was observed in the uterine stroma of \(Stat3^{f/f}\) mice on day 5 of pregnancy (Figure 3.5B, panel c), its levels were drastically reduced in the stromal cells of \(Sw^{d/d}\) uteri (Figure 3.5B, panel d), indicating a marked reduction in stromal EGF signaling in the absence of epithelial \(Stat3\). We also analyzed the expression of ERBB4 and phospho-ERBB4, which are located in the submyometrial stroma. No significant differences in their levels were observed between \(Stat3^{f/f}\) and \(Sw^{d/d}\) mice on day 5 of pregnancy (data not shown).

We further examined whether LIF, acting upstream of STAT3 activation, is indeed the key factor controlling the production of the EGF family ligands in the epithelial cells. To test this possibility, we again used the delayed implantation model in which embryo attachment to the uterine epithelium is initiated in response to LIF. As shown in Figure 3.5C, administration of LIF to mice undergoing delayed implantation stimulated the synthesis of \(Egf, Hbegf,\) and \(Areg\) in uterine luminal epithelium. These results strongly supported our hypothesis that LIF-mediated activation of luminal epithelial STAT3 drives the production of the EGF family ligands in this tissue.
Paracrine control of EGF receptor signaling is critical for stromal proliferation during decidualization

To address whether the impaired production of the EGF family ligands in the luminal epithelium of \( SW^{dd} \) mice was responsible for the observed defect in stromal cell proliferation during decidualization, we conducted an experiment in which these mutant mice were subjected to experimentally-induced decidualization with or without supplementation of the EGF family ligands. Since \( Egf, Hbegf, \) and \( Areg \) were collectively down regulated in uteri of \( SW^{dd} \) mice, we administered a combination of these factors into the mutant uteri and then assessed their ability to rescue stromal proliferation. As shown in Fig. 3.6A, the stroma of vehicle-treated \( SW^{dd} \) uteri showed significantly reduced proliferative activity (BrdU immunostaining) and expression of activated pERBB1 relative to control \( Stat3^{ff} \) uteri (compare panels a and b, d and e). The levels of both BrdU-positive cells and pERBB1 were markedly elevated in the uterine stroma of \( SW^{dd} \) mice treated with the mixture of EGF family ligands (Figure 3.6A, panels c and f). It is of interest to note that this treatment, which also increased the levels of pERBB1 in the luminal epithelium, did not trigger proliferation of these cells. Consistent with these observations, we found a marked upregulation of mRNAs corresponding to cell cycle regulators \( Ccne1 \) and \( Ccne2 \) in uterine stromal cells in response to stimulation by the EGF ligands (Figure 3.6B). Taken together, these results uncovered a paracrine regulatory mechanism in which the EGF family ligands, produced downstream of LIF-induced activation of STAT3 in luminal epithelial cells, trigger EGF receptor signaling in the stromal cells to critically control the proliferation of these cells during decidualization.

DISCUSSION

Previous studies showed that the production of LIF by uterine glands is obligatory for attachment of the embryo to uterine luminal epithelium (17). While it was reported that LIF induces phosphorylation of STAT3 in uterine epithelial cells during implantation, the downstream molecular pathways that mediate its effects on uterine functions remain largely unknown. Previous studies, using inhibitors of STAT3 pathway, have indicated a role of STAT3 in implantation (45,46). However, these studies did not provide any insight into the mechanisms via which STAT3 regulates uterine function. In this study, we investigated the molecular mechanisms via which the LIF-STAT3 signaling pathway controls uterine functions during implantation. Our studies provide unique insights into the pathways that operate downstream of STAT3 to control uterine epithelial remodelling and stromal proliferation and differentiation during implantation.
In mice, STAT3 is expressed in both uterine luminal epithelium and stroma during implantation (Fig. 3.1). This expression occurs in a biphasic pattern that overlaps with a similar biphasic expression of LIF, mainly in the uterine glandular epithelium (25,26). Since LIF receptors are primarily located in the luminal epithelium at the time of implantation, a detailed examination of the molecular events regulated by STAT3 would require a uterine epithelial-specific knockout of this gene. We, therefore, created $SW^{d/d}$ mice in which conditional deletion of Stat3 occurred specifically in these cells. While ovarian function was unaltered and embryogenesis occurred normally in $SW^{d/d}$ mice, the uteri of these animals displayed an inability to implant the embryos. Interestingly, many of the hallmarks of uterine receptivity remained unaffected in these mutant mice. For example, the circulating levels of estrogen and progesterone and the expression patterns of their cognate receptors in uterine compartments were indistinguishable between control and mutant mice (data not shown). The expression status of MUC-1, a glycocalyx protein, which is considered an important indicator of uterine receptivity, was examined (47,48). As the uterus achieves receptivity, MUC-1 expression declines in normal luminal epithelium. We found that downregulation of epithelial MUC1 within the implantation window also occurred in $SW^{d/d}$ mice (Figure 3.10A, panel a-d). Additionally, other critical functional markers of uterine receptivity, such as Indian hedgehog (14,49) and LIF exhibited unaltered expression in $SW^{d/d}$ uteri compared to Stat3$^{ff}$ uteri (Figures 3.10B and 3.10C). Although LIF expression was unaffected in the $SW^{d/d}$ mice, this cytokine was unable to act on the uterine luminal epithelial cells in the absence of its downstream effector STAT3.

Since uterine stromal Stat3 remained intact in $SW^{d/d}$ mice (Fig 3.8), our study specifically addressed the uterine changes dictated by epithelial Stat3. In contrast, recent studies by Lee et al. (50) and Sun et al. (51) used conditional mutant mice, developed by crossing floxed Stat3 mice with PR-Cre mice, which resulted in Stat3 gene deletion in both epithelial and stromal compartments of the uterus. Loss of Stat3 in the stromal compartment apparently led to alterations in pathways in the stromal cells with consequent dysregulation of estrogen and progesterone signaling in the uterus as reported by these investigators. These effects are not observed when targeted deletion of Stat3 is carried out specifically in the epithelium, allowing us to uncover epithelium-specific roles of Stat3 and its paracrine effects on the stroma during implantation.

Our study showed, for the first time, that STAT3 directs the cellular remodeling that alters the molecular organization of epithelial junctional complexes at the time of implantation. In the preimplantation phase, the integrity of the polarized luminal epithelium is
primarily maintained via adherent and tight junctions, which form a continuous seal of junctional complexes in the apical and basolateral regions of the epithelial cells (31). These junctions help maintain epithelial cell polarity by preventing the lateral diffusion of integral membrane proteins between the apical and basolateral surfaces. E-cadherin, a Ca\(^{2+}\)-dependent transmembrane adhesion molecule, connects adjacent epithelial cells by linking their cytoskeletons via \(\alpha\) and \(\beta\) catenins to maintain cell-cell adhesion and apical-basal polarity (31,52). In addition, components of tight junctions, such as the claudin family proteins, help establish a paracellular barrier between the epithelial cells and the intercellular space. They also participate in regulating the flow of diffusible molecules between adjacent polarized epithelial cells (53,54). The loss of apical-basolateral polarity of the luminal epithelial cells with consequent redistribution of plasma membrane proteins is long considered a prerequisite for successful implantation (55). It is believed that the uterus achieves receptivity by downregulating the tight and adherent junction complexes in the luminal epithelium. This loss of epithelial polarization allows the proper organization and presentation of cell surface adhesion molecules that allow interaction with the blastocyst trophectoderm initiating the process of implantation. Indeed, we observed a striking down regulation of several components of these junctional complexes in luminal epithelium as the uterus progressed from non-receptive (day 1) to receptive phase (day 5, morning) during normal pregnancy (Figure 3.11, compare panels a and c; panels d and f).

It is evident that STAT3 signaling is critical for suppressing the expression of claudin-1, -3 and -4 transcripts within the implantation window. It also down regulates the expression of \(\alpha\) and \(\beta\)-catenin, which likely contributes to the redistribution of E-cadherin away from the lateral junctional complexes during implantation. These concerted changes in the junctional complexes, driven by STAT3 signaling, critically alters uterine epithelial phenotype by disrupting cell-cell linkages and altering cell polarity to allow the embryonic trophectoderm to attach to and invade through the epithelial cell layer into the underlying stromal tissue. Further support for this view comes from the observation that administration of exogenous LIF, which induces embryo attachment in progesterone-primed mice in a delayed implantation model, also specifically downregulates the same epithelial junctional proteins. Taken together, our results established that STAT3 acting downstream of LIF signaling dictates uterine epithelial reprogramming during implantation.

The precise mechanisms by which STAT3 controls the expression of specific members of the claudin family and the \(\alpha\) and \(\beta\) catenins in the uterine epithelium remain
unclear. Our preliminary analysis of the regulatory regions of claudin-1 gene indicated putative binding sites for STAT3, pointing to a possible direct mode of regulation of this gene by STAT3. Claudin-1 gene expression is also influenced by tyrosine kinase signaling in a variety of cancers (56,57). Therefore, it is possible that binding of LIF to the LIF receptor in the luminal epithelium, which activates JAK kinase-mediated tyrosine phosphorylation, contributes to the suppression of expression of a specific subset of claudin genes during implantation. The mechanism by which STAT3 promotes downregulation of E-cadherin from luminal epithelial junctional complexes at the time of implantation is also unclear. Previous studies indicated that, in certain tumour cells, STAT3 signaling induces downregulation of E-cadherin mRNA via a pathway involving the transcription factor ZEB1 (58,59). We, however, did not observe a down regulation of E-cadherin mRNA in uterine epithelium of SW\textsuperscript{\textit{d/d}} mice, indicating that a STAT3-ZEB1-dependent transcriptional repression of the E-cadherin gene is unlikely to operate in these mutant uteri. Instead, we found that the levels of α and β catenin, which associate with E-cadherin in junctional complexes, are repressed by STAT3 signaling. These findings raise the possibility that the disappearance of E-cadherin from epithelial junctional complexes is partly due to the downregulation of α and β catenins in these complexes. Alternatively, signaling via STAT3 may alter E-cadherin post-translationally, leading to its disappearance from the epithelial cell membrane. Further studies are needed to understand the exact mechanism by which STAT3 downstream of LIF signaling controls the expression and remodelling of the junctional protein complexes in the uterine epithelium.

Another novel and important finding of this study is the discovery of an indispensable role of uterine epithelial STAT3 in regulation of stromal cell decidualization via a paracrine mechanism. Deletion of epithelial \textit{Stat3} in SW\textsuperscript{\textit{d/d}} uteri without altering STAT3 expression in the stromal compartment impaired stromal cell decidualization in an experimentally-induced decidualization model, which does not require the presence of an embryo to induce the decidual response. Recent studies from our laboratory and elsewhere indicated a role for uterine stromal STAT3 in controlling human and mouse decidualization (50,51,60). In this paper, we report that uterine epithelial STAT3 signaling is also critical for this differentiation process. Specifically, we found that, in the absence of uterine epithelial STAT3, the stromal cells exhibit a significant reduction in their capacity to undergo proliferation in response to a decidual stimulus. These results suggested a role of epithelial STAT3 in promoting production and/or secretion of a paracrine factor(s) from the uterine epithelium, which in turn
induces stromal cell proliferation. Indeed, a marked reduction in the levels of a subset of EGF family growth factors was observed in the uterine epithelium of SW<sup>dd</sup> mice on day 5 of gestation compared to Stat3<sup>f/f</sup> controls, indicating that these growth factors are likely downstream paracrine effectors generated by epithelial STAT3 signaling.

The EGF family ligands induce cellular proliferation by binding to the ERBBs, which belong to a family of receptor tyrosine kinases (61). Upon ligand binding, the ERBB receptors dimerize, triggering autophosphorylation of several tyrosine residues in the C-terminal domain. These phosphorylation events help activate several downstream pathways involved in cellular proliferation (62,63). ERBB1 is the predominant form of ERBB in the uterine stroma (41,44). Consistent with our hypothesis that the EGF family growth factors, synthesized in response to epithelial STAT3 signaling, control stromal proliferation during decidualization, we noted a marked reduction in the level of active, phosphorylated ERBB1 in the uterine stroma of SW<sup>dd</sup> mice. The role of the EGF family growth factors as paracrine mediators of epithelial STAT3 was further confirmed when we administered a mixture of EGF family ligands to the uteri of SW<sup>dd</sup> mice during experimentally-induced decidualization. This treatment was effective in rescuing the uterine stromal proliferation defect in SW<sup>dd</sup> mice. This result strongly supported our view that STAT3-mediated production of EGFs in the luminal epithelium is critical for stromal proliferation. Our studies further demonstrated that administration of an implantation-inducing dose of LIF promotes the synthesis of several members of the EGF family in the luminal epithelium of mice undergoing delayed implantation. These results lend further support to our hypothesis that activation of STAT3 downstream of LIF critically regulates epithelial-stromal crosstalk during implantation via a paracrine mechanism involving ERBB signaling.

In summary, the SW<sup>dd</sup> mice, in which Stat3 is exclusively deleted in the luminal epithelium, provided a unique model to delineate the signaling pathways downstream of LIF, an essential regulator of uterine function during implantation (Figure 3.7). Our study revealed a novel mechanism by which STAT3 activation in luminal epithelium, in response to glandular LIF, regulates epithelial cell-cell junctions, polarity, and function during the receptive phase. It also promotes stromal proliferation at the time of decidualization via paracrine growth regulatory signals originating in the epithelium. Although this study focused on the role of STAT3 in uterine epithelium, further studies, utilizing both uterine epithelial- and stromal-specific knockouts of Stat3 will enable us to fully address the intricate and complex molecular mechanisms controlled by this critical transcription factor in the pre- and post-implantation uterus.
MATERIALS AND METHODS

Mice, hormone treatments, and tissue collection

All experiments involving mice were conducted in accordance with National Institutes of Health standards for the use and care of mice. The animal protocols were approved by the University of Illinois Institutional Animal Care and Use Committee. The Stat3 floxed (Stat3\textsuperscript{floxed}) mice were kindly provided by Dr Shizuo Akira of Osaka University, Osaka, Japan, and Dr Hua Yu of City of Hope, Duarte, CA. Stat3\textsuperscript{floxed} mice were crossed with Wnt7a-Cre mice to generate SW\textsuperscript{dd} mice. For mating studies, Stat3\textsuperscript{floxed} and SW\textsuperscript{dd} female mice of C57BL/6 background were housed with wild-type C57BL/6 male mice (Charles Rivers). The presence of a vaginal plug after mating was designated as day 1 of pregnancy.

In superovulation experiments, 7 to 8 week old female mice were injected intraperitoneally with 5 IU of pregnant mare serum gonadotrophin (PMSG) and 48 hours later with 5 IU of human chorionic gonadotropin (hCG). The mice were killed 16-18h post-hCG administration, and oocytes were flushed from the oviducts and counted.

To induce delayed implantation, mice were ovariectomized in the morning (0700-0800 h) of day 4 of pregnancy and maintained by daily injections of P (2 mg per mouse) from days 5 to 7. To terminate delayed implantation and induce blastocyst attachment, the P-primed delayed implanting mice were given 5 injections of LIF (1 ug, Santa Cruz) at 2 h intervals on the fourth day of the delay (day 8). Mice were killed at 24 h after last LIF injection and uteri were collected.

Decidualization was experimentally induced in non-pregnant mice as described previously (24). Briefly, mice were first ovariectomized. Two weeks following ovariectomy, mice were injected with 100 ng of E in 0.1 ml of sesame oil for 3 consecutive days. This was followed by daily injections of 1 mg of P and 10 ng of E for 3 consecutive days. Decidualization was then initiated in one horn by injection of 50ul oil. The other horn was left unstimulated. The mice were treated with E+P for additionally post stimulation and then killed to collect the uterine tissue either 20 h post stimulus or 72 h post stimulus. For the rescue of proliferation during decidualization, 100 ul of vehicle (HBSS) or an EGF family cocktail, containing EGF (20ug, Sigma-Aldrich), HB-EGF (5ug, R&D systems), AREG (100ug, R&D systems), was added intraperitoneally every 2 h for 6 h prior to decidual stimulation. BrdU (100ul) was injected 2 h prior to collection and uteri were collected 20 h post stimulus.

For analysis of gene expression during normal pregnancy, uteri were collected at different days of pregnancy for RNA isolation and IHC. One horn of the uterus was fixed in
10% formalin prior to IHC, and the other horn was flash frozen in liquid nitrogen prior to RNA isolation.

**Uterine epithelial and stromal cell isolation**

Uteri were obtained from pregnant mice on day 4 or day 5. With the aid of a dissecting microscope, each uterine horn was cut transversely into two equal segments. The tissues were then incubated in 10 ml of a solution of 0.5% bovine pancreatic trypsin (Cooper Biomedical, Malvern, PA) in Ca- and Mg-free PBS, pH 7.4. Tissues were first incubated at 4°C for 1 h and then at 37°C for 40 min. After incubation, the uterine tissue was squeezed with forceps, dissociating the epithelium from the uterine pieces. The epithelial fragments in the supernatant were collected with a pipette; this procedure was repeated three or four times. The epithelial fragments were collected by centrifugation at 100 X g for 5 min. The resulting cell pellet was subjected to RNA isolation protocol.

The remaining uterine tissue was incubated with 0.5g/L collagenase at 37°C for 40 min. After incubation, the tube was shaken vigorously and the supernatant was passed through a 70 um mesh to remove tissue debris. Stromal cells were collected by centrifugation at 1700 X g for 5 min.

**DNA microarray analysis**

Uterine epithelial cells were isolated from day 4 pregnant Stat3flo/flo and SWd/d mice and total RNA was prepared. The RNA was hybridized to Affymetrix mouse arrays (GeneChip Mouse Genome 430A 2.0 array) containing probes that represented ~14,000 known genes. They were processed and analyzed, according to the Affymetrix protocol.

**Real-time PCR analysis**

Uterine tissue was either homogenized or uterine epithelium was isolated and total RNA was extracted by using TRIZOL reagent, according to the manufacturer’s protocol. cDNA was prepared by standard protocols. The cDNA was amplified to quantify gene expression by real-time PCR, using gene-specific primers and SYBR Green (Applied Biosystems, Warrington, UK). The expression level of Rplp0 (36B4) or Cytokeratin 18 (Ck18) was used as the internal control. For each treatment, the mean Ct and standard deviation were calculated from individual Ct values obtained from three replicates of a sample. The normalized ΔCt in each sample was calculated as mean Ct of target gene subtracted by the mean Ct of internal control gene. ΔΔCt was then calculated as the difference between the ΔCt values of the control and treatment sample. The fold change of
gene expression in each sample relative to a control was computed as $2^{-\Delta\Delta Ct}$. The mean fold induction and standard errors were calculated from three or more independent experiments.

**Immunohistochemistry**

Formalin-fixed uterine pieces were processed for paraffin embedding. Cross sections (5μm thickness) were mounted onto microscope slides (Fisher Scientific). For immunostaining, uterine sections were deparaffinized in xylene (three times for 5 min each), rehydrated through a graded series of treatment with ethanol (100%, 95%, 85%, and 70% for 5 min each), and rinsed in tap water. For all samples, antigen retrieval was performed by boiling the sections in 0.01 M sodium citrate buffer (pH 6.0) for 20 min, followed by incubation at RT for 30 min. A 5% solution of normal donkey serum (Jackson Immunoresearch) in PBS was used as a blocking buffer. Sections were incubated with the following primary antibodies diluted in blocking solution (0.25% bovine serum albumin [BSA], 0.3% Triton X-100, sterile PBS) overnight at 4°C: Ki67 (BD Pharmingen), STAT3 – 79D7 (Cell Signaling), pSTAT3-Y705 (Abcam), ERBB1 (Santa Cruz), pERBB1( Cell Signaling), E-cadherin (Santa Cruz), β-Catenin (Santa Cruz) CLDN-1 (Santa Cruz). The sections were washed and incubated with either biotinylated secondary antibodies (Jackson Immunoresearch Laboratories Inc.) or Cy3/Dylite conjugated secondary antibodies for 60 min. For biotinylated samples this was followed by incubation with streptavidin-conjugated horseradish peroxidase (Histostain kit; Zymed Laboratories Inc.) for 45 min. Sections were stained with 3- amino-9-ethyl carbazole (AEC) solution (Zymed Laboratories Inc.) and counterstained with Mayer’s hematoxylin (Sigma). Stained sections were mounted in diazabicyclo (2, 2, 2) octane (DABCO), Tris-Cl (pH 8.5), and sterile double-distilled water. Immunofluorescence samples were mounted in Prolong GOLD with DAPI and cured for 24h before imaging. Negative controls included incubation with donkey serum and omission of the primary antibody for all samples.

**Image capture and quantitation of immunostaining**

The images of immunohistochemical staining were captured by using a Leica (Nussloch, Germany) DM2500 light microscope fitted with a Qimaging Retiga 2000R camera (Qimaging, British Columbia, Canada). For counting, at least 5 to 6 individual 20x fields from each sample were captured. The numbers of positively stained uterine nuclei in each field were averaged and expressed as a percentage of the total number of these cells. Images were processed on ADOBE Photoshop version 8. The standard deviation was
determined for each averaged total. ANOVA single-factor analysis was conducted on the grouped means to determine statistical significance at a significance of a $p$ value of $< 0.01$. Immunofluorescence imaging was performed on a LEICA 700 confocal microscope. These images were directly documented from the scope with minimal processing.

**Steroid hormone assay**

The serum P4 and E levels were measured by radioimmunoassay by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

**Statistical analysis**

Statistical analysis was performed by t-test or ANOVA. The values were expressed as mean ± SEM and considered significant if $p < 0.05$. To test data in some experiments a Bonferroni's Multiple Comparison Test was applied to compare multiple data points.
Figure 3.1. STAT3 is expressed and activated in the preimplantation uterus. Real-time PCR was performed to monitor the expression of mRNAs corresponding to Stat3 in uterus on days 1 to 5 of gestation. The relative levels of gene expression on different days of pregnancy were determined by setting the expression level of Stat3 mRNA (A, Left panel) on day 1 of pregnancy at 1.0. Rplp0, encoding a ribosomal protein, was used to normalize the level of RNA. Uterine sections from day 1 to day 5 (a–e) of pregnancy were subjected to
immunohistochemical analysis using anti-pSTAT3 (B, Right panel) antibody. Panel f shows uterine sections from day 3 pregnant mice treated with non-immune IgG. L, G and S indicate luminal epithelium, glandular epithelium and stroma, respectively.
Figure 3.2 Deletion of Stat3 from uterine epithelium prevents attachment of embryo. A. Embryo implantation sites were examined in Stat3<sup>f/f</sup> and SW<sup>d/d</sup> mice by the vascular permeability assay, which can be scored as distinct blue bands (black arrows) following an injection of Chicago blue dye on day 5 of pregnancy (D5, n = 5) of pregnancy. The graph represents the quantification of implantation sites in Stat3<sup>f/f</sup> and SW<sup>d/d</sup> mice on day 5 of pregnancy.
Figure 3.2 (cont.) B. Failure of embryo attachment in \( SW^{d/d} \) uteri. Histological analysis of uterine sections obtained from \( Stat3^{+/+} \) (a) and \( SW^{d/d} \) (b) mice on day 5 (\( n = 4 \)) of pregnancy by Haematoxylin and Eosin staining. Note the intimate contact between embryo and luminal epithelium in \( Stat3^{+/+} \) mice and the free floating embryo in the uterine lumen of \( SW^{d/d} \) mice. L and E indicate luminal epithelium and embryo respectively.
Figure 3.3 STAT3 regulates junctional remodelling in uterine luminal epithelium. A. Real-time PCR was performed to analyze the expression of selected adherent and tight junction components in uterine epithelial cells isolated from uteri of Stat3^f/f and SW^d/d mice on day 4 of pregnancy. The level of Ck18 was used as internal control to normalize gene expression. The data are represented as the mean fold induction ± SEM *p<0.01, **p<0.001. B. Confocal images showing expression of E-cadherin (panels a and d, green) and β-catenin (panels b and e, red) in uterine epithelial cells of Stat3^f/f and SW^d/d mice on day 4 of pregnancy. Arrows indicate the co-localization of these proteins (panels c and f, orange, merge) in the intercellular lateral junctions in LE of SW^d/d mice while E-cadherin expression is seen only in apical tips in uterine luminal epithelial cells of Stat3^f/f mice.
Figure 3.3 (cont.) C. Confocal images of CLDN1 protein (panels b, c, e, and f, red) expression. Arrows indicate persistent CLDN1 expression in intercellular junctions in LE of $SW^{+/d}$ uteri (panel f) on day 4 of pregnancy as opposed to overall down regulation in the LE in $Stat3^{+/}$ uteri (panel c).
Figure 3.3(cont.) D. Confocal images showing rescue of implantation upon administration of LIF to progesterone-primed mice undergoing delayed implantation. LIF induces nuclear localization and phosphorylation of STAT3 (red) in LE cells at the implantation site (panel f, white arrows mark STAT3 phosphorylation), total STAT3 protein levels in the luminal epithelium remain unaltered between control and LIF treated uteri (compare panel a and e), though STAT3 expression is more pronounced in the nuclei of LIF treated animals (white arrows, co-localization with DAPI appears as purple). Note the absence of E-cadherin (green, compare panels c and g) and down regulation of CLDN-1 (red, compare
panels d and h) in the intercellular junctions of LE at the implantation site of LIF-treated mice, while the expression of these proteins is maintained in the control uteri (indicated by white arrows). L and E indicate luminal epithelium and embryo, respectively. Note that embryo shows staining of E-cadherin, β-catenin and CLDN-1.
Figure 3.4 Ablation of uterine epithelial Stat3 leads to a defect in decidualization. A. SW\(^{ff}\) and SW\(^{dd}\) mice were subjected to experimentally-induced decidualization. The right horn (R) was stimulated, while the left horn (L) was left unstimulated. (a) Stat3\(^{ff}\), 72 h after decidual stimulation. Robust decidualization was observed in the stimulated right horn. (b) SW\(^{dd}\), 72 h after decidual stimulation. Minimal or no decidualization was observed in the stimulated right horn. B. Ratio of wet weight gain in right vs left horn. *p, 0.001 (t-test, n=5).
Figure 3.4 (cont.) C. Cell proliferation was measured by Ki67 immunostaining in uterine sections of $SW^{ff}$ and $SW^{dd}$ mice. (a) $Stat3^{ff}$, 20 h after decidual stimulation; (b) $SW^{dd}$, 20 h after decidual stimulation. (c) $Stat3^{ff}$, day 5 of gestation (d) $SW^{dd}$, day 5 of gestation. D. Percentage of Ki67-positive cells in the stromal compartment of $SW^{ff}$ and $SW^{dd}$ mice, 20 h after decidual stimulation. The data represents average number of cells from multiple fields of multiple uterine sections.*p<0.01 (t-test, n=5).
Figure 3.4 (cont.) E. Stromal cells were isolated from uteri on day 5 of pregnancy and total RNA was prepared. Real-time PCR was performed to monitor the expression of mRNAs corresponding to the cell cycle genes *Ccne1*, *Ccne2*, and *Cdkn1A*. The data are represented as the mean fold induction ± SEM, *p < 0.01. F. Alkaline phosphatase activity was detected in the decidualized stroma. (a) sections of Stat3<sup>ff</sup> uteri (b) sections of SW<sup>dd</sup> uteri. Purple color indicates alkaline phosphatase activity.
Figure 3.5 Uterine epithelial STAT3 controls EGF receptor signaling in the stroma. A. Real-time PCR was performed to monitor the expression of EGF family growth factors in the uterine epithelia of Stat3<sup>f/f</sup> and SW<sup>d/d</sup> mice on day 5 of pregnancy. Epithelial cells were isolated from pregnant mice as described in the Materials and Methods and total RNA was prepared. Ck18, an epithelial marker, was used to normalize the level of RNA. The data are represented as the mean fold induction ± SEM, **p < 0.001, *p < 0.01. B. The levels of ERBB1 and pERBB1 were examined in the uterine sections of Stat3<sup>f/f</sup> and SW<sup>d/d</sup> mice on day 5 of pregnancy by IHC. Black arrows indicate the presence of pERBB1, an activated form of the receptor in secondary decidual zone of SW<sup>f/f</sup> mice ((panel c). This immunostaining was dramatically reduced in secondary decidual zone of SW<sup>d/d</sup> mice (panel d).
Figure 3.5 (cont.) C. Mice undergoing delayed implantation were treated with or without LIF. The uteri were collected, epithelial cells were isolated. Total RNA was prepared from these cells and subjected to real time PCR to monitor the expression of transcripts corresponding to various members of the EGF family. LIF induces the expression of several EGF family members in uterine epithelium at 24 h post treatment. The data are represented as the mean fold induction ± SEM, **p < 0.001.
Figure 3.6 Paracrine control of EGF receptor signaling is critical for stromal proliferation during decidualization. A. Rescue of stromal cell proliferation defect in SW^{d/d} mice upon administration of the EGF family ligands. Stat3^{f/f} and SW^{d/d} mice were subjected to experimentally induced decidualization with or without treatment with vehicle (HBSS) or a cocktail of the EGF family ligands EGF, HBEGF, and AREG. Sections of decidualized uterine horns at 20h post stimulus were analysed by IHC employing antibodies against BrdU and pERBB1. (A, d) Stat3^{f/f} mice treated with vehicle; (b, e) SW^{d/d} mice treated with vehicle; (c, f) SW^{d/d} mice treated with the EGF family ligands. BrdU immunostaining is strong in (a), reduced in (b), and restored in (c). pERBB1 immunostaining is strong in (d), absent in (e), and restored in (f). B. Real time PCR analysis of Ccne1 and Ccne2. Stromal cells were isolated from uteri of SW^{d/d} mice treated with vehicle or EGF cocktail. Total RNA was prepared from these cells and analysed by real time PCR. The data are represented as the mean fold induction ± SEM, *p < 0.01.
Figure 3.7 Conceptual model of epithelial STAT3 action in the mouse uterus during implantation. In the preimplantation uterus, LIF, secreted from the glands, acts via the LIF receptors on the luminal epithelium to activate epithelial STAT3 via a phosphorylation event. The activated STAT3 then functions as a downstream effector of LIF to repress critical components of adherent and tight junctional complexes in the luminal epithelium, such as E-cadherin, α and β-catenin, and a subset of Claudins. This remodelling of epithelial junctions is critical for altering cell polarity and promoting embryo attachment. Activated epithelial STAT3 also induces the expression of several members of the EGF family in the luminal epithelium. These growth factors function as paracrine mediators to promote stromal proliferation and differentiation, which are key events during early pregnancy.
Figure 3.8 Deletion of Stat3 gene in the uterine epithelium of SW\textsuperscript{d/d} mice. A. Total RNA was extracted from epithelial and stromal cells isolated from uteri of Stat3\textsuperscript{f/f} and SW\textsuperscript{d/d} mice on day 4 of pregnancy (n= 4) as described in the Materials and Methods and analyzed by real-time PCR. Relative levels of Stat3 mRNA expression in uterine epithelium and stroma of SW\textsuperscript{d/d} mice are compared to those of Stat3\textsuperscript{f/f} mice. The data are represented as the mean fold induction ± SEM, **p < 0.001. B. Immunohistochemical analysis of total STAT3 protein expression in the uterine sections of Stat3\textsuperscript{f/f} (left panel) and SW\textsuperscript{d/d} mice (right panel) on day 4 of pregnancy. Notice the absence of STAT3 expression in luminal and glandular epithelium of SW\textsuperscript{d/d} mice, white arrows. L, G and S indicates luminal epithelium, glandular epithelium and stroma respectively.
Figure 3.9 Ovarian functions and preimplantation events remain unaffected in SW$^{d/d}$ mice. A. Age-matched prepubertal Stat3$^{ff}$ (n= 3) and SW$^{d/d}$ mice (n= 4) were subjected to superovulation. The oocytes were recovered and counted at 18 h following hCG administration (values are mean ± SEM). B. Preimplantation embryos were recovered from uteri of Stat3$^{ff}$ (n= 6) and SW$^{d/d}$ mice (n= 6) in the morning of day 4 of pregnancy, counted (values are mean ± SEM) and imaged. C & D: P and E levels in serum of Stat3$^{ff}$ (n= 4) and SW$^{d/d}$ mice (n= 3) mice on day 4 of pregnancy.
Figure 3.10 Assessment of uterine receptivity markers in SW^{d/d} mice. A. Immunohistochemical analysis of MUC-1 protein expression in the uterine sections of Stat3^{f/f} (left panel) and SW^{d/d} mice (right panel) on day 1 (a and b) and day 4 (c and d) of pregnancy. L indicates luminal epithelium. B, C. Expression of Ihh and Lif mRNA in SW^{d/d} uteri. Real-time PCR was performed to monitor the expression of Ihh and Lif in the uterine epithelium of Stat3^{f/f} and SW^{d/d} mice on day 4 of pregnancy. The level Ck18 was used as internal control to normalize gene expression.
Figure 3.11 Downregulation of epithelial junctional complexes during implantation. Immunohistochemical analysis and confocal imaging of E-cadherin/β-catenin (upper panel) and CLDN1 (lower panel) expression in the uterine sections of Stat3<sup>fl/fl</sup> mice on day 1 (a and d), day 4, morning (b and e) and day 5, morning (c and f) of pregnancy. Orange color represents merged E-cadherin (green) and β-catenin (red) signals, indicating co-localization of these proteins. White arrows point toward intercellular junctions and indicate changes in expression of junctional factors. L and E indicate luminal epithelium and embryo, respectively.


Matsuzaki S, Darcha C, Maleysson E, Canis M, Mage G. Impaired down-regulation of E-cadherin and beta-catenin protein expression in endometrial epithelial cells in the


45. Catalano RD, Johnson MH, Campbell EA, Charnock-Jones DS, Smith SK, Sharkey AM. Inhibition of Stat3 activation in the endometrium prevents implantation: a


CHAPTER IV

Ongoing/Future Studies
Role of Endothelial PAS Domain Protein – 1 (EPAS-1) in
Uterine Decidual Angiogenesis
ABSTRACT

Estrogen (E) and Progesterone (P) acting through their cognate nuclear receptors ESR1 and PGR are master regulators of critical events during pregnancy. Their role in embryo implantation and subsequent stromal cell decidualization is well documented. Concomitant with decidualization, an extensive vascular network develops in the stromal compartment through a process known as angiogenesis that supports the development of the implanted embryo. The role of E and P in this process remains unclear and the molecular mechanisms underlying steroid hormone control of uterine angiogenesis remain unknown. E can regulate uterine stromal cells differentiation and angiogenesis through one of its downstream target genes, Connexin-43 (Cx-43). Deletion of Cx-43 in uterine stromal cells resulted in down-regulation of hypoxia inducible pathway, including its master regulator Endothelial PAS Domain Protein – 1 (EPAS-1), a transcription factor known to promote angiogenesis in other tissues. EPAS-1 is widely expressed in uterine stromal cells during decidualization and is induced by E in the ovariectomized mouse model. To address the functional role of this protein, we used the Cre-Lox strategy to create conditional mutant mice in which $Epas-1^{−/−}$ expression is abolished in the uterus ($Epas-1^{−/−}$). These transgenic mice are infertile and exhibit an implantation and angiogenesis defect. In this study we are trying to assess the molecular mechanism behind the angiogenic defect seen in $Epas-1^{−/−}$. Preliminary findings indicate that $Epas-1$ promotes angiogenesis in the uterus by regulating the expression of pro-angiogenic factors like Vascular Endothelial Growth Factor – A (Vegf-A), Sphingosine kinase-1 (Sphk-1) and Angiopoetin-2 (Angpt-2). Further studies will reveal the exact mechanism of regulation of these factors by EPAS-1 and their role in uterine decidual angiogenesis.
INTRODUCTION

Steroid hormones estrogen (E) and progesterone (P) orchestrate the structural and functional changes in the mammalian uterus during early stages of pregnancy that enable blastocyst attachment, initiating the process of implantation [1, 2]. In mouse and human, as the embryo invades the stromal compartment, the fibroblastic stromal cells undergo differentiation into a unique secretory tissue, termed decidua [3]. This transformation process, known as decidualization, is crucial for executing extensive tissue remodeling that ensures proper maternal-fetal interactions, leading to the establishment of pregnancy.

Decidualization is also accompanied by the creation of an extensive vascular network within the stromal bed that supports embryonic and placental growth and maintains early pregnancy [4, 5]. During angiogenesis, new blood vessels are generated by the extension of pre-existing vessels into avascular space. This process involves the local degradation of the vascular basal membrane by proteases, proliferation and migration of endothelial cells and assembly of these cells into new vessels [6, 7]. In the female reproductive system, an active angiogenesis is required to support the cyclic remodeling of the uterus. Formation of these new maternal blood vessels in the stromal compartment at the time of embryonic implantation is critical for the establishment and maintenance of pregnancy [5, 8]. Although uterine angiogenesis is known to be influenced by the steroid hormones estrogen (E) and progesterone (P), the underlying molecular pathways remain poorly understood [9-11].

Estrogen, acting via its nuclear receptors, exerts a profound influence on uterine functions during early pregnancy. However, only a few E-regulated genes important for implantation have been discovered so far. One such protein is, Cx-43 [12]. We have recently discovered a novel link between Cx-43 signaling and the expression of EPAS-1, within uterine stromal cells. EPAS-1 is a transcription factor that has been shown to be regulated by hypoxic conditions and regulates molecules essential for angiogenesis [13-15]. In order to study the functional role of Epas-1 we have created a conditional knockout of the Epas-1 gene which has Epas-1 knocked out in all cells expressing progesterone receptor (PGR). This conditional knockout mouse, termed Epas-1^d/d, is infertile (data not shown) and exhibits an angiogenesis defect. Our preliminary findings indicate that Epas-1 regulates angiogenesis by regulating the expression of angiogenic factors such as Vegf-A, Sphk-1, and Angpt-2. Vegf-A, a potent endothelial cell mitogen that mediates rapid endothelial proliferation, is expressed in the decidua on morning of day 5 during the initiation of vascular permeability [16-18]. It is widely believed that VEGF-A plays a key role in angiogenesis within the uterine decidua during pregnancy. Similarly Sphk-1 has been shown to be of critical importance for fully
functional uterine decidual angiogenesis [19]. Future studies will reveal the exact mechanism of regulation of these angiogenic factors by *Epas-1* and their role in uterine angiogenesis.

RESULTS

Downregulation of hypoxia induced angiogenic pathway in *Cx-43<sup>+++</sup>* mice uterine stromal cells

We have previously shown that E acting through its downstream target *Cx-43* controls crucial aspects of stromal decidualization and subsequent angiogenesis [12]. To gain insights into the mechanism underlying the angiogenesis defect observed in *Cx-43<sup>+++</sup>* mice, we isolated uterine stromal cells from *Cx-43<sup>+/+</sup>* and *Cx-43<sup>+++</sup>* uteri on day 7 of pregnancy and performed gene expression profiling, using Affymetrix Mouse GeneChip arrays. Interestingly, our study revealed down-regulation of transcripts corresponding to genes involved in hypoxia induced angiogenic response in *Cx-43<sup>+++</sup>* mice uteri compared to *Cx-43<sup>+/+</sup>* control uteri (Figure 4.1A). The microarray data was validated by real-time PCR analysis. As shown in Figure 4.1B, we confirmed a marked down-regulation of mRNAs corresponding to *Siah2*, *Epas-1*, *Sphk-1*, *Cx-43* and *Vegf-A* in *Cx-43<sup>+++</sup>* mice uterine stromal cells.

Genes involved in hypoxia mediated angiogenic response are expressed in uterine stromal cells during pregnancy

Hypoxia-inducible factors (*Epas-1*, *Epas-2*, *Epas-3*) are central regulators of the cellular response to hypoxia [20]. Prolyl-hydroxylation of EPAS proteins by PHD enzymes is prerequisite for EPAS protein degradation [21, 22]. E3 ubiquitin ligase family member SIAH2 targets PHDs for degradation which results in increased stability of EPAS protein during hypoxia [23, 24]. Once stabilized EPAS protein can act on downstream targets to induce their expression and promote angiogenesis (Figure 4.2A).

In order to understand the regulation of genes involved in hypoxia mediated signaling during pregnancy we looked at the expression profile of *Siah2*, *Epas-1*, *Sphk-1*, and *Vegf-A* on different days of pregnancy. *Siah2* is expressed in the uterus in a progressive fashion; *Siah2* levels are low on day 4-6 of pregnancy, its levels increase by day 7 and then remain high until day 10 of pregnancy (Fig. 4.2B). Levels of *Epas-1* show a similar expression pattern to *Siah2*, with lower levels from D4-D6 followed by high expression through D7-D10 of pregnancy (Fig. 4.2C). Levels of *Sphk1* increase in uterine stromal cells by D8 of pregnancy and remain high through D10 of pregnancy (Fig. 4.2D). However, *Vegf-A* shows a biphasic pattern of expression with levels declining from D4-D6 of pregnancy, followed by high expression on D7 which subsequently declines to low levels by D10 of pregnancy.
pregnancy (Fig. 4.2E). This overlapping expression pattern of Siah2, Epas-1, Sphk-1 and Vegf-A indicates a regulatory network wherein SIAH2 inhibits the PHDs promoting the expression of EPAS-1 which in turn regulates the expression of angiogenic factors like Sphk-1 and Vegf-A in the uterine stromal cells to promote angiogenesis.

**Epas-1 is essential for uterine angiogenesis**

Epas-1\(^\text{dd}\) mice are infertile and our preliminary analysis indicated that these mice exhibit an implantation defect (data not shown). Interestingly, uterine stromal cells in these mutant animals do undergo initial decidualization reaction and have comparable wet weight gain compared to Epas-1\(^\text{f/f}\) mice uteri. We proceeded by analyzing the angiogenic capacity of Epas-1 deficient uteri during pregnancy. When the uterine sections of pregnant Epas-1\(^\text{f/f}\) mice were immunostained using an antibody against platelet/endothelial cell adhesion molecule (PECAM), a marker of endothelial cells, they displayed a well-developed vascular network spanning the endometrial bed that surrounds the implanted embryo on day 7 of pregnancy (Figure 4.3, a). The PECAM immunostaining, however, was reduced drastically in uterine sections of Epas-1\(^\text{dd}\) mice on day 7, indicating that only a rudimentary vasculature formed in mutant uteri (Figure 4.3, compare panel a & b).

This result indicated that Epas-1\(^\text{dd}\) mice exhibit an angiogenesis defect during pregnancy. However, the embryos in Epas-1\(^\text{dd}\) mice do not survive in utero beyond day 7 of pregnancy. Therefore, it was important to address whether Epas-1 controls uterine decidual angiogenesis independently of embryonic development. Non-pregnant mice were subjected to experimentally induced decidualization in which a mechanical perturbation of the steroid-primed uteri triggers a decidual response in absence of the implanting embryo [25]. Uteri of ovariectomized Epas-1\(^\text{f/f}\) and Epas-1\(^\text{dd}\) mice were prepared by treating these animals with a well-established regimen of E and P, and then decidualization was initiated in the left uterine horn by injecting 50ul oil while the right horn was left unstimulated. Immunohistochemical analysis of Epas-1\(^\text{f/f}\) and Epas-1\(^\text{dd}\) uteri with a PECAM antibody revealed that the artificially stimulated Epas-1\(^\text{f/f}\) horn displayed an extensive endothelial cell network spanning the endometrial bed (Figure 4.3, c). By contrast, similarly treated uterine horns of Epas-1\(^\text{dd}\) mice displayed drastically reduced PECAM staining, indicating that only a rudimentary vasculature was formed (Figure 4.3, d). These studies clearly indicate that even in the absence of the conceptus, EPAS-1 plays a crucial role in uterine decidual angiogenesis in the uterus.
**Epas-1 regulates uterine decidual angiogenesis**

We next wanted to identify the downstream targets of EPAS-1 which play a role in uterine decidual angiogenesis. It is known that EPAS proteins regulate the expression of their downstream targets *Vegf-A* and *Sphk-1* in order to promote angiogenesis in various tissue [26-29]. Additionally, *Epas-1* has been shown to directly regulate the expression of *Vegf-A* by binding to its promoter in certain tissues [30]. Therefore, we analyzed the expression of these angiogenic factors in uterine stromal cells isolated from *Epas-1^{flo}/* and *Epas-1^{d/d}* mice. Uterine stromal cells were isolated from day 4 pregnant mice and subjected to *in vitro* decidualization protocol in the presence of E+P for 72h or 96h. As shown in figure 4.4A, the levels of angiogenic factors such as *Vegf-A*, *Sphk-1*, and *Angpt-2* were markedly reduced in the stromal cells of mutant mice 96h post decidualization. Interestingly, *Angpt-1*, which antagonizes the action of *Angpt-2* was upregulated. *Prl8a2*, a terminal differentiation marker, was also down-regulated in *Epas-1^{d/d}* stromal cells, indicating defective terminal differentiation (Figure 4.4A). These results suggested that the impaired angiogenesis observed in *Epas-1^{d/d}* mice uteri might be due to the lack of regulation of angiogenic factors by EPAS-1. In order to test if this downregulation of *Vegf-A* at the mRNA level translates into decreased protein expression, we checked the levels of VEGF-A protein in the same conditions by immunofluorescence and ELISA. As shown in figure 4.4B, the basal expression level of VEGF-A was similar between *Epas-1^{flo}/* and *Epas-1^{d/d}* mice uterine stromal cells (compare panels a and b), but there was dramatic decrease of VEGF-A protein in the mutant stromal cells when compared to *Epas-1^{flo}/* at 96h post decidualization (Figure 4.4B, compare panels c and d). VEGF-A is a secreted protein and hence it can be detected in the surrounding medium by ELISA. As shown in figure 4.4C, levels of VEGF-A protein were significantly reduced in mutant stromal cells when compared to control at 72h post decidualization, and declined further at 96h post decidualization. These results clearly show that EPAS-1 regulates the expression of angiogenic factors in the uterine stromal cells during pregnancy to promote angiogenesis.

**DISCUSSION**

Angiogenesis is a physiological mechanism leading to growth of new blood vessels from pre-formed ones. The endothelium goes through several different processes including cell proliferation, cell migration, tube formation, endothelium repair, etc. A number of publications have described a link between impaired angiogenesis in the uterus with pregnancy related diseases such as pre-eclamptic pregnancies, gestational diabetes, and
intrauterine growth restriction. [5, 31, 32]. According to these studies, faulty angiogenesis is due to compromised secretion and activity of pro-angiogenic factors such as VEGF, SPHK-1, ANGPT-2, interleukin 8 (IL-8), as well as anti-angiogenic factors such as soluble receptor of VEGF (sFlt-1), ANGPT-1, etc. [16, 18, 33].

E, acting through its nuclear receptor ESR1, is known to play a critical role during implantation, but its role in uterine angiogenesis remains unknown. Our group has recently identified a novel link between E signaling and angiogenesis in the uterus. Cx-43 and Epas-1 are downstream targets of E which mediate its effects on uterine angiogenesis. Studies involving Cx-43<sup>dd</sup> mice revealed a link between gap junction signaling and hypoxia induced signaling which leads to angiogenesis (Figure 4.4 A, B). The complementary expression profile of genes involved in regulation of hypoxia mediated signaling and its downstream targets in the uterus suggested active signaling. However, it is known that EPAS proteins turnover is regulated not only at transcriptional level, but also at a post translational level by an intricate mechanism which involves Siah2’s inhibition of PHD enzymes thereby limiting EPAS proteins proteasomal degradation during hypoxia [34, 35]. Therefore, in order to further understand this regulatory mechanism operational during hypoxia we are also studying the protein expression profile of these genes involved in hypoxia mediated responses during pregnancy.

In order to understand the molecular basis of the effects of hypoxia mediated signaling in the uterus we generated Epas-1<sup>dd</sup> animals in which Epas-1 has been knocked out of all uterine cells expressing PGR. Even though Epas-1<sup>dd</sup> mice ovulate, they are infertile. Surprisingly, we found that embryos attach to Epas-1<sup>dd</sup> luminal epithelium but are unable to penetrate through the luminal epithelial cells (data not shown). It is remarkable that Epas-1<sup>dd</sup> stromal cells are able to begin decidualization even though the embryo does not come into contact with stromal cells. Stromal cell expression of alkaline phosphatase activity is unaltered in the Epas-1<sup>dd</sup> uterus and uterine wet weight gain is statistically the same as in Epas-1<sup>ff</sup> mice 72 hours after artificial decidual stimulus. However, Epas-1<sup>dd</sup> decidual cells do not express PRP, a classic decidualization marker which is induced on day 7 of pregnancy (data not shown). This indicates that Epas-1<sup>dd</sup> stromal cells are unable to fully differentiate and may never reach a terminal differentiated status.

We began to study Epas-1<sup>dd</sup> function in the uterus with the hypothesis that this transcription factor mediates angiogenesis in the uterus. As marked by PECAM staining, we can see that angiogenesis is impaired in Epas-1<sup>dd</sup> mice on day 7 of pregnancy and during
experimentally induced decidualization. It will be imperative to identify the downstream molecular pathways of Epas-1 during stromal cell decidualization. Initial results suggest that EPAS-1 regulates the expression of proangiogenic molecules in the uterus that contribute positively to uterine angiogenesis. Further studies into the exact mechanism of action of EPAS-1 and its regulation of Vegf-A and Sphk-1 will enable us to decipher the signaling mechanism underlying hypoxia induced uterine decidual angiogenesis.

MATERIALS AND METHODS
Animals and Tissue Collection

All animals were maintained in University of Illinois animal facilities. The health of the animals was monitored by Division of Animal Resources veterinarians. All procedures involving the use of animals were approved by the University IACUC committee. Epas-1 floxed mice were obtained from Jax Laboratories. Transgenic female mice were mated to mature C57 wild-type males. The copulatory plug indicates day 1 of pregnancy. Female mice were then subsequently euthanized by carbon dioxide overdose on different days of pregnancy. The uterus was collected and either fixed in 10% buffered formalin for paraffin blocks, frozen or used to isolate stromal cells.

Artificial Decidualization

Decidualization was artificially induced as previously described [12]. Mice were ovariectomized and rested for 2 weeks to remove circulating hormones. Animals then were injected with 100ng estrogen in 0.1ml corn oil s.c. daily for three days and then rested for two days. Following rest, mice were injected with E (10ng) and P (2mg) in 0.1ml corn oil for three days. On the third day of E+P injection mice were anesthetized and corn oil was injected into 1 uterine horn. Mice were then injected daily with E+P until mice were euthanized.

Uterine Stromal Cell Isolation

Uterine stromal cells were isolated as previously described [12]. Briefly, uteri were removed from mice after euthanasia with carbon dioxide. The uterus was cut open and digested with 6 g/liter dispase (Invitrogen) and 25g/liter pancreatin for 1hr at room temperature. The enzymes were quenched with 10% FBS and cells were washed with Hanks balanced salt solution (HBSS). Cells were then incubated in 0.5 g/liter collagenase in HBSS for 1hr at 37°C. After incubation the tubes were vortexed for 10-12 s until the supernatant became turbid with dispersed cells. The contents of the tube were then passed through a 80-
μm gauze filter (Millipore). Cells were resuspended in Dulbecco's modified Eagle's medium-F12 medium (DMEM-F12; with 100 unit/liter penicillin, 0.1 g/liter streptomycin, 1.25 mg/liter fungizone) containing 10% heat-inactivated fetal calf serum and live cells were assessed by trypan blue staining using a hemocytometer.

**Immunofluorescence**

Immunofluorescence was carried out as previously described [36]. Frozen sections or cells in a four well chamber slide were used. Briefly, uterine sections/cells were fixed in 10% formaldehyde for 10 minutes followed by 3 x (1x PBS) washes. These were then blocked for 1 h at room temperature with serum. Sections were then incubated with primary antibodies against PECAM (BD), VEGF-A (Santa Cruz) overnight at 4°C. Sections were then incubated with secondary antibodies conjugated to Cy3 at room temperature followed by coversliping with Prolong Gold reagent containing DAPI.

**Quantitative PCR and RNA Isolation**

RNA was isolated from tissues or cells using trizol followed by RNA cleanup kit (Qiagen). RNA was converted to cDNA using a kit (Applied Biosystems). Oligonucleotides specific for genes of interest were developed. QPCR reaction was carried out using SYBR-Green master mix (Applied Biosystems) on either 7000 or 7500 Applied Biosystems Real-time PCR machines.

**Microarray Analysis**

Uterine stromal cells were isolated from D7 pregnant Cx-43floxed and Cx-43disrupted mice. Messenger RNA was isolated from cells using trizol and purified using a Qiagen kit. Messenger RNA was submitted to the Keck Biotechnology Center at UIUC and hybridization on Affymetrix chips was performed. Data were analyzed using Panther and Ingenuity Pathway Analysis software.

**ELISA – VEGFA**

Secretion of mouse VEGF by uterine stromal cells was detected by Quantikine mouse VEGF immunoassay (R&D, Minneapolis, MN) following the manufacturer’s instructions. The detection limit for VEGF-A was 3 ng/liter.
Figure 4.1 Downregulation of hypoxia induced angiogenic pathway in Cx43$^{d/d}$ mice uterine stromal cells. A. Tabular representation of selected data obtained from microarray analysis of RNA obtained from uterine stromal cells isolated from uteri of Cx43$^{f/f}$ and Cx43$^{d/d}$ mice on day 7 of pregnancy. B. Real-time PCR was performed to analyze the expression of selected hypoxia mediated pathway genes and Cx43 in uterine stromal cells isolated from uteri of Cx43$^{f/f}$ and Cx43$^{d/d}$ mice on day 8 of pregnancy. The level of Rplp0 was used as internal control to normalize gene expression. The data are represented as the mean fold induction ± SEM *p<0.01, **p<0.001.
Figure 4.2 Genes involved in hypoxia mediated angiogenic response are expressed in uterine stromal cells during pregnancy. A. Hypothetical model of hypoxia induced cellular signaling in mouse uterine stromal cells during pregnancy Real-time PCR was performed to monitor the expression of mRNAs corresponding to Siah2 (B), Epas-1 (C), in uterus on days 4 to 10 of gestation. The relative levels of gene expression on different days of pregnancy were determined by setting the expression level of specific mRNA on day 4 of pregnancy at 1.0. Rplp0, encoding a ribosomal protein, was used to normalize the level of RNA.
Figure 4.2 (cont.) Real-time PCR was performed to monitor the expression of mRNAs corresponding to *Sphk-1* (D) and *Vegf-A* (E) in uterus on days 4 to 10 of gestation. The relative levels of gene expression on different days of pregnancy were determined by setting the expression level of specific mRNA on day 4 of pregnancy at 1.0. *Rplp0*, encoding a ribosomal protein, was used to normalize the level of RNA.
Fig. 4.3 *Epas-1* is essential for uterine angiogenesis. Immunofluorescence staining for PECAM (red) in uterine sections. Uterine sections of *Epas-1<sup>ff</sup>* (a, c) and *Epas-1<sup>d/d</sup>* (b, d) mice on day 7 of pregnancy (a, b) and 72h post decidual stimulus (c, d) are shown.
Fig. 4.4 *Epas-1* regulates uterine decidual angiogenesis. A. Real-time PCR was performed to monitor the expression of mRNAs corresponding to various angiogenic and decidual factors in isolated stromal cells from D4 pregnant *Epas-1^ff^* and *Epas-1^dd^* mice uteri treated with E+P and cultured for 96h. *Rplp0*, was used as internal control to normalize gene expression.
Figure 4.4 (cont.) B. Immunofluorescence staining for VEGF-A and Phalloidin in *in vitro* decidualized uterine stromal cell. Epas-1^{f/f} (a,c) and Epas-1^{d/d} (b,d) cells, basal (a,b) and 96h after *in vitro* decidualization (c,d) are shown.
Figure 4.4 (cont.) C. Epas-1^{ff} and Epas-1^{dd} uterine stromal cells were grown for 72h and 96h in the presence of E+P. The VEGF-A in supernatant was measured in duplicate by ELISA.
Bibliography


CHAPTER V

Conclusion
During early pregnancy, the maternal steroid hormones E and P promote blastocyst implantation and uterine stromal cell decidualization. E and P act on their target cells in the uterus via their receptors ESR1 and PGR. Global receptor knockout studies in mice have firmly established the importance of ESR1 and PGR during implantation. Although these studies prove that steroid mediated signaling is indispensable for successful pregnancy, they do not shed light on the underlying mechanism of action of these hormones. Also, these global hormone receptor knockout mice lack hormone mediated signaling in the whole uterus, which makes it impossible to understand the role of hormone mediated signaling in a uterine compartment specific fashion.

The aim of my thesis work was to investigate the compartmental specific role of ESR1 during pregnancy, the underlying mechanism of action, and the process of epithelial-mesenchymal interactions. To this end, we used a conditional knockout of ESR1 in which ESR1 was deleted solely from the epithelial compartment of the uterus. This deletion would allow us to investigate the loss of ESR1 in the epithelium, the stromal function of ESR1 remained unaffected. Using this mouse model, we first investigated whether the epithelial function was maintained in these animals. Loss of epithelial ESR1 did not affect E mediated epithelial proliferation, indicating there is a paracrine signaling mechanism which originates from the stroma that regulates epithelial proliferation. These results align with our previous observation in Hand2-null mice, in which uterine stromal FGF’s drive uterine epithelial proliferation. Hand2 is a transcription factor under the control of PGR. Hand2 blocks this proliferation by inhibiting FGF secretion during the receptive phase. We next investigated whether the loss of epithelial ESR1 affects uterine stromal decidualization. This decidualization is one of the prerequisites for successful implantation which entails differentiation of endometrial stromal cells into a unique secretory tissue, known as the decidua. We discovered that a loss of epithelial ESR1 results in a lack of stromal cell decidualization. Further studies indicated that E acting on glandular ESR1 promotes the secretion of the cytokine LIF, which is responsible for initiating the downstream signaling pathway leading to decidualization. We have shown that LIF, acting through its receptor on the epithelial cells, activates the MAPK pathway to induce the transcription of a well-known target of P signaling and a promoter of decidualization, IHH. IHH then acts in a paracrine manner on the stromal cells to promote their decidualization. This study elucidated a novel functional link between E and P signaling in the uterus during pregnancy. We have shown that during early pregnancy LIF and P act in concert to induce the expression of Ihh which then aids in stromal cell proliferation and differentiation leading to successful pregnancy.
This work identified LIF as a downstream target of glandular epithelial ESR1, which aids in stromal cell decidualization by acting through the MAPK pathway in the luminal epithelium around D2-D3 of pregnancy. It is known that during the receptive phase there is a second surge in expression of *Lif* around day 4.5 of pregnancy. At this point LIF binds to its receptor on the luminal epithelium and activates the JAK-STAT3 pathway to promote uterine receptivity. There is limited knowledge of the genes regulated by the LIF-JAK/STAT3 pathway which makes the uterus receptive to the incoming embryo. In order to understand LIF mediated signaling through the JAK-STAT3 pathway we generated and studied an epithelial knockout of STAT3. Interestingly, loss of epithelial STAT3 renders the uterus non-receptive to blastocyst implantation. We first assessed the effect of loss of epithelial STAT3 on uterine epithelial function during pregnancy. Our studies revealed that STAT3 negatively regulates cell-cell connections in the epithelium by regulating the expression of a set of junctional adhesion complex proteins, catenins and claudins. This was the first time that STAT3 was shown to direct the cellular remodelling that alters the molecular organization of epithelial junctional complexes at the time of implantation. Another novel and important finding of this study was the discovery of the indispensable role of uterine epithelial STAT3 in regulation of stromal cell decidualization via a paracrine mechanism. We found that loss of epithelial STAT3 inhibits the expression EGF family members from the luminal epithelium. These EGFs act in a paracrine manner through their cognate receptors on the stromal cells to promote proliferation and differentiation. Further studies will reveal the nature of regulation of junctional adhesion proteins and EGF family members by STAT3.

Together, these studies shed light on complex and intricate signaling mechanisms driven by epithelial-stromal interactions at play in the uterus during early pregnancy. Our data reveal that the proliferative response of the uterine epithelium in response to E is independent of epithelial ESR1, and in fact is stimulated by growth factors released from the stroma. During the receptivity phase E acting through ESR1 promotes the secretion of LIF, which then activates the JAK/STAT3 pathway in the luminal epithelium to trigger the cellular remodeling that makes the epithelium receptive to the implanting embryo (Fig 5.1). One of the most interesting discoveries that emerged from these projects is that epithelial ESR1 is indispensable for uterine stromal cell decidualization. During early pregnancy E acting through glandular ESR1 promotes the secretion of LIF which along with P acts on the luminal epithelial cell through the MAPK pathway and induces the expression of *Ihh*. *IHH* binds to its receptor PTCH1 on the stromal cell and promotes decidualization by inducing the expression of molecules like NR2F2. Around day 4.5 of pregnancy, E once again promotes
the secretion of LIF which now acts on the luminal epithelial cell via the JAK-STAT3 pathway. This induces the expression of EGF family members, which are secreted into the stroma and help in stromal proliferation and differentiation (Fig 5.2).

The current challenge is to understand how these studies in mice translate into human uterine function. A recent study in our lab has shown the importance of ESR1, as well as STAT3 in human endometrial stromal decidualization. It would be interesting to determine if the dysregulation of these factors affects fertility and other reproductive pathologies, such as endometriosis and endometrial cancer. Impaired stromal-epithelial interactions may also contribute to endometrial hyperplasia, a precursor to endometrial cancer which is associated with unregulated E signaling. Treatment regimens for endometrial hyperplasia often include progestin therapy to inhibit E mediated proliferation. Recently, Jones et al. identified HAND2 as the most commonly hypermethylated and silenced gene in endometrial hyperplasia and cancer and the degree of HAND2 methylation was positively correlated with the severity of hyperplasia. It is possible that molecules like LIF, IHH and STAT3, which we have discovered play an important role in epithelial stromal interaction, may be dysregulated in different reproductive pathologies. Further exploration of these complex signaling mechanisms regulating epithelial stromal interactions in the uterus during pregnancy, and their dysregulation in certain reproductive pathologies would help in developing a targeted approach in treating infertility as well as certain endocrine disorders.
Figure 5.1 Prior to implantation, E, acting through ESR1 in the stroma (pink), drives epithelial (purple) proliferation through the secretion of paracrine acting FGFs. During the periimplantation period, P, acting on PGR in the stroma, promotes the expression of HAND2. HAND2 inhibits expression of FGFs and blocks E induced epithelial proliferation. E induced LIF in the glandular epithelium (blue) acts in a paracrine manner on the luminal epithelium (purple) to activate the JAK/STAT pathway to mediate a shift in junctional integrity required for implantation. Activation of LIF activation of JAK/STAT also promotes proliferation of the stroma (pink) via expression and secretion of EGFs from the luminal epithelium. Dashed lines represent indirect events. E, estrogen; ESR1, E receptor; FGF, fibroblast growth factor; FGFR, FGF receptor; ERK1/2, extracellular signal regulated kinase1/2; P, progesterone; PGR, P receptor; HAND2, heart and neural crest derivatives expressed transcript 2, LIF, leukemia inhibitory factor; LIFR, LIF receptor; JAK/STAT3, Janus kinase/signal transducers and activators of transcription 3; EGF, epidermal growth factor; EGFR, EGF receptor.
Figure 5.2 In the preimplantation uterus around day 1-2 of pregnancy, E acting through its receptor ESR1 induces *Lif* production in the glands. LIF secreted from the glands, acts via the LIF receptors on the luminal epithelium and activates ERK signaling synergistically with P which acts through its receptor PGR. The JAK-STAT3 pathway is not activated by LIF in the luminal epithelial cells at this time. Activated MAPK then functions as a downstream effector of LIF and P to induce the expression of *Ihh* in the luminal epithelium. IHH secreted from the luminal epithelium activates the IHH-PATCH1-NR2F2 signaling cascade in the uterine stromal cells (pink). Around day 4-4.5 of pregnancy a second wave of LIF production activates JAK/STAT signaling in the epithelium. This promotes proliferation of the stroma via expression and secretion of EGFs from the luminal epithelium. Dashed lines represent indirect events. E, estrogen; ESR1, E receptor; LIF, leukemia inhibitory factor; LIFR, LIF receptor; MAPK, MAP kinase; IHH, Indian hedgehog; PTCH1, patch-1; NR2F2, Nuclear Receptor Subfamily 2, Group F, Member 2; JAK/STAT3, Janus kinase/signal transducers and activators of transcription 3; EGF, epidermal growth factor; EGFR, EGF receptor. These chain of signaling events help promote stromal proliferation and differentiation, which are key events during early pregnancy.