ANALYSIS OF THE ROLE OF THE TRANSCRIPTION FACTOR C/EBPβ IN
CONTROLLING UTERINE FUNCTIONS DURING EARLY PREGNANCY

BY

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ABSTRACT

Embryo implantation into the endometrium is a complex biological process involving the integration of steroid hormone signaling, endometrial tissue remodeling and maternal-fetal communications. A successful pregnancy is the outcome of the timely integration of these events during the early stages of implantation. The involvement of ovarian steroid hormones, estrogen (E) and progesterone (P), acting through their cognate receptors, is essential for uterine functions during pregnancy. The molecular mechanisms that control the process of implantation are undergoing active exploration. Through our recent efforts, we identified the transcription factor, CCAAT Enhancer Binding Protein Beta (C/EBPβ) as a prominent target of estrogen and progesterone signaling in the uterus. The development of a C/EBPβ-null mouse model, which is infertile, presented us with an opportunity to analyze the role of this molecule in uterine function. We discovered that C/EBPβ functions in two distinct manners: (i) by acting as a mediator of E-induced proliferation of the uterine epithelium and (ii) by controlling uterine stromal cell differentiation, a process known as decidualization, during pregnancy. My studies have delineated important mechanisms by which E regulates C/EBPβ expression to induce DNA replication and prevent apoptosis of uterine epithelial cells during E-induced epithelial growth. In subsequent studies, I analyzed the role of C/EBPβ in decidualization and uncovered a unique mechanism by which C/EBPβ regulates the synthesis of a unique laminin-containing extracellular matrix (ECM) that supports stromal cell differentiation and embryo invasion. In order to better define the role of laminin in implantation, we developed a laminin γ1-conditional knockout mouse model. This is currently an area of ongoing investigation. The information gained from our analysis of C/EBPβ function in

the uterus provides new insights into the mechanisms of steroid hormone action during early pregnancy. Ultimately, our findings may aid in the understanding of dysregulation of hormone-controlled pathways that underlie early pregnancy loss and infertility in women.

To my parents, Edwin and Dorothy, and my brothers

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CHAPTER 1

HORMONAL REGULATION OF UTERINE FUNCTIONS: BACKGROUND AND SIGNIFICANCE [1]

1.1.   INTRODUCTION

Hormonal regulation of growth and proliferation of the endometrium.

The ovarian steroid hormones estrogen (E) and progesterone (P) play critical roles in the maintenance of the mammalian uterus through cyclical rounds of cell proliferation and differentiation during the reproductive cycle (1). In rodents, ovarian E produced during the proestrus stage stimulates uterine luminal and glandular epithelial cell proliferation, preparing the uterus for potential pregnancy. During this growth phase, E causes distinct physiological changes, such as increased uterine wet weight and structural remodeling of the epithelial cell layer, while also accelerating their entry into S-phase of the cell cycle (1-4). At the onset of pregnancy, increasing levels of P produced from the newly formed corpora lutea in the ovaries suppresses E-stimulated proliferation of the uterine luminal epithelium. The actions of E and P in these epithelial cells are mediated via their respective nuclear receptors, estrogen receptor alpha (ERα) and progesterone receptor (PR) (5, 6). Acting in concert, these hormones control early events that are essential for providing a suitable environment for blastocyst attachment to luminal epithelium and

successful implantation. In the adult female mouse, administration of exogenous E and P to ovariectomized mice faithfully reproduces the uterine epithelial responses seen during the estrous cycle and early pregnancy. Therefore, the mouse uterine luminal epithelium offers as an excellent model in which one may explore the molecular mechanisms by which the steroid hormones control cell proliferation. These mechanisms will be discussed in more detail in Chapter 2.

Endometrial stromal differentiation and the process of decidualization.

In mammals, the onset of pregnancy is heralded by the implantation of an embryo into the uterus, initiating a complex and reciprocal relationship between the mother and the fetus. This maternal-fetal dialogue involves an intimate interaction between the specialized trophectodermal cells of the embryo and the receptive uterine lining of the mother. The steroid hormones estrogen (E) and progesterone (P) play a pivotal role in directing early uterine events during pregnancy. These hormones orchestrate the changes in the uterine epithelium that makes it competent to attach to the blastocyst and initiate the process of implantation (7, 8). Subsequently, E and P regulate a series of complex interactions at the interface between the developing embryo and the cells in the stromal compartment, leading to the formation of a differentiated maternal tissue, known as the "decidua", which supports embryo growth and maintains early pregnancy. Although the details of these events vary in different species, the central roles played by E and P in controlling various phases of early pregnancy are common to many mammals (9). Ultimately, the timely and ordered regulation of the cellular and genetic changes in the

endometrial tissue surrounding the implanting embryo is critical for the establishment of early pregnancy.

The mouse blastocyst reaches the uterus four days after fertilization (10, 11). The attachment of the blastocyst to the uterine epithelium initiates the process of decidualization that involves differentiation of the underlying fibroblastic stromal cells into morphologically distinct cells, termed decidual cells (12-14). These differentiated cells have unique biosynthetic and secretory properties. There are also substantial changes in the extracellular matrix. This cellular differentiation, induced by P following a brief priming with E, is a prerequisite for successful implantation. The decidua is a transient tissue, which begins to develop at the time of blastocyst attachment on day 4.5 of pregnancy. During the next 3 days of gestation, decidual cells surrounding the site of embryo attachment proliferate and differentiate extensively, eventually becoming larger, and often acquire a bi-nucleated or polyploid status (15). Stromal cell polyploidy during differentiation eventually results in apoptosis and the latter process is thought to limit the life span of decidual cells, allowing placental expansion and development (13, 14). By the end of the invasive period (day 10.5), the decidua is totally regressed. A variety of functions have been attributed to the decidua, such as providing a source of growth factors and cytokines that support embryo development, serving an immunoregulatory role during pregnancy and regulating trophoblast invasion (7, 12). An additional role for decidual cells is to support maternal blood vessel formation in order to perfuse and nourish the developing embryo. Despite these critical functions, steroid-induced signaling molecules that participate in the formation and function of the decidual tissue remain

poorly understood. In the next section, we will discuss the role of important steroid-hormone regulated molecules that have been identified through our efforts and others and are involved in early pregnancy by controlling uterine decidualization.

Steroid hormone-regulated pathways involved in decidualization and implantation.

Role of estrogen and progesterone receptors pathways

Despite the abbreviated reproductive cycle in mice relative to that of humans, this species has served as an important animal model to study the regulation of uterine functions by E and P. The cellular actions of these hormones are mediated through intracellular estrogen receptor (ER) and progesterone receptor (PR) proteins, which are hormone-inducible transcription factors (16). Hormone-occupied ER or PR is recruited to specific DNA response elements in the promoters of target genes. These genomic actions trigger the expression of specific gene networks in different cell types within the uterus and the products of these genes in turn mediate the hormonal effects during the reproductive cycle and pregnancy. The development of mutant mouse models lacking ER and PR has established the requirement of these hormones and their downstream signaling pathways for successful establishment and maintenance of pregnancy (5, 6).

The PR knockout (PRKO) mice display a non-receptive uterus that is refractory to an artificial deciduogenic stimulus, such as the intrauterine injection of oil (6). Thus, the PRKO mouse uterus cannot support embryo implantation. When these mutant mice are

placed under chronic E stimulation, increased hyperplasia and an inflammatory response is observed, due to unopposed action of E in the uterus (8). On the other hand, ERα-null mice have impaired uterine growth and fail to prepare for blastocyst attachment (5). Due to the pleiotropic and multisystem effects of global ERα and PR gene ablation, the interpretation of the mutant phenotypes is complicated. Thus, we used the LoxP/Cre strategy in our recent studies and have uncovered a novel role for ERα in mediating stromal cell decidualization (Mary Laws, Indrani Bagchi, and Milan Bagchi, unpublished results). Conditional targeting of ERα deletion is achieved through expression of Cre recombinase under the control of the endogenous PR promoter, avoiding the embryonic and developmental effects of global ERα ablation and allowing significant recombination activity only in the PR expressing tissues of the mature animal (17). The ablation of ERα in PR-positive uterine cells resulted in a failure of the mutant animals to exhibit a decidual response to an artificial stimulus, indicating an important role for ERα in decidualization. In summary, mediation of murine endometrial decidualization requires ERα and PR proteins to effect uterine stromal differentiation. The current effort in the field is to identify and characterize the molecular pathways underlying the actions of E and P and their respective receptors.

Downstream mediators of E and P actions during decidualization

A number of studies employing gene expression profiling have identified steroid-regulated pathways that control epithelial and stromal functions during implantation in mice (18, 19). Here, we describe the physiological relevance of three major ER- and PR-

target genes in embryo implantation and decidualization, with special emphasis on the role of the transcription factor C/EBPβ, which will be the focus of this dissertation.

Roles of Bone Morphogenetic Protein-2 (BMP2) and Wingless 4 (Wnt4)

BMPs are the largest family of morphogens belonging to the TGF-β superfamily of growth modulators. They were initially identified by their ability to induce ectopic formation of cartilage and bone, and were subsequently shown to influence a broad spectrum of cellular functions including proliferation, differentiation, apoptosis, migration, and adhesion in a large variety of cell types during embryonic development (20). Recently, the decidual stage-specific expression of BMP2 and its receptor in the uterine stroma during early pregnancy was uncovered, providing a potential link between BMP2 signaling and the steroid-dependent changes underlying stromal differentiation during decidualization (21).

BMP2 is expressed in the stromal cells surrounding the implanted embryo (22). Studies by S.K. Dey and coworkers demonstrated that when beads coated with heparin-binding epidermal growth factor were placed into pseudopregnant uteri, they induced decidualization concomitantly with BMP2 expression (23). Administration of the antiprogestin RU486 downregulated BMP2 expression in uterine stromal cells, indicating that the expression of BMP2 is downstream of pathways mediated via PR (24). The functional role of BMP2 during embryo implantation was demonstrated using transgenic mice carrying a conditional deletion of this gene in mouse uterus (22). BMP2-null mice

are infertile due to the absence of a decidual response. Although the embryos attach to the uterine epithelium, the stromal cells fail to undergo decidualization. The decidual phenotype could be partially rescued through the addition of recombinant BMP2 into the uterine lumen, ruling out the possibility that a developmental defect contributes to the implantation failure.

In parallel with the creation of the BMP2-null mice, mouse primary stromal cultures were utilized to provide novel insights into the role of BMP2 and its downstream signaling pathways in uterine decidualization (24). The addition of recombinant BMP2 to the undifferentiated mESC significantly increased Smad signaling which in turn led to an increase in ALP activity and markedly accelerated the stromal differentiation program. Furthermore, when an siRNA targeted to BMP2 mRNA was transfected into undifferentiated mESCs, it efficiently suppressed BMP2 expression and also inhibited stromal differentiation as indicated by the drastically reduced expression of the well-established decidual markers. These studies indicated that BMP2-mediated canonical Smad signaling in the uterus plays a critical role in stromal cell differentiation during early pregnancy.

Microarray analyses conducted with BMP2-null mESCs revealed the involvement of Wnt4 in BMP2 signaling (24). The Wnt family of signaling proteins exerts pleiotropic effects similar to Bmp2 (25). Overlapping expression of BMP2 and Wnt4 was noted *in vivo* in the uterine stromal compartment during early pregnancy (23). Most importantly, a marked induction of Wnt4 in mESC cultures was observed when Bmp2 was added to

induce differentiation, suggesting that it is a direct downstream target of Bmp2 action (24).

Roles of Indian Hedghog (Ihh) and COUP-TFII

Studies by DeMayo and coworkers revealed a novel role for another morphogen, Indian Hedgehog (Ihh), in mediating epithelial and stromal cross-talk that triggers decidualization. The regulation of this factor by P and PR appears to be confined to the luminal epithelium of the receptive uterus during the window of implantation (26, 27). The expression of Patched-1 (Ptch1), the receptor for Ihh, exhibited a similar temporal pattern in the stromal compartment, supporting an epithelial-stromal communication. Conditional ablation of Ihh expression in the uterus yielded reproductive defects that are consistent with a role for epithelial Ihh in mediating PR-controlled decidualization of the subjacent stroma (28). Recent studies using tissue recombinants indicated that the expression of Ihh in the epithelium is controlled by stromal PR (29), indicating complex interactions between epithelial and stromal compartments that require further exploration.

Prominent among the potential target genes mediating Ihh function in the uterine stromal cells is the orphan nuclear receptor family member COUP-TFII. Insights into the reproductive functions of COUP-TFII were gained by Cre recombinase-mediated conditional excision of this gene in mouse uterus (30). The loss of COUP-TFII in the uteri rendered these mice infertile primarily due to a loss of embryo attachment in the luminal epithelium. Furthermore, a failure of the artificially induced decidual reaction

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revealed a stromal impairment. Interestingly, administration of recombinant BMP2 *in vivo* led to a partial rescue of the decidualization phenotype in COUP-TFII-null uteri, indicating that BMP2 is a downstream mediator of COUP-TFII function (30). Collectively, these findings broadly defined a dynamic pathway in which PR regulates epithelial Ihh expression and secretion into the stromal compartment. Ihh then acts via the Ptch1 receptor to induce a decidual response, which is mediated by COUP-TFII and BMP2.

Identification of (C/EBPβ) as a novel target of steroid regulation during implantation

Gene expression profiling conducted in our laboratory identified CCAAT/Enhancer Binding Protein-β (C/EBPβ) as a novel target of E and P actions in the mouse endometrium (18, 31, 32). This transcription factor belongs to a family of basic leucine zipper (bZIP) proteins, which regulate numerous biological processes, including cell proliferation, differentiation, metabolic homeostasis, acute phase inflammation and apoptosis (33). While C/EBPβ has been shown to be a critical regulator of proliferation in mammary gland (34, 35), liver (36) and now uterine epithelium (37), this molecule has also been linked to anti-proliferative actions in separate tissues, such as epidermal keratinocytes (54). This suggests that C/EBPβ can exert both positive and negative control of the cell cycle and its function is dependant on the cellular environment.

Role of C/EBPβ in the mouse endometrium during implantation

Among the C/EBPs, C/EBPβ has been identified as a critical mediator of the biological actions of E and P in mouse uterus (32). The administration of E or P to ovariectomized, non-pregnant mice caused a robust induction of C/EBPβ mRNA and protein expression in both epithelial and stromal compartments (32). During normal murine pregnancy, a robust induction of C/EBPβ occurs predominantly in the stromal compartment during the decidualization phase. Similarly, C/EBPβ expression is up regulated upon experimentally induced decidualization. The decidual stage-specific expression of C/EBPβ likely arises from a complex interplay of E and P within the uterine compartments. Although a transient rise in E in the preimplantation period induces C/EBPβ in the stromal compartment of pregnant uterus, PR becomes a critical regulator of this gene as these cells become progressively differentiated. This view is strongly supported by the observation that RU486, an antagonist of PR, efficiently suppressed stromal C/EBPβ expression when administered on day 6 of pregnancy (32).

The essential role for C/EBPβ in decidualization was uncovered when mice carrying a deletion in this gene were examined. Peter Johnson and coworkers first reported that female mice lacking C/EBPβ were infertile, due to complications in ovulation (39). However, upon further examination, functional abnormalities in the uterus also were evident in mutant females (32). Wild-type (WT) embryos transferred to pseudopregnant uteri of C/EBPβ-null mice failed to implant. Uterine defects noted in the mutant mice included a reduced epithelial cell proliferation in response to E, and more importantly, an

impaired stromal response to a deciduogenic stimulus. There was a striking lack of expression of alkaline phosphatase (ALP), a classical early marker of stromal cell differentiation, in C/EBPβ-null stroma. These phenotypic defects were observed in the presence of exogenously administered E and P, indicating that they were independent of ovarian malfunction, and intrinsic to the uterus.

In addition to its role in decidualization of the uterus, C/EBPβ appears to be important for the proliferation of the stromal fibroblasts, an event that precedes their differentiation to decidual cells. The morphology of C/EBPβ-null uteri in response to a decidual stimulus showed a significantly reduced mass of stromal cells as compared to WT uteri, indicating that stromal cells were not proliferating efficiently in the mutant. This was evidenced by a drastic reduction in Ki67 staining at 24 h following a decidual stimulus (32). Further investigation of this observation revealed that DNA synthesis was intact in the mutant stromal cells but they are eventually arrested at the G2-M checkpoint (Wang, W. et. al, unpublished). This is supported by microarray analysis, where the expression of cyclins B1 and B2 and two other G2-M regulators, E2F3 and 14-3-3 zeta is downregulated in C/EBPβ-null stroma.

C/EBPβ is a critical regulator of decidualization in the mouse endometrium

The expression pattern of C/EBPβ closely overlaps with the onset of decidualization during implantation. Additionally, mice nullizygous for C/EBPβ are infertile (32, 39). To investigate whether the mutant mice could support pregnancy, Mantena *et al* performed

embryo-transfer experiments. In these experiments, blastocysts were harvested from WT females on day 4 of pregnancy and were subsequently placed into the uteri of pseudopregnant WT or C/EBPβ-null females, on day 4 of pseudopregnancy. After a period of five days, the number of implanted embryos and the size of the decidual tissue were assessed. It was found that a substantial number of blastocysts did not implant into the C/EBPβ-null recipients (>90%) and there was a remarkable lack of decidual mass formed in the mutant (32). These findings indicated a fundamental defect in uterine function during pregnancy.

To address whether this defect could be a result of inadequate steroid hormone levels synthesized by the ovary, E and P were administered to C/EBPβ-null mice and a artificial decidual stimulus given. In this protocol, WT and C/EBPβ-null mice are ovariectomized, treated with E and P and then decidualization is experimentally induced with mechanical stimulation of the uterus (18, 32). When the uterine horns were examined within 72 h after receiving a stimulus, the WT mice displayed a robust decidual response, evidenced by an enlarged horn packed with decidual cells (32). In contrast, the similarly treated C/EBPβ-null uterine horns showed a significantly reduced mass of decidual cells. Further analysis was conducted using well-established biochemical markers of decidualization. The expression of one such marker, alkaline phosphatase (ALP), was markedly diminished in the C/EBPβ-null uteri 72 h after artificial decidual stimulation (32). Meanwhile, the levels of ERα and PR remained unchanged between WT and mutant uteri, indicating that steroid hormone signaling pathways were not disrupted.

Collectively, these observations highlight the important role of C/EBPβ in decidualization and implantation.

Gene networks downstream of C/EBPβ involved in decidualization: modulation of extracellular matrix (ECM)

The striking defect in decidualization witnessed in C/EBPβ-null mice prompted the examination of gene networks that might be regulated by this transcription factor in the endometrium. Using a DNA microarray approach, we were able to identify putative targets of C/EBPβ action in purified uterine stromal cells isolated from WT and C/EBPβ-null mice. There were several prominent categories of genes uncovered from this analysis, among which there was one class of genes encoding molecules involved in the formation of the extracellular matrix (ECM) (Wang, W. et. al, unpublished). The extracellular matrix is a complex and dynamic structure that controls cell function by regulating the interactions between cells and their substrates and modulating external stimuli such as growth factors and cytokines (40). The most significantly regulated ECM-related molecules in our analysis included hyaluronan synthase I, which synthesizes hyaluronic acid, and two proteins that function in partnership with the hyaluronan network, Ptx3 and Tnfip6 (41-43). More interestingly, a group of ECM molecules involved in basement membrane-like matrix formation were identified by our gene profiling studies, namely, collagen type IV α2 subunit, laminin subunits α1 and γ1 and fibulins 1 and 2. These molecules assemble together to form a classical basement membrane or basal lamina-like structure, which then recruits other ECM proteins such as nidogen, fibronectin and entactin. The integrity of this multi-molecular structure depends

on the self-polymerization of laminin subunits and the subsequent binding interactions between these laminins and other ECM molecules (40, 44-47). The potential regulation of this unique ECM by C/EBPβ is intriguing for two major reasons. Firstly, the observations that notable basal lamina components were being synthesized in the uterine stoma, a non-classical basal lamina setting, are counter-intuitive to current concepts about ECM function. Secondly, there are previous reports of basement membrane proteins, such as laminins and collagen type IV, being robustly expressed in decidual tissue during implantation (48, 49). Collectively, these findings raise the possibility that the C/EBPβ-regulated ECM molecules participate in the formation of a unique basement membrane-like environment that supports decidualization and pregnancy in the mouse. The availability of a powerful *in vitro* primary culture system in which we can differentiate uterine stromal cells to decidual cells presented us with an opportunity to address the above hypothesis. This work will be discussed in Chapter 3. Furthermore, in Chapter 4, we describe ongoing studies in which we created a conditional *laminin γ1* knockout mouse using a loxP/Cre strategy to evade the embryonic and developmental effects of global *laminin γ1* ablation.

Conclusions

The involvement of steroid hormones E and P acting through their cognate receptors is critical to the precise and timely regulation of the endometrial events required for pregnancy. These events comprising embryo attachment, stromal decidualization and vascularization of the endometrium require the appropriate participation of a host of

genes that are regulated by E and P (Fig. 1.1). This chapter has focused on the theme of decidualization as a biological event that is both unique and essential to pregnancy in mice and humans. The use of powerful mouse genetics and manipulation of mouse culture systems, combined with informative gene expression profiling strategies in murine samples, has made it feasible to pinpoint specific molecular cues to decidualization. Our studies have centered on the role of one such molecule, C/EBPβ, as an important regulator of uterine functions. These and future studies will serve to illuminate the mechanisms by which E and P regulate uterine function and will serve as a framework in which to explore new therapeutic approaches to human endometrial diseases, particularly those associated with aberrant steroid hormone signaling such as excessive E action or reduced P sensitivity.

FIG. 1.1.
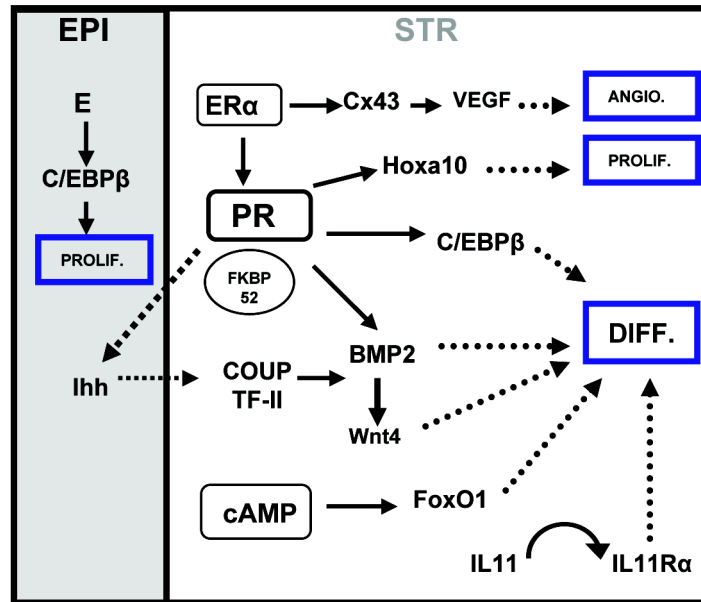
Figure 1.1. The molecular pathways controlling decidualization

An emerging blueprint of the molecular pathways underlying the mouse and human endometrial functions, leading to decidualization, is shown. EPI and STR represent uterine epithelial and stromal compartments, respectively. The bold arrows represent the hypothetical linear relationships between the indicated factors. The dashed arrows point to functional links for which the mechanisms are still unknown. The schematic describes only those factors that are discussed in this article. BMP2, bone morphogenetic protein 2; cAMP, cyclic adenosine monophosphate; C/EBPβ, factor CCAAT/enhancer binding protein-β; COUP-TF II, chicken ovalbumin upstream promoter-transcription factor II; Cx43, connexin 43; E, estrogen; ERα, estrogen receptor α; FoxO1, Forkhead Box O1; Hoxa-10, Homeobox A10; Ihh, Indian hedgehog; IL, interleukin; IL11Rα, interleukin 11 receptor α; PR, progesterone receptor; VEGF, vascular endothelial growth factor; Wnt4, Wingless 4.

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CHAPTER 2

C/EBPβ IS AN ESSENTIAL MEDIATOR OF ESTROGEN-INDUCED PROLIFERATION OF THE UTERINE EPITHELIUM [1]

2.1. ABSTRACT

Female mice lacking the transcription factor C/EBPβ are infertile and display markedly reduced estrogen (E)-induced proliferation of the uterine epithelial lining during the reproductive cycle. The present study showed that the E-stimulated luminal epithelial cells of C/EBPβ-null uterus are able to proceed through the G1 phase of the cell cycle before arrest in the S phase. Markedly reduced expression of E2F3, an E2F family member, and lack of nuclear localization of cyclin E, a critical regulator of cdk2, accompanied this cell-cycle arrest. An increased nuclear accumulation of p27, an inhibitor of the cyclin E/cdk2 complex, was also observed in the mutant epithelium. Gene expression profiling of C/EBPβ-null uterine epithelium revealed that the blockade of E-induced DNA replication triggers the activation of several well-known components of DNA damage response pathway, such as ATM, ATR, histone H2AX, checkpoint kinase 1 and tumor suppressor p53. Activation of p53 by ATM/ATR kinase led to increased expression of p21, an inhibitor of G1-S progression, which helps maintain the cell-cycle arrest. Additionally, p53-dependent mechanisms

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contributed to an increased apoptosis of replication-defective cells in the C/EBPβ-null epithelium. C/EBPβ, therefore, is an essential mediator of E-induced growth and survival of uterine epithelium of the cycling mice.

2.2. INTRODUCTION

The physiology of estrogen-induced uterine epithelial proliferation

In preparation for pregnancy, the uterine epithelium of the adult cycling mouse undergoes several synchronized rounds of cell proliferation (1). Ovarian estrogen (E) and progesterone (P) production regulate this cell proliferation process in a carefully controlled manner, allowing for appropriate uterine growth. Rising E levels during the proestrus stage of the reproductive cycle stimulate uterine epithelial cells to undergo DNA replication, resulting in the overall growth of the epithelial lining (1-4). At the onset of pregnancy, increasing production of P from the ovary inhibits E-induced epithelial proliferation. The dynamic actions of these two hormones in the uterine epithelium are mediated via their cellular receptors, estrogen receptor α (ERα) and progesterone receptor (PR) (5, 6). As a result of the dynamic interplay between E and P, the uterine epithelium is well prepared for the arrival of a blastocyst. The use of ovariectomized mice, which have been administered exogenous E and P, presents a valuable model with which to explore the molecular regulation of uterine epithelial proliferation.

The mechanisms of estrogen-regulated proliferation in the endometrium

Several previous studies documented the mitogenic effects of E on rodent uterine epithelium and examined the mechanisms underlying this steroid-stimulated proliferation (1, 4). The administration of E to ovariectomized mice led to transcriptional induction of proto-oncogenes such as c-fos, c-myc, and n-myc, the epidermal growth factor (EGF) and its receptor EGFR, transforming growth factor (TGF) α, and insulin-like growth factor 1 (IGF-1), concomitant with uterine epithelial proliferation (1, 4, 7, 8). Based on these findings, it was proposed that proto-oncogene and growth factor pathways mediate E-induced growth response in the uterus (9-12). However, later studies using knockout mouse models failed to provide unequivocal evidence in support of a role of certain of these factors in the female reproductive tract (13, 14). For example, the EGF-null mice exhibited no reproductive abnormalities, suggesting that E-mediated growth of the uterine epithelium is not completely abrogated in these mice (13). Similarly, mice lacking TGFα did not display any reproductive deficiency (14). Interestingly, administration of P to ovariectomized mice, which down-regulated E-induced uterine epithelial proliferation, did not alter the expression profiles of many of these growth-promoting factors, indicating that these factors may not directly participate in E-induced cell cycle entry. Collectively, these observations raised the possibility that additional factors are involved in transmitting the E-induced growth response to the cell cycle machinery (1, 9, 12).

In recent studies, we identified and characterized the transcription factor, CCAAT Enhancer Binding Protein Beta (C/EBPβ) as a steroid-hormone regulated gene that is

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critical for uterine functions (15). C/EBPβ is a member of the bZIP family of leucine-zipper proteins and has been implicated as a regulator of proliferation and differentiation in a diverse set of tissues (16-20). The importance of C/EBPβ in female fertility was revealed when the C/EBPβ-null females were found to have severely compromised ovarian and uterine functions. In addition to a failure in ovarian follicle rupture (21), the C/EBPβ-null uterus is refractory to embryo attachment to uterine luminal epithelium (LE), as demonstrated by embryo transfer experiments performed in ovariectomized mice supplemented with E and P (15). The expression of C/EBPβ is induced rapidly in the uterine LE during the early phases of E-induced proliferation of this tissue (15). Its importance in uterine growth was highlighted by the markedly diminished expression of the Ki67 antigen, a widely used marker of cell proliferation, in E-treated uterine epithelia of C/EBPβ-null mice (15).

In this study, we noted that simultaneous treatment with P, which reduces E-induced uterine LE proliferation, also decreased the expression of C/EBPβ, thereby positioning this transcription factor as a biologically relevant mediator of E action in this tissue. We postulated that E-induced expression of C/EBPβ and its downstream targets control the production and/or activity of one or more major cell-cycle regulatory molecules in the uterine epithelium. Using stage-specific cell-cycle markers, we determined that the loss of C/EBPβ leads to a significant impairment in DNA replication in the uterine LE. We also identified alterations in the expression and cellular localization of cyclin E, E2F3, and p27, key molecules that modulate the G1-S transition of the cell cycle. We further showed that the proliferation defect in C/EBPβ-null uterine LE is associated with the

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activation of well-known DNA damage response pathways involving ataxia telengiectasia mutated (ATM), ATM-Rad3-related (ATR), checkpoint kinases Chk1/2, and p53. The activated p53 helps to maintain the G1-S blockade of the cell cycle by inducing the synthesis of the inhibitor p21. P53 also promotes apoptosis of replication-defective uterine LE cells. Collectively, these studies identified the molecular pathways by which C/EBPβ mediates E-induced proliferation and survival of uterine LE during the reproductive cycle and early pregnancy.

2.3.   MATERIALS AND METHODS

Animals, hormone treatments and tissue collection.

All experiments involving animals were conducted in accordance with the National Institutes of Health standards for the use and care of animals. The animal protocols were approved by the University of Illinois Institutional Animal Care and Use Committee. Heterozygous mice carrying a mutation in C/EBPβ gene were provided by Dr. Peter F. Johnson of the National Cancer Institute. Female mice carrying a null mutation in the C/EBPβ gene were derived from crosses of heterozygous females with nullizygous male mice as described previously (21). Female WT and C/EBPβ-null mice of 129Sv background were ovariectomized at 10-11 weeks of age and rested for two weeks. These mice were then treated with a single dose of E (250 ng in sesame oil) by intraperitoneal injection (i.p.) for various durations. In experiments where P was used, mice were injected with a single dose of P (1 mg in sesame oil) along with E (250

ng). For each treatment group, at least five mice were used at each time point. In some experiments, animals were injected i.p. with BrdU (2 mg/animal; BD Pharmingen) 1 h prior to sacrifice. Uteri were collected and fixed in 10% formalin prior to immunohistochemistry (IHC). Alternatively, uteri were pooled for isolation of primary epithelial cells.

Isolation of mouse primary uterine epithelial cells

Hormone-treated uterine horns were excised, trimmed of fat and dissected longitudinally to expose the uterine lumen. Dissected horns were then cut into 4 to 5 mm long pieces and washed in Hank's Balanced Salt Solution (HBSS; Gibco). Uterine tissue pieces were then placed in HBSS containing 6 g/L dispase (Invitrogen) and 25 g/L pancreatin (Sigma) for 1 h at RT followed by 10 min at 37 ºC. The tubes were gently agitated to release luminal epithelial cells from the rest of the uterine tissue. The cell suspension was filtered through a 100 μm pore-size sieve (BD) to remove any tissue debris. The filtrates containing luminal epithelial cells were centrifuged at 2000 rpm for 5 min to pellet the cells. The cells were washed twice in PBS and re-pelleted. The pellets were lysed with Trizol for RNA extraction or with RIPA lysis buffer for preparation of whole cell protein lysates.

Real-time PCR analysis

Total RNA was isolated from uterine cells by standard Trizol-based protocols and

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converted to cDNA. The cDNA was amplified by real-time PCR to quantify gene expression using gene-specific primers and SYBR® Green (Bio-Rad Laboratories) in an iCycler (BioRad) instrument. As a loading control, the expression level of the 36B4 gene, which encodes a ribosomal protein, was determined. For each treatment, the mean threshold cycle (Ct) and standard deviation were calculated from Ct values obtained individually from 3 to 4 replicates of that sample. Each sample was subjected to three independent real-time PCR trials. Normalized Ct (ΔCt) values were determined by subtracting the mean Ct values of 36B4 from the mean Ct of each gene. The $\Delta\Delta$Ct values were then calculated as the differences between ΔCt values of genes from treated samples and control samples. Fold change was derived by applying the formula $2^{-\Delta\Delta Ct}$ to the $\Delta\Delta$Ct values. The error bars indicate $2^{-\Delta\Delta Ct} \pm$ S.D. ANOVA single factor analysis was conducted on the grouped means to determine statistical significance at a significance level of $P<0.05$.

The real-time PCR primer sequences are: C/EBPβ, AGCGACCAGTACAAGATG (forward) and CTGCTCCACCTTCTT (reverse); cyclin E, CCTGGATGTTGGCTGCTTAG (forward) and CACCACTGATAACCTGAGACC (reverse); cyclin D1, GCATCTACACTGACAACTCTATC (forward) and GCCAGGTTCCACTTGAGC (reverse); cyclin A, TCTTCCTTCCTTGGTTGCTG (forward) and ACTTCTCCCTGATTGCTTGC (reverse); cyclin B, CATTCCAAGTTTCGGCTTCT (forward) and CGTCAGCTCCATCAGGTCATT (reverse); E2F1, GTACTCCAGCCGCATGTATC (forward) and GCCATAGGAAGGACGCATAC (reverse); E2F2, GGCAACTTCAAGGAGCAGAC

(forward) and GGCACAGGTAGACTTCAATGG (reverse); E2F3,

GAACAAGGCAGCAGAGGTG (forward) and GACTTCTTCTTAATGAGGTGGATG

(reverse); cdk2, ACAGGGCAAGGTGAAAGAC (forward) and

AGGAGGACGGCAATGAGG (reverse); cdk4, TTGCCTCCAGAAGACGAC (forward)

and GCTCCAGACTCCTCCATC (reverse); cdk1, CTGGGCACTCCTAACAACGAAG

(forward) and TCCAAGCCGTTCTCGTCCAG (reverse); Lactoferrin,

GGACCGCAGGCTGGAACATC (forward) and

ACAGGGCACAGAGATTGGATTTGG (reverse); p27,

GTTGCTTTGTGTGGCTTCTG (forward) and TGGTTGCTTGGCTAATAAATGC

(reverse); p21, GTTCCTTGCCACTTCTTAC (forward) and

ACTGCTTCACTGTCATCC (reverse); 36B4, CTGCTGCCACCACTGCTG (forward)

and TCCTCATCTGATTCCTCCGACTC (reverse); ATM,

CCAGCATCTCCTCCTCCAC (forward) and CGCACCACTCTCGTCTTTC (reverse);

DNA microarray analysis

WT and C/EBPβ-null mice were ovariectomized and treated with E, and after 18 h, the

LE cell layer was isolated and total RNA was prepared using Trizol-based protocols.

Total RNA was incubated with DNase I (DNA-free ®, Ambion Inc.) to eliminate

genomic DNA contamination. RNA concentration and purity were assessed by

measuring the A260/280 OD ratios in a spectrophotometer. The integrity of

the RNA samples was analyzed by using a Bio-Analyzer gel electrophoresis

method to distinguish bands of 18S and 28S ribosomal RNA. The RNA was

then processed to generate biotinylated cRNA probes that were subsequently hybridized to high-density Affymetrix oligonucleotide arrays (GeneChip®Mouse Genome 430 2.0 Array) representing ~14,000 known murine genes. Fluorescent hybridization signals from the arrays were analyzed by GeneChip® Scanner® 3000 and data files processed for comparison analysis using GeneChip® Operating Software (GCOS). The hybridization signals were analyzed by comparing arrays hybridized with WT and C/EBPβ-null epithelial RNA samples, respectively, using the WT signals as a baseline. Using the signal-log ratios for each gene, the fold change in the expression of that gene was determined. Finally, the comparison analysis yielded a list of genes that were upregulated (+) or downregulated (-) in C/EBPβ-null uterine epithelium relative to WT samples.

Potential C/EBPβ-regulated genes obtained from microarray analysis were further categorized by biological functions using two major gene ontology softwares: Panther (http://www.pantherdb.org) and Ingenuity Pathway Analysis (Ingenuity ® Systems).

Immunohistochemistry (IHC)

Formalin-fixed uterine pieces were processed for paraffin embedding. Cross sections (5 μm thickness) were mounted onto microscope slides (Fisher Scientific). For immunostaining, uterine sections were deparaffinized in xylene (thrice, 5 min each), rehydrated through a graded series of treatment with ethanol (100%, 95%, 85%, and 70%, 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% BSA, 0.3% Triton X-100, sterile PBS) overnight at 4ºC: BrdU & Ki67 (BD Pharmingen), phospho-Ser[10] histone H3 (Upstate Biotechnology), cyclin E, cyclin A & Rad18 (Abcam), cyclin D1 (LabVision NeoMarkers), E2F-3 (Santa Cruz Biotechnology), p27 (BD Transduction Laboratories), phospho-(Ser1981)-ATM, phospho-(Ser139)-H2AX, Caspase 3 & cleaved Caspase 3 (Cell Signaling Technology). The sections were washed and incubated with biotinylated secondary antibodies (Jackson Immunoresearch Laboratories Inc.) for 60 min followed by incubation with streptavidin-conjugated horseradish peroxidase (Histostain Kit; Zymed Laboratories Inc.) for 45 min. Sections were stained with AEC solution (Zymed Laboratories Inc.) and counterstained with Mayer's Hematoxylin (Sigma). Immunofluorescence staining was performed by incubating sections with the Cy3-conjugated Streptavidin complex (Jackson Immunoresearch Laboratories) following incubation with secondary antibodies. The counterstaining was done using 4',6'-diamidino-2-phenylindole (DAPI; Invitrogen Inc.). Stained sections were mounted in fluorescence mounting media (20% Glycerol, 8% w/v Poly-vinyl alcohol (PVA), Diazabicyclo [2.2.2] octane (DABCO), Tris-HCl pH 8.5 and sterile water). Negative controls included incubation with donkey serum and omission of the primary antibody for all samples.

TUNEL staining

To detect apoptotic cells containing fragmented DNA, *in situ* labeling of free 3' OH of nicked DNA was carried out, using the In Situ Cell Death Detection Kit (Roche Applied Science), following the manufacturer's protocol. Briefly, deparaffinized and rehydrated sections were incubated at RT for 15 min with proteinase K (20 μg/mL in 10 mM Tris-HCl, pH 8.0). Sections were washed and incubated for 1 h at 37ºC in the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase enzyme (TdT), fluorescein-labeled nucleotide mixture and diluent buffer (30 mM Tris-HCl, pH 8.0). For negative controls, TdT enzyme was omitted from the reaction mixture. After rinsing in PBS, sections were counterstained with DAPI and mounted in fluorescence mounting media.

Image capture and quantitation of immunostaining

The images of immunohistochemical staining were captured using a Leica (Nussloch, Germany) DM2500 light microscope fitted with a Qimaging Retiga 2000R camera (Qimaging, British Columbia, Canada). For counting, at least 5-6 individual 40x fields from each sample were captured. The number of positively stained uterine LE nuclei in each field were averaged and expressed as a percentage of the total number of these cells. 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 $P<0.05$. Fluorescent images for phospho-histone H3,

cyclin E, p27 and E2F-3 were captured using the Leica DM2500. For all other antibodies, fluorescence microscopy and image capture were performed using a Leica DMR microscope and a Retiga EXI monochrome CCD camera (Qimaging). All images were processed using Adobe Photoshop CS version 8.0.

Western Blotting

Whole cell lysates were prepared from primary LE cells isolated from uteri collected from control (untreated) or E-treated WT and C/EBPβ-null mice. The cells were homogenized using a RIPA lysis buffer. Protein concentrations of cell lysates were determined using the Bradford assay from Bio-Rad. Proteins in 20 μg of each cell lysate was separated by SDS-PAGE (10%) and transferred to Hybond-P membranes (Amersham Pharmacia Biotech). The membranes were blocked in Tris-buffered saline with 0.1% (v/v) Tween-20 (TBS-T) and 5% (w/v) nonfat dry milk for 1 h. They were then incubated overnight at 4ºC with primary antibodies diluted 1:1000 in TBS-T containing 5% (w/v) BSA. These antibodies were directed against Chk1, p53, phospho-Thr[187]-p27 and calnexin (Santa Cruz Biotechnology), p21 (BD Pharmingen), phospho-Ser[296]-Chk1 and phospho-Ser[15]-p53 (Cell Signaling Technology) and cdk2 and phospho-Thr[160]-cdk2 (Abcam). Membranes were then incubated for 1 h at RT with secondary antibodies conjugated to horseradish peroxidase and diluted 1:5000. After thorough washes with TBS-T, chemiluminescence detection was performed using SuperSignal Femto and Pico reagents from Pierce (Thermo Scientific).

Chromatin Immunoprecipitation (ChIP)

The ChIP analysis was conducted using the EZ-ChIP (Upstate Biotechnology), according to the manufacturer's protocol. Briefly, mouse uterine LE cells were isolated from 11-week old, ovariectomized WT mice of 129sv background that were subjected to E treatment (250 ng) for 12h. The cells (7.5×10^6) were washed in PBS and cross-linked with 1% formaldehyde at room temperature for 10 min. The cross-linked cells were lysed using SDS lysis buffer and sonicated 5 times for 10 sec each pulse. Lysates were precleared with salmon sperm DNA-protein A and the DNA-protein complexes were subsequently immunoprecipitated using antibodies against RNA polymerase II, rabbit IgG or C/EBPβ (Santa Cruz Biotechnology). The immune complexes were recovered with protein A agarose. The beads were then repeatedly washed and protein complexes were eluted. The cross-linking was reversed and proteins were digested using 0.5 mg/ml proteinase K. Purified DNA were used as templates for real-time PCR using various primer sets to amplify specific regions of the E2F3 promoter.

***In silico* Promoter Analysis**

Putative C/EBPβ binding sites at the E2F3 promoter were determined by *in silico* analysis of the proximal promoter region (-1 bp to -1900 bp) using TESS (22), TFsearch (23) and Consite (24) softwares. Primers were designed to amplify specific regions containing putative C/EBPβ binding sites as follows: -329 bp to -340 bp

(TTCATACCCCTCCCACAAGA & TTTTATTGTCCTTCTAGCCATGA); -458bp to -473 bp (CCAGTGATTCAGCATACATTACA & TTATTGCCCTCACCACCTTC); -584 bp to -595 bp (ACAGTCTTGGGTGAGCTGGT & CCCTACACACTCGGTTCCT) and -1054 bp to -1066 bp (AGCCTCTTTGACTGGGACTG & TCAGTATTTTGCTGGGGTCTC).

Statistical Analysis:

Statistical significance was assessed by ANOVA analysis at a significance level of $P < 0.05$ and is indicated by an asterisk in the figures. NS indicates non-significant changes ($P > 0.05$).

2.4. RESULTS

P suppresses E-induced C/EBPβ expression in uterine epithelial cells

It is well known that P antagonizes the E-induced proliferation of uterine LE cells (1, 25, 26). Our previous studies showed that C/EBPβ expression is markedly induced in the uterine LE of ovariectomized mice in response to an acute administration of E (15). We report here that simultaneous administration of P inhibited acute E-induced uterine LE proliferation as measured by Ki67 immunostaining (Fig. 2.1, A). We also determined the levels of C/EBPβ mRNA and protein in uterine LE cells of ovariectomized mice after treatment with E or E plus P for 2 and 11 h. We noted that treatment with E alone leads to

a robust increase in C/EBPβ mRNA and protein levels within 2 h followed by a decline in its expression to basal levels by 11 h (Fig 2.1, B and C). When mice were treated with a combination of E and P, the E-induced increase in C/EBPβ mRNA levels at 2 h was strongly suppressed. P, therefore, counteracts the transcriptional induction of C/EBPβ by E. These results are consistent with our hypothesis that C/EBPβ is a critical downstream mediator of E-stimulated uterine epithelial cell proliferation and P exerts its antiproliferative effects, at least in part, by suppressing C/EBPβ expression in these cells.

E-dependant S-phase activity is impaired in C/EBPβ-null uterine epithelium

Earlier studies demonstrated that E-induced proliferation of uterine LE is significantly reduced in the C/EBPβ-null mice compared to the wild-type (WT) mice of same genetic background (15). The goal of the present study was to determine the precise stage at which the cell cycle progression is impaired in the mutant uterine LE. To assess cell cycle entry and progression through the G1 phase, we monitored the expression of two cell cycle markers, minichromosome maintenance molecule 3 (MCM3) and Ki67, by IHC analysis. MCM3 is a major component of the "pre-replicative licensing complex" that is highly expressed during G1 phase in preparation for entry into S phase (27). Ki67 is a nuclear antigen that is expressed at all stages of active cell cycle (27, 28). At 8 and 12 h after E treatment, which correspond to early and late G1, both MCM3 and Ki67 were expressed at comparatively equal levels in WT and C/EBPβ-null epithelia (Fig 2.2, A & B). This observation suggested that both WT and mutant LE cells are competent to enter

G1 from G0 phase upon E stimulation and able to progress through G1 phase of the cell cycle.

To examine the G1-S transition and S-phase activity, ovariectomized WT and C/EBPβ-null mice were treated with E for durations of 8, 12, 15 and 18 h. A one-hour pulse of BrdU, a thymidine analogue, was administered to these mice immediately prior to collection of uterine tissue. Nuclear immunostaining of BrdU, which is indicative of S phase activity, was absent in untreated ovariectomized WT and C/EBPβ-null mice (data not shown). Sporadic staining of BrdU was visible in ~5% WT LE cells at 8 h following E treatment (Fig 2.3 A, panel a, and 2.3 B). This staining was significantly increased by 12 h, indicating the entry of a substantial number (~16%) of uterine LE cells into S-phase (Fig 2.3 A, panel c, and Fig. 2.3 B). The BrdU incorporation increased further at 15 h (Fig 2.3 A, panel e). By 18 h of E treatment, almost all uterine LE cells are actively undergoing DNA replication in WT mice (Fig 2.3 A, panel g). In comparison, in the uterine LE of C/EBPβ-null mice, BrdU incorporation was drastically reduced at all time points tested following E treatment (Fig 2.3 A, panels b, d, f, h; Fig 2.3 B). While greater than 90% of WT LE cells showed BrdU-positive staining at 18 h following E treatment, only 10% were BrdU positive in the mutant epithelium (Fig 2.3, A & B). These results suggested that in the absence of C/EBPβ, E-stimulated uterine LE cells are severely impaired in their ability to undergo DNA replication.

One would predict that the blockade in S phase activity in C/EBPβ-null uterine LE would result in a loss of E-induced mitotic activity in these cells. To test this prediction, we

employed an antibody that specifically recognizes phosphorylated serine 10 of histone H3. This marker typically localizes to distinct regions of chromosome condensation during mitosis, appearing as punctuate loci in the nucleus (29). As expected, the levels of phospho-histone H3 staining steadily increased from 18 to 20 h and declined modestly from 20-24 h, in the E-treated WT uterine LE (Fig 2.4 A). However, in the absence of C/EBPβ, the phospho-histone H3 staining was absent or drastically reduced in LE when compared to WT uteri. (Fig 2.4, A & B). Consistent with the S-phase impairment that we had observed, the C/EBPβ-null mice displayed virtually no evidence of mitotic activity throughout the LE cell layer. The mitotic block was further confirmed by examining the mRNA and protein levels of cyclin B, a well-defined regulator of mitosis. As expected, the expression of cyclin B2 mRNA (Fig 2.4 C, panel a) and protein (Fig 2.4 C, panel b) was greatly reduced in C/EBPβ-null LE compared to the WT tissue (Fig 2.4 C).

Altered expression and activities of multiple G1-S phase regulatory factors in C/EBPβ-null uterine epithelium

The progression of the cell cycle from G1 to S-phase is orchestrated by stage-specific cyclins, cyclin-dependent kinases and other cell cycle regulatory factors (28). Cyclin D1 is typically expressed early in the G1 phase and associates with cdk4 to promote S-phase entry. Cyclins E and A then associate with cdk2 and directly regulate the S-phase progression. cdk2 achieves full functionality through a Cdk-Activating Kinase (CAK)-dependant phosphorylation of a threonine residue (Thr160) (30, 31). To analyze the basis of the lack of E-induced proliferation in the C/EBPβ-null epithelium, we, therefore,

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examined the expression levels of each of these S-phase-regulatory cyclins and cdks in WT and C/EBPβ-null LE cells at 15 h following E-treatment.

When the expression of cyclin D1 and cyclin A mRNAs was examined in WT and C/EBPβ-null uterine LE cells, no significant difference in their levels in WT and mutant epithelia was seen (Fig 2.5 A, panels a & c). Immunohistochemistry of uterine sections at 15 h following E treatment revealed that the levels of the cyclin D1 protein and its nuclear localization were unaltered (Fig. 2.5 A, panel b). Interestingly, we noted a modest decrease in the levels of cyclin A protein in the nuclei of mutant LE cells (Fig 2.5 A, panel d). We did not observe any significant difference in the expression levels of cdk2 and cdk4 mRNAs in WT and C/EBPβ-null uterine epithelial cells at 15 h following E treatment (Fig 2.5 A, panel e, data not shown). In contrast, we noted an approximately 50% reduction in the expression of cyclin E mRNA in the C/EBPβ-null uterine epithelial cells compared to the WT cells (Fig 2.5 B, upper panel). This decrease in cyclin E levels in the mutant epithelium was further confirmed by IHC of tissue sections collected at 15 h following E-treatment (Fig 2.5 B, lower panels). Most notably, we failed to detect nuclear localization of cyclin E in mutant epithelial cells. While the presence of this cyclin was prominent in the nuclei of WT epithelial cells (Fig 2.5 B, lower panels a and c), its immunostaining was markedly reduced and predominantly cytoplasmic in the C/EBPβ-null epithelium (Fig 2.5 B, lower panels b and d). Coincident with the reduced nuclear presence of cyclin E, we observed that the activation of cdk2 via phosphorylation of its Thr160 residue was diminished in C/EBPβ-null epithelial cells (Fig 2.5 A, panel f). The reduced expression of cyclin E, combined with a lack of its nuclear localization in

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the epithelial cells, therefore, contributes to impaired activation of cdk2, leading to the defect in E-induced S-phase activity in the C/EBPβ-null uterine LE.

The E2Fs 1, 2 and 3 belong to a family of six known E2Fs, and have been implicated in several studies as being the most important regulators of the G1-S checkpoint (32). Upon mitogenic stimulation, activated E2F factors transactivate downstream genes, including cyclin E, promoting progression through the cell cycle into S-phase. The mRNA levels of these three E2Fs were investigated in WT and C/EBPβ-null LE cells. While the expression levels of E2F-1 and E2F-2 displayed no statistically significant difference between WT and mutant epithelial cells, the E2F-3 mRNA level was diminished by approximately 50% in the LE cells lacking C/EBPβ (Fig. 2.6 A, panels a-c). This observation was further confirmed by IHC analysis, showing reduced nuclear expression of E2F-3 protein in the LE of WT and C/EBPβ-null tissue sections following 15 h of E treatment (Fig. 2.6 B). This finding is consistent with previous reports that E2F-3 plays an essential role in promoting the S-phase activity (33, 34). It is of interest to note that the steady state expression level of E2F3 mRNA is considerably higher than that of E2F1 or E2F2 mRNA in E-treated uterine LE (Fig. 2.6 A). Therefore, the reduced levels of E2F-3 may have a strong impact, resulting in diminished expression of cyclin E mRNA and the consequent impaired ability of C/EBPβ-null uterine epithelial cells to progress through the S-phase of the cell cycle.

C/EBPβ is recruited to the E2F3 promoter in response to E

C/EBPβ manifests its gene regulatory role by binding to the promoters of its target genes. To explore the possibility that C/EBPβ directly mediates the E-induced expression of E2F-3 and/or cyclin E, we examined the promoter regions of these genes by *in silico* analysis. We identified four putative binding sites for C/EBPββ in the E2F3 promoter and two such sites in the cyclin E promoter as indicated in the Materials and Methods. We then performed ChIP analysis to test for C/EBPβ occupancy at these sites. While we did not detect C/EBPβ recruitment to any of the putative binding sites in the cyclin E promoter sites (data not shown), we found a robust recruitment of this transcription factor to the −584 region of the E2F3 promoter following E treatment (Fig 2.6 C).

Alterations in the G1-S inhibitory factors p27 and p21 in C/EBPβ-null uterine epithelium

The cyclin-dependant kinase inhibitors (CKIs) block the cell cycle progression by negatively regulating the actions of cyclin-cdk complexes (28, 35). The predominant CKIs belong to the INK4 and Cip/Kip family of inhibitors, the latter of which includes p21 and p27, two well characterized G1-S regulators (35). Both p21 and p27 directly inhibit the cyclin E/cdk2 and cyclin A/cdk2 complexes, thereby controlling the S-phase activity. We found that the levels of p27 mRNA were only modestly elevated in C/EBPβ-null LE cells compared to WT cells at 0, 12 and 15 h after E treatment (Fig 2.7 A). Strikingly, however, prominent nuclear localization of the p27 protein was observed in

the C/EBPβ-null epithelium at 15 and 18 h of E-treatment (Fig 2.7 B, panels b and d). This nuclear accumulation of p27 protein coincided with the block in S-phase progression and lack of G2-M transition of these cells. Phosphorylation of p27 at threonine 187 by cdk2 serves as a signal for the ubiquitin-mediated degradation of this inhibitor (36). Consistent with the lack of cdk2 activity in the C/EBPβ-null LE cells, we observed that phosphorylation of p27 at Thr187 was reduced in these cells compared to WT cells, as indicated by IHC and western blotting (Fig 2.7 C & Fig 2.7 D).

We also detected an increased level of p21 mRNA in C/EBPβ-null LE cells relative to WT cells at all times following E treatment (Fig. 2.7 E). Of particular note was the greater than two-fold higher expression of p21 mRNA (Fig. 2.7 E) and a marked enhancement in p21 protein level in the mutant epithelium compared to the WT tissue at 18 h after E treatment (Fig. 2.7 F). It is likely that this increased expression of p21 in C/EBPβ-null epithelial cells contributes to the maintenance of the G1-S arrest.

Activation of DNA damage checkpoint machinery and expression of repair proteins in the C/EBPβ-null uterine epithelium following the G1-S arrest

To further investigate the gene networks underlying C/EBPβ function during E-induced proliferation of the uterine LE, we compared the gene expression profiles of WT and C/EBPβ-null uterine LE cells. Briefly, ovariectomized mice were treated with E, and after 18 h, the LE cell layer was isolated and total RNA was subjected to DNA microarray analysis using Affymetrix murine arrays as described in the Materials and

Methods. The results indicated that the expression of 620 genes was upregulated and that of 116 genes was downregulated greater than 2-fold in C/EBPβ-null uterine LE cells compared to WT cells (C. R. Ramathal and M. K. Bagchi, unpublished results). Using gene ontology analysis, we identified a subset of genes with well-established roles in DNA damage response among those markedly up regulated in the LE lacking C/EBPβ. These genes encoded factors regulating cell cycle checkpoints (such as *Atm, Atr, Birk5, Skp2, Cdc20*), DNA repair (such as *Rad18, Rad51, Rad54B, Rpa1, Mre11A, Exo1, Xrcc5*) and apoptosis (such as *Casp3, Cdh2, Tp73l*). A summary of genes regulated by C/EBPβ in E-treated uterine epithelial cells is provided in Appendix A.

A potential consequence of compromised DNA replication is collapse of replication forks or creation of aberrant structures at the replication forks, making the DNA prone to damage, such as single-strand or double-stand break formation (37, 38). These DNA lesions are detected by ATM and ATR proteins, which are primary sensors and transducers of the DNA damage response (37, 38). These proteins signal downstream to the checkpoint kinases, Chk1 and Chk2, and the tumor suppressor p53 in an attempt to correct the defect and remove damaged cells by triggering apoptosis (Fig. 2.8 A). An additional mechanism involves the recruitment of the E3 ubiquitin ligase Rad18 to stalled replication forks. Rad18, in partnership with Rad6, is thought to assist in disassembly of the aberrant fork (39). Consistent with the results of our microarray analysis, real-time PCR confirmed the up-regulation of mRNAs corresponding to *Atm* and *Atr* in the C/EBPβ-null uterine LE (Fig. 2.8 B). Activation of the ATM and ATR kinases via unique phosphorylation events signals to the cell cycle checkpoint molecules that govern the G1-

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S transition (37, 38). As shown in Fig. 8C, in the E-treated WT uterine LE, ATM exists in an inactive non-phosphorylated form, indicating the absence of any DNA damage response. In contrast, prominent nuclear staining for the active phosphorylated (Ser1981) form of ATM was detectable throughout the C/EBPβ-null uterine LE at 18 h following E-treatment (Fig. 2.8 C). In addition, accumulation of phosphorylated (Ser139) histone H2AX, a well-established marker of ATM-dependent DNA damage response (40, 41), was observed at 18-20 h in C/EBPβ-null uterine epithelial cells (Fig 2.8 E, panels a & b). As further evidence of replication fork lesions, we observed an increased nuclear expression of Rad18 in C/EBPβ-null epithelial cells compared to WT cells (Fig 2.8 E, panels c & d).

We next assessed the occurrence of a DNA damage response downstream of ATM/ATR activation by monitoring other markers, such as checkpoint kinase 1 (Chk1), and p53 in the C/EBPβ-null epithelium. Phosphorylation of Chk1 at Ser296 by ATM indicates that the DNA damage response and checkpoint activation are initiated (37). Phosphorylation of p53 at the Ser-15 residue by the activated ATM-Chk1 pathway causes p53 transcriptional activation (37). Western blotting experiments were performed, using soluble lysates prepared from LE cells obtained from uteri collected from WT and C/EBPβ-null mice following E treatment for 18 h. The data revealed a marked elevation in the levels of phosphorylated forms of Chk1 (Ser296) and p53 (Ser15) (Fig 2.8 D). There was also a significant increase in the level of total p53 protein in the C/EBPβ-null LE cells relative to WT cells at 18 h of E treatment (Fig. 2.8 E). It was previously reported that a consequence of up regulation and activation of p53 is elevated expression

of its transcriptional target p21 (37, 42). We indeed saw a significant rise in the level of

p21 protein (Fig. 2.7 E), which presumably helps maintain the cell-cycle arrest in the

mutant epithelium. Collectively, our results established that the lack of C/EBPβ, which

leads to a block in E-mediated proliferation of the uterine epithelium, triggers the

ATM/ATR-dependent DNA damage response pathway, and activates p53-mediated

signaling that helps maintain the cell cycle block.

Increased apoptosis in the C/EBPβ-null uterine epithelium following the G1-S arrest

Phosphorylation of p53 at Ser15 by ATM-Chk1/Chk2 pathway promotes its stabilization

by preventing ubiquitination and degradation (37, 43). P53 is known to promote the

expression of several pro-apoptotic genes, which in turn stimulate the mitochondrial

pathways leading to caspase activation (41). We, therefore, investigated the possibility

that the activation of an ATM/ATR/Chk1/Chk2 DNA damage response mechanism in

C/EBPβ-null uterine LE cells, which results in activation and accumulation of p53,

commits these cells to enter programmed cell death. In order to assess the initiation of

apoptosis in E-treated uterine LE cells of WT and C/EBPβ-null mice, we first examined

the activation of caspase 3 (Casp3), a well-known marker of this process (41, 44). We

performed IHC analysis to determine the levels of Casp3 and its proteolytically cleaved

active form (cl-Casp3) in these cells. Examination of the levels of full-length Casp3 at 48

h following E-treatment detected a higher expression of this protein in the cytosolic

compartment of C/EBPβ-epithelial cells compared to the WT epithelial cells (data not

shown). When we assayed for the presence of cl-Casp3 at the 48 h time point, an increased presence of this apoptosis marker was evident in the C/EBPβ-null epithelium (Fig 2.9 A).

We next performed TdT-mediated dUTP nick-end-labeling (TUNEL) staining to assess the extent of DNA degradation in apoptotic cells of the uterine epithelia of WT and C/EBPβ-null mice. We detected a notable increase in TUNEL staining at discrete foci in the LE of mutant mice compared to WT mice at 48 h of E-treatment (Fig 2.9, B & C). These data suggested that the arrest of the E-stimulated C/EBPβ-null LE cells at the S phase of the cell cycle triggers the activation of p53-dependent mechanisms that increase their susceptibility to programmed cell death and affect their survival.

2.5.   DISCUSSION

C/EBPβ is an essential mediator of E-induced S-phase entry of the uterine epithelial cells.

Our previous studies revealed that C/EBPβ is a major downstream target of E regulation in the uterus (15). The discovery that the uterine epithelium in C/EBPβ-null mice is non-receptive to embryo implantation (15) prompted us to utilize this animal model to investigate the functional role of C/EBPβ and its downstream pathways in uterine epithelial biology. The studies presented here demonstrated that the absence of C/EBPβ

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results in a significant defect in the ability of uterine LE cells to proliferate in response to E stimulation. This is evidenced by a major impairment in DNA replication activity and multiple dysregulated cell cycle components in the C/EBPβ-null uterine LE. We determined that C/EBPβ-null uterine LE cells are able to enter the cell cycle and progress through the G1 phase before getting arrested in the S-phase (Fig. 2.2 & 2.3). Ablation of the C/EBPβ gene, therefore, led to a complete loss of E-induced DNA replication and subsequent mitotic activity in the uterine LE. We, therefore, uncovered a role for C/EBPβ as an essential mediator of E-induced DNA replication in the uterine epithelium. Interestingly, we uncovered in separate studies that C/EBPβ-null uterine stromal cells progress normally through S-phase but encounter a block in mitosis (Wang, W. *et al.,* In press). This observation suggests that C/EBPβ may regulate the two critical cell cycle checkpoints in independent mechanisms based on its cellular context.

Previous reports documented that E administered to ovariectomized mice promotes the G1 to S phase transition of the uterine LE cells (1, 4, 26). Studies by Pollard and coworkers indicated that E-induced nuclear localization of cyclin D1 is an essential event that permits S-phase entry of the uterine LE cells (1, 26). It was shown that in the presence of E, the activation and nuclear translocation of cyclin D1 occurs via the inhibition of GSK-3β activity (25). It was further proposed that P opposes the proliferative actions of E by allowing phosphorylation of cyclin D1 by GSK-3β and subsequent nuclear export of this cyclin. In the present study, we noted that the nuclear presence of cyclin D1 remained unaltered in the S-phase-arrested LE cells of C/EBPβ-null uteri (Fig 2.5 B). These results suggested that C/EBPβ regulation of uterine LE cell

proliferation occurs via a mechanism that is unrelated to the nuclear translocation of cyclin D1. Our findings are corroborated by previous reports that the nuclear entry of cyclin D1 occurs in the absence of C/EBPβ in the liver and mammary epithelium (16, 45).

A major finding of our study is that, upon E treatment, the level of cyclin E mRNA is notably less in the C/EBPβ-null LE cells compared to the WT LE cells and the cyclin E protein failed to localize to the nucleus (Fig. 2.5 B). It is likely that the C/EBPβ-mediated entry of LE cells into the S phase of the cell cycle primarily involves regulation of cyclin E-cdk2 function. During the S phase of the cell cycle, the active cyclin E/cdk2 complex is localized in the nucleus of WT LE cells and coordinates the assembly of DNA replication factors onto the origins of replication (28, 47). In contrast, in C/EBPβ-null uteri, a combination of reduced expression of cyclin E and its absence from the nucleus of LE cells would prevent its partnership with cdk2 and its S-phase promoting activities. Consequently, inadequate cdk2 activation in C/EBPβ-null LE cells would also contribute to an S-phase blockade (Fig 2.5 B). Tong and Pollard previously reported that P abolishes E-induced cyclin E-cdk2 activity in the uterine epithelium (26). This report is consistent with our observation that simultaneous administration of P suppresses E-induced C/EBPβ expression, which in turn controls the expression and nuclear presence of cyclin E. Our finding is also consistent with a previously published microarray study conducted using whole uteri of mice (2). In that study, the acute administration of E stimulated the expression of uterine cyclin E and cdk2, although the cell-type(s) in which these molecules are expressed remained unclear.

Previous reports indicated that the loss of C/EBPβ results in reduced cyclin E expression, reduced cdk2 functionality and a decreased proliferative activity in mammary epithelia and in hepatocytes during liver regeneration (16, 45). The precise mechanism by which C/EBPβ regulates the expression and nuclear localization of cyclin E is presently unknown. Several studies have proposed that cyclin E is a target of transcriptional and post-translational regulation by the E2F transcription factors, which are important regulators of the G1-S transition and DNA replication (32-34, 48-50). Interestingly, among the E2F family members that we have examined, E2F3 is the most highly expressed in the WT LE cells (Fig. 2.6 A, panel d). It displayed reduced expression levels in the C/EBPβ-null uterine epithelium (Fig 2.6 A, panel c), while the levels of E2F1 and E2F2 remained unaltered. ChIP analysis demonstrated the recruitment of C/EBPβ to the proximal promoter region of E2F3, strongly suggesting that E2F3 is a primary target of C/EBPβ in uterine LE cells. We postulate that E2F3 regulates cyclin E expression, which in turn controls the entry of uterine LE cells into the S phase of the cell cycle.

We found that the CDK inhibitors, p21 and p27, also play important roles in blocking E-induced cell cycle progression in C/EBPβ-null uterine LE. In G0 and early G1 phases, a high level of p27 is present in hormone-withdrawn uterine LE. P27 binds to the cyclin E-cdk2 complex and inhibits its activity. Upon E-stimulation, p27 protein levels decline via ubiquitin-mediated degradation. In late G1, p27 becomes a substrate of phosphorylation by the cyclin E-cdk2 complex. Phosphorylated p27 is released from the cyclin E-cdk2 complex, exits its nuclear location and undergoes degradation, resulting in cyclin E-cdk2

activation (28, 36, 46, 51). Consistent with this scenario, we observed that the E-stimulated WT uterine LE cells efficiently targeted p27 for ubiquitin-mediated degradation by phosphorylation thereby depleting nuclear p27 protein upon S phase entry (Fig 2.7 C & 2.7 D). In contrast, a robust nuclear accumulation of p27 was detected in C/EBPβ-null epithelia, coincident with the drastically reduced cyclin E levels in the nuclear compartment and the cell-cycle blockade in S-phase (Fig 2.7 B). Our results supported the view that the accumulation of p27 protein arises from inefficient phosphorylation of p27 by the cdk2/cyclin E complex and consequent lack of ubiquitin-mediated degradation of p27 in the mutant LE cells (Fig 2.7 C & 2.7 D). The nuclear retention of p27 suggests that it may interact with and inactivate any residual cyclin E-cdk2 or cyclin A-cdk2 complex that might form in the nuclei. We also noted a remarkable rise in the level of the p21 inhibitor at later stages of the cell cycle arrest. The sustained presence of p27 and p21 inhibitors during early and late S-phase maintains the blockade of cyclin/cdk2 activities, preventing uterine LE cells from completing DNA replication and entering mitosis.

E stimulation in the absence of C/EBPβ activates the DNA damage checkpoint pathway in the uterine epithelium

Dysregulation of cyclin E was previously reported to cause destabilization of pre-replication complexes necessary for DNA synthesis (47). We postulate that a defect in cyclin E/cdk2-controlled replication fork assembly is encountered when E-stimulated C/EBPβ-null LE cells traverse through G1 and attempt to progress through S-phase. We

favor the hypothesis that the onset of a replication defect in E-stimulated C/EBPβ-null uterine LE triggers the DNA damage checkpoint activation. In mammalian cells, stalled replication forks or replication-blocking lesions are typically recognized by the ATM /ATR kinase checkpoint pathways as DNA damage signals, triggering a series of well-defined molecular events, culminating in cell cycle arrest and DNA repair (37, 38, 41, 43, 52). Stalled replication forks also recruit the ubiquitin ligase Rad18, a key component of the replication repair pathway (39, 53). In our studies, the ATM-dependent checkpoint pathway is activated in C/EBPβ-null uterine LE cells as evidenced by the enhanced accumulation of active, phosphorylated forms of ATM kinase and its downstream targets, histone variant H2AX, Chk1 and p53 (Fig 2.8). The increased presence of Rad18 in nuclei of replication-arrested C/EBPβ-null epithelial cells further confirms the replication fork lesions in mutant LE cells. Activation of Chk1 via phosphorylation at Ser296 is known to affect S-phase progression (43, 51). Furthermore, ATM/ATR-dependent phosphorylation of p53 at Ser15 leads to its activation and stabilization. The transcriptionally active p53, in turn, promotes the synthesis of p21, which directly inhibits the cdk2/cyclin E complex (26, 35, 37, 46). In this manner, activation of p53-dependent mechanisms downstream of DNA damage response helps maintain the DNA replication arrest in hormone-stimulated C/EBPβ-null epithelial cells.

Loss of C/EBPβ expression in uterine epithelium triggers apoptosis: implications for E-dependent cell survival

In addition to its mitogenic effects, E is also known to promote cell survival by acting as an anti-apoptotic agent (54, 55). A block in E-induced uterine LE cell proliferation in the

absence of C/EBPβ may trigger apoptosis, which is the culmination of a cascade of molecular steps including cell cycle withdrawal and DNA fragmentation (44). We also considered apoptosis as a potential biological end-point for S-phase-arrested C/EBPβ-null uterine LE cells since there is a strong connection between the ATM/p53-dependent checkpoint response and removal of damaged cells via programmed cell death (37, 41). One of the essential players in apoptosis is the serine-protease Casp3, which plays a central role in coordinating a variety of downstream events leading to cell death (41, 44). Our studies revealed that a subpopulation of E-treated C/EBPβ-null LE cells blocked in S-phase entered the apoptotic pathway as evidenced by the increased presence of the cleaved, active form of Casp3 in these cells compared to WT LE cells (Fig 2.9 A). Additionally, we noted a significant increase in cell death due to DNA fragmentation in C/EBPβ-null epithelium compared to the WT tissue (Fig 2.9, B & C). The genetic deletion of C/EBPβ has been previously linked to increased apoptosis and reduced tumorigenesis in skin keratinocytes (56, 57). It is likely that the majority of the C/EBPβ-null uterine LE cells undergo DNA repair and recovery, thereby preventing widespread apoptosis in the epithelium. In support of this concept, the expression of several proteins associated with the DNA repair pathway such as *Rad18, Rad51, Rad54B, Rpa1, Mre11A, Exo1, Xrcc5* was elevated in uterine epithelium lacking C/EBPβ.

In conclusion, C/EBPβ is an early and critical mediator of the E-controlled proliferative response in the uterine LE. The functional link between E and C/EBPβ provides a novel mechanism by which this hormone controls the expression, localization and activity of specific cell cycle regulatory molecules, such as E2F3, cyclin E, p27 and p21, to

influence DNA synthesis in the LE cells (Fig 2.10). The downstream actions of this key transcription factor are also indispensable for DNA repair and cell survival, allowing the preparation of a functional uterine epithelium for establishment of pregnancy. Furthermore, the role of C/EBPβ as a mediator of proliferative as well as anti-apoptotic effects of E in the uterine epithelium presents it as a potential target for anti-cancer therapeutics in preventing E-dependent endometrial cancers.

2.6. ACKNOWLEDGEMENTS

FIG. 2.1.
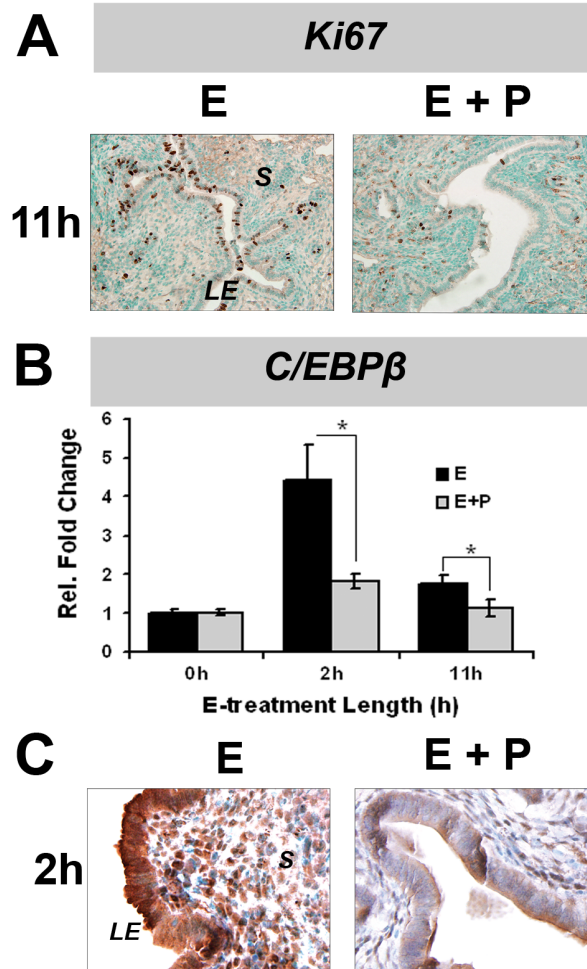
Figure 2.1. Uterine epithelial cell proliferation and C/EBPβ expression in response to E & P

WT mice were ovariectomized and treated with E or E plus P as described in Materials and Methods. Uteri were collected from these mice at 2 and 11 h after hormone administration.

A. IHC analysis of Ki67 expression in 11 h E- and E+P-treated uterine sections.

B. Primary uterine LE cells were isolated from uteri of WT mice treated with E or E plus P. Total RNA was analyzed by real-time PCR using C/EBPb-specific primers. The fold changes indicate gene expression levels at different times relative to E-treated cells at 0 h. Statistically significant differences ($P<0.05$) are indicated by *.

C. IHC analysis of C/EBPb expression in 2 h E- and E+P-treated uterine sections. LE:luminal epithelium, S:stroma.

FIG. 2.2.

A | **MCM3**

WT *C/EBPβ -/-*

8h

LE

S

12h

B | **Ki67**

WT *C/EBPβ -/-*

12h

S

LE
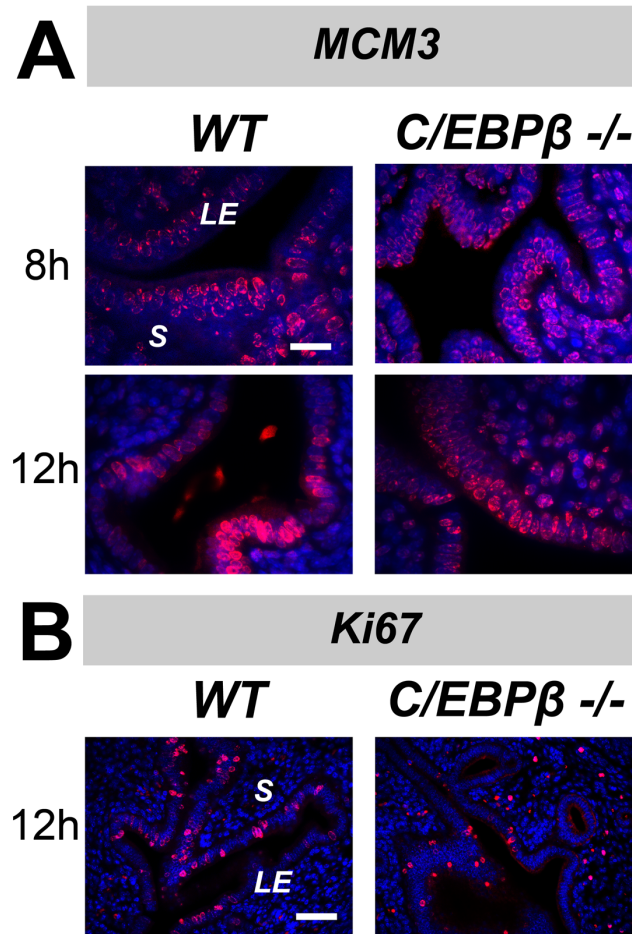
Figure 2.2. The C/EBPβ-null uterine epithelial cells enter into G1 phase in response to E

WT or C/EBPb (-/-) mice were ovariectomized and treated with E for 8 or 12 h.
LE: epithelium, S:stroma.

A. IHC analysis of MCM3 expression in 8 h and 12 h E-treated WT and C/EBPβ-null uteri. *Bar,* 20 mm.

B. IHC analysis of Ki67 expression in 12 h E-treated WT and C/EBPβ-null uteri.

Staining for MCM3 or Ki67 is indicated in red and DAPI-stained nuclei are shown in blue. *Bar,* 50 mm.

FIG. 2.3.

A

B
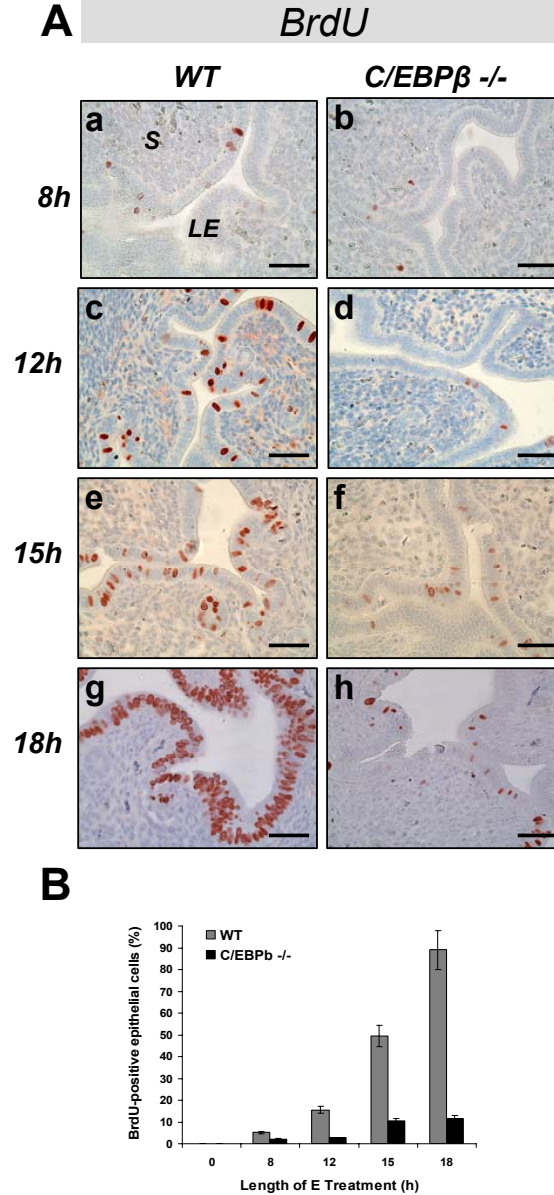
Figure 2.3. E-induced S-phase activity is impaired in C/EBPβ-null uterine epithelium

WT or C/EBPb (-/-) mice were ovariectomized and treated with E. Uteri were collected at 8, 12, 15 and 18 h following E administration. Mice were injected with BrdU 1 h prior to sacrifice.

A. IHC of uterine sections using an anti-BrdU antibody. *Bar,* 50 mm. Red deposits indicate positive staining for BrdU.

B. For quantitation of BrdU incorporation, positively stained nuclei of LE cells were counted as described in the Materials and Methods. Statistically significant differences (*P<0.05*) are indicated by *.

FIG. 2.4.
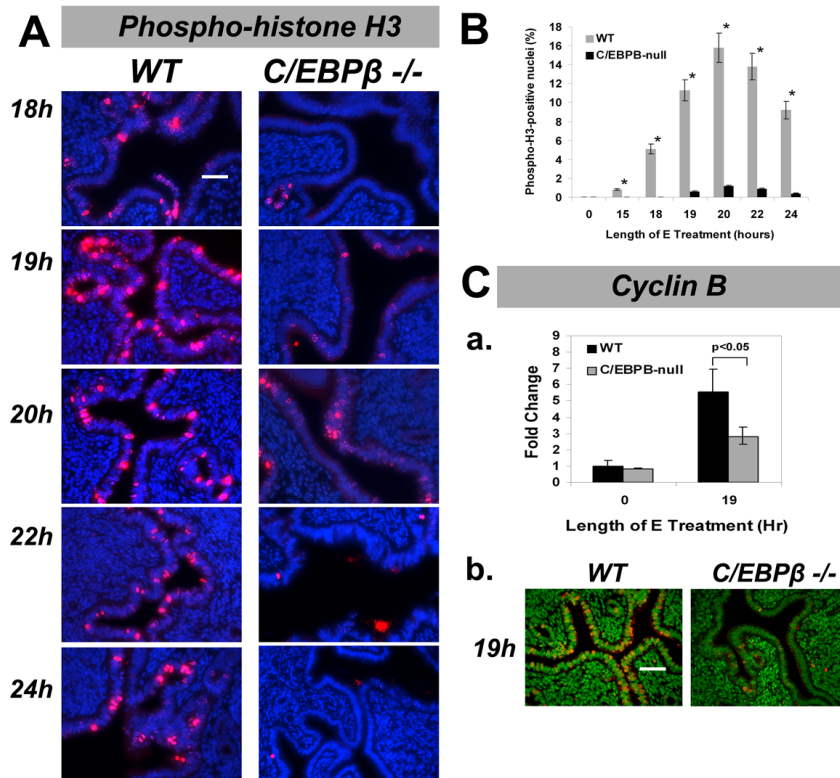
Figure 2.4. Mitotic activity is absent in E-treated C/EBPβ-null uterine epithelium

WT or C/EBPb (-/-) mice were ovariectomized and treated with E. Uteri were collected at 18, 19, 20, 22 and 24 h following E administration, and sections were subjected to IHC.

A. Analysis of mitotic activity in the uterine LE using an anti-phospho-histone H3 (Ser[10]) antibody. Specific staining is indicated in red and DAPI-stained nuclei are shown in blue. *Bar,* 50mm.

B. Quantitation of nuclei stained positively for phospho-histone H3.

C. Expression of cyclin B2 mRNA and protein in the uterine LE upon E treatment. (panel a) Primary uterine LE cells were isolated from uteri obtained from mice following 19 h of E-treatment. Total RNA was prepared and the expression level of cyclin B2 mRNA was determined by real-time PCR. The fold changes indicate cyclin B2 mRNA expression level relative to E-treated WT cells at 0 h. (panel b) Uterine sections of mice treated with E for 19 h were subjected to IHC using an anti-cyclin B2 antibody. Positive staining for cyclin B2 is indicated in red and DAPI-stained nuclei are shown in green. *Bar,* 50 mm. Statistically significant differences (*P<0.05*) are indicated by *.

FIG. 2.5.

A
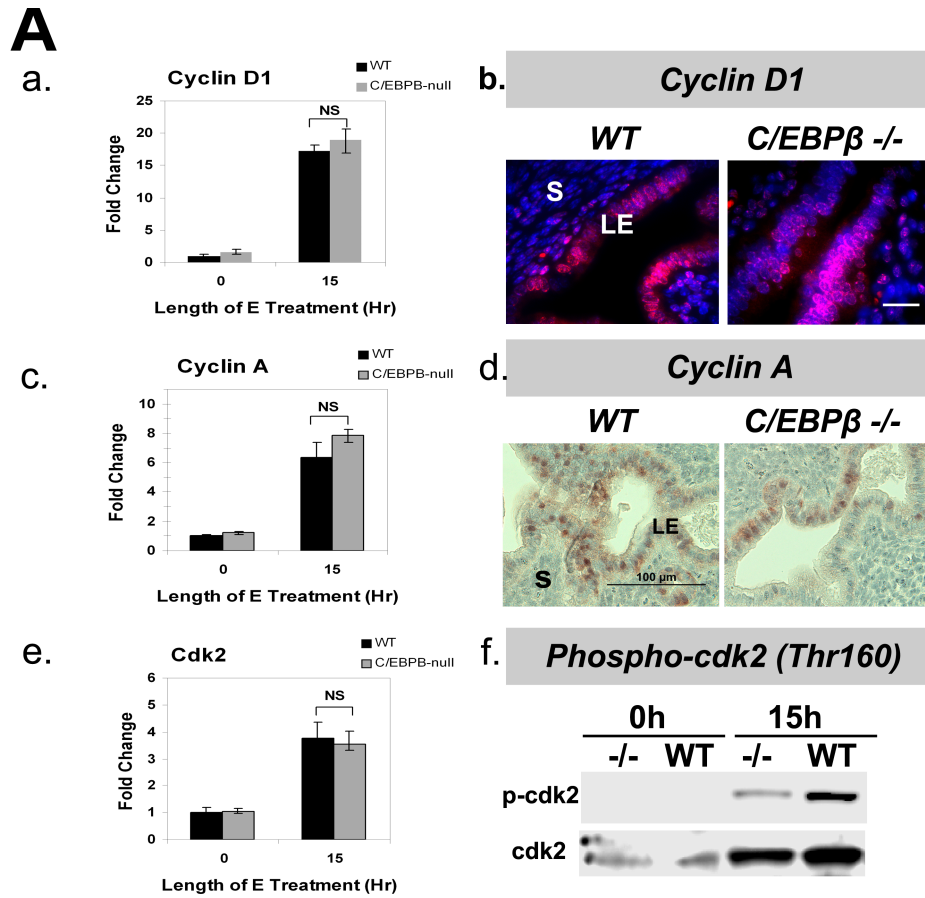
Figure 2.5. Expression and localization of G1-S cyclins and cdks in E-treated C/EBPβ-null uterine epithelium

A. LE cells were isolated from uteri of ovariectomized WT or C/EBPb (-/-) mice treated with E for 0 and 15 h. Expression levels of cyclin D1 (panel a), cyclin A (panel c) and cdk2 (panel e) mRNAs were determined by real-time PCR. NS indicates differences that are not statistically significant (*P>0.05*). Sections of uteri collected at 15 h following E treatment were subjected to IHC using antibodies against cyclin D1 (panel b) and cyclin A (panel d). Positive staining is indicated in red and DAPI-stained nuclei are shown in blue*.* (panel f) Lysates of LE cells were prepared at 0 and 15 h of E treatment and analyzed by western blotting using antibodies against cdk2 and phosphorylated cdk2 (Thr160).

Figure 2.5. continued.
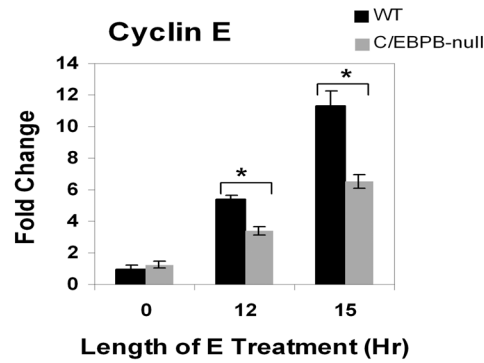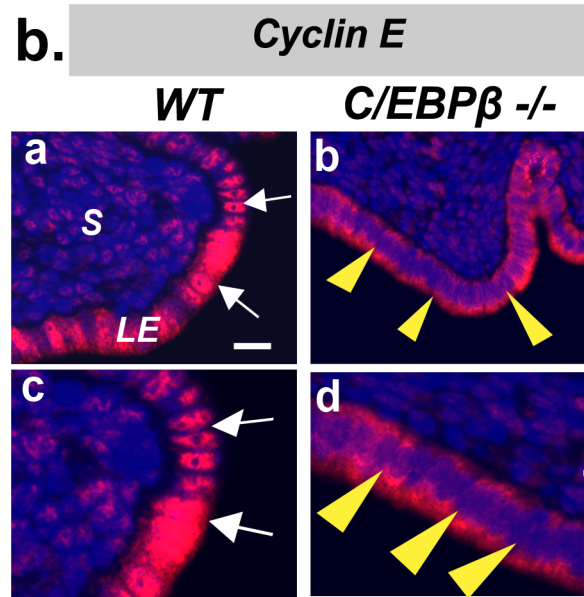
B. LE cells were isolated from uteri of mice treated with E for 0, 12 and 15 h. Expression of cyclin E mRNA (upper panel) was determined by real-time PCR. The fold changes indicate cyclin E mRNA expression level relative to E-treated WT cells at 0 h. Statistically significant differences (*P<0.05*) are indicated by *. Lower panels: Sections of uteri obtained from mice treated with E for 15 h were subjected to IHC using anti-cyclin E antibody. Panels c and d are displayed at a magnification of 100mm. White arrows indicate nuclear staining for cyclin E, while yellow arrowheads indicate cytosolic localization. Positive staining for cyclin E is indicated in red and DAPI-stained nuclei are shown in blue*.* LE:luminal epithelium, S:stroma.

FIG. 2.6.
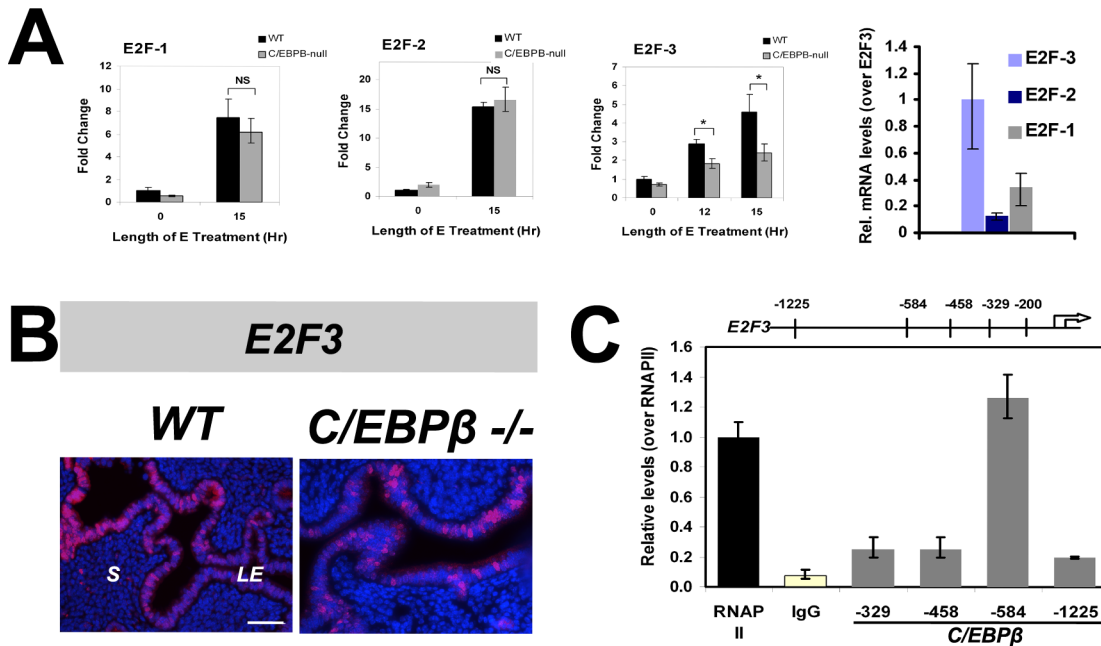
Figure 2.6. C/EBPβ regulates expression of the E2F family genes in the E-treated uterine epithelium.

A. LE cells were isolated from uteri of ovariectomized WT or C/EBPb (-/-) mice following treatment with E for 0, 12 and 15 h. Expression levels of E2F-1 (panel a), E2F-2 (panel b) and E2F-3 (panel c) were determined by real-time PCR. The fold changes indicate mRNA expression levels of the E2F genes relative to E-treated WT cells at 0 h. Panel d: Relative steady state levels of mRNAs for E2Fs 1, 2, and 3 in primary LE cells, obtained from uteri treated with E 15 h, are shown following normalization with 36B4 mRNA level. Statistically significant differences (*P<0.05*) are indicated by *.

B. Uteri from WT and C/EBPb-null mice treated with E for 15 h were collected and sections were subjected to IHC using anti-E2F-3 antibody. Positive staining for E2F-3 is indicated in red and DAPI-stained nuclei are shown in blue.

C. ChIP analysis: LE cells were isolated from uteri of ovariectomized WT mice following treatment with E for 12 h. ChIP was performed as described in Materials & Methods using antibodies against C/EBPb, RNAP polymerase II (RNAP II) and rabbit IgG. Relative levels of recruitment at various sites on the E2F3 promoter were determined by real-time PCR and normalized to input DNA and RNAP II values.

60

FIG. 2.7.

p27 (Kip1)

A

B

C *Phospho-p27 (Thr187)*

D

p21 (cip1)

E

F
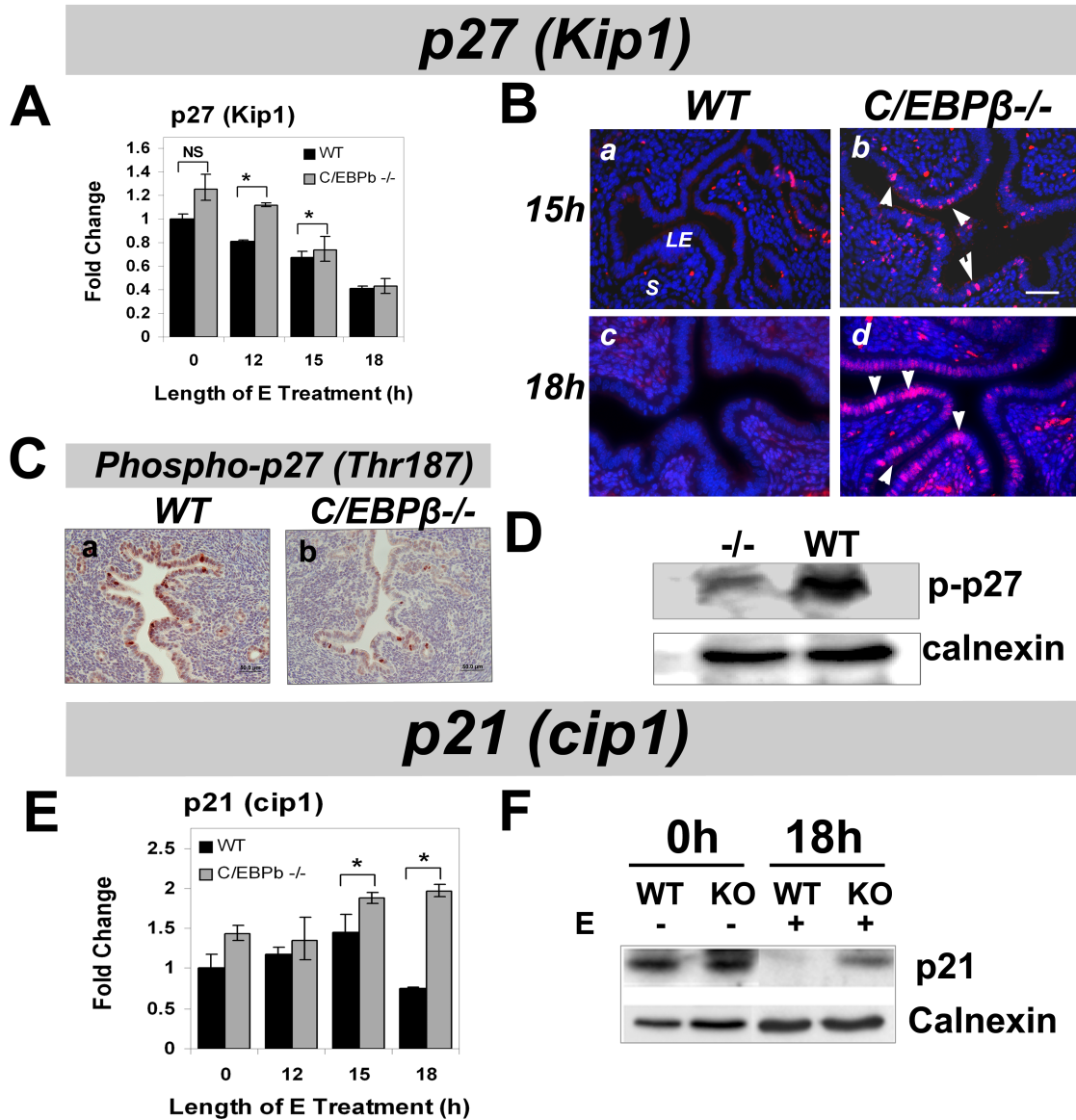
Figure 2.7. Expression of the cdk inhibitors p27 and p21 in the C/EBPb-null uterine epithelium in response to E

A. LE cells were isolated from uteri of ovariectomized WT or C/EBPb (-/-) mice following treatment with E for 0, 12, 15 and 18 h. Expression levels of p27 mRNA were determined by real-time PCR. The fold changes indicate expression levels of p27 and p21 mRNA relative to E-treated WT cells at 0 h. Statistically significant differences (*P<0.05*) are indicated by * and non-significant differences are indicated by N.S.

Figure 2.7. continued.

B. Uterine sections of WT and C/EBPb-null mice treated with E for 15 h (B, panels a & b) and 18 h (B, panels c & d) were subjected to IHC using an anti-p27 antibody Positive staining for p27 is indicated in red and DAPI-stained nuclei are shown in blue. Nuclear presence of p27 is indicated by white arrowheads in panels b and d. *Bar,* 50 m.

C. IHC analysis of phosphorylated p27 (Thr 187). Positive staining for phospho-p27 is indicated by red deposits.

D. Lysates of LE cells isolated from uteri of mice treated with E for 15 h were analyzed by western blotting using the anti-phospho p27 (Thr187) antibody. Immunostaining of calnexin served as a loading control.

E. Expression levels of p21 mRNA were determined by real-time PCR.

F. LE cells were isolated from uteri of mice treated with E for 0 and 18 h. Cell lysates were analyzed by western blotting using anti-p21 antibodies.

FIG. 2.8.
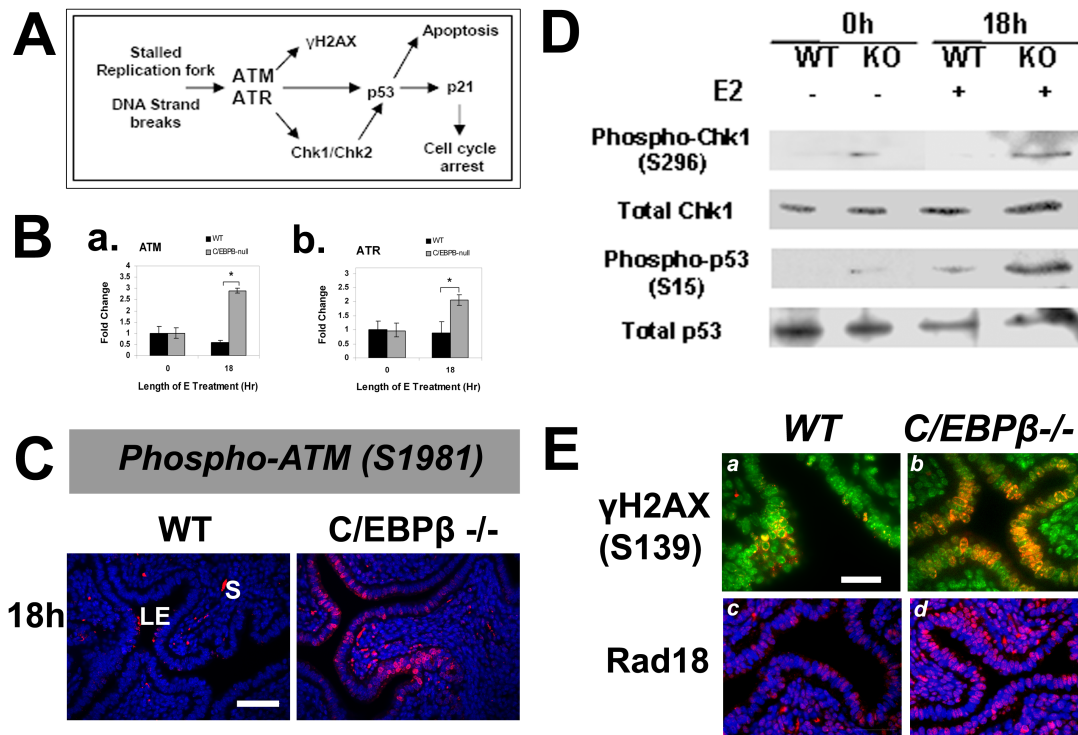
Activation of the DNA damage checkpoint pathway in E-treated C/EBPb-null uterine epithelium

A. A schematic of the major DNA damage checkpoint pathways leading to p53-dependent apoptosis or p21-mediated cell cycle arrest.

B. LE cells were isolated from uteri of WT or C/EBPb (-/-) mice treated with E for 0 and 18 h. Expression levels of ATM (a) and ATR (b) mRNAs were determined by real-time PCR. Statistically significant differences (*P<0.05*) are indicated by *.

C. Uterine sections of WT and C/EBPb-null mice treated with E for 0 and 18 h were subjected to IHC using an antibody specific for phosphorylated Ser 1981 of ATM. Positive staining for phospho-ATM is indicated in red and DAPI-stained nuclei are shown in blue*.* LE:luminal epithelium, S:stroma. *Bar*, 50 mm.

D. Lysates of LE cells, isolated from uteri of mice treated with E for 0 and 18 h, were analyzed by western blotting using antibodies specific for Chk1, phosphorylated Chk1 (Ser296), p53, phosphorylated p53 (Ser15), and p21. Immunostaining of calnexin indicated equal loading in the lanes (data not shown).

E. Sections of uteri collected from WT and C/EBPb-null mice treated with E for 20 h were subjected to IHC using antibodies specific for phosphorylated histone H2A, variant X (g-H2AX, panels a & b) and Rad18 (panels c & d). Positive staining is indicated in red and DAPI-stained nuclei are shown in green. *Bar,* 20 μm.

FIG. 2.9.
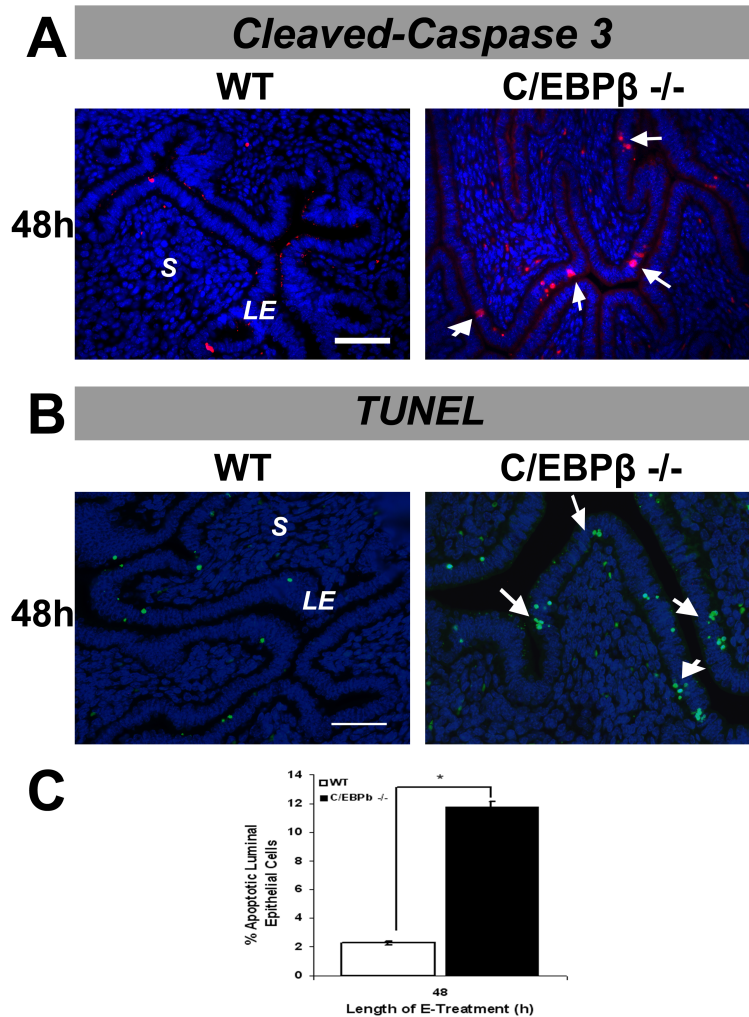
Evidence for increased apoptosis in E-treated C/EBPb-null uterine epithelium

A. Uteri were collected from ovariectomized WT and C/EBPb-null mice treated with E for 48 h. Uterine sections were subjected to IHC using an antibody specific for the cleaved form of caspase 3. Positive staining for cleaved-caspase 3 is indicated in red and DAPI-stained nuclei are shown in blue. Arrowheads indicate cells that stained positive for cleaved-caspase 3 in the uterine LE.

B. Uteri collected from WT and C/EBPb-null mice treated with E for 48 h were sectioned and subjected to a TdT-mediated dUTP nick-end-labeling (TUNEL) assay. Positive staining for apoptotic cells is indicated in green and DAPI-stained nuclei are shown in blue. Arrows indicate apoptotic cells in the uterine LE.

C. Quantitation of epithelial nuclei stained positively in TUNEL assay was performed. The data were plotted as percentage of total epithelial cells present in the fields. Statistically significant differences (*P<0.05*) are indicated by *.

FIG. 2.10
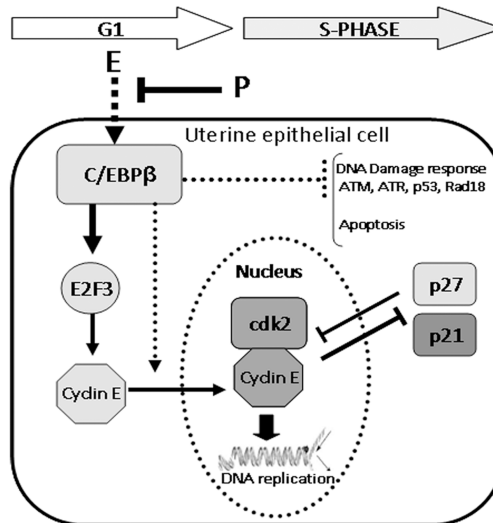
Molecular pathways regulated by C/EBPb during E-induced proliferation of uterine epithelial cells

In mouse uterine epithelium, expression of C/EBPb is stimulated by E and opposed by simultaneous treatment with P. C/EBPb controls entry of E-stimulated epithelial cells into the S phase of the cell cycle by up regulating the expression of E2F3 and cyclin E. It also promotes the nuclear localization of cyclin E, which enables the formation of an active cyclin E-cdk2 complex critical for DNA replication. Lack of C/EBPb allows nuclear accumulation of p27, which contributes to cell-cycle arrest. In the E-stimulated C/EBPb-null uterine epithelium, stalled DNA replication activates a DNA damage response pathway involving ATM/ATR, Chk1/Chk2, Rad18 and p53. The activated p53 maintains the block in DNA replication by enhancing the synthesis of the cell cycle inhibitor p21 and also promotes the removal of damaged cells via caspase-dependent apoptosis.

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CHAPTER 3

C/EBPβ REGULATES THE SYNTHESIS OF A UNIQUE BASAL LAMINA-LIKE EXTRACELLULAR MATRIX THAT PLAYS A CRITICAL ROLE DURING IMPLANTATION

3.1.   ABSTRACT

During implantation, the uterine stroma undergoes a remodeling process known as decidualization. *In vitro*, stromal fibroblasts differentiate into large, polyploid cells that secrete vast amounts of extracellular matrix proteins such as laminins, collagen IV and entactin. Laminins, in particular, are instrumental in the formation of a unique, peri-cellular basement membrane around decidualized cells. We recently uncovered an essential role for the transcription factor C/EBPβ in decidualization. Gene expression profiling of stromal cells revealed that ECM genes such as laminins γ1 and α1, and collagen type IV alpha 2 were regulated by C/EBPβ during decidualization. The present study shows that C/EBPβ transcriptionally regulates the synthesis of laminin γ1 by decidual cells. Additionally, siRNA-mediated silencing of laminin γ1 expression rendered stromal cells incapable of forming a peri-cellular basement membrane and consequently, unable to undergo differentiation. Co-immunoprecipitation studies revealed that laminin γ1 putatively interacts with the integrin β1 receptor on decidual cells. Interruption of laminin γ1-integrin β1 interactions by echistatin or silencing of integrin β1 inhibited stromal differentiation and activation of FAK, a regulator of actin remodeling. To understand the role of laminin γ1 in modulating maternal-fetal dialogue, pre-implantation

uterine stromal cells were co-cultured with blastocysts. We found that blockade of C/EBPβ function prevented embryo outgrowth and invasion in these co-cultures. SiRNA-mediated silencing of laminin γ1 expression was also characterized by loss of an invasive phenotype in trophoblasts. C/EBPβ, therefore, regulates laminin γ1-dependent basement membrane assembly, a structure essential for proper decidualization of stromal cells and pivotal in modulating embryo invasion.

3.2.   INTRODUCTION

The process of embryo implantation into the endometrium involves a series of complex and reciprocal interactions between maternal and fetal tissues. The steroid hormones E and P play a pivotal role in coordinating the events surrounding implantation, by controlling endometrial proliferation and differentiation in a dynamic manner (1). The endometrial stromal differentiation, a process known as decidualization, is triggered by embryo implantation and is unique to the mammalian species (2-4). Terminally differentiated stromal cells undergo remodeling to form a transient, highly specialized tissue mass that completely surrounds the invading embryo and modulates the survival and growth of the fetus. It is hypothesized that the decidual cells synthesize a diverse set of proteins and hormones that signal to the fetus in a paracrine or juxtacrine manner (5-7). During the uterine remodeling process, a variety of extracellular matrix (ECM) proteins are deposited in a pericellular pattern in the decidual tissue (8-10). Prominent among these molecules are laminins, collagen type IV and desmin, which are components

of a prototypical basement membrane or basal lamina and are not found outside this structure. The present study aims to address the regulation and function of this unique basement membrane during decidualization.

The basement membrane is an intricate and dynamic structure that controls cellular functions by regulating the interactions between cells and their surroundings and modulating external stimuli such as growth factors and cytokines (11). Laminins, a large family of glyoproteins, are vital to the biological functions of the basal lamina in many different mammalian tissues and organs. The integrity of this multi-molecular structure depends on the self-polymerization of laminin subunits and the subsequent interactions between the laminins and other ECM molecules (11-14). The assembly of laminins and collagen IV forms a polymeric and insoluble structure, which then recruits other ECM proteins such as nidogen, fibronectin and entactin. Multiple distinct isoforms exist for laminin α, β, and γ subunits. Latest reports indicate that at least fifteen different laminin heterotrimers can be assembled from five α, three β and three γ isoforms (11, 13, 15). Of the laminin subunits, laminin γ1 is especially important because it is found in 10 out of the 15 laminin trimers. Inactivating mutations in the *Lamc1* gene caused early lethality in developing embryos (16). Laminins, collagen type IV, and fibronectin are abundant in pregnant uteri (9, 10). They are also synthesized by endometrial stromal cells cultured *in vitro* (17, 18). It was speculated that the uterine basement membrane serves as a platform for uterine remodeling and embryo invasion during implantation. However, the precise function of this unique basement membrane in endometrial stromal differentiation and implantation still eludes us.

Our lab has recently identified the steroid hormone-regulated transcription factor CCAAT Enhancer Binding Protein Beta (C/EBPβ) as a critical regulator of endometrial function (19-21). We observed that female mice lacking C/EBPβ are infertile due to an impaired decidual response. A closer examination revealed that C/EBPβ-null uterine stromal cells are unable to undergo proper proliferation and differentiation (19). This observation prompted the examination of gene networks that are regulated by this transcription factor in the uterine stromal compartment. Using a DNA microarray approach, we were able to identify putative targets of C/EBPβ in uterine stromal cells isolated from WT and C/EBPβ-null mice. We uncovered several categories of genes from this analysis. Prominent among these genes were those encoding ECM-related molecules involved in basement membrane formation, namely, laminin subunits α1 and γ1, collagen type IV α2 subunit, and fibulins 1 and 2 (Wang, W. et. al, unpublished). These findings raised the possibility that the C/EBPβ-regulated ECM molecules participate in the formation of a unique basement membrane that supports decidualization and implantation in the mouse. We further postulated that the stromal basement membrane critically controls the dialogue between maternal decidual cells and the invading embryo during implantation. Indeed, the current literature is suggestive of a major role of ECM molecules in the basal lamina in modulating embryo growth and survival, underlining the need for further investigation (5, 22, 23). The availability of a powerful *in vitro* primary culture system in which uterine stromal cells undergo differentiation into decidual cells allowed us to address the above hypotheses (24). In this in vitro system, the stromal cells

express high amounts of basal lamina ECM components, making it a suitable experimental model for our studies.

We discovered that C/EBPβ regulates the expression of two key laminin subunits, laminin γ1 and laminin α1, which participate in the formation of a pericellular basal lamina-like ECM. Our study provided evidence for a novel role for this ECM in endometrial stromal differentiation and cytoskeletal remodeling. Furthermore, we found that the basal lamina containing laminin γ1 signals via an integrin β1 complex in the decidual cell membrane to control differentiation. Additionally, we designed a functional *in vitro* culture system in which pre-implantation blastocysts spread into differentiating uterine stromal cells. Using this system, we demonstrated that C/EBPβ-directed formation of uterine stromal basal lamina-like ECM is critical for embryo invasion.

3.3. MATERIALS AND METHODS

Materials

Recombinant mouse laminin-1 was purchased from Trevigen ® (Gaithersburg, MD). The integrin inhibitor, Echistatin, α1 variant, was purchased from Sigma Aldrich (St. Louis, MO). Laminin- and Matrigel™-coated plates (BD-BioCoat™) Cellware were purchased from BD Biosciences (Bedford, MA).

Animals, treatments and tissue collection.

All experiments involving animals were conducted in accordance with the National Institutes of Health standards for the use and care of animals. The animal protocols were approved by the University of Illinois Institutional Animal Care and Use Committee. Female mice (CD-1) were sacrified on day 4 of gestation and uteri collected for isolation of primary stromal cells (PSC). Artificial decidualization (ADR) was induced in WT and C/EBPβ-null mice as previously described (25). Mice subjected to ADR protocol were killed after 48 h and uteri collected for isolation of PSCs.

Isolation of mouse primary uterine stromal cells and *in vitro* decidualization.

Isolation of primary uterine stromal cells (PSC) was carried out as described previously (24). Briefly, uterine horns of day 4 pregnant uteri were dissected longitudinally to expose the lumen and cut into 3-5 mm pieces. Uterine tissue pieces were washed in Hanks Balanced Salt Solution (HBSS) and subsequently incubated in HBSS containing 6 g/L dispase (Invitrogen) and 25 g/L pancreatin (Sigma) for 1 h at RT followed by 10 min at 37°C. Uterine pieces were gently mixed to release uterine epithelial clumps into the supernantant and the supernantant discarded. The partially digested uterine tissue pieces were washed in HBSS and incubated in HBSS containing 0.5 g/L collagenase (Sigma) for 45 min at 37°C. After incubation, tissue pieces were vortexed to release stromal cell clumps into the supernantant. The turbid supernatant containing stromal cells was filtered through a 70 μm cell sieve (BD) to separate digested uterine tissue pieces from cells.

Filtered cells were resuspended in Dulbecco's modified Eagle's Medium-F12 medium (DMEM-F12A; with 100 unit/liter penicillin, 0.1 g/liter streptomycin, 1.25 mg/liter fungizone) containing 2% heat-inactivated fetal bovine serum and live cells were assessed by trypan blue staining using a hemocytometer. Cells (2.5×10^5) were seeded in 6-well cell culture plates. Alternatively, approximately 4×10^4 cells were seeded in 2- or 4-well slide chamber dishes. After attachment for 2-3 h, unattached cells were removed by multiple washes with sterile PBS. Cell culture was continued with addition of fresh medium supplemented with 10 nM E and 1 μM P.

Co-culturing of blastocysts and stromal cells

Female mice (CD-1) were sacrificed on day 4 of gestation and pre-implantation blastocysts were collected by flushing uterine horns and oviducts with sterile PBS. Blastocysts were cultured for ~24 h in 30 μl microdrops of M16 embryo culture medium (Sigma) under mineral oil, at 37°C in 5 % CO_2. PSCs or immortalized Mouse Embryonic Fibroblasts (MEF) were plated into 2-well slide chamber dishes and grown in fresh medium (DMEM-F12A; with 100 unit/liter penicillin, 0.1 g/liter streptomycin, 1.25 mg/liter fungizone, 2% heat-inactivated fetal bovine serum) supplemented with E and P as described above. After 24 h, unhatched blastocysts were carefully transferred onto confluent layers of PSCs or MEFs in culture dishes, at one 1 blastocyst per well. Co-cultures were continued for up to 96 h and outgrowth was examined at various times during the culture period with an Olympus CKX41 inverted microscope and photographed. Embryo attachment was verified by gently shaking the culture dish.

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Immunofluorescence staining for cytokeratin (CK) was performed post-fixation of co-cultures as described below. The total area of embryo outgrowth and spreading was determined from CK-stained samples using Image J software analysis. The final value for each condition was derived from the average of 3-5 independent embryos.

Adenoviral Transductions

An adenoviral vector, expressing the dominant negative A-C/EBP mutant {{183 Olive,M. 1996; }} and under the control of the CMV promoter, was a gift of Dr. Charles Vinson {{184 Bonovich,M. 2002; }}. Control adenoviral vectors lacking an A-C/EBP insert were also obtained from this source. Adenoviral stocks were purified using the Adeno-X™ Virus Mini Purification Kit (Clontech Laboratories Inc.). Concentrations of viral titers were determined in infective units/mL (Ifu/mL) using the Adeno-X™ Rapid Titer Kit (Clontech Laboratories Inc.). PSCs were infected with equal amounts of control or A-C/EBP adenoviral viruses at a multiplicity of infection (MOI) ranging from 2-60 Ifu/cell. After 24 h of infection, cells were induced to differentiate in virus-free medium containing E and P as defined above.

SiRNA transfections

SiRNAs corresponding to mouse laminin γ1 (AAAGGTGTTCAGGCGATTG), integrin β1 (TGCTTGTATACATTCTCCG) and negative control siRNAs that did not anneal to any specific target were pre-designed and synthesized by the Ambion siRNA delivery

resource (Ambion Inc.). Following isolation of PSCs and attachment for 3 h, annealed siRNA duplexes were transfected in the PSCs following the manufacturer's protocol for siLentFect™ Lipid Reagent (Bio-Rad). Briefly, in 6-well plates, 4 μl of SiLentFect transfection reagent was mixed with 20-40 nM of siRNA duplexes in normal differentiation medium (containing E and P) to form complexes and dispersed. In 2- or 4-well slide chambers, 2 ul of SilentFect transfection reagent was mixed with 20-40 nM of siRNA duplexes and dispersed. The transfection was repeated every 24 h.

Real-time PCR analysis

Total RNA was isolated from PSCs by standard Trizol-based protocols and converted to cDNA as described previously in Chapter 2. The cDNA was amplified by real-time PCR to quantify gene expression using gene-specific primers and SYBR® Green (Bio-Rad Laboratories). As a loading control, the expression level of the 36B4 gene, which encodes a ribosomal protein, was determined. For each treatment, the mean threshold cycle (Ct) and standard deviation were calculated from Ct values obtained individually from 3 to 4 replicates of that sample. Each sample was subjected to three independent real-time PCR trials. Fold change was derived from the mean Ct values as described previously in Chapter 2. ANOVA single factor analysis was conducted on the grouped means to determine statistical significance at a significance level of $P<0.05$.

The real-time PCR primer sequences are: C/EBPβ, AGCGACCAGTACAAGATG (forward) and CTGCTCCACCTTCTT (reverse); laminin γ1,

GATGACGCCGACCAGGAC (forward) and GCTGAGGAGGCTGCTGAC (reverse);

laminin α1, CTGAGAGGCGGCTATGTGGAGATG (forward) and

CTGCTGTTCTTGGTGGCGAATGTG (reverse); laminin α2,

TGTATTGGAAGGTCTGGAAG (forward) and GGTCACACTCACATTCTTG

(reverse); laminin α3, GAACTCCGCCTGGTGAAG (forward) and

TGACTTGAGGTGGCAGAAC (reverse); laminin α4, CGCCAGGACAGCCAAGAAC

(forward) and CGCAGGTCATCGGTCAAGTC (reverse); laminin α5,

CTCTTCTATGACTTCGGCTCTG (forward) and CTCCACACGCACCAACAC

(reverse); laminin β1, TGAATGCCTCCACCACAGACC (forward) and

TCCTCCTGCTGCTCCTTGAAC (reverse); PRP, AGTCTGAACTCATCCTGCTTGG

(forward) and TTGATGCAGCTTTCTCCCACAG (reverse); ALP,

CATATAACACCAACGCTCAG (forward) and TGGATGTGACCTCATTGC (reverse);

PR, AGCCAGCCAGAGCCCACAG (forward) and

CCCACAGGTAAGCACGCCATAG (reverse); integrin α1,

CAGCAGCCACTCCAGCAATG (forward) and GGATAACGGTGAGAAGCCAGATG

(reverse); integrin α2, GGGACCTCACAAACACCTTCAG (forward) and

GCTATGCCGAACCTCAGTATCTC (reverse); integrin α6,

CTCTCGTTCTTCGTTCCAGGTTG (forward) and GCAGCAGCGGTGACATCTATG

(reverse); integrin α7, AGCCACTGCCTAACATCTG (forward) and

ACTTACTCCACTATCTCCATCC (reverse); integrin β1,

AGTGAATGGCAACAATGAAGC (forward) and AATCAGCAGCAAGGCAAGG

(reverse); integrin β3, TTGCTACTCTGCTCATCTGG (forward) and

GCTCTGGCTCGTTCTTCC (reverse); 36B4, CTGCTGCCACCACTGCTG (forward)

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and TCCTCATCTGATTCCTCCGACTC (reverse).

Immunocytochemistry (ICC)

Mouse PSCs and blastocysts were fixed (10% formalin) and blocked with a 5% solution of normal donkey serum (Jackson Immunoresearch) in PBS. Sections were incubated with the following primary antibodies diluted in blocking solution (5% NDS, sterile PBS) overnight at 4°C: laminin γ1 (Chemicon & Abcam), laminin α1 (Chemicon), C/EBPβ & ERα (Santa Cruz), PRP (Chemicon), integrin β1 (BD), integrin α6 (Abcam), phospho-Tyr397 FAK (Cell Signaling Technology) and pan-Cytokeratin (Sigma-Aldrich). Samples were washed and incubated with biotin-, Cy3- and DyLight™488-conjugated secondary antibodies (Jackson Immunoresearch Laboratories Inc.) for 60 min. For PRP immunostaining, PSCs were incubated with avidin/biotin complex (Vectastain kit; Vector Laboratories Inc.) for 30 min and subsequently stained with DAB solution (Sigma Aldrich) and counterstained with 3% Methyl Green (Sigma). For immunofluorescence, counterstaining was done using 4',6'-diamidino-2-phenylindole (DAPI; Invitrogen Inc.). Stained cells were mounted in Vectashield fluorescence mounting media (Vector laboratories Inc.). 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 immunocytochemical staining were captured using an Olympus BX51 model light microscope fitted with a Olympus DP71 digital camera (Olympus). In some

samples, fluorescent imaging was performed using a Leica DMR microscope and a Retiga EXI monochrome CCD camera (Qimaging). All images were processed using Adobe Photoshop CS version 8.0.

Western Blotting

Whole cell lysates were prepared from PSCs isolated from day 4 pregnant uteri. Whole cell lysates were subjected to Western blotting as previously described in Chapter 2 using primary antibodies directed against laminin γ1 (Abcam), phospho-Tyr397 FAK (Cell Signaling Technology), ERα & calnexin (Santa Cruz Biotechnology). Chemiluminescence detection was performed using SuperSignal Femto and Pico reagents from Pierce (Thermo Scientific).

Stromal cell membrane isolation and co-immunoprecipitation

Membrane extracts were prepared from PSCs isolated from day 4 pregnant uteri and cultured for 72 h in the presence of E and P. Fractionation of cells into membrane and cytoplasmic fractions was carried out using a well-established protocol. Briefly, cells were homogenized using a non-denaturing lysis buffer C (20 mM HEPES, pH 7.9, 0.05% glycerol, 50 mM sucrose, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA & 2x protease inhibitor cocktail (Roche)). Homogenates were centrifuged at 2000 rpm to separate nuclei from cell extracts. Remaining extracts were centrifuged at 45,000 rpm to separate membrane proteins from cytoplasm. Membrane proteins were resuspended in buffer M (20 mM HEPES, pH 7.9, 0.05% glycerol, 50 mM sucrose, 1.5 mM $MgCl_2$, 20 mM KCl,

0.2 mM EDTA, 2x protease inhibitor cocktail & 1% sodium dodecyl beta-maltoside) and subjected to a well established immunoprecipitation protocol using monoclonal antibodies raised against integrin β1 (BD) or laminin γ1 (Abcam). Immune complexes were recovered with protein A sepharose or protein G sepharose and separated by SDS-PAGE (8%). A western blotting approach was used as described above using antibodies recognizing integrin β1 (BD) or laminin γ1 (Abcam).

Chromatin Immunoprecipitation (ChIP)

The ChIP analysis was conducted using the EZ-ChIP kit (Upstate Biotechnology), according to the manufacturer's protocol. Briefly, mouse PSCs were cultured for 24 h in medium supplemented with E and P, washed in PBS and cross-linked with 1% formaldehyde at room temperature for 10 min. The cross-linked cells were lysed using SDS-lysis buffer and sonicated 5 times for 10 sec each pulse. Lysates were precleared with salmon sperm DNA-protein A and the DNA-protein complexes were subsequently immunoprecipitated using antibodies against RNA polymerase II, rabbit IgG or C/EBPβ (Santa Cruz Biotechnology). The immune complexes were recovered with protein A agarose. The beads were then repeatedly washed and protein complexes were eluted. The cross-linking was reversed and proteins were digested using 0.5 mg/ml proteinase K. Purified DNA were used as templates for real-time PCR using various primer sets to amplify specific regions of the E2F3 promoter.

***In silico* Promoter Analysis**

Putative C/EBPβ binding sites at the *Lamc1* promoter were determined by *in silico* analysis of the proximal promoter region (-1 bp to -1900 bp) using Consite (27) and TESS (28) softwares. Primers were designed to amplify specific regions containing putative C/EBPβ binding sites as follows: -15bp to -23bp (5'-CTG TCA TTT AAC CGG GCA AG-3' & 5'-AGG TCC GAA GAG GAG GAT GT-3'); -94bp to -105bp (5'-GGA TGT TCA AAG CGA GAT GA-3' & 5'-ACC TGG GTA AGC GAT GAC AG-3'); -499bp to -501bp (5'-GTA GCC ACC ACG GTC ACA TT-3' & 5'-AGG ATC GGC CTC GGG ATA C-3'); and -1533 bp to -1544 bp (5'-CTG TCA TTT AAC CGG GCA AG-3' & 5'-AGG TCC GAA GAG GAG GAT GT-3')

Statistical Analysis

Statistical significance was assessed by ANOVA analysis at a significance level of $P < 0.05$ and is indicated by an asterisk in the figures. NS indicates non-significant changes ($P > 0.05$).

3.4.   RESULTS

Primary cultures of uterine stromal cells express classical basal lamina ECM molecules

It was previously reported that in pregnant rodents uterine decidual tissue expresses high amounts of basal lamina ECM proteins such as laminins, collagen type IV and entactin

(9). In order to address the functional role of these unique basal lamina components in decidualization, we utilized a well-established *in vitro* model in which uterine stromal cells differentiate into decidual cells in response to E and P. We hypothesized that decidual cells derived from uterine stromal cells *in vitro* synthesize the same basal lamina ECM proteins produced *in utero*. To confirm this prediction, we measured the mRNA levels of several laminin genes that are reported to be expressed *in vivo*. We observed that the levels of laminins γ1, α1 and α5 mRNAs were specifically and markedly induced during *in vitro* decidualization (Fig. 3.1 A). In contrast, the expression of laminin β1as well as several other laminin α subunits remained largely unchanged. Additionally, we showed that the decidual cells secreted high levels of laminin and collagen type IV proteins into the peri-cellular spaces (Fig. 3.1 B).

Laminin γ1 is a direct target of regulation by C/EBPβ in vivo

We have previously demonstrated that C/EBPβ is robustly induced during early pregnancy and controls decidualization of uterine stromal cells during implantation (19). A DNA microarray analysis conducted using WT and C/EBPβ-null uterine stromal cells revealed that the expression of two laminin subunits, laminin γ1 and laminin α1, is regulated by C/EBPβ (Wang, W. unpublished). We confirmed this observation by measuring the mRNA levels of both laminin subunit genes in stromal cells isolated from WT and C/EBPβ uteri subjected to an artificial decidual stimulus. The expression of laminin γ1 and laminin α1 mRNAs was markedly reduced in C/EBPβ-null uterine stromal cells collected from uteri after 48 h of decidual stimulation (Fig 3.2 A). Due to

the robust down-regulation of laminin γ1 in C/EBPβ null stromal cells, we investigated whether C/EBPβ directly controls the expression of this gene. We examined the *Lamc1* promoter using *in silico* analysis and identified four putative binding sites for C/EBPβ as indicated in Materials and Methods. We then performed chromatin immunoprecipitation (ChIP) analysis to examine C/EBPβ occupancy at these sites. We discovered that there was significant recruitment of this transcription factor to the -94 region of the *Lamc1* promoter during differentiation of stromal cells (Fig 3.2 B).

Inhibition of C/EBPβ function impairs differentiation of primary uterine stromal cells *in vitro*

In order to assess whether C/EBPβ plays a critical role in uterine stromal cells subjected to differentiation *in vitro*, we employed a loss of function approach. A dominant negative mutant (A-C/EBP), previously constructed in Dr. Charles Vinson's laboratory, forms a heterodimer with the endogenously expressed C/EBPβ and prevents its interaction with the target genes (26). To block the transcriptional activities of C/EBPβ, we introduced the A-C/EBP mutant into stromal cells via adenoviral infection methods. Using this strategy, we were able to efficiently suppress C/EBPβ-mediated differentiation in uterine stromal cells infected with adenovirus at an MOI of 6:1 or more (Fig 3.3 A). This was evidenced by the dose-dependant reduction in mRNA levels of two well-characterized biochemical markers of decidualization, prolactin-related protein (PRP) and alkaline phosphatase (ALP). When a control adenovirus containing an empty adenoviral vector (Ad-CTRL) was used, no significant effect on PRP and ALP levels was observed (Fig 3.3 A). As an

additional read-out of differentiation *in vitro*, we examined the morphology and cytoskeletal organization of stromal cells transduced with Ad-CTRL or A-C/EBP (Ad-DN). In decidual cells, the actin cytoskeleton organizes into bundles of short to intermediate filaments that lie parallel to the long axis of the cell (18). When we labeled actin filaments with Alexa Fluor 488-conjugated phalloidin to visualize actin remodeling, cells transduced with Ad-CTRL showed significant cytoskeletal remodeling and appeared large, flattened and cuboidal-shaped, which are characteristics of decidualized cells (Fig 3.3 B, left panel). In contrast, A-C/EBP-treated cells retained a relatively fibroblastic, undifferentiated phenotype and displayed a poorly formed, diffuse network of actin cytoskeleton (Fig 3.3 B, right panel). In control experiments, we ascertained that the levels of endogenous C/EBPβ and estrogen receptor (ERα) proteins remained unchanged in cells transduced with Ad-CTRL or A-C/EBP (Fig 3.3 C).

C/EBPβ regulates the expression of laminin γ1 and laminin α1 in primary stromal cultures

To examine whether the expression of laminin γ1 and laminin α1 is regulated by C/EBPβ in primary stromal cultures, we inhibited C/EBPβ function using the A-C/EBP mutant as described previously. When we compared the mRNA levels of laminin γ1 and laminin α1 in cells transduced with Ad-CTRLor A-C/EBP, we found that there was a significant reduction in expression of both genes in A-C/EBP-treated cells at 24 h and 48 h of the differentiation process (Fig 3.4 A & B). We next examined the levels of laminin γ1 and laminin α1 proteins. Both laminin subunits were deposited in a pericellular fashion by

stromal cells transduced with Ad-CTRL (Fig 3.4 C, panels a & c). We also observed a peri-nuclear deposition of both laminins, the significance of which is unclear to us. The expression levels of both laminin proteins were dramatically reduced when C/EBPβ function was blocked by A-C/EBP (Fig 3.4 C, panels b & d). Collectively, these data suggested that laminin γ1 and laminin α1 are both regulated by C/EBPβ during differentiation of uterine stromal cells *in vitro*.

Laminin γ1 expression is critical for differentiation and cytoskeletal remodeling of uterine stromal cells

The regulation of laminin γ1 by C/EBPβ in uterine stromal cells raised the possibility that this ECM molecule is important for the process of decidualization. Fifteen laminin heterotrimers are known to arise from combinations of single α, β and γ subunits (11, 13, 14). Laminin γ1 is uniquely shared among 10 out of 15 of these combinations, making it a key subunit for laminin assembly. Furthermore, the *Lamc1* null mutation in mice was peri-implantation lethal due to a failure in embryonic basement membrane formation (16). Therefore, it is critical to examine the role of this important laminin gene in decidualization. We began by testing whether siRNA-mediated silencing of Laminin γ1 expression would affect the process of differentiation and cytoskeletal remodeling. This approach would effectively eliminate assembly of all ten laminin γ1-containing trimers in the basement membrane.

Uterine stromal cells were transfected with scrambled siRNA (siCTRL) or siRNA targeted to exon 3 of the laminin γ1 transcript (siLAMC1). The levels of laminin γ1 mRNA and protein were efficiently down-regulated (>80%) upon administration of siLAMC1 (Fig 3.5 A, upper & lower panels). Interestingly, this reduction in laminin γ1 expression caused mRNA levels of two decidual biomarkers, PRP and ALP, to decline by ~50% (Fig 3.5 C), while the levels of C/EBPβ, ERα and progesterone receptor (PR) remained unaffected, (Fig 3.5 B). The decline in the level of PRP mRNA was also accompanied by the reduction in the level of the corresponding protein in siLAMC1-treated cells (Fig 3.5 D). The down-regulation of PRP and ALP is a strong indication that differentiation of stromal cells is impaired when laminin γ1 expression is decreased.

Concomitant with the decrease in differentiation markers, we also observed that the actin cytoskeleton is disrupted when we attenuated laminin γ1 expression. The actin cytoskeleton was disorganized in siLAMC1-treated cells as compared to the siCTRL-treated cells (Fig 3.5 E, panels c & f). Additionally, the overall morphology of siLAMC1-treated cells was not representative of the typical decidual phenotype (Fig 3.5 E, panel f). Collectively, these observations indicated that the siRNA-mediated attenuation of laminin γ1, a critical component of the uterine basement membrane, perturbs the process of decidualization. To test whether we could rescue the loss of a decidual phenotype after laminin γ1 knockdown, uterine stromal cells were treated with recombinant laminin-1 (Laminin α1β1γ1), following siRNA treatment. Upon addition of laminin-1, PRP and ALP mRNA levels could be recovered in a dose-dependent fashion in siLAMC1-treated cells (Fig 3.5 F, panels b & c).

Decidual cells express integrin β1 receptors, which interacts with laminin γ1 during decidualization

The integrin family of receptors serve as receptors for laminin in the cell membrane (11, 29, 30). The α and β subunits of integrins form heterodimers, which transmit signals from the ECM to the cytoplasm. This is made possible through adhesive interactions between integrin receptors and the ECM, and is largely mediated by laminin polymers positioned on the cell membrane. , We hypothesized that the laminins deposited around the decidual cells interact with integrin receptors to activate intracellular signaling pathways.

Integrin β1 is the most prevalent integrin subunit among all reported laminin receptors in the basement membrane (11, 31). When we examined the levels of integrin β1 protein in decidual cells, we found that its localization on the cell membrane (Fig 3.6 B, panels a & b) coincided with sites of focal adhesions indicated by F-actin staining in the same cells (Fig 3.6 B, panels c & d). To identify the integrin alpha subunit that potentially partners with integrin β1 on decidual cell surface, we analyzed the expression levels of several alpha integrins known to interact with integrin β1 and laminin. We found that the mRNAs corresponding to integrins α2 and α6 were significantly up-regulated (Fig 3.6 A). Like integrin β1, integrin α6 was expressed at the cell periphery and in the cytoplasm of differentiating stromal cells (Fig 3.6 B, panels e & f). Based on these observations, it is likely that integrin α2β1 and/or α6β1 are the membrane receptors for laminins during decidualization. To directly test whether laminin γ1 and integrin β1 co-exist in a complex at the cell membrane, we employed a coimmunoprecipitation approach. Using non-

denatured membrane extracts prepared from decidual cells after 72 h of differentiation, we performed immunoprecipitations for either integrin β1 (Fig 3.6 C, panel a) or laminin γ1 (Fig 3.6 C, panel b) and then assessed the presence of each protein in the coimmunoprecipitate. We confirmed by Western blotting that both integrin β1 and laminin γ1 are present in a complex formed at the cell membrane of decidual cells (Fig 3.6 C, arrows).

The interaction between laminin γ1 and integrin β1 is important for stromal differentiation

To test whether an association between the laminin ECM and integrin β1 complex is necessary for decidualization, we utilized two different loss-of-function approaches. In the first approach, cells were treated with the disintegrin, echistatin, a potent antagonist of integrin β1 or β3 binding to ECM (32). Treatment with a non-toxic dose of this inhibitor significantly impaired stromal differentiation (Fig 3.6 D). Actin cytoskeleton remodeling was defective in echistatin-treated cells, and the typical morphology of the decidual cells failed to appear. In parallel experiments, BSA-treated cells displayed cellular architecture characteristic of the decidual phenotype. Additionally, the levels of the differentiation marker, PRP, were reduced upon echistatin treatment (Fig 3.6 E). In the second approach, we silenced integrin β1 expression with a siRNA targeted to its transcript. This intervention led to a moderate decrease in the levels of both differentiation markers, PRP and ALP (Fig 3.6 F). Collectively, these data indicated that when integrin β1 function is abrogated, stromal differentiation and actin remodeling are impaired. We postulate that

the lack of laminin- integrin interactions at the stromal cell membrane contributes to this defect.

Downstream signaling pathways are disrupted when laminin γ1 is silenced in decidual cells

To further test our hypothesis that the association between the laminin-containing ECM and the integrin β1 complex at the stromal cell surface is important for decidualization, we examined the activation of a classical integrin-dependent signaling molecule, focal adhesion kinase (FAK). Among a diverse set of intracellular signaling molecules that are modulated by integrin receptors, FAK is a prominent target, making it an obvious candidate for further investigation in decidual cells (33, 34). FAK is activated through phosphorylation of multiple residues, of which a major one is Tyr^{397}. We first examined the levels of phospho-Tyr^{397}-FAK in decidual cells treated with either siCTRL or siLAMC1. While control cells displayed a typical punctuate distribution of phospho-Tyr^{397}FAK at the cell membrane (Fig 3.7 A, panel a, arrows), the levels of phospho-FAK were significantly reduced in cells where laminin γ1 is silenced (Fig 3.7 A, panel b). This finding was confirmed by Western blotting of decidual cell lysates collected at various times following siRNA treatment (Fig 3.7 B). The levels of ERα, an important decidual factor, did not change with either siRNA treatment. These observations suggest that FAK activation is dependant on the accumulation of the laminin ECM and its interaction with integrin in decidual cells.

Co-culture of pre-implantation blastocysts with uterine stromal cells

The process of embryo implantation is highly dependant on the ability of uterine stromal cells to differentiate and interact with the invading trophoblasts. The current thinking is that factors such as ECM proteins, produced and secreted by the decidua, assist in the establishment of a maternal-fetal interphase that ultimately leads to formation of the uterine vasculature (5, 6). It is challenging to fully decipher the molecular mechanisms underlying this complex *in vivo* process, necessitating the use of *in vitro* models to replicate the process of implantation (5, 22, 23). In order to recapitulate the process of embryo invasion into the decidua, we employed an *in vitro* strategy of co-culturing blastocysts and primary uterine stromal cells (Fig 3.8 A). Using this approach, we were able to monitor the outgrowth of embryos by examining the migration of the outer trophoblast cell layer into the surrounding decidual cell layers (Fig 3.8 B & C). We documented the outgrowth of the embryo at regular intervals for approximately 80 h and observed that viable blastocysts placed on a confluent bed of stromal cells (Fig 3.8 B, panels i & ii) underwent hatching, as evidenced by the shedding of the protective zona pellucida (Fig 3.8 B, panel ii), and attached to the bed of stromal cells by 21 h of co-culture (Fig 3.8 B, panel iii). The attached embryos continued to spread steadily, as evidenced by the increase in overall area of the embryonic tissue (Fig 3.8 B, panels IV-VI). Between 51 h and 80 h of co-culture, embryo outgrowth was especially prominent, as evidenced by the extent of trophoblast migration into the surrounding decidual cells (Fig 3.8 B, panels v and vi). To further define embryo outgrowth, we monitored the spreading of trophoblast cells using antibodies against cytokeratin, an established

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biochemical marker of the trophoblast cell layer (Fig 3.8 C). We observed that cytokeratin-positive trophoblasts migrate away from the embryo and penetrate into the uterine stromal cells over time (Fig 3.8 C, arrows). After 72 h of culture, the migrating trophoblasts closely interfaced with stromal cells (Fig 3.8 C, panel b) and this phenomenon persisted until 96 h of co-culture (Fig 3.8 C, panel c). Collectively, these observations suggested that the co-culturing of maternal and embryonic cells is likely to recapitulate the fundamental aspects of embryo implantation in the mouse. We then utilized this model to understand the functions of C/EBPβ -mediated basal lamina in embryo implantation.

Embryo spreading is most efficient on ECM synthesized by decidual cells

It is thought that embryo outgrowth is dependent on interactions between the trophoblast and the laminin-based ECM produced by the decidualized uterine stromal cells. To determine the specificity of embryo spreading on a decidual ECM, we compared the efficiency of different substrates in promoting embryo spreading. When cultured on glass surface without any matrix coating, blastocysts were able to attach but did not spread (Fig 3.9). However, when placed on undifferentiated mouse embryonic fibroblasts (MEF), blastocysts showed a modest amount of spreading. We next tested embryo outgrowth on various ECM substrates (Fig 3.9). On plates coated with laminin alone, the spreading was about ~40% as effective as on decidual cells (Fig 3.9). We found that collagens type I and type IV are more effective than laminin in promoting embryo spreading and about twice as effective as fibronectin in promoting outgrowth (Fig. 3.9). However, embryo

outgrowth on a collagen IV-coated surface was ~70% as effective as on decidual cells. Finally, we tested embryo outgrowth on matrigel, a solubilized tissue basement membrane substrate containing laminins, collagen type IV, entactin and heparan sulfate proteoglycan. Surprisingly, this combination of ECM molecules was only ~60% as effective as the decidual cells (Fig 3.9). These data strongly suggested that embryos develop and spread most effectively on the ECM of fully decidualized stromal cells. The absence of properly reconstituted ECM limits embryo outgrowth. Collectively, these data suggested that decidual cells synthesize a full complement of ECM components that effectively support embryo penetration.

Blockade of C/EBPβ function in stromal cells prevents embryo spreading and trophoblast invasion

We previously demonstrated that C/EBPβ function is critical for uterine stromal decidualization (Fig 3.3). We next tested the hypothesis that C/EBPβ-mediated differentiation of stromal cells is important for supporting trophoblast outgrowth in our co-culture model. First, we blocked C/EBPβ activity in differentiating stromal cells by introducing the dominant negative mutant, A-C/EBP. Following pre-treatment of stromal cells with Ad-CTRL or A-C/EBP (Ad-DN), we placed unhatched, preimplantation-stage blastocysts on to pre-treated stromal cells. We then closely monitored embryo hatching, attachment and spreading over time. After 48 h of co-culture, blastocysts grown on Ad-CTRL–treated cells exhibited an efficient penetration of trophoblasts into the decidual cells, resulting in embryo spreading (Fig 3.10 A, panel a). However, blastocysts grown on

stromal cells in which C/EBPβ function is inhibited by Ad-DN displayed significantly diminished trophoblast penetration and embryo outgrowth (~60%) (Fig 3.10 A, panel b). These differences were quantified across five independent experiments by measuring the overall area of embryo outgrowth (Fig 3.10 A, panel c). These data served as a proof of principle that the maternal contributions to embryo implantation can be studied in this co-culture model by manipulation of gene function in stromal cells.

Disruption of stromal basal lamina-like ECM inhibits embryo spreading and trophoblast migration

We next investigated whether trophoblast migration and embryo spreading are affected by altering the assembly of basal lamina-like ECM in stromal cells. Stromal cells were transfected with control siRNA or siLAMC1 targeting the laminin γ1 mRNA prior to co-culturing them with blastocysts. Following placement of blastocysts on siRNA-treated cells, the co-cultures were monitored for 48 h after which they were fixed and analyzed for embryo outgrowth and trophoblast migration. Blastocysts grown on cells treated with control siRNAs displayed an expected extent of spreading and trophoblast penetrance into the decidual cells (Fig 3.10 B, panel a). In contrast, blastocysts displayed a pronounced reduction in spreading when placed on stromal cells in which laminin γ1 was silenced by siLAMC1 (Fig 3.10 B, panel b). This observation was repeated in five independent experiments and the embryo spreading was quantified. The results revealed that blastocysts cultured on siLAMC1-treated stromal cells were spreading ~60% less efficiently than on cells treated with control siRNA (Fig 3.10 B, panel c). Collectively,

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these results demonstrated the importance of the basal lamina-like stromal ECM in promoting embryo growth and trophoblast migration.

3.5.   DISCUSSION

The process of endometrial remodelling during implantation involves the differentiation of stromal cells, establishment of maternal vasculature and a remarkable transformation of the ECM within the stromal compartment. In this study, we have attempted to shed new insights into the role of the uterine basal lamina in implantation by utilizing *in vitro* approaches. We have established a novel molecular link between C/EBPβ, a major regulator of endometrial function, and laminin synthesis in uterine stromal cells. We demonstrated that when laminin synthesis is interrupted, uterine stromal differentiation is perturbed due to morphological and biochemical defects. We further showed that C/EBPβ and laminin are both critical for embryo invasion into decidual cells *in vitro*.

C/EBPβ is an important regulator of uterine stromal differentiation and stromal ECM remodeling.

Upon embryo penetration into the uterus, fibroblastic stromal cells transform into morphologically and biochemically distinct decidual cells that synthesize large amounts of basement membrane ECM molecules. Previous reports have documented that coincident with decidua formation, the formation of a unique basal lamina-like ECM, containing laminin γ1, laminin α1 and collagen type IV α2 subunit, was markedly

induced (8-10). The expression of these molecules appeared to spread throughout the decidual zone. They were assembled into a peri-cellular ECM network, which surrounded the invading embryo. Laminin γ1, in particular, was ubiquitously expressed throughout the entire decidual transformation (10). Because of the unique setting of this basement membrane ECM in the uterine tissue, it is plausible that a major function of this ECM is to support the differentiation of uterine stromal cells. Another possibility is that the uterine basal lamina provides the guidance cues that an embryo needs in order to implant correctly in the uterus (5, 10). In this study, we focused our efforts in understanding the role of C/EBPβ in stromal basal lamina formation. C/EBPβ regulates the expression of laminin γ1 and laminin α1 and is recruited to the *Lamc1* promoter during stromal differentiation (Fig 3.1). Furthermore, the expression levels of both laminin γ1 and laminin α1 were down-regulated upon blockade of C/EBPβ function (Fig 3.4).

These observations are consistent with the notion that ECM production by decidual tissue is a highly regulated process. Indeed, it is well known that steroid hormones induce stromal cells to produce paracrine factors that drive decidualization and regulate ECM synthesis and secretion. However, to date, the gene regulatory mechanisms controlling the production of this ECM and its function have remained unclear. Our study indicates that the steroid hormone-regulated production of the basal lamina-like ECM is mediated via C/EBPβ. We demonstrated that uterine stromal cells failed to differentiate adequately *in vitro* when C/EBPβ function is inhibited (Fig 3.3). Gene expression profiling of wild type and C/EBPβ-null stromal cells has led to the identification a subset of genes involved in stromal ECM formation during decidualization. These data, together with our

microarray findings, are the first demonstration that C/EBPβ regulates ECM production during stromal differentiation. The contribution of this unique decidual ECM to the differentiation process is likely to be an important aspect of uterine functions. To specifically test this concept, we investigated the role of laminin γ1 in uterine stromal differentiation.

Laminin γ1 is a critical component of the stromal basal lamina-like ECM and is necessary for decidualization.

The basement membrane is a highly specialized and complex structure that interacts with cell surfaces within a wide variety of mammalian tissues. Previously thought to be an inert structure, the basement membrane has been receiving more attention recently due to its dynamic regulatory roles in a variety of cellular processes such as proliferation, differentiation, morphogenesis and apoptosis (11, 36, 37). Several lines of evidence have indicated that laminin heterotrimers ($\alpha_x\beta_x\gamma_x$) catalyze the assembly of basement membranes (11, 38). The laminins polymerize with other laminin heterotrimers through their NH_2-terminal arms, forming a continuous layer upon which collagen type IV, nidogen, entactin and perlecan assemble (36). Furthermore, the genetic ablation of the *laminin γ1* gene, an essential laminin subunit in most of the laminin heterotrimers, leads to failures in embryonic differentiation, embryonic tissue organization and Schwann-cell differentiation (16, 37, 39). Previous reports also showed that trimeric laminins could only acquire full functionality upon the assembly of laminin α chains with their β and γ chain partners (42). These observations underline the pivotal role of laminin γ1 in basal lamina formation and further emphasize the importance of this ECM structure in diverse

cellular processes. In the present study, we have used a uterine stromal cell culture system to uncover the role of laminin γ1 and the basal lamina-like matrix in stromal decidualization. When we silenced laminin γ1 gene expression by RNAi, we observed that uterine stromal cells were unable to differentiate properly into decidual cells (Fig 3.5). This defect was characterized by severe morphological abnormalities in stromal cells, a failure in actin cytoskeletal remodeling and a reduction in the levels of classical biochemical markers of decidualization. When laminin-1 ($\alpha_1\beta_1\gamma_1$) was added exogenously to cells in which laminin γ1 was silenced, stromal differentiation was partially rescued, as measured by the recovery of PRP and ALP levels (Fig 3.5). This result further underscored the importance of laminins in uterine stromal differentiation. Collectively, these observations support the concept that the assembly of the basal lamina-like ECM is critical for stromal differentiation. In previous reports by Klaffky *et al*. and Miner *et. al.*, the composition of laminins in the uterine basal lamina was surveyed during the early post-implantation period. It was observed that laminins α5, β1 and γ1 were especially enriched in the decidualized stromal matrix (40, 41). These studies concluded that the laminin heterotrimers present at the onset of implantation are laminins 10 and 11. Most strikingly, both of these trimeric laminins contain laminin γ1. We have also observed that laminin α5, a subunit of laminins 10 and 11, is expressed in decidual cells and its spatiotemporal expression overlaps with that of laminin γ1 (Fig 1, data not shown). We postulate that the production of laminins γ1 and α5 during stromal differentiation may permit formation of laminins 10 and 11 by decidual cells. Therefore, in the context of earlier observations, our findings are supportive of an important role for laminin γ1 in *in vitro* stromal differentiation.

Signaling mechanisms controlled by laminins during decidualization.

Laminins in the basal lamina ECM interact with membrane-bound receptors, such as integrins, to modulate intracellular signaling pathways and influence cell behavior. There is considerable redundancy among the potential integrin receptors for laminins in the basement membrane. However, integrin β1 is the most abundant subunit among the laminin-interacting integrin receptors (11). Based on the previously published reports that differentiating uterine stromal cells synthesize laminins 10 and 11, we reasoned that these laminins serve as ligands for integrin receptors present on the cell membranes to drive stromal differentiation. A survey of the expression levels of the individual integrin subunits that are able to interact with these two laminin trimers predicted that integrin subunits α1, α2, α6 and β1 are likely to be the candidate laminin-binding components on the decidual cells (Fig 3.6). These integrin subunits can dimerize into active integrin αβ membrane-bound receptors (α1β1/α2β1/α6β1) (11). Laminin heterotrimers will then interact with integrin heterodimers via the globular tail domains present in the laminin α chain. Based on the abundance of integrin α6 and β1 proteins in decidual cells (Fig 3.6 A), and in accordance with previous literature, we propose that integrin α6β1 is a major receptor for laminin (30). To support this notion, we have demonstrated via co-immunoprecipitation studies that laminin γ1-containing laminins interact with integrin β1 on decidual cell membranes (Fig 3.6 C). Specifically, we propose that laminin γ1 heterotrimerizes with laminin α1 or α5 chains and that the association with integrin β1 is mediated by the globular domain of the alpha chains. On a functional level, the inhibition of laminin-integrin interactions by echistatin, or the silencing of integrin β1 expression

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resulted in the loss of differentiation of stromal cells. These findings nicely correlate with integrin β1's proposed role as a laminin receptor. Additionally, mice carrying deletions in integrin β1 or laminin γ1 are both peri-implantation lethal due to failures in embryonic basement membrane formation, suggesting a strong connection between the functions of these two proteins (16, 43, 44). The impairment in decidual phenotype observed upon interruption of laminin γ1 or integrin β1 function in stromal cells is further evidence that this integrin subunit is required for proper laminin assembly and function.

We also demonstrated that an attenuation of laminin γ1 function resulted in the down-regulation of FAK activity (Fig 3.7). FAK is a critical component of the integrin-mediated signaling network. Upon activation by phosphorylation, it is responsible for the modulation of actin remodeling via Src kinase and/or MAP kinase pathways in various cell types (31, 33, 34). We propose that the association of laminin and integrin triggers FAK activation that permits actin remodeling. Coincidentally, the localization of integrin β1 on decidual cells closely overlaps with sites of actin bundling and focal adhesion formation (Fig 3.6 B). When we interrupted laminin γ1 expression in uterine stromal cells by RNAi, we also disrupted laminin-integrin interaction. As a result, FAK activation was inhibited and stromal cells were unable to assemble actin cytoskeleton properly, failing to form focal adhesions. Therefore, our studies uncovered a signaling mechanism by which stromal ECM containing laminin γ1 transmits information to the interior of the cell via the integrin receptor. It was proposed previously that as cells synthesize increasing amounts of laminin, cooperative interactions between laminin and integrin receptors are strengthened and promote cytoskeletal re-organization to instruct cell behavior (36). We

hypothesize that a similar ECM-driven transformation occurs in differentiating uterine stromal cells during the decidualization process.

Laminin plays a vital role in mediating maternal-fetal interactions during implantation.

From our experiments involving co-cultures of blastocysts and uterine stromal cells, we have obtained three major insights. First, we were able to show that placement of pre-implantation blastocysts with differentiating uterine stromal cells successfully recapitulates certain aspects of the *in vivo* implantation, such as embryonic growth and trophoblast migration. Second, we demonstrated that C/EBPβ-mediated differentiation of stromal cells is critical for embryo spreading and penetration into these cells. The loss of C/EBPβ funtion in stromal cells, in addition to its profound effect on decidualization, appears to affect the ability of trophoblasts to invade into and interact with the stromal matrix. Finally, our studies have revealed that basal lamina in the stromal ECM plays a critical role, in modulating embryo spreading and invasion.

It was proposed by several groups that the uterine basement membrane is a major facilitator of maternal-fetal interactions during the post-implantation phase. By directly interacting with the mural trophoblast layer of the blastocyst, the laminins and collagen IV networks in the decidualized matrix modulate trophoblast migration and infiltration into the stromal compartment (5, 22). During these events, trophoblast cells express integrin receptors, such as integrin α7β1, which are the primary embryonic receptors for

the laminin trimers present in the uterine basement membrane (40). A majority of these predictions were confirmed through the use of *in vitro* models in which blastocysts were cultured on surfaces coated with laminin, collagen IV or fibronectin and subsequently, trophoblast behavior was assessed. To add to these studies, we analyzed peri-implantation events by examining how blastocysts behave when cultured together with differentiating stromal cells harvested from the same uterine tissue (Fig 3.8). This approach was previously utilized when human endometrial stromal cells were differentiated and grown together with blastocysts (45, 46).

A major challenge in the field is to decipher the mechanisms by which the trophoblasts adhere to and interact with uterine stromal ECM proteins. We reasoned that as differentiation progressed, stromal cells would secrete laminin γ1 and other basal lamina components into the pericellular space, allowing for their direct contact with mural trophoblast cells of the blastocyst. As a result of this intimate interaction, a reciprocal relationship is established between decidual cells and the embryo, permitting trophoblast invasion into the decidual cells and the advancement of both the decidual and embryonic development programs (5, 6). In support of this concept, we observed that if we inhibit stromal differentiation by blocking the function of C/EBPβ, the trophoblast cells do not receive the maternal signals that allow them to acquire an invasive behavior (Fig 3.10A). We interpreted this defect as a cumulative outcome of several molecular mechanisms disrupted by the blockade of C/EBPβ function. The interruption of C/EBPβ-regulated laminin γ1 synthesis would result in defective basement membrane formation and the loss of an intimate association between trophoblasts and decidual cells. Our results confirmed

this idea when we silenced laminin γ1 expression and witnessed a significant loss of embryo spreading and trophoblast invasion (Fig 3.10 B & C). Collectively, our studies regarding the role of laminin γ1 in embryo invasion provide novel mechanistic insights about how trophoblasts interact with uterine cells. Furthermore, our results provide a view of the interactions observed between trophoblasts and uterine ECM *in vitro*, which would likely correspond to interactions observed between invading trophoblast giant cells and decidua *in vivo*. It will be of further interest to understand how the laminins in the stromal basement membrane interact with integrin receptors in the embryo.

The uterine basement membrane is a complex mixture of ECM proteins and the contribution of each component to embryo invasion is of tremendous interest. A study by Sutherland *et. al.* showed that when blastocysts were placed on culture plates coated with laminin or collagen or fibronectin, they displayed fairly similar patterns of outgrowth and spreading (23). Our studies showed that blastocysts adhered to and spread on a decidualized stromal matrix most efficiently, followed by collagen type IV-coated surfaces. They also showed a moderate ability to spread on laminin-1 alone or on Matrigel, which resembles a basal lamina type of ECM (Fig 3.9). Our studies are generally in agreement with previous reports by Sutherland *et. al.,* describing the spreading of blastocysts on collagen IV and laminin. Most interestingly, blastocysts did not spread very effectively when placed on proliferating mouse embryonic fibroblasts (MEF), underlining the requirement for specific maternally-derived matrices or signals to modulate embryo invasion.

In conclusion, this study shows that uterine stromal cells undergo decidualization, producing a unique basal lamina-like ECM. This process is controlled by the steroid hormone-regulated factor, C/EBPβ. Our study has uncovered a novel mechanism by which C/EBPβ regulates the assembly of the stromal basement membrane by controlling the synthesis of laminin γ1, a critical component of this structure. Our results further demonstrated that the basal lamina-like ECM containing laminin γ1 has multiple functional roles during decidualization. It controls stromal differentiation and the accompanying cellular architecture necessary for decidualization. It is also a key regulator of the critical interactions between maternal and fetal tissues during implantation. In the future, it would be important to develop mouse models with uterus-specific mutations in various ECM components in the stroma to precisely delineate the mechanisms that regulate maternal-fetal interactions during pregnancy.

3.6. ACKNOWLEDGEMENTS

FIG. 3.1.
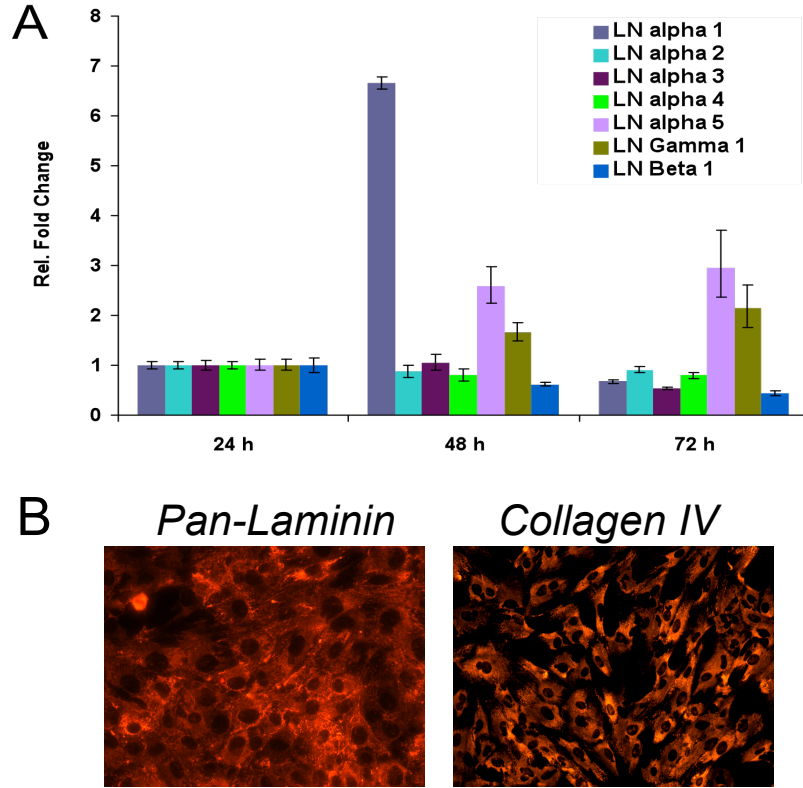
Figure 3.1 Uterine decidual cells express basal lamina ECM *in vitro*

PSCs were isolated from pre-implantation day 4 (D4) pregnant mouse uteri, plated for 3 h and subsequently differentiated with E and P for 24, 48 and 72 h.

A. After E and P treatment, total RNA was isolated for real-time PCR analysis. Relative expression levels of laminins γ1, a1, a2, a3, a4, a5 and β1 mRNAs were determined. The fold changes for each timepoint indicate gene expression relative to 24h E+P-treated-PSC.

B.. Cells were fixed after 48 h of E and P treatment and subjected to immunofluorescence using antibodies recognizing all laminins (Pan-Laminin) and Collagen type IV (Collagen IV).

FIG. 3.2.

A

B
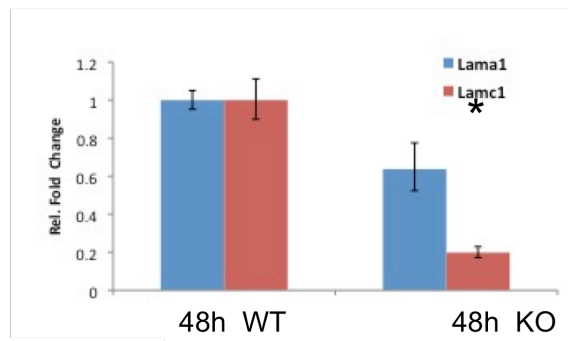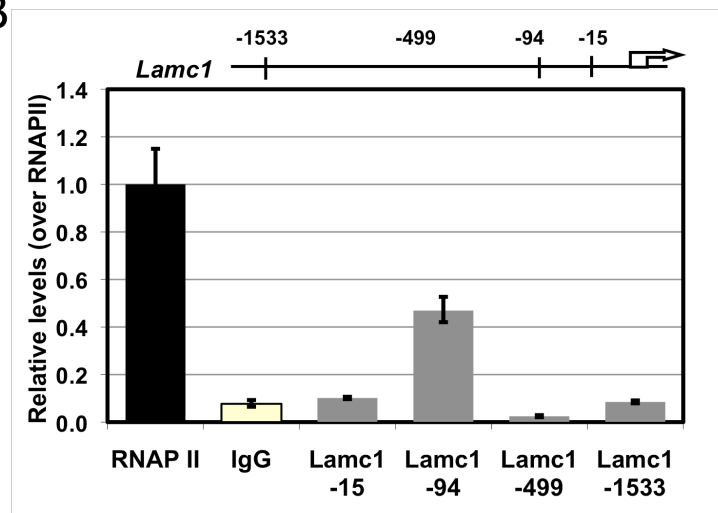
Figure 3.2. C/EBPβ regulates laminin γ1 and α1 during uterine stromal differentiation

A. WT and C/EBPβ-null mice were subjected to artifical decidualization protocol as described in Materials and Methods. Uteri were collected after 48 h after decidual stimulation and primary stromal cells (PSC) isolated as described in Materials and Methods. Total RNA was analyzed by real-time PCR using specific primers against laminin γ1 (Lamc1) and laminin α1 (Lama1). The fold changes indicate gene expression levels in C/EBPβ-null cells (KO) relative to wild-type (WT) cells. Statistically significant differences (*P<0.05)* are indicated by *.

B. ChIP analysis: PSCs were isolated from pre-implantation day 4 (D4) pregnant mouse uteri, plated for 3 h and subsequently differentiated with E and P for 24 h. ChIP was performed as described in Materials and Methods using antibodies against C/EBPβ, RNA polymerase II (RNAP II) and rabbit IgG. Relative levels of recruitment at various sites on the Lamc1 promoter were determined by real-time PCR and normalized to input DNA and RNAP II values.

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FIG. 3.3.

A

B

C

Figure 3.3. C/EBPβ regulates uterine stromal cell differentiation

PSC were isolated from D4 pregnant uteri and attached for 3 h. Cells were then transduced with adenovirus particles carrying either an empty vector (Ad-CTRL) or a vector expressing a dominant negative mutant of C/EBPβ, A-C/EBP (Ad-DN) for 24 h. Adenoviral treatment was conducted at MOIs of 0, 6, 12, 25, 50 and 60 as described in Materials and Methods. After adenovirus removal, PSC were treated with E and P for 72h and total RNA isolated for real-time PCR analysis.

A. Relative expression levels of PRP and ALK-P mRNAs were determined. The relative fold change for each adenoviral treated sample is represented as compared to PSC treated with an MOI of 0.

B. Cells were fixed and subjected to immunofluorescence using Alexa-fluor-Phalloidin to label actin filaments (F-actin). DAPI-stained nuclei are shown in blue. **C.** Cells were fixed and subjected to immunofluorescence using antibodies recognizing C/EBPβ and ERα.

FIG. 3.4.
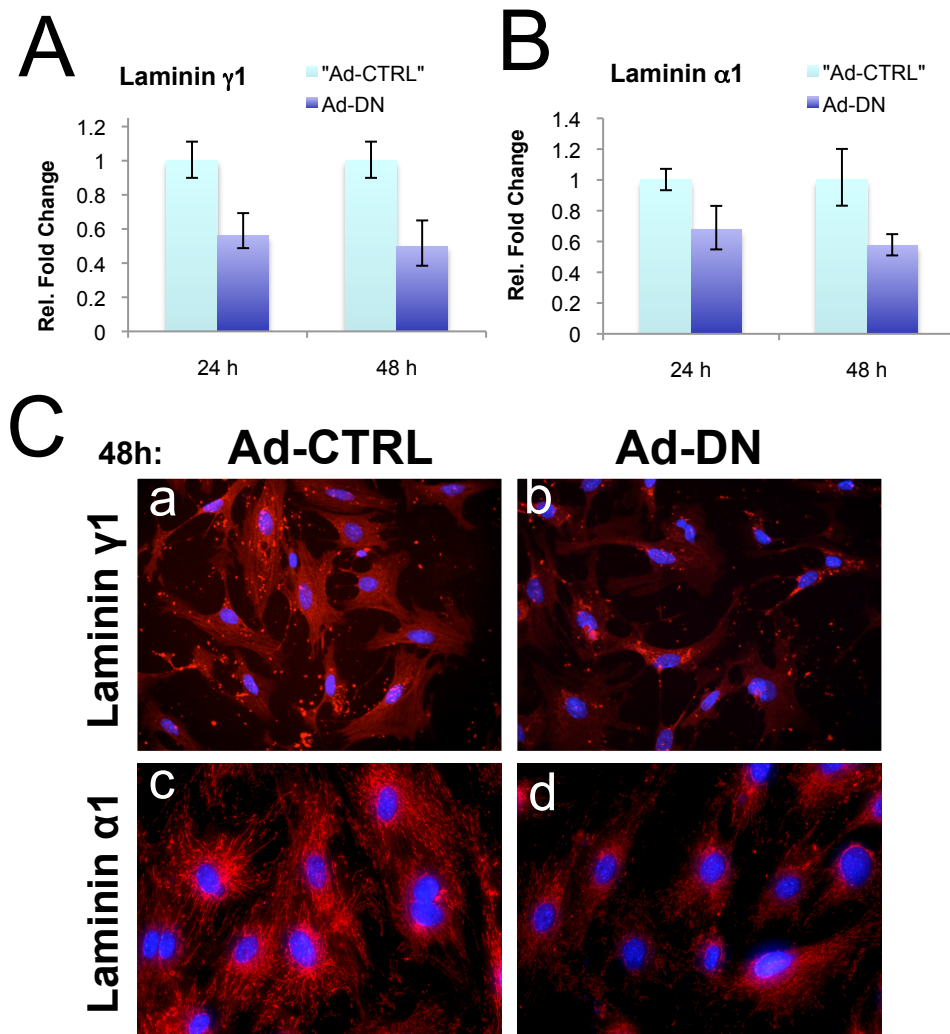
Figure 3.4. C/EBPβ regulates the synthesis and secretion of laminins γ1 and α1 during stromal cell differentiation
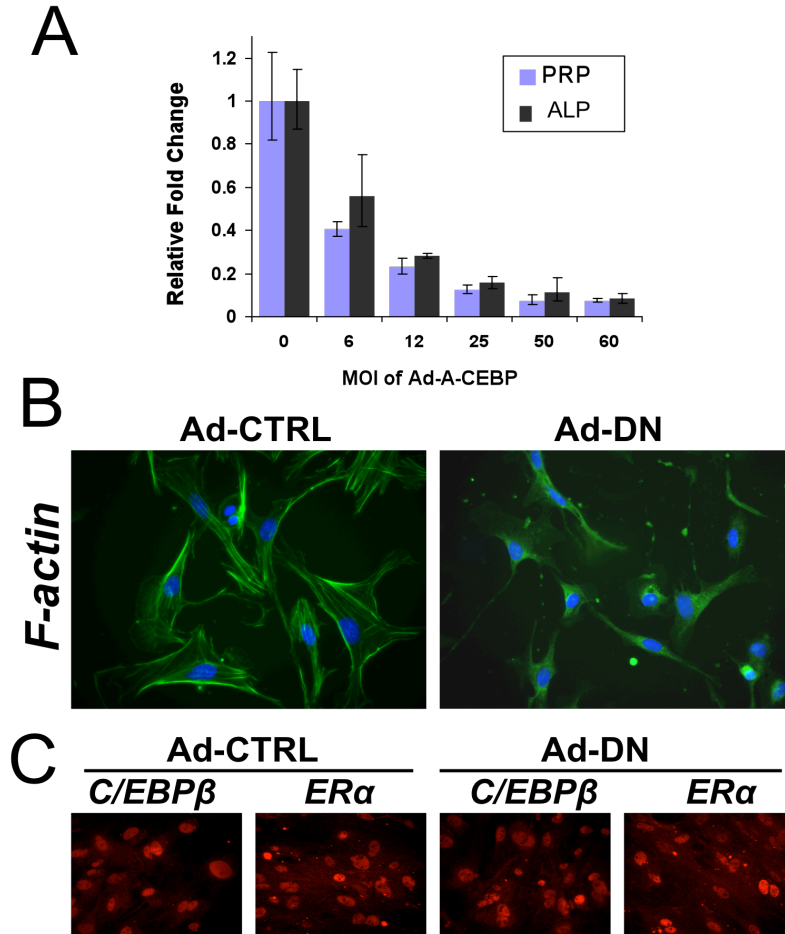
PSC were isolated from D4 pregnant uteri and attached for 3 h. Cells were then transduced with adenoviral particles carrying either an empty vector (Ad-CTRL) or a vector expressing a dominant negative mutant of C/EBPβ, A-C/EBP (Ad-DN) for 24 h. After adenovirus removal, PSC were treated with E and P for 24, 48 and 72 h and total RNA isolated for real-time PCR analysis using primers specific to laminin γ1, **A,** and laminin α1, **B**.

C. Alternatively, after adenovirus removal, PSC were treated with E and P for 48 h and subsequently fixed and subjected to immunofluorescence using antibodies recognizing laminin γ1 and laminin α1.
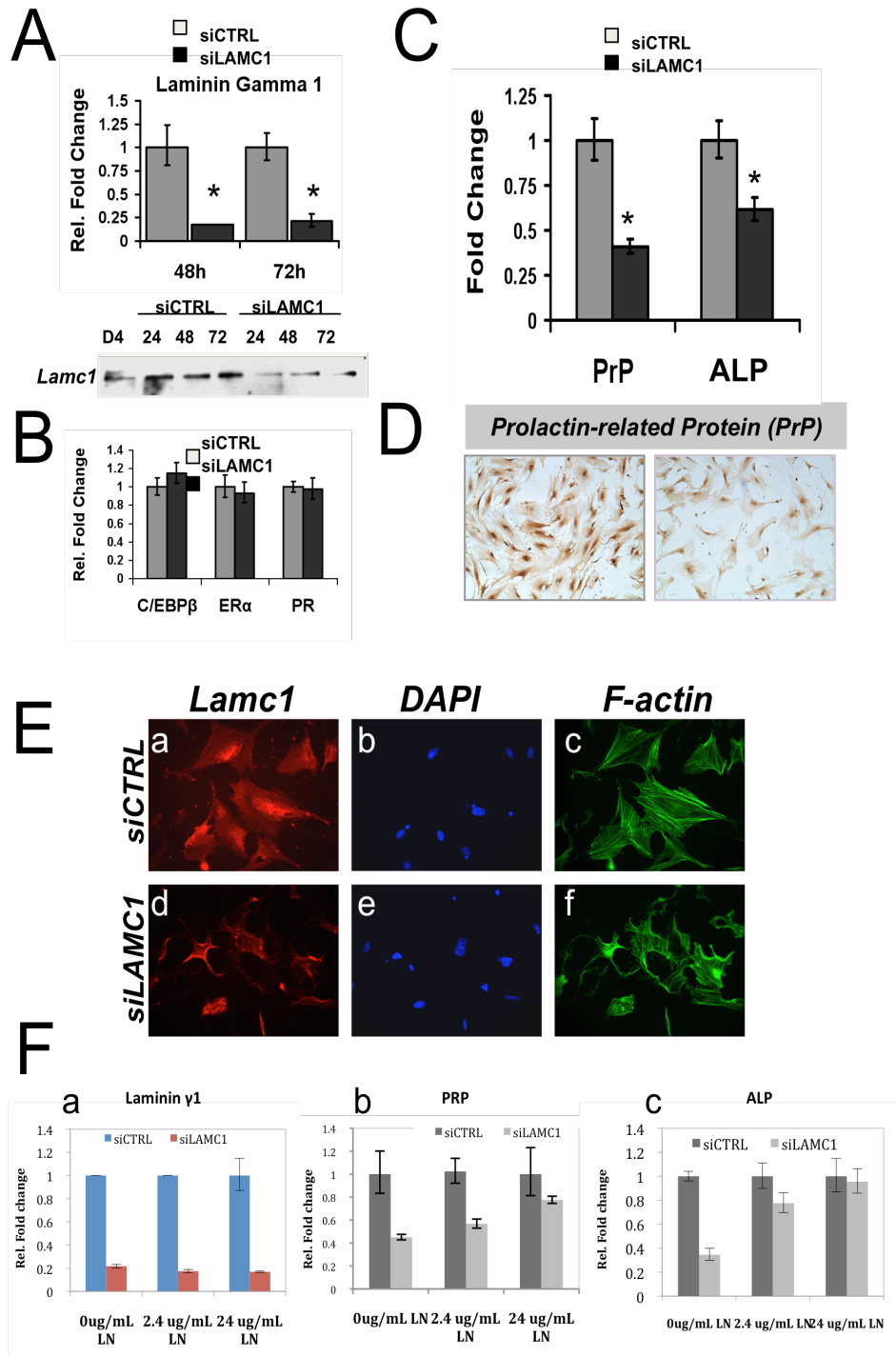
FIG. 3.5.

Figure 3.5. SiRNA-mediated silencing of laminin γ1 expression inhibits stromal differentiation and cytoskeletal remodeling.

Figure 3.5. continued:

PSC were isolated from D4 pregnant uteri and attached for 3 h. Cells were then transfected with 20 nM negative control siRNAs (siCTRL) or siRNAs targeted against laminin γ1 (siLAMC1) as described in Materials and Methods.

A, *Upper* After 48 and 72 h, total RNA was collected and real-time analysis was conducted using primers specific to laminin γ1. Fold changes indicate gene expression levels in siLAMC1-treated PSCs relative to siCTRL-treated PSCs. ***Lower,*** Western blotting analysis of laminin γ1 protein levels in whole-cell lysates prepared from PSC isolated from D4 pregnant uteri, and from siRNA-transfected PSC after 24, 48 and 72 h.

B. Real-time analysis of C/EBPβ, ERα and PR mRNA levels at 72 h, using RNA collected from PSCs transfected with siCTRL or siLAMC1.

C. Real-time analysis of PRP and ALP mRNA levels at 72 h, using RNA collected from PGCs transfectd with siCTRL or siLAMC1.

D. SiRNA-treated PSCs were fixed after 72 h of transfection and subjected to immunocytochemical analysis to monitor PRP protein levels.

E. PSCs were transfected with siCTRL or siLAMC1 for 48 h, fixed and subjected to immunofluorescence analysis using an antibody recognizing laminin γ1 (panels a & d). DAPI-stained nuclei are shown in blue (panels b & e). Actin filaments (F-actin) were labeled with Alexa-fluor-phalloidin (panels c & f).

F. PSCs were transfected with siCTRL or siLAMC1 for 48 h following which siRNAs were removed and stromal culture continued in the absence or prescence of 2.4 ug/mL and 24 ug/mL of recombinant laminin-1 for an additional 24 h as described in Materials and Methods. Total RNA was collected and subjected to real-time PCR using primers specific to laminin γ1 (panel a), PRP (panel b) and ALP (panel c).

FIG. 3.6.
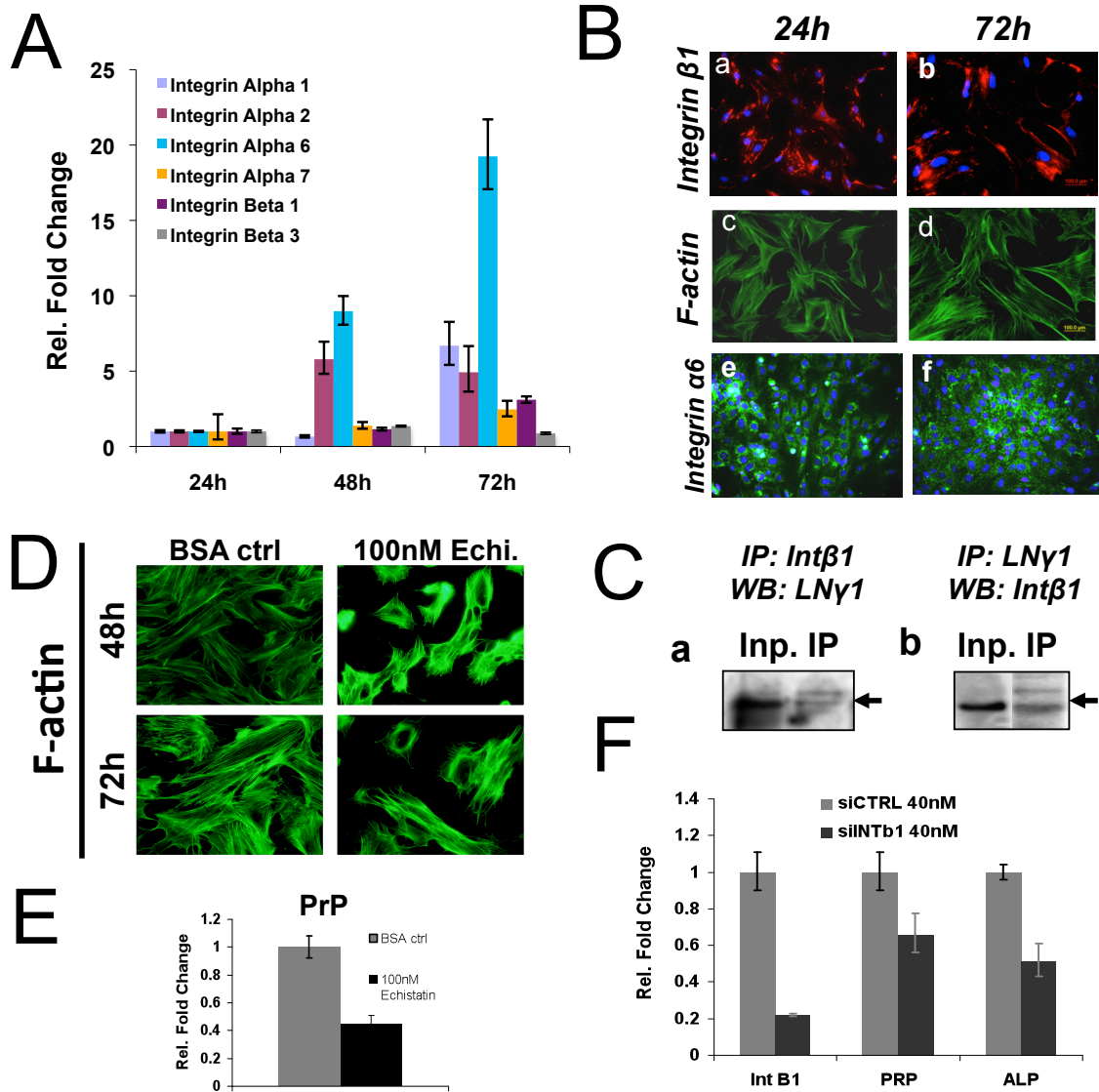
Figure 3.6. Differentiating stromal cells express integrins receptors for laminin γ1

PSC were isolated from D4 pregnant uteri and attached for 3 h. Cells were then treated with E and P for 24, 48 and 72 h.

A. Total RNA was collected at each timepoint for real-time PCR analysis using primers specific to integrin α1, integrin α2, integrin α6, integrin α7 and integrin β3. Fold changes indicate relative gene expression levels at each timepoint relative to 24 h.

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Figure 3.6. continued:

B. E and P-treated PSC were fixed and subjected to immunofluorescence analysis using antibodies recognizing integrin β1 (panels a & b) and integrin α6 (panels e and f). Actin filaments (F-actin) were labeled with Alexa-fluor-phalloidin (panels c & d). DAPI-stained nuclei are shown in blue.

C. PSC were treated with E and P for 72 h, following which, non-denatured, solubilized cell membrane extracts were prepared as described in Materials and Methods. Immunoprecipitation was performed on extracts using monoclonal antibodies recognizing integrin β1 (panel a) and laminin γ1 (panel b). Subsequently, Western blotting analysis was performed using antibodies recognizing laminin γ1 (panel a) and integrin β1 (panel b). Arrows indicate the presence of a co-immunoprecipitated protein.

D. PSC were isolated from D4 pregnant uteri and attached for 3 h. Subsequently, cells were treated with growth medium containing either BSA (100nM) or echistatin (Echi.), (100 nM). After 48 h and 72 h, cells were fixed and subjected to immunofluorescence using Alexa-fluor-phalloidin to label F-actin.

E. Real-time PCR analysis of PRP mRNA levels in BSA and echistatin-treated PSCs.

F. PSC were isolated from D4 pregnant uteri and attached for 3 h. Cells were then transfected with siRNA (40 nM) targeted against a scrambled, non-coding sequence (siCTRL) or integrin β1 (siINTb1) as described in Materials and Methods. After 48 h, total RNA was collected and real-time analysis was conducted using primers specific for integrin β1, PRP and ALP. Fold changes indicate gene expression levels in siLAMC1-treated PSCs relative to siCTRL-treated PSCs.

FIG. 3.7.

A Phospho-FAK (Y397)

siCTRL   siLAMC1

B

siCTRL   siLAMC1

D4   24   48   72   24   48   72

Lamc1

p-FAK (Y397)

ERα

Calnexin
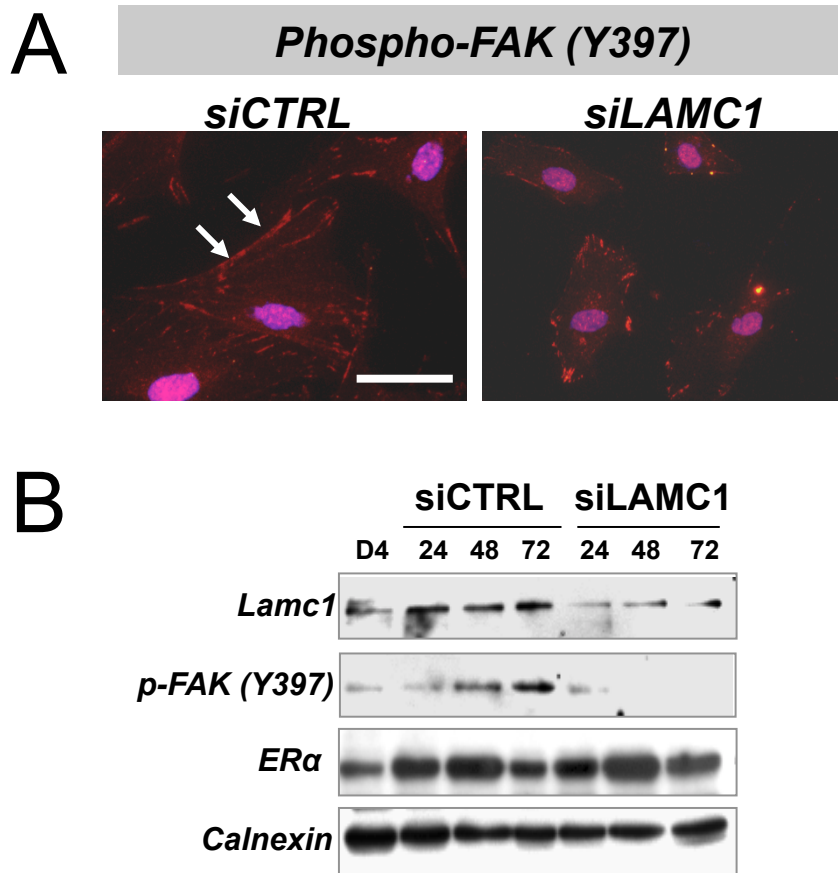
Figure 3.7. Focal Adhesion Kinase (FAK) activation is controlled by laminin γ1 during stromal differentiation.

PSC were isolated from D4 pregnant uteri and attached for 3 h. Cells were then transfected with siRNA (20 nM) targeted against a scrambled, non-coding sequence (siCTRL) or laminin γ1 (siLAMC1) as described in Materials and Methods.

A. After 48 h transfection with siCTRL or siLAMC1, cells were fixed and subjected to immunofluorescence using antibodies recognizing phosphorylated Tyr397-FAK. DAPI-stained nuclei are shown in blue.

B. Whole-cell lysates were prepared from PSC isolated from D4 pregnant uteri and from siRNA-transfected PSC after 24, 48 and 72 h of E and P treatment. Protein levels of laminin γ1, phosphorylated Tyr397-FAK, ERα and calnexin were analyzed by Western blotting using specific antibodies.

FIG. 3.8.
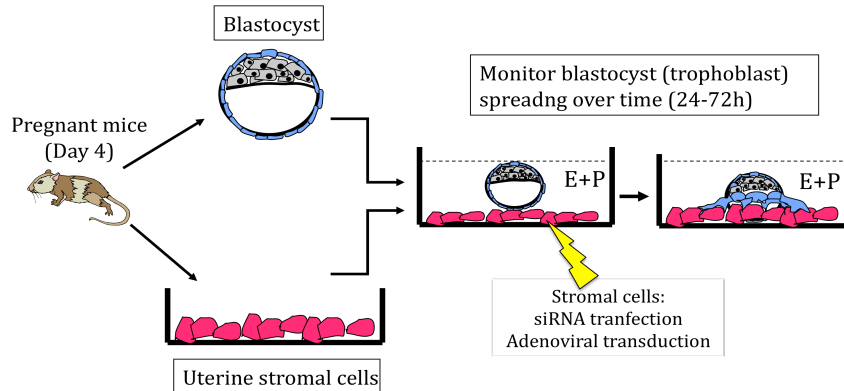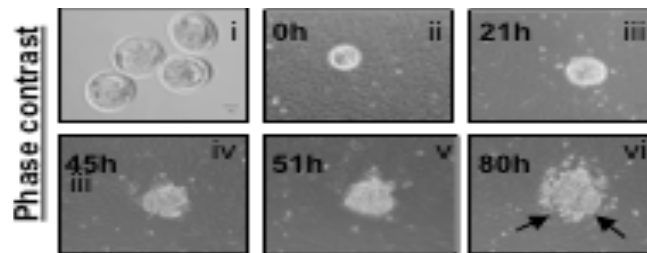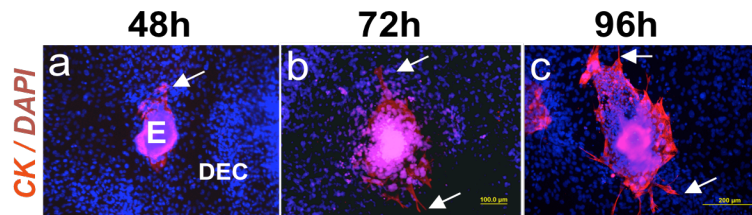
Figure 3.8. Co-culture of pre-implantation blastocysts with uterine stromal cells

A. Schematic representation of the experimental strategy utilized to isolate and co-culture pre-implantation blastocysts and uterine stromal cells.

B. Pre-implantation blastocysts (panel i) and PSCs were harvested from D4 pregnant uteri as described in the Materials and Methods. Blastocysts were placed onto a bed of PSCs (panel ii) and cultured together for durations of 21 h (panel iii), 45 h (panel iv), 51 h (panel vi) and 80 h (panel v). Live-cell images were captured at each timepoint.

C. Blastocyst and PSCs were co-cultured for 48 h (panel a), 72 h (panel b) and 96 h (panel c), fixed and subjected to immunofluorescence using an antibody recognizing cytokeratin (CK). DAPI-stained nuclei are shown in blue. Migrating trophoblasts are indicated by arrows.

FIG. 3.9.
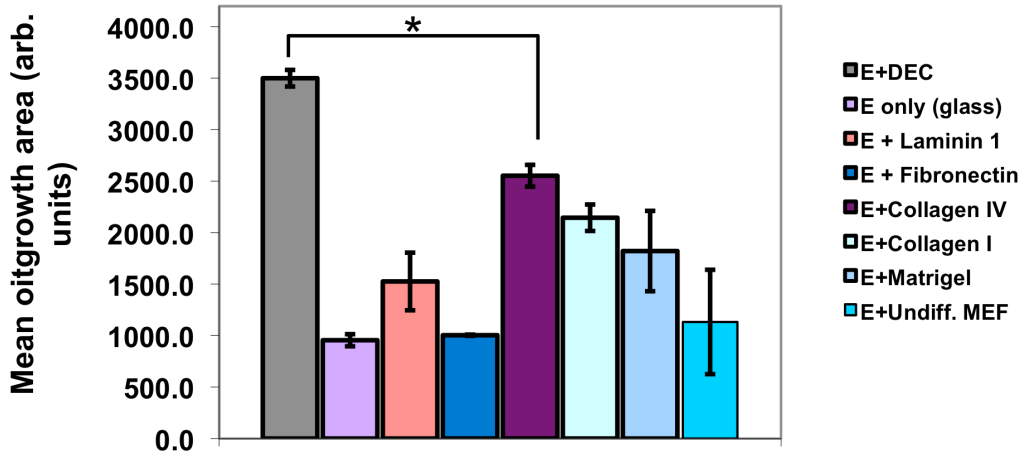
Figure 3.9. Embryo invasion into decidual cells is controlled by the stromal basement membrane.

Blastocysts were isolated from D4 pregnant uteri and cultured for 48 h on PSCs (E+DEC), glass alone (E only), MEFs (E+undiff. MEF), laminin-1-coated glass (E+laminin-1) and matrigel-coated glass (E+matrigel). Bars represent the area of embryo outgrowth, which was determined using Image J software as described in Materials and Methods.

FIG. 3.10.
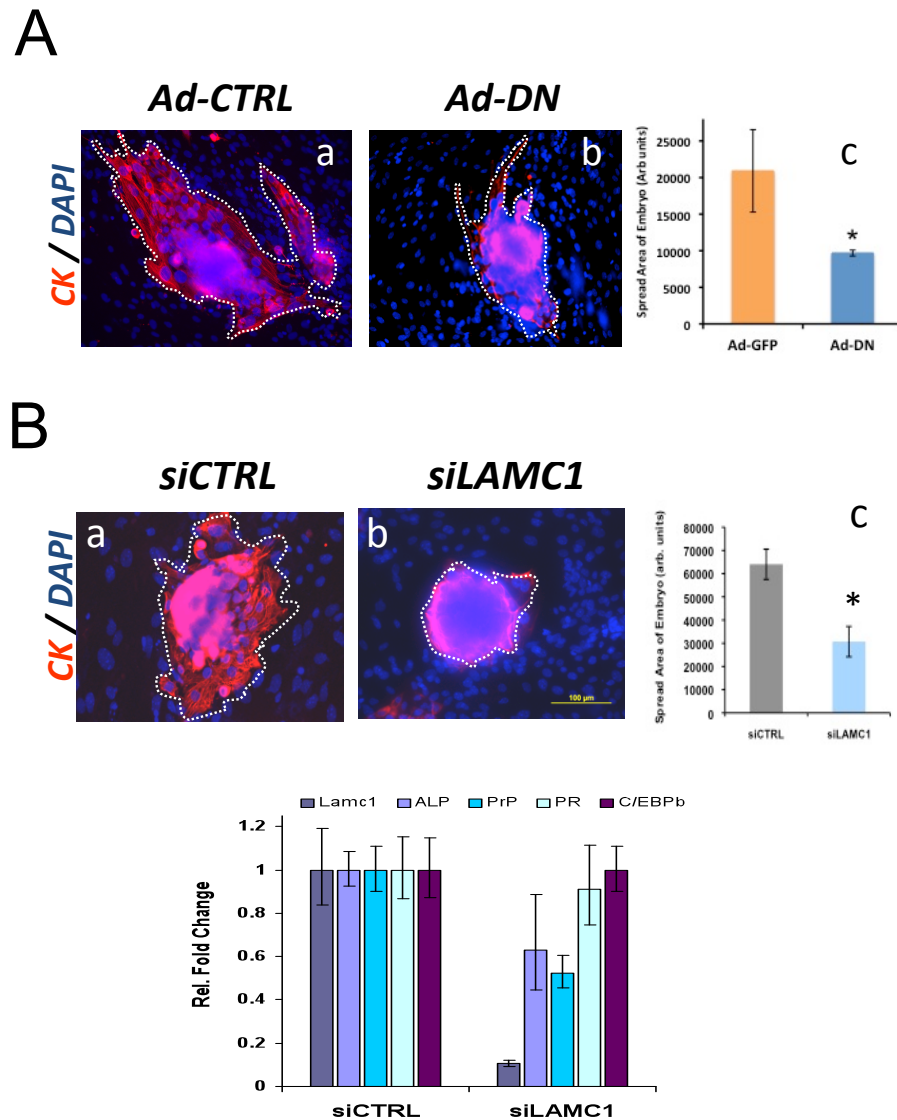
Figure 3.10. Embryo invasion into decidual cells is controlled by C/EBPβ and the laminin γ1-containing ECM

A. Blastocysts and PSCs were harvested from D4 pregnant uteri. PSCs were then transduced with adenoviral particles carrying either an empty vector (Ad-CTRL) or a vector expressing a dominant negative mutant of C/EBPβ, A-C/EBP (Ad-DN) for 24 h. After adenovirus removal, blastocysts were placed on Ad-CTRL-treated PSCs (panel a) or Ad-DN-treated PSCs (panel b). Cells were fixed and subjected to immunofluorescence using an antibody against cytokeratin (CK). DAPI-stained nuclei are shown in blue. Area of embryo outgrowth on PSCs was determined using ImageJ software as described in Materials and Methods (panel c) and is indicated by dashed line. DEC, decidual cells.

Figure 3.10. continued:

B. Blastocysts and PSCs were isolated from D4 pregnant uteri. PSCs were then transfected with siRNA (40 nM) targeted against a scrambled, non-coding sequence (siCTRL) or laminin γ1 (siLAMC1) as described in Materials and Methods. After siRNA removal, blastocysts were placed on siCTRL-treated PSCs (panel a) or siLAMC1-treated PSCs (panel b). Cells were fixed and subjected to immunofluorescence using an antibody against cytokeratin (CK). DAPI-stained nuclei are shown in blue. Area of embryo outgrowth was determined using ImageJ software as described in Materials and Methods (panel c) and is indicated by dashed line. DEC, decidual cells.

C. Real-time PCR analysis of mRNA levels of Lamc1, ALP, PRP, PR and C/EBPβ using total RNA isolated from siCTRL or siLAMC1-transfected PSCs, prior to co-culture with blastocysts.

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CHAPTER 4

CREATION AND ANALYSIS OF MUTANT MICE LACKING UTERINE LAMININ γ1 (*LAMC1*) TO ADDRESS THE ROLE OF LAMININ-CONTAINING ECM DURING PREGNANCY

4.1.   ABSTRACT

During implantation, the uterine stromal tissue undergoes significant remodeling and synthesizes a unique basal lamina-like ECM containing laminins, collagen IV and other ECM proteins. Laminin γ1 helps drive the polymerization of other laminin subunits and catalyzes the assembly of the basal lamina, underlining its critical participation in this cellular structure. We have previously shown in Chapter 3, using *in vitro* mouse culture systems, that laminin γ1 synthesis is regulated by the important decidual factor, C/EBPβ. Furthermore, production of laminin γ1 by uterine stromal cells is vital for their ability to enter differentiation and acquire a decidual phenotype. In order to address the role of laminin γ1 in the uterine remodeling process, we created a mutant mouse model in which the laminin γ1 gene function was conditionally ablated by the use of the loxP/Cre strategy. Mice carrying loxP inserts flanking an essential exon of *laminin γ1* (*Lamc1$^{flox./flox}$*) were crossed with progesterone receptor (PR)-Cre transgenic mice to generate *laminin γ1*-null female mice (*Lamc1$^{d/d}$*). We confirmed that laminin γ1 gene expression was specifically extinguished in uterine stromal cells of *Lamc1$^{d/d}$* mice during early pregnancy (day 6). In an ongoing breeding study, - *Lamc1$^{d/d}$* female mice (n=10)

were mated with wild type males and found to be subfertile in comparison to *Lamc1$^{flox/flox}$* mice. - While certain of the *Lamc1$^{d/d}$* mice became pregnant and produced a reduced number of pups per litter, a majority of mutants failed to give birth despite successful mating. These results indicated that laminin γ1 plays an important role in the uterus during implantation. Future efforts will be directed toward understanding the precise nature of the reproductive defect in *laminin γ1*-null mice.

4.2. INTRODUCTION

During embryo implantation, an intricate relationship is established between the maternal and embryonic tissues. This interaction relies on an intimate association of endometrial epithelial and stromal cells with the blastocyst proper thereby permitting bi-directional crosstalk between the two tissues. The ability of the uterine tissue to support and modulate embryo invasion is a carefully coordinated process requiring the participation of the steroid hormones estrogen (E) and progesterone (P). As a result of their regulation of endometrial proliferation and differentiation, E and P play a pivotal role in coordinating the events surrounding implantation (1). A key maternal event, known as decidualization, is triggered by embryo implantation (2-4). Decidualization is a cell differentiation process whereby stromal cells undergo cellular remodeling to form a transient, highly specialized tissue that modulates survival and growth of the fetus. Many studies have suggested that the production and paracrine functions of numerous growth factors, cytokines, and hormones in the decidua are required for the induction of the embryonic developmental program (5-7). Simultaneously, several groups have

documented the increased peri-cellular deposition of a variety of extracellular matrix (ECM) proteins within the decidua (8-10). Among these molecules, laminins, collagen type IV and desmin are notable since all three of these molecules are exclusively found in the classical basement membrane or basal lamina. In addition to being abundant in pregnant uteri (9, 10), laminins, collagen type IV and fibronectin were also synthesized by mouse uterine stromal cells cultured *in vitro* (11, 12). Collectively, these studies raised the possibility that the uterine basal lamina-like ECM serves as a platform for tissue remodeling and embryo invasion during implantation. However, despite the identification of novel ECM components produced by the decidua, the precise role of these ECM proteins during early pregnancy remains unclear. In this chapter, we address the role of the laminin-containing ECM in controlling decidualization and embryo implantation *in vivo.*

The basement membrane is an intricate structure that controls the interactions between cells and their microenvironments, providing a local source of growth factors and cytokines to cells (13). Laminins, a large family of glyoproteins, are vital to the biological functions of the basement membrane and the basal lamina in many different mammalian tissues and organs. The functions of this multi-molecular structure largely depends on the self-polymerization of laminin subunits and the subsequent recruitment of other ECM molecules such as collagen IV and nidogen by the laminins (13-16). Latest reports indicate that at least 15 laminin heterotrimers can be derived from five α, three β and 3 γ subunits (13, 14, 16). Of these, laminin γ1 is especially important because it is found in 10 out of the 15 trimers. Furthermore, mutations in the *Lamc1* gene caused early

lethality in developing embryos due to a failure in embryonic basement membrane formation (16). Additionally, a study by *Murray et. al.* showed that embryoid bodies differentiated from *Lamc1*-null mice lacked appropriate tissue organization, leading to fundamental developmental defects that inhibit embryo growth (18). Mutations in two other laminin genes, laminin α1 (*Lama1*) and laminin β1 (*Lamb1*), were created using a gene-trap method and displayed embryonic defects in Reichert's membrane formation and basement membrane formation, respectively (19). Collectively, these studies underline the importance of all three laminin genes for fetal survival and development. Furthermore, these studies necessitate the creation of transgenic mouse models in which the functions of *Lamc1, Lama1* and *Lamb1* can be conditionally ablated to understand their time- and tissue-specific functions. A recent study by Chen *et. al.* utilized a LoxP/Cre strategy to ablate *Lamc1* functions in Schwann cells and uncovered a novel role for this ECM protein in Schwann cell function.

Recent efforts in our lab have identified the steroid hormone-regulated transcription factor C/EBPβ as a critical regulator of endometrial function and laminin synthesis in the stromal compartment (20-23). Using a DNA microarray approach, we identified several putative targets of C/EBPβ action in uterine stromal cells among which a prominent group of genes encoded ECM proteins (Wang, W. et. al, unpublished). In Chapter 3, the functions of laminin subunits α1 and γ1 were closely examined, using *in vitro* approaches, and the results indicated a close relationship between C/EBPβ and laminin γ1 function during decidualization. These studies suggested that C/EBPβ-regulated laminin γ1 initiates formation of a unique basal lamina-like ECM that supports

decidualization and initiates a dialogue between maternal decidual cells and the invading embryo during implantation. In support of our findings, present literature is suggestive of a major role of uterine ECM molecules in modulating embryo growth and survival (5, 24, 25). We propose that the assembly of a uterine basal lamina-like ECM network containing laminin γ1 provides the necessary environmental setting in which the stromal tissue differentiates and supports embryo invasion. To advance our understanding of the role of laminin γ1 in implantation, we developed a mouse model in which we selectively ablated *laminin γ1* gene function using a PR-driven *Cre* recombinase. We and others have successfully used this strategy to understand the role of a wide variety of genes involved in decidualization (26-28). Briefly, mice harboring 'floxed' alleles of *laminin γ1* (*Lamc1*$^{f/f}$) were crossed with transgenic PR-Cre mice to generate *laminin γ1*-deficient mice. The PR promoter attains maximal activity in the uterine stroma during the early post-implantation stages of pregnancy, inducing Cre recombinase and effectively eliminating expression of laminin γ1 in these cells. In this chapter, we describe the creation of the laminin γ*1*-null mice and the ongoing breeding studies using these mice.

4.3.   MATERIALS AND METHODS

Animal handling and tissue collection

All experiments involving animals were conducted in accordance with the National Institutes of Health standards for the use and care of animals. The animal protocols were

approved by the University of Illinois Institutional Animal Care and Use Committee. For mating studies, *Lamc1*^{flox/flox} and *Lamc1*^{flox/flox}*PR-Cre*^{cre/+}(*Lamc1*^{d/d}) mice were housed with wild type C57/BL6 mice (Charles River). The presence of a vaginal plug after mating was designated as the first day of pregnancy. Animals were sacrificed at various days of pregnancy for uterine tissue collection and for primary stromal cell isolation.

Generation of conditional *laminin γ1*-null mice

Conditional *laminin γ1*-null mice (*Lamc1*^{flox/flox} PR^{cre/+}) were generated by crossing *laminin γ1*-floxed mice (a gift from Z. Chen and S. Strickland), (29) with PR-Cre knock-in mice (25). Briefly, female homozygous *laminin γ1*-floxed mice (*Lamc1*^{flox/flox}) were mated with male homozygous PR-Cre mice (PR^{cre/cre}) to produce offspring (*Lamc1*^{flox/+} PR^{cre/+}) that are heterozygous for both mutations. These heterozygous mice (*Lamc1*^{flox/+} PR^{cre/+}) were then bred with each other or with homozygous *laminin γ1*-floxed mice (*Lamc1*^{flox/flox}) to produce conditional mutant mice with the genotype *laminin γ1*^{flox/flox} PR^{cre/+} (*Lamc1*^{d/d}). Additionally, homozygous mice obtained from the second cross (*Lamc1*^{flox/flox}*PR*^{cre/cre}) were bred with homozygous *laminin γ1*-floxed mice (*Lamc1*^{flox/flox}) to produce conditional mutant mice (*lamc1*^{d/d}). In these mice, Cre-mediated excision of floxed *laminin γ1* led to a null mutation of this gene in PR-expressing tissues. Genomic DNA isolated from tail biopsy samples was used for PCR screening to verify the genotypes of mice. The primer sequences used were P1 (5'-CTCAGAGCTGGCTTCTCACAT-3') & P2 (5'-GATTTTCAAAGAAGCAGAGTGTG-3'). The PR-Cre-mediated excision of floxed *laminin γ1* alleles in *Lamc1*^{d/d} mice was

assessed by PCR analysis of genomic DNA isolated from primary stromal cells of pregnant uteri (day 6).

Primary uterine stromal cell isolation

Primary uterine stromal cells were harvested from pregnant uteri on day 6 of pregnancy as previously described in Chapter 3.

Real-time PCR analysis

Total RNA was isolated from primary stromal cells of pregnant uteri by standard Trizol-based protocols and converted to cDNA as described previously in Chapter 2. The cDNA was amplified by real-time PCR to quantify gene expression using gene-specific primers and SYBR® Green (Bio-Rad Laboratories). As a loading control, the expression level of 36B4 gene, which encodes a ribosomal protein, was determined. For each treatment, the mean threshold cycle (Ct) and standard deviation were calculated from Ct values obtained individually from 3 to 4 replicates of that sample. Each sample was subjected to three independent real-time PCR trials. Fold change was derived from the mean Ct values as described previously in Chapter 2. ANOVA single factor analysis was conducted on the grouped means to determine statistical significance at a significance level of $P<0.05$.

The real-time PCR primer sequences are: laminin γ1, GATGACGCCGACCAGGAC (forward) and GCTGAGGAGGCTGCTGAC (reverse); laminin γ1:exon 2, CCGAGTGCCTACAACTTTGA (forward) and GCTGGCATGTCCGTTACATT (reverse); laminin γ2 TGCCACGCCTCTGCCGACTTC (forward) and

ACCGCCTTCCAACCATCCACATCC (reverse); PR, AGCCAGCCAGAGCCCACAG (forward) and CCCACAGGTAAGCACGCCATAG (reverse); 36B4, CTGCTGCCACCACTGCTG (forward) and TCCTCATCTGATTCCTCCGACTC (reverse).

Immunohistochemistry

For all proteins labeled, uterine sections were obtained, flash frozen and embedded in OCT. Cryosections were taken at 10 μm and subjected to immunostaining. Uterine sections were fixed using a 10% formalin solution (Sigma) and then blocked using a 5% solution of normal donkey serum (Jackson Immunoresearch) in PBS. Sections were incubated with the following primary antibodies diluted in blocking solution (0.25% BSA, 0.3% Triton X-100, sterile PBS) overnight at 4ºC: laminin γ1 (Abcam) and pan-laminin (Sigma). The sections were washed and incubated with biotinylated secondary antibodies (Jackson Immunoresearch Laboratories Inc.) for 60 min followed by incubation with streptavidin-conjugated horseradish peroxidase (Histostain Kit; Zymed Laboratories Inc.) for 45 min. Sections were stained with AEC solution (Zymed Laboratories Inc.) and counterstained with Mayer's Hematoxylin (Sigma). Immunofluorescence staining was performed by incubating sections with Cy3-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). The counterstaining was done using 4',6'-diamidino-2-phenylindole (DAPI; Invitrogen Inc.). Stained sections were mounted in Vectashield fluorescence mounting media (Vector Laboratories). Negative controls included incubation with donkey serum and omission of the primary antibody for all samples.

4.4. RESULTS

Expression of *laminin γ1* in the uterus during early pregnancy.

We monitored the expression of laminin γ1 in wild type pregnant uterine tissue and found that this ECM molecule is robustly expressed in various compartments of the uterus throughout early pregnancy. On day 5 of pregnancy, soon after embryo implantation, laminin γ1 is expressed in the uterine stroma and deposited in the pericellular spaces between stromal cells (Fig. 4.1, panel a & d). We also observed high levels of laminin γ1 in the epithelial basement membrane and the basement membrane of developing endothelial blood vessels. Additionally, high levels of laminin γ1 were observed in the myometrial compartment of the uterus. As pregnancy advanced to days 6 -8, laminin γ1 expression intensified in the primary decidual zone of the uterus surrounding the implanted embryo. During this time, the laminin γ1 expression was highly organized and appeared to radiate in a complex, network-like fashion towards the secondary decidual zone (Fig. 4.1, panel b, c, e & f). The high levels of laminin γ1 in the myometrium were maintained on days 6 and 8. The laminin γ1 expression was also localized in endothelial basement membrane on day 8, which is in agreement with previous reports about endothelial laminin expression. The close spatio-temporal association of laminin γ1 expression with the progression of decidualization strongly suggests that the laminin γ1-containing basal lamina is important for this biological transformation.

Generation of mice with uterine stromal cell-specific excision of the *laminin γ1* gene.

It was previously discovered that mice carrying a global *laminin γ1*-null mutation die at the embryonic stage due to failure of basement membrane formation early in embryogenesis (17). To address the functional role of *laminin γ1* during early pregnancy in adult mice, we employed the LoxP/Cre strategy to generate a conditional mutant lacking the expression of this ECM protein in stromal cells of the pregnant uterus. We achieved the deletion of *laminin γ1* gene by crossing female mice harboring a "floxed" *laminin γ1* gene (29) with PR-Cre (26) male mice in which the gene encoding Cre is inserted at the PR gene locus and under the control of the PR promoter. When the resulting *laminin γ1*$^{\text{flox/flox}}$ PR$^{\text{cre/+}}$ (*Lamc1*$^{d/d}$) female progenies were bred with WT males of the same background (C57/BL6), the expression of Cre, which is controlled by the promoter regions of the endogenous PR gene, was maximally induced during early pregnancy. This led to the Cre-mediated excision of the "floxed" *laminin γ1* gene in PR-expressing uterine cells, as evidenced by the significant loss of *laminin γ1* mRNA expression in the PR-positive stromal cells of the conditional mutant mice during early pregnancy (day 6) (Fig. 4.2 A). The excision of *laminin γ1* did not produce any changes in PR mRNA levels (Fig. 4.2 B). Additionally, we did not observe any compensatory increase in the mRNA levels of laminin γ2, a related laminin γ subunit (Fig. 4.2 C). Immunohistochemical analysis confirmed the Cre-mediated excision of *laminin γ1* in uterine stromal cells near the site of embryo implantation, consistent with the fact that these cells express high levels of PR during pregnancy (Fig. 4.2 D, panels d-f). In contrast, excision of the "floxed" *laminin γ1* gene was not detected in uterine endothelial

basement membrane or the embryonic basement membrane, where PR is not expressed, indicating the cell-specific nature of the conditional mutation (Fig. 4.2 D, panel e, arrows). We also noted that laminin γ1 expression was significantly reduced in the myometrium of pregnant uteri (Fig. 4. 2D, panel f).

A limited breeding study was initiated in September 2009 in which female mice lacking laminin γ1 exhibited reduced fertility (Table 1). Our studies indicated that out of ten *Lamc1$^{d/d}$* females placed in the breeding program, four (40%) became pregnant and delivered litters. Only two of these litters survived beyond the first two days of their birth. Interestingly, the four mice, which had delivered pups in their first pregnancy, never produced a second litter. They exhibited normal mating behavior but failed to give birth. Additionally, our analysis revealed that six out of ten female mice that were originally placed in the breeding program (60%) never gave birth despite mating normally with WT males. Overall, mice carrying a conditional ablation of *laminin γ1 (Lamc1$^{d/d}$)* gave birth to approximately 95 % lesser number of pups than *Lamc1$^{flox/flox}$* mice (15/10 vs. 81/8).

4.5. DISCUSSION AND ON-GOING STUDIES

During embryo implantation, the uterine stroma synthesizes a unique basal lamina-like ECM composed of laminins and collagen IV that assembles into a well-organized pericelluar network (3, 5, 9). Although the precise function of this ECM is presently

unclear, it is thought to be important in supporting decidualization and embryo invasion (5). In chapter 3, we made the significant observation that the expression of laminin γ1, an important component of the basal lamina-like ECM, is directly regulated by the transcription factor C/EBPβ during decidualization. Furthermore, we have shown that the function of laminin γ1 is important for stromal differentiation and embryo invasion *in vitro*. The next challenge is to evaluate the importance of laminin γ1 function *in utero* by employing a conditional laminin γ1 knockout mouse model. In this section, we discuss our efforts towards this aim and future strategies for defining the phenotype of laminin γ1-null mice.

The spatio-temporal expression of laminin γ1 closely overlaps with the decidualization process in uterine stromal compartment. When we examined laminin γ1 expression on days 5 through 8 of pregnancy, we found increasing levels of laminin expression in the stromal cells underlying the luminal epithelium and surrounding the implanted embryo. The peri-cellular deposition of the laminins in the stromal compartment coincides with the progress of decidualization, strongly suggesting that the laminin γ1-containing ECM has a major function in the differentiation process. Additionally, high levels of laminin γ1 were observed in the uterine myometrial compartment during early pregnancy. The functional significance of myometrial laminin expression is presently unclear.

We observed that only a small subset (20%) of *laminin γ1*-deficient female mice gave birth to healthy offspring, indicating that female mice lacking this gene are sub-fertile (Table 1). Immunohistochemical assays revealed that the expression of laminin γ1

mRNA and protein was efficiently ablated in uterine stroma and myometrium of *Lamc1$^{d/d}$* mice at an early stage of pregnancy (Fig. 2). The presence of laminin γ1 in the basement membrane of the endothelial cell network is consistent with the fact that these blood vessel structures do not express PR (30), thereby avoiding the Cre-mediated excision of laminin γ1. It was also noted that laminin γ1 is highly expressed in the embryonic basement membrane of both *Lamc1$^{flox/flox}$* and *Lamc1$^{d/d}$* mice, in accordance with previous findings (19). Furthermore, the presence of implanted embryos on day 6 of pregnancy in laminin γ1-null uteri indicated that embryo attachment to the uterine wall was not affected by this mutation. Taken together, our preliminary observations are strongly suggestive of a reproductive failure in laminin γ1-null mice. The ongoing efforts are to identify the precise nature of the reproductive defect in these mice and to identify the molecular mechanisms by which laminin γ1 controls uterine function.

In order to pinpoint the mechanisms underlying the reproductive defect in laminin γ1-null mice, we are assessing the integrity of the female reproductive axis. The ovarian function in laminin γ1-null mice will be analyzed through the use of superovulation experiments conducted with mutant mice. Based on our preliminary findings that a subset of laminin γ1-null mice gave birth, we predict that ovarian function is preserved in these mutants. Additionally, we examined whether the adult laminin γ1-null females (n=5) displayed estrous cycling and found that these mice progressed normally through the estrous cycle stages (data not shown). If ovarian function is found to be intact in these mice, a detailed and systematic analysis of developmental landmarks in pregnant laminin γ1-null mice would have to be conducted. For example, an initial strategy would involve the

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harvesting of uterine tissue from *Lamc1*^{flox/flox} and *Lamc1*^{d/d} on days 8, 10, 12, 18 and 21 of pregnancy. These time points would inform us about important stages of the uterine remodeling process during pregnancy, including embryo attachment, embryo invasion, decidualization, neo-vascularization, placentation and parturition. Using these approaches, we would eventually understand the contribution of the laminin γ1-containing ECM to the uterine remodeling process.

4.6.   ACKNOWLEDGEMENTS

FIG. 4.1
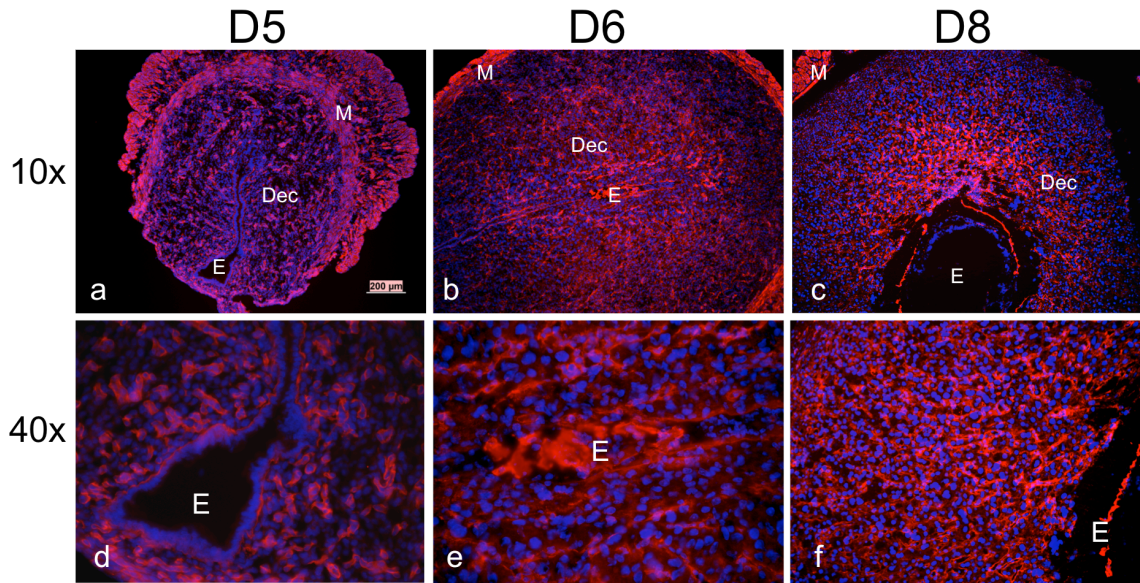
Figure 4.1. Induction of Laminin γ1 expression in uterine stromal cells during implantation

Frozen uterine tissue sections were collected on days 5 (***panels a, c)***, 6 (***panels b, e***) and 8 (***panels c, f***) of pregnancy and subjected to immunohistochemical analysis using a rat monoclonal antibody against laminin γ1. Positive immunfluorescent staining for laminin γ1 is indicated in red and DAPI-stained nuclei are indicated in blue. ***Panels a-c***, lower magnification areas. ***Panels d-f***, higher magnification views of the images in *panels a-c.*

FIG. 4.2
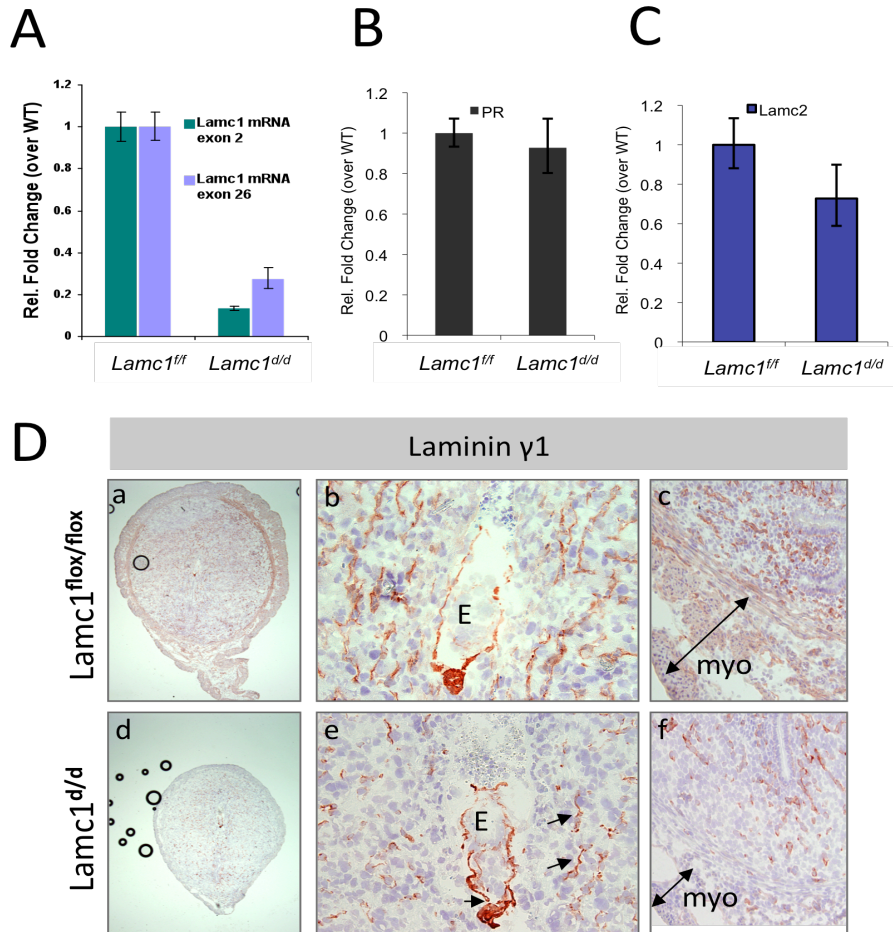
Figure 4.2. Loss of laminin γ1 expression in the uterine stromal cells of *Laminin γ1*-conditional knockout mouse

Lamc1^flox/flox mice and *Lamc1^d/d* mice were subjected to timed matings as described in the *Materials and Methods*. Mice were sacrificed and uteri collected on day 6 of pregnancy.

A. Primary stromal cells were harvested from pregnant uterine tissue as described in *Materials and Methods*. Real-time PCR analysis was employed to measure gene expression of laminin γ1 in *Lamc1^d/d* mice relative to *Lamc1^flox/flox* mice

B. Real-time PCR analysis of PR mRNA levels in *Lamc1^flox/flox* and *Lamc1^d/d* mice.

C. Real-time PCR analysis of laminin γ2 mRNA levels in *Lamc1^flox/flox* and *Lamc1^d/d* mice.

D. Uterine cross sections from pregnant mice (day 6) stained with an antibody recognizing laminin γ1. In *Lamc1^flox/flox* mice, laminin γ1 expression was abundant throughout the uterine stromal compartment (***panels a-c)***. In contrast, laminin γ1 expression was efficiently ablated in the uterine stromal cells of laminin γ1-null mice (***panels d-f***). Laminin γ1 expression in the endothelial and embryonic basement membranes was intact in laminin γ1-null uteri and is indicated by black arrows (***panel e***). Myometrial expression of laminin γ1 is also ablated in *Lamc1^d/d* mice and is indicated by two-headed arrows (***panels c & f***). E denotes the implanted embryo.

TABLE 4.1

Genotype	Number of Mice	Number of litters	Number of healthy pups	Number of pups/mouse
Lamc1$^{flox/flox}$	11	18	98	8.9
Lamc1$^{d/d}$	10*	2‡	15	1.5
* 10 mice were put into the breeding program, 6 did not give birth				
‡ Out of 4 litters born, 2 litters died within 2 days of birth				

Table 4.1. Impact of Laminin γ1 ablation on female fertility

Lamc1$^{flox/flox}$ and *Lamc1*$^{d/d}$ mice were placed into a six-month breeding study to assess female fertility.

4.8. REFERENCES

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CONCLUSIONS

Embryo implantation is a critical biological program that determines a successful pregnancy. This important event can be dissected into the pre-implantation preparation of the uterine lining and the post-implantation uterine remodeling process, involving stromal decidualization and neo-vascularization. Through the efforts of many groups, the mechanisms by which the steroid hormones E and P control these landmark events are beginning to be understood. In this dissertation, the actions of an important steroid hormone-regulated transcription factor, C/EBPβ, have been analyzed through the use of a C/EBPβ-null mouse model and an *in vitro* primary cell culture system.

Our studies established an essential role for C/EBPβ in the E-dependant growth and preparation of the uterine epithelial lining. C/EBPβ performs its important functions in the epithelium by controlling the DNA replication step of the cell cycle. By allowing the epithelial cells to undergo proper cell division in response to the hormone, it preserved genomic integrity and prevented apoptosis of these cells. Our *in vitro* studies, using a primary stromal cell culture model, established that C/EBPβ is also a major regulator of uterine stromal differentiation (decidualization). It does so by controlling the formation of a unique laminin-containing ECM. The laminin family member, laminin γ1 was shown to be directly regulated by C/EBPβ and is a critical component of this unique ECM. The basal lamina-like ECM modulates integrin-dependant cell signaling and differentiation. By modeling uterine events employing embryo-stromal co-cultures, we identified a laminin-dependent mechanism by which embryos can invade into uterine stromal cells.

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Finally, we set out to define a role for the laminin-containing stromal ECM *in vivo* by creating a laminin γ1-conditional knockout mouse model. Based on our preliminary results and the ongoing studies with this mouse model, the role of the laminin-containing uterine ECM will be further clarified. In conclusion, the studies presented in this dissertation have provided important contributions to the overall understanding of how the steroid hormones control the growth and differentiation of the uterus to regulate female fertility. Furthermore, the knowledge gained about the proliferative role of C/EBPβ during uterine epithelial growth may be of clinical relevance as it may help us to understand the role of this factor in endometrial cancer.

APPENDIX A

SUMMARY OF GENES UPREGULATED IN E-TREATED C/EBPβ-NULL UTERINE EPITHELIAL CELLS

TABLE A.1. Summary of genes upregulated in 18 h E-treated C/EBPβ-null uterine epithelial cells

Biological Process		Gene information	
Cell Cycle Arrest		**Gene Description**	**Fold Change**
Atm	NM_007499	Mus musculus ataxia telangiectasia mutated homolog (human)	2.46
Atr	AF236887.1	Mus musculus protein kinase ATR	2.00
Birc5	BC004702.1	baculoviral IAP repeat-containing 5	2.14
Cdc25b	NM_023117.1	cell division cycle 25 homolog B (S. cerevisiae)	2.00
Cdkn2c	NM_007671.1	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	2.14
Cse1l	NM_023565.1	chromosome segregation 1-like (S. cerevisiae)	2.00
Cul3	NM_016716.1	cullin 3	2.14
Mad2l1	NM_019499.1	MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)	2.14
Maged1	NM_019791.1	melanoma antigen, family D, 1	2.14
Rbbp4	BF011461	retinoblastoma binding protein 4	2.14
Pa2g4	AI152156	proliferation-associated 2G4, 38kD	2.00
Skp2	NM_013787.1	S-phase kinase-associated protein 2 (p45)	2.00
Trp63	NM_011641.1	transformation related protein 63	3.48
Apoptosis		**Gene Description**	**Fold Change**
Casp3	D86352.1	Caspase 3	2.46
Tnfrsf5	AI385482	tumor necrosis factor receptor superfamily, member 5 (new name: CD40)	4.00
Ptgs1	BB520073	prostaglandin-endoperoxide synthase 1	2.46
Tcf3	NM_009332.1	transcription factor 3	2.46
Tcf4	U16321.1	basic transcription factor MITF-2A	2.00

TABLE A.1 CONTINUED –

DNA Repair & Recombination		Gene Description	Fold Change
Exo1	BC006671.1	exonuclease 1	2.00
Fen1	NM_007999.1	flap structure specific endonuclease 1	2.00
Ku80	AF166486.1	Ku autoantigen	2.46
Mre11a	NM_018736.1	meiotic recombination 11 homolog A (S. cerevisiae)	2.14
Pola1	NM_008892.1	DNA polymerase alpha 1, 180 kDa	2.14
Pold1	BB385244	DNA polymerase delta 1, catalytic domain	2.00
Rad18	BC011120.1	Similar to RAD18 homolog (S.cerevisiae)	2.46
Rad23a	NM_009010.1	RAD23a homolog (S. cerevisiae)	3.25
Rad51	NM_011234.1	RAD51 homolog (S. cerevisiae)	2.14
Rev1l	NM_019570.1	REV1-like (S. cerevisiae)	2.00
Rpa1	BC019119.1	replication protein A1 (70 kDa)	2.00
Rpa2	AK011530.1	replication protein A2	2.00

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PROFILE

Ph.D. level biologist with background in steroid hormone biology, endocrinology, molecular biology and animal research work. Experience in primary cell culture models and DNA microarray analysis.

EDUCATION

Ph.D., Endocrinology & Cell Biology University of Illinois, Urbana, IL   2004-present

B.A., Biochemistry   Knox College, Galesburg, IL   2001-2004
(*Minor: Economics*)

RESEARCH EXPERIENCE

Department of Molecular & Integrative Physiology,   *2004-present*
University of Illinois-Urbana
Research Adviser: Milan Bagchi
Major findings/achievements:

- Molecular and biochemical analysis of Estrogen-controlled cell proliferation in uterus.

  o Determined the importance of C/EBPβ in growth and survival of uterine epithelium in transgenic mouse model lacking C/EBPβ function.
  o Established the regulatory role of C/EBPβ (transcription factor) in uterine response to estrogen.
  o Utilized DNA microarray technology to illuminate novel gene networks downstream of C/EBPβ in mouse uterine epithelium.
  o Conducted a systematic molecular analysis of cell cycle and survival genes involved in proliferative actions of C/EBPβ in the uterus.
  o Published findings in Molecular and Cellular Biology journal and was acknowledged as a "Spotlight" article in 2010.

- Molecular and genetic analyses of extracellular matrix during uterine differentiation.

  o Characterized the role of C/EBPβ in controlling 'decidualization' (differentiation) of uterine cells during pregnancy.
  o Developed and implemented *in vitro* uterine stromal cell cultures to analyze role of Laminin in controlling embryo implantation.
  o Established a functional role for Laminin in uterine stromal differentiation.
  o Designed and implemented an *in vitro* model of embryo and uterine cell co-culture to study embryo implantation.
  o Created a laminin-knockout mouse model to understand gene function of lamnin in the endometrium during pregnancy.

SKILLS

Technical:

- Cell Biology: Primary cell isolation & culture from murine uterus; human endometrial cell line & breast cancer cell line culture; tissue isolation; cell purification; cell proliferation and differentiation assays; fluorescence & confocal microscopy

- Molecular Biology: Immunohistochemistry; RNA extraction; Real-time PCR; RT-PCR; reverse transcription; RNA purification; electrophoresis; plasmid cloning and amplification

- Animal: Transgenic mouse breeding & husbandry; genotyping; surgery; dissection

Software Implementation:

- Bioinformatics: Affymetrix Gene-Chip analysis; PANTHER Gene Analysis; Ingenuity Systems pathway analysis

- Bio-imaging: Adobe Photoshop & Illustrator; GIMP photo analysis

COMMITTEES, HONORS & AWARDS

- Publication in *Molecular and Cellular Biology* selected as a "spotlight" article **2010**
- Organizing Committee: Annual Minisymposium on Reproductive Biology, Evanston IL **2008**
- Merck/AAAS Research Fellowship, Galesburg, IL **2003-04**
- Richter Memorial Trust Grant, Galesburg, IL **2002-04**

PUBLICATIONS

- Ramathal, C., Bagchi, I., Bagchi, M., 2009. "Lack of CCAAT Enhancer Binding Protein Beta (C/EBPβ) in uterine epithelial cells impairs estrogen-induced DNA replication, induces DNA damage response pathways and promotes apoptosis" *Mol. Cell. Biol.* **30:** 1607-1619

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SELECTED PRESENTATIONS

- Ramathal, C., Wang, W., Bagchi, I.C., Bagchi, M.K. "The transcription factor C/EBPβ controls endometrial stromal differentiation and remodeling during implantation by promoting the formation of a unique extracellular matrix". Poster presentation at Annual meeting for Society for the Study of Reproduction in Pittsburg, PA., 2009.

- Ramathal, C., Wang, W. Bagchi, I.C., Bagchi, M.K. "The transcription factor C/EBPβ controls endometrial stromal differentiation and remodeling during implantation by promoting the formation of a unique extracellular matrix". Oral presentation at Research Meeting for Specialized Cooperative Centers Program in Reproduction & Infertility Research in Chicago, IL, 2009.

- Ramathal, C., Wang, W., Bagchi, I.C., Bagchi, M.K. "The CCAAT Enhancer Binding Protein Beta (C/EBPβ) controls stromal cell differentiation and remodeling during decidualization by promoting the formation of a unique basal lamina-like extracellular matrix**.** Oral presentation at the Annual Minisymposium on Reproductive Biology in Evanston, IL, 2008.

- Ramathal C., Bagchi, I.C., Bagchi, M.K. "The CCAAT Enhancer Binding Protein Beta is an Essential Mediator of Estrogen-induced Cell Proliferation and Survival in the Uterine Epithelium of Mice. Poster presentation at the Gordon Conference: Reproductive Tract Biology, in Andover, NH, 2008.

- Ramathal C., Bagchi, I.C., Bagchi, M.K. "The CCAAT Enhancer Binding Protein Beta is an Essential Mediator of Estrogen-induced Cell Proliferation in the Uterine Epithelium of Mice. Oral presentation at the Cell & Molecular Biology Training Grant Research Symposium at Urbana, IL, 2007.

TEACHING EXPERIENCE

Teaching Assistant   **University of Illinois-Urbana, IL   2005-present**
Molecular & Cellular Biology
- Courses taught:
    - Molecular Genetics
    - Cells Tissues and Development.
- Independently led group discussions for over 500 students for 9 semesters
- Achieved teaching excellence by student selection (4 semesters)

INDUSTRIAL EXPERIENCE

Research Intern   Colloidal Environmental Technologies, Arlington Hts, IL   2003
- Performed chemical and mechanical quality control analysis of polymer designs.
- Evaluated data using statistical software and MS Excel for supervisor.

ACTIVITIES
- Member, Illini Biotechnology Organization (IBO)
- Member of soccer team in park district league, music production, cooking.