Dynamic Assembly of Basement Membrane Components in Tissue Development during Pregnancy

By

Celestial R. Jones-Paris

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Approved:

Greg Sephel, Ph.D.

Billy G. Hudson, Ph.D.

Joey Barnett, Ph.D.

Bibhash C. Paria, Ph.D.

Mary Zutter, M.D.

To my loved ones and ancestors that progressed beyond circumstances,

held their heads high in spite of surrounding hardships and oppression,

and instilled the faith that led the march toward new horizons.

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PREFACE

Multicellular organisms are harmonious integrations of extracellular matrix (ECM) and cells. ECM was originally thought to only serve as the framework that organized cells, and cells were the constituent regarded to be chiefly responsible for governing tissue function. Later, cell responses to environmental cues via direct cell-matrix interactions became recognized as the central mechanisms in many aspects of biology. Currently, the molecular intricacies of ECM are increasingly accepted as fine-tuned drivers of cellular and molecular function. Discoveries from the Hudson Laboratory have been at the forefront of a paradigm shift that establishes ECM, particularly basement membrane, as an active contributor influencing evolution, development, physiology, and disease.

Basement membranes (BMs) are specialized forms of ECMs that underlie polarized cells of vascular, ductal, and glandular tissues and encase essential cells of the reproductive, nervous, muscular systems and fat tissues. BMs are sheet-like, insoluble scaffolds that form a semi-permeable barrier between cell populations and control cell behaviors. The utilities of BMs in tissue function lie in their complex supramolecular arrangement of a collection of proteins that include collagen IV and laminin families, perlecan, and nidogen. Peroxidasin and Goodpasture-antigen binding protein (GPBP) are emerging as members of the BM that have important roles in overall BM function. The works described in this dissertation addresses the dynamic interplay of these components in the physiological progression of pregnancy and adds to the fundamental understanding of BMs assembly in tissue development with potential implantations in pathological processes. Hurdles in studying BMs in physiology include: 1) in most circumstances, once BMs are produced they are continually maintained unless disrupted by injury or disease, and 2) functions of different BMs and their components are not easily dissociated experimentally. In the present studies, we overcome these hurdles by tracking spatial and temporal modulations of BM components during assembly of extracellular scaffolds in tissues developed for pregnancy.

In this dissertation, I sought to test the hypothesis that BM structure and components are dynamically regulated during uterine, embryonic, and extraembryonic tissue development for pregnancy. This dissertation is arranged in chapters that emphasize maternal, early embryonic, and extraembryonic

tissues that employ BMs for reproductive success. Chapter I, provides background knowledge on BM structure, function, and composition. Chapter II, presents the novel finding that BM components are have distinct and integrated patterns of localization in uterine tissues during the transitional reprogramming phenomenon called decidualization, and the components collectively localize in both preimplantation epithelium and postimplantation decidua. Chapter III, advances the understanding of embryonic BMs by revealing specific BM components are utilized for the early arrangement of tissues. Chapter IV, describes alterations in BM protein localization during the growth and establishment of extraembryonic tissues and propose roles in pertinent reproductive pathologies, focusing specifically on fetal membranes and placenta. The findings from these studies provide links to physiological contexts that may translate to other cellular and molecular physiological and pathological processes, such as tissue morphogenesis and cancer that are described in Chapter V.

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LIST OF ABBREVIATIONS

AFM	atomic force microscopy
BM(s)	basement membrane(s)
BMPs	bone morphogenic proteins
dpc	days post coitum
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
EPI	epiblasts
ES cells	embryonic stem cells
FGF10	fibroblast growth factor 10
FM	fetal membranes
GPBP	Goodpasture antigen-binding protein
HB-EGF	heparin-binding epidermal growth factor-like growth factor
HPV	human papillomavirus
ICM	inner cell mass
KO	knockout
LOX(s)	lysyl oxidase(s)
LOXL2	LOX-like 2
MET	mesenchymal-epithelial transition
MMP(s)	matrix metaloproteinase(s)
NC1	non-collagenous 1 domain
PE	primitive endoderm
PECAM	platelet/endothelial cell adhesion molecule
PHG	phloroglucinol
PROMs	premature rupture of membranes
qPCR	quantitative polymerase chain reaction
SNPs	single nucleotide polymorphisms
TE	trophectoderm
TEM	transmission electron microscopy
TGFβ	transforming growth factor-beta
VE	visceral endoderm
VWFC	von Willerbrand factor type C
ZO-1	zonula occludens-1

LIST OF PUBLICATIONS

Jones-Paris CR, Paria S, Berg T, Saus J, Bhave G, Paria BC, Hudson BG. Basement membrane ultrastructure and component localization data from uterine tissues during early mouse pregnancy. Data in Brief. 2016 Oct 31; in press.

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CHAPTER I

Introduction

Extracellular matrix (ECM) is the vast network of proteins organized in spaces around cells and between tissues. ECM supports multicellularity of organisms by enabling 3-dimenional organization, modulating cell behaviors, and acting as a barrier and sensor between cells and environmental stress. Basement membranes (BMs) are specialized ECM scaffolds that compartmentalize and reinforce tissue architecture, govern growth factor gradients, direct cell migration and adhesion, and stimulate cell differentiation (Vracko, 1974; Pöschl *et al.*, 2004; Hynes, 2009; Pastor-Pareja & Xu, 2011; Yurchenco, 2011; Daley & Yamada, 2013).

Anatomical Aspects of Basement Membranes

Basement membranes are ubiquitously present in all organ systems and have diverse molecular and spatial characteristics that collectively support tissues that withstand physiological demands for congruent biological outcomes. BM expression is restricted to the tissues chiefly responsible for the operations associated with essential organ functions (Vracko, 1974). It underlies epithelia of glands and ducts of the digestive, respiratory, and urogenital tracts; defines endothelia of blood vessels (Sephel *et al.*, 1996; Hagios *et al.*, 1998; Hynes, 2009; Yurchenco, 2011); and encapsulates decidua cells (Wewer *et al.*, 1985; Farrar & Carson, 1992), Schwann cells (Scherer, 1997; Court *et al.*, 2006), adipocytes (Kubo *et al.*, 2000; Sillat *et al.*, 2012), and myocytes (Campbell & Stull, 2003; Sanes, 2003; Boonen & Post, 2008) (Figure 1.1).

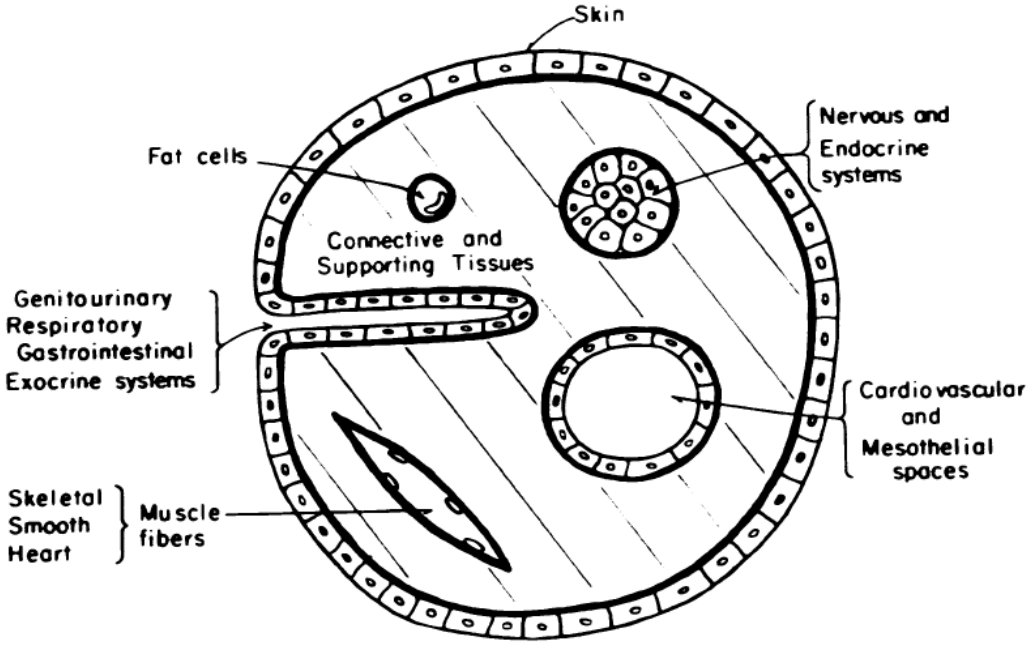


Figure 1.1: Hybrid diagram of the anatomic distribution of basement membranes. Basement membrane displayed as a heavy black line. Adapted from (Vracko, 1974).

Over the past half-century, BMs have been the subject of intense investigation. BMs were first insinuated in 1840 as anatomical sheaths in muscles (Bowman, 1840). Since ground-breaking discoveries of several BM components in the 1970s and 1980s, citations have rapidly expanded and now more than 41,000 research and review articles having explored and reported the biomedical significance of BMs (based on pubmed.gov search for “basement membrane”, October 2016). The functions of BMs are governed by 1) the solid scaffold structure and 2) composition. These properties are integrated and provide distinctive effects on cell biology and physiology.

Structural Characteristics of Basement Membranes

Basement membranes are inherent laminations of tissues. They are constructed as dense membranes with 5-100 nm pores that inhibit migration of cells, which are typically 10 μm in diameter, and constrain the permeability of macromolecules that exceed 40 kDa (Abrams *et al.*, 2000; Danysh *et al.*, 2010; Wolf & Friedl, 2011; Hohenester & Yurchenco, 2013; Kelley *et al.*, 2014; Gaiko-Shcherbak *et al.*, 2015; Morrissey & Sherwood, 2015). Thus, BMs function as barriers that control cell migration and regulate paracrine signaling involved in many processes including, tissue morphogenesis, intravasation/extravasation of inflammatory cells, and invasion of metastatic tumor cells (Wang *et al.*, 2008; Voisin *et al.*, 2009; Yu *et al.*, 2009; Friedl & Alexander, 2011; Lu *et al.*, 2012; Wallingford *et al.*, 2013; Enemchukwu *et al.*, 2016). Filtration features are enhanced by layering BMs such as observations of the effects of the triple-layered BMs that form the filtration network of kidney glomeruli (Casotti & Braun, 1996). BMs also immobilize molecules by tightly binding growth factors through specific submolecular protein-protein interactions and/or electrostatically charged regions and form reservoirs that alter cell behaviors upon contact (Reddi, 2000; Lieleg *et al.*, 2009; Oschman, 2009; Yang *et al.*, 2014b).

BM scaffolds establish architectural support for tissues by providing appropriate resilience and tensile strength in response to physiological forces, such as blood pressure, and peristalsis. Through mechanoreceptors, individual cells sense the rigidity of neighboring BMs and distal BMs up to 20 μm away within the tissue environment (Discher *et al.*, 2005; Butcher *et al.*,

2009). Together, these properties create 3-dimensional functionality based on the BM scaffold structure. The collective diversity of BM scaffolds is based on the components that also provide individual functionalities.

Components of the Basement Membrane

The basement membrane is composed of an assortment of proteins that assemble into supramolecular arrangements that establish BM form and function. The BMs characteristically include collagen IV, laminin, perlecan or agrin, nidogen, and other minor collagens (including types VII, XV, and XVIII) (Hynes, 2012). Network assembly is dictated by stoichiometric ratios of its components, cell-matrix interactions, matrix-matrix binding sites, other environmental conditions such as elemental availability and pH (Figure 1.2) (Grant *et al.*, 1989; Hynes, 1992; Pedchenko *et al.*, 2004; Shimizu *et al.*, 2007; Xu *et al.*, 2009; Dohn *et al.*, 2013; McCall *et al.*, 2014; Kubow *et al.*, 2015). Two additional proteins, peroxidasin and Goodpasture antigen-binding protein (GPBP), have recently emerged as potential members of BM components by meeting the criteria of being identified in several BMs and influencing scaffold functions.

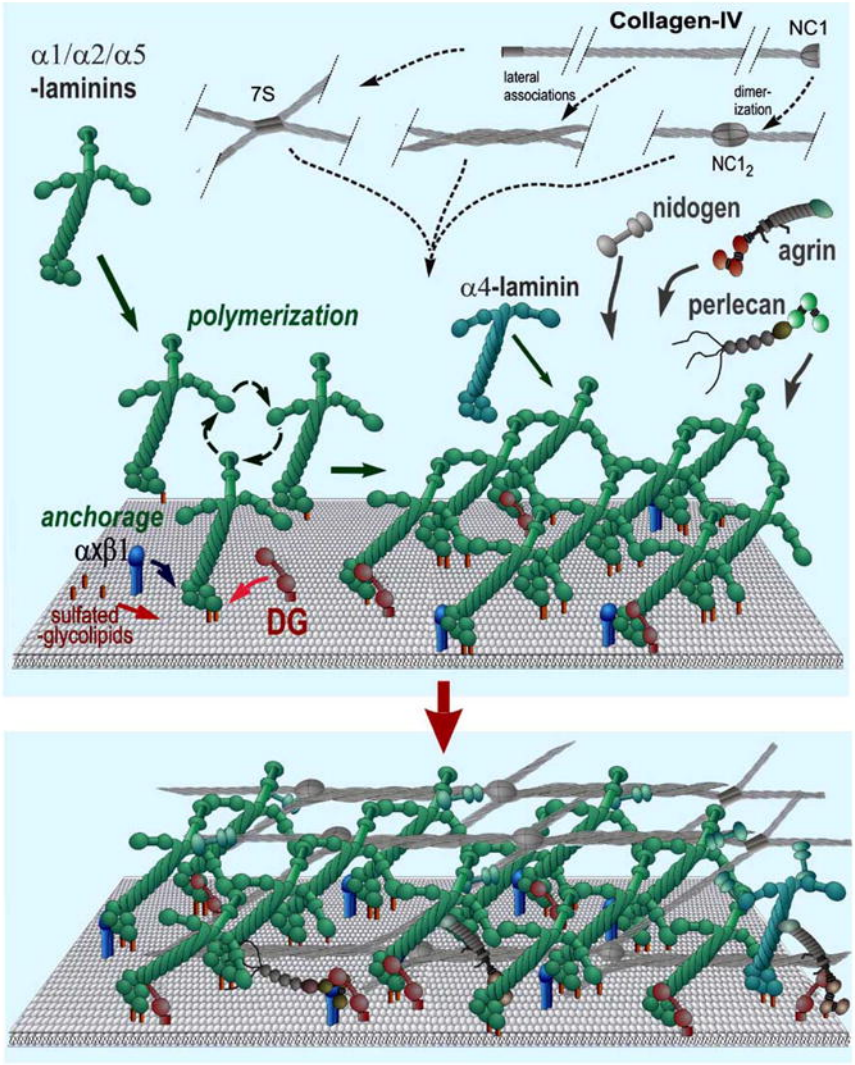


Figure 1.2: Diagram of the assembly basement membrane structural proteins highlighting specific interactions and binding of components. Adapted from (Yurchenco & Patton, 2009).

Collagen IV is an essential collagen of the BM (Khoshnoodi *et al.*, 2008). At present, there are 28 known types of collagens in the collagen superfamily (Shoulders & Raines, 2009). Groundbreaking investigations by Hodge and Schmitt et al. in the late 1950s led to the discovery and classification of the first fibrillar collagen (collagen I), which was followed by discoveries of collagens 2 and 3 in the late 1960s and early 1970s (Glimcher *et al.*, 1957; Hodge & Schmitt, 1958; Miller, 1976). The most recently discovered collagen is collagen XXVIII is a beaded filament-forming collagen first identified in 2006 in BMs around nerve cells (Veit *et al.*, 2006). Collagen IV is the most abundant collagen of BMs and was first discovered in the Kefalides laboratory in the late 1960s as a special collagen that forms the extracellular scaffold of glomerular tissues of the kidney (Kefalides, 1966, 1972, 1973). All major collagens, including collagen IV, have the defining feature of triple helical coiled polypeptide strands that establish trimeric protomers which arrange into supramolecular structures (Shoulders & Raines, 2009).

Collagen IV is expressed as alpha helical protomer molecules that are assembled by first interacting at C-terminal non-collagenous (NC1) regions that initiate the zipping of the collagenous backbone. Chain-specific expression results in one of three distinct trimer patterns known to occur in nature: α1α1α2(IV), α3α4α5(IV), and α5α5α6(IV) (Khoshnoodi *et al.*, 2008) (Figure1.3). These heterotrimeric protomers are stabilized by disulfide crosslinks generated by lysyl oxidases and are secreted into the extracellular space where they arrange into a lattice (Siegel *et al.*, 2002). Collagen IV scaffolds are present during early embryogenesis and are required for development (Pöschl *et al.*, 2004). Our laboratory has discovered a novel sulfilimine crosslink that stabilizes interfacing collagen IV protomers by covalently bonding noncollagenous (NC1) domains (Vanacore *et al.*, 2009). We have recently found collagen IV crosslinking to be critical for tissue development in *Drosophila melanogaster* eggs, larvae, and *Danio rerio* embryos. Interference with the crosslinking results in embryonic morphological anomalies and dysfunction of BM mechanical integrity (Fidler *et al.*, 2014; McCall *et al.*, 2014).

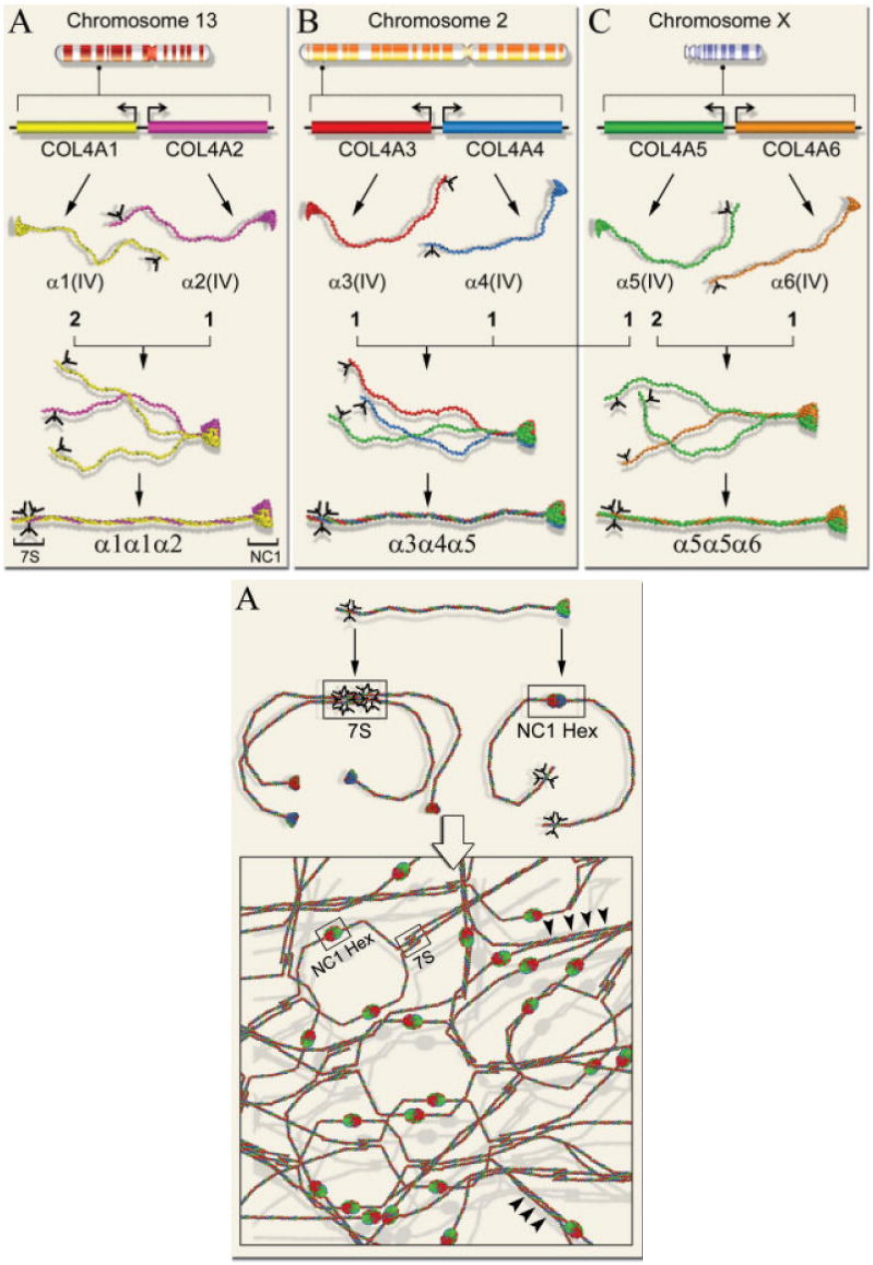


Figure 1.3: Schematic of Collagen IV Expression and Assembly. (Top) The genetic organization, protein production, and chain assembly of six different isoforms of collagen IV chains. (Bottom) Collagen IV network assembly by interactions at C-terminal NC1 domains and N-terminal 7S domains. Adapted from (Khoshnoodi *et al.*, 2008).

Laminin is a glycoprotein comprised of heterotrimeric subunits whose intracellular assembly are supported by C-terminal coil-coiled interactions (Beck *et al.*, 1990; Burgeson *et al.*, 1994; Hamill *et al.*, 2009). It is thought to be the first component that spontaneously polymerizes into a network during BM assembly (Hohenester & Yurchenco, 2013). Of the sixteen known types, laminin-111 is the most well-characterized as a result of extensive studies of embryonal carcinoma cell lines and the Engelbreth-Holm-Swarm (EHS) tumor BM-rich matrix (Chung *et al.*, 1979; Timpl *et al.*, 1979; Beck *et al.*, 1990; Burgeson *et al.*, 1994). Laminin is essential for gastrulation and organogenesis and is defective in multiple diseases including those that cause loss of neuromuscular junction functions and kidney disorders, such as Pierson syndrome (Aumailley & Smyth, 1998; Miner *et al.*, 2004; Rogers & Nishimune, 2016). In cooperation with collagen IV, laminin gives rise to the distinguishing ultrastructure of the scaffold, often referred to as basal lamina in electron microscopic studies.

Peroxidasin is a heme-containing peroxidase of the peroxidase-cycloxygenase superfamily (Zamocky *et al.*, 2008). It docks into the BM network where it has direct contact with collagen IV. The C-terminal von Willebrand factor type C (VWFC) domain of peroxidasin promotes its interactions with matrix proteins and facilities oligomerization that enhances catalytic activity (Hynes, 2012; Colon & Bhave, 2016). The peroxidase domain of peroxidasin consumes hydrogen peroxide and bromide (or chloride) to catalyze the formation of covalent sulfilimine (-S=N-) crosslinks between collagen IV NC1 protomers (Bhave *et al.*, 2012; McCall *et al.*, 2014). Peroxidasin function and the resulting sulfilimine crosslinks are essential for the reinforcement of collagen IV NC1 networks of BMs. Perturbation of peroxidasin expression or function leads to early developmental disorders and dysfunction of several tissues, including eyes and intestine. This has been demonstrated in *Nematostella vectensis*, *Drosophila melanogaster*, *Danio rerio*, *Mus musculus*, and *Homo sapiens* (Khan *et al.*, 2011; Bhave *et al.*, 2012; Fidler *et al.*, 2014; McCall *et al.*, 2014; Yan *et al.*, 2014).

Goodpasture antigen-binding protein (GPBP) contains diverse conserved domains which permit the protein to function in many capacities. The function of GPBP presumably depends on its localization patterns, whether it is in the ECM, cell membrane, cytosol, or nucleus (Raya *et al.*,

1999). Several domains of GPBP function in intracellular transport of lipids between organelles, yet its extracellular function remains unclear (Mencarelli *et al.*, 2010). Still, studies have shown GPBP localized to BMs in several tissues and implied extracellular functionality (Raya *et al.*, 1999; Granero-Moltó *et al.*, 2008; Revert-Ros *et al.*, 2011). Moreover, its overexpression is associated with collagen IV and BM ultrastructure expansion in autoimmune pathogenesis in humans and mouse models (Raya *et al.*, 2000; Revert *et al.*, 2007).

Development of Tissues During Pregnancy as a Model System to Investigate Basement Membrane Dynamics

In this dissertation, we sought to test the hypothesis that BM structure and components are dynamically regulated during uterine, embryonic, and extraembryonic tissue development for pregnancy. Pregnancy is an ideal system to investigate BM dynamics because it involves sequentially predictable events that allow feasible tracking of various tissues supported by BM scaffolds. Placental mammals evolved mechanisms of vivipary to enable sustained interactions between the embryo and mother through reprogramming of uterine tissues and development of extraembryonic tissues (Martin, 2007; Wagner *et al.*, 2014). In Chapters II through IV of this dissertation, essential biological processes are described in maternal, embryonic, and extraembryonic tissues that work in concert during pregnancy (Figure 1.4). Observations provide evidence of dynamic properties of BMs and establish that some components have distinct roles during development of tissues for pregnancy. Overall, the studies described in this dissertation have advanced our understanding of BM dynamics in a variety of tissues.

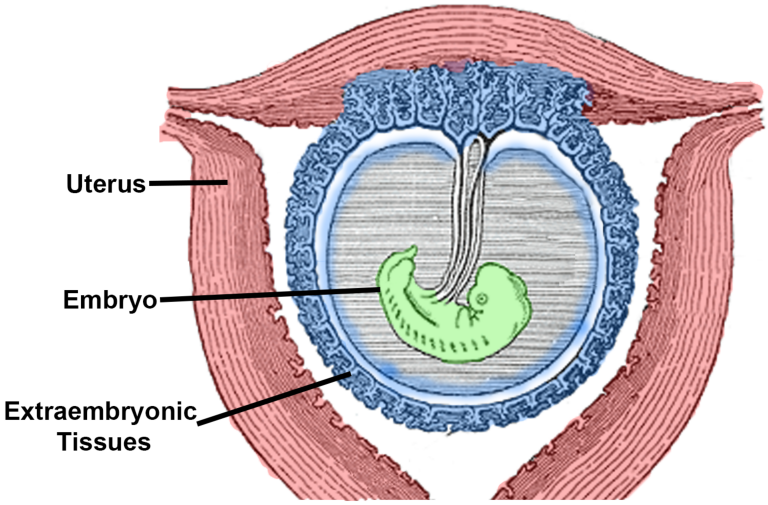


Figure 1.4: Illustration of an *in utero* embryo with extraembryonic tissues. Development of these tissues is addressed in Chapters II through IV of this dissertation. Highlighted red, uterus; green, embryo; blue, extraembryonic tissues. Adapted from (Gray, 1918).

CHAPTER II

Embryo Implantation Triggers Dynamic Spatiotemporal Expression of Basement Membrane Components During Uterine Reprogramming

Portions of work and research from this chapter were published in manuscripts authored by Jones-Paris et al. (Jones-Paris *et al.*, 2016b; c)

Introduction

In uterine tissues, basement membranes (BMs) function as scaffolds that circumscribe cell populations, modulate cell behaviors, and provide architectural and mechanical support to tissues (Hynes, 2009; Yurchenco, 2011). Basement membranes play active roles in regulating uterine biology by being positioned adjacent to key cell types: 1) between the epithelial or endothelial cells and their underlying stromal compartment (Hagios *et al.*, 1998; Davis & Senger, 2005); and 2) encapsulating cells such as myocytes (Campbell & Stull, 2003; Sanes, 2003; Boonen & Post, 2008), adipocytes (Kubo *et al.*, 2000; Sillat *et al.*, 2012), Schwann cells (Scherer, 1997; Court *et al.*, 2006), and decidua cells (Wewer *et al.*, 1985; Farrar & Carson, 1992; Diao *et al.*, 2011; Oefner *et al.*, 2015). These scaffolds activate signal transduction by directly interacting with transmembrane cell receptors, modulate paracrine signaling by regulating chemokine gradients and immobilizing growth factors, and establish tissue tension and integrity by bearing compression and expansion tissue forces (Lukashev & Werb, 1998; Khoshnoodi *et al.*, 2008; Yurchenco & Patton, 2009; Fang *et al.*, 2014). Basement membranes are comprised of a group of proteins, including collagen IV, laminin, perlecan, and nidogen (Miner *et al.*, 2004; Pöschl *et al.*, 2004; Kelley *et al.*, 2014). Emerging concepts argue that BMs undergo dynamic physiological adaptation by being fine-tuned with specific composition and localization throughout the life of organisms (Xu *et al.*, 2009; Isabella & Horne-Badovinac, 2015; Morrissey & Sherwood, 2015), though the fundamental mechanisms remain unknown.

Additional proteins have emerged as potential components of BMs: peroxidasin, Goodpasture antigen-binding protein (GPBP), and lysyl oxidases (LOX). Peroxidasin catalyzes the formation of sulfilimine crosslinks at interfacing NC1 domains of collagen IV protomers and therein reinforces networks of BMs. The lack of these reinforcements led to early developmental disorders and dysfunction in several tissues and organisms (Khan *et al.*, 2011; Bhave *et al.*, 2012; Fidler *et al.*, 2014; McCall *et al.*, 2014; Yan *et al.*, 2014). However, observations of the dynamic distribution of peroxidasin in BMs is limited to *Caenorhabditis elegans* tissues (Gotenstein *et al.*, 2010) and embryonal PFHR9 cell lines (Cummings *et al.*, 2016), and has been implied in embryonic mouse tissues (Yan *et al.*, 2014).

Extracellular GPBP was discovered through its binding to kidney BM (Raya *et al.*, 1999) and has since been shown to bind major BM components laminin and collagen IV (Revert *et al.*, 2008; Mencarelli *et al.*, 2012). Overexpression of GPBP in renal tissues is associated with collagen IV rearrangement and ultrastructure expansion of glomerular BM in immune complex-mediated pathogenesis in mice and humans (Raya *et al.*, 2000; Revert *et al.*, 2007).

Lysyl oxidases are a family of five enzymes, LOX and LOX-like (1-4), that catalyze the formation of reactive aldehyde from lysine residues that result in intermolecular crosslinks across collagenous helixes. LOX-like 2 (LOXL2) preferentially mediates collagen IV assembly in BMs (Grau-bové *et al.*, 2014; Anazco *et al.*, 2016). Inhibition of LOXL2 disrupts BM assembly which arrest angiogenic sprouting (Bignon *et al.*, 2011) and chondrocyte differentiation (Kvist *et al.*, 2008; Iftikhar *et al.*, 2011), while erroneous expression in fibroblasts is associated stroma activation that supports malignant progression (Barry-Hamilton *et al.*, 2010; Barker *et al.*, 2011). While LOX family enzymes have been studied in early-term fetal membranes and placenta (Hein *et al.*, 2001; Polettini *et al.*, 2016), their localization in uterine tissues during early pregnancy remains unknown. Although peroxidasin and GPBP have been identified in several BMs, whether they undergo dynamic regulation in healthy adult tissues is unknown.

I explored the BM dynamics that occur in adult uterine tissues in early pregnancy. Embryo implantation triggers the endometrium to undergo rapid and extensive changes in cell

populations and extracellular matrix (ECM) for development of the decidua, a cocoon-like tissue barrier between mother-embryo throughout pregnancy (Mester *et al.*, 1974; Wang *et al.*, 2004; Zhang & Paria, 2006; Gellersen *et al.*, 2007; Wagner *et al.*, 2014). These changes include apoptosis of endometrial epithelial and endothelial cells, restriction of immune cell infiltration, and proliferation of mesenchymal stromal cells that differentiate into cells with select epithelial-like features, thus they are often referred to as epithelioid decidua cells (Fig. 2.1). Studies of endometrial and decidual ECM show collagen IV and laminin change from limited localization underlying epithelial and endothelial cells to broad localization surrounding epithelioid cells throughout the mature decidua (Wewer *et al.*, 1985; Farrar & Carson, 1992; Diao *et al.*, 2011; Oefner *et al.*, 2015). However, colocalization of these two proteins has not been examined upon the initial switch to decidua tissue, during the periimplantation period. Here, our findings further reveal that the uterus reacts to blastocyst implantation by triggering the dynamic regulation of peroxidasin, GPBP, and LOXL2, as well as collagen IV and laminin, with distinct spatiotemporal expression and localization patterns in uterine tissues, suggesting both individual and collective functions of these BM proteins.

Results

Histology of uterine tissues during early pregnancy

Previous studies of decidualization have established the mouse as a reliable species to model changes that occur in the uterus during human reproduction, even though some aspects of anatomy and timing differ between mice and humans (Lim & Wang, 2010; Ramathal *et al.*, 2010; Rashid *et al.*, 2011). In mice, endometrium epithelial and mesenchymal cell behaviors are altered upon the initiation of pregnancy through ovarian-derived hormones and cellular compartment cross-talk (Grant, 2003; Yoshinaga, 2013; Hantak *et al.*, 2014; Pawar *et al.*, 2015). By day 4 of the 21-day term of pregnancy in mice, the uterus is conducive to blastocyst implantation and decidualization is triggered upon embryo attachment (Paria *et al.*, 2001b; Dey *et al.*, 2004; Sroga

et al., 2012). The majority of decidual tissue develops by day 7 of pregnancy and persists as the embryo grows and develops placenta that merges with the decidua for nutrient and waste exchange (Serman & Serman, 2011; Wagner *et al.*, 2014).

Tissue arrangement and morphological alterations were tractable by histological assessment of uterine horns and implantation sites. Hematoxylin and Eosin (H&E) staining of tissue sections revealed the gradual reduction of epithelial glandular and luminal tissues preimplantation (Figure 2.1 A and B). Following embryo attachment, the implantation site lacked glandular structures. The remaining endometrial tissues included nonpolar fibroblast-like stromal cells surrounded by unstained extracellular matrix and limited luminal tissue that was laterally restricted to the mesometrial region, the side of the endometrium that is nearest the uterine blood supply (Figure 2.1 C). Throughout this dissertation, data of histological cross-sections of uterine tissues will be relatively aligned top-to-bottom mesometrial to antimesometrial regions. On days 6 through 8 of pregnancy, the nonpolar stromal cells were replaced with a high-density of cuboidal cells that resulted in a relatively continuous pink cytoplasmic staining with few interruptions of unstained areas (Figure 2.1 D-E). The tissues beyond the circumference of the endometrium maintained consistent morphology, aligned myofibroblasts in the myometrium and a spongy connective tissue in the perimetrium (Figure 2.1 A'-E').

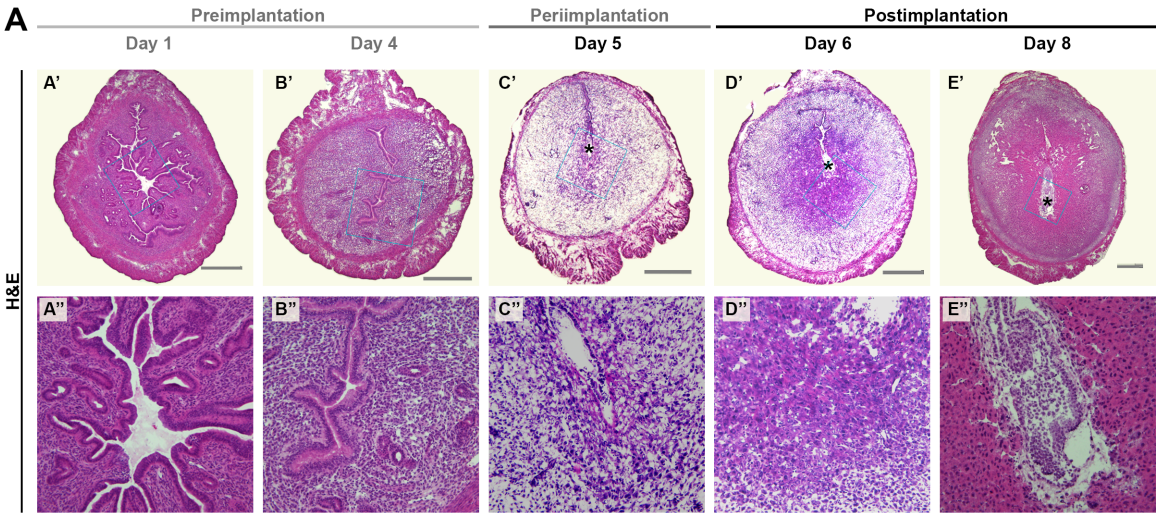


Figure 2.1: Histological representation of uterine tissue organization during early pregnancy. Brightfield micrographs of H&E-stained mouse uterus during early pregnancy. Scale bar = 500 µm. Blue inset squares in A'-E' are indicative of the field of view in A"-E". Asterisk (*) indicates the location of the embryo.

Ultrastructure of BM during decidua development

Although BMs have been observed in both pre- and post- implantation uterine tissues (Wewer *et al.*, 1985; Farrar & Carson, 1992; Diao *et al.*, 2011; Oefner *et al.*, 2015), little is known about the BM ultrastructure properties. Here, these properties were investigated by comparing the BM of the luminal epithelium on day 4 of pregnancy (Figure 2.2 A) and decidual zone of day 7 implantation sites (Figure 2.2 B) by transmission electron microscopy (TEM) that displays electron-dense layers of BMs called the lamina densa.

In the endometrium on day 4 of pregnancy, a smooth and narrow layer of the lamina densa was observed basal to cells of the luminal epithelium, but the lamina densa was not within the stroma (Figures 2.2 A). The location and structure of uterine epithelial the lamina densa displayed characteristics of BM oriented basal to polarized cells with apical-lateral localized cell-cell junctions (Hagios *et al.*, 1998; Nelson & Bissell, 2006). In the implantation site on day 7 of pregnancy, the lamina densa was positioned within the pericellular space of cells throughout the decidua. However, the lamina densa of the decidua had intermittent disruptions where cell-cell contacts and electron dense junctions were observed between cells (Figures 2.2 B). These observations led us to further analyze characteristics of the lamina densa of uterine luminal epithelium during the periimplantation period, also known as the window of implantation, and during the course of decidua development.

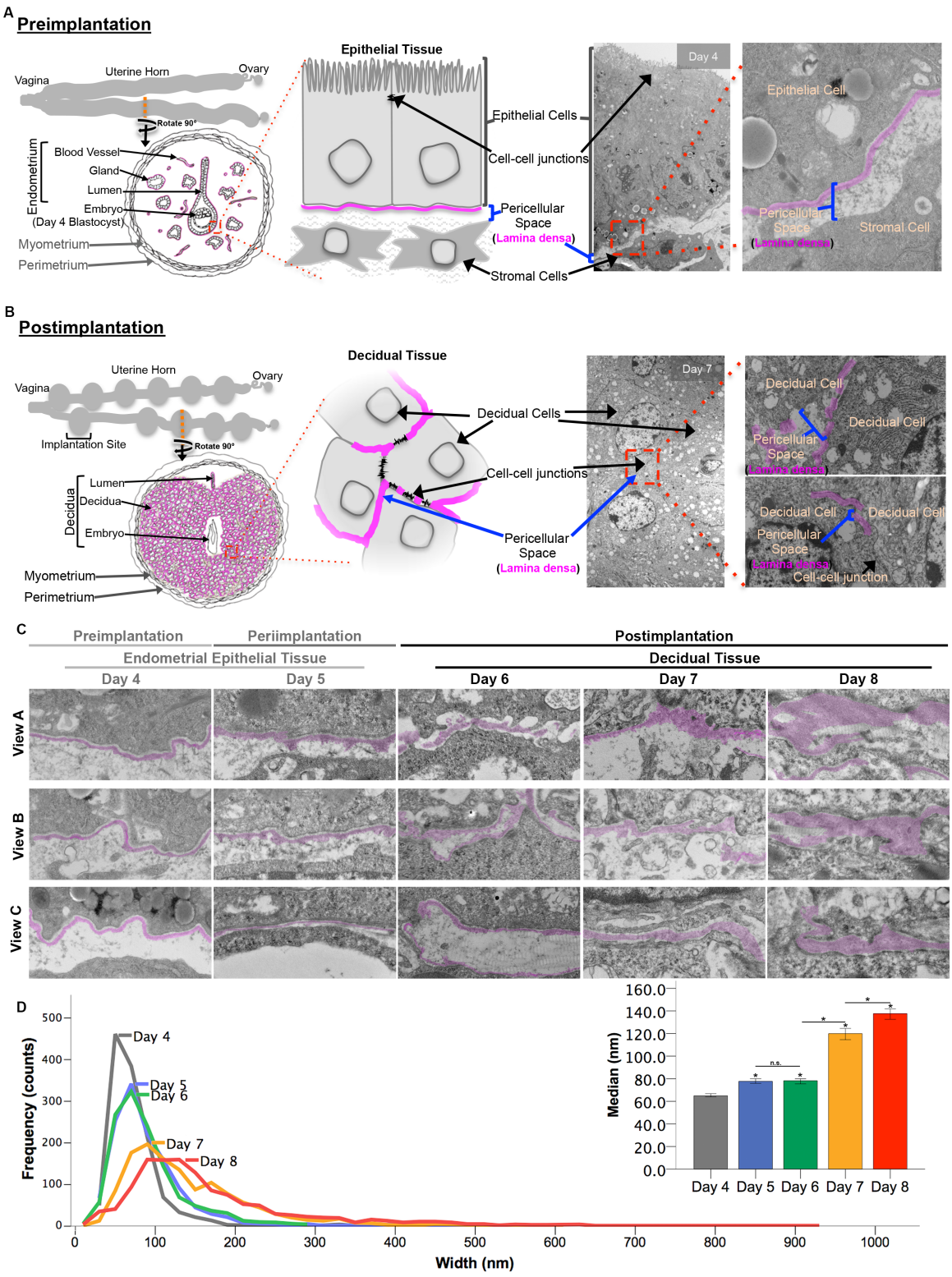


Figure 2.2: Ultrastructure of basement membrane in the endometrium and deciduum during early pregnancy. Transmission electron microscopy of the mouse uterus from days 4–8 of pregnancy. The lamina densa (basement membrane) is highlighted with pink. A) The lamina densa of day 4

uterine endometrium with a diagram showing a cross-section of the uterine horn (orange dashed line), tissue anatomy, and the position of the lamina densa highlighted pink between the uterine epithelium and stroma; electron microscopic images at 9500x and 15,000x. B) The lamina densa of day 7 decidua with a diagram showing a cross-section of implantation site horn (orange dashed line), tissue anatomy, the position of the lamina densa highlighted pink within the deciduum; electron microscopic images at 9500x and 15,000x). C) Micrographs (15,000x) of the pericellular lamina densa from day 4–8 of pregnancy (views 1–3 represent the variation of the thickness of the lamina densa). D) Quantitation of the lamina densa thickness. Frequency plot with lines representing distribution of widths out of 1300 measurements (counts) per day of pregnancy, and the same data as bars representing medians and 95% confidence intervals; Kruskal–Wallis test determined differences among groups (p-value b 0.001), and Post-hoc pairwise comparisons determined intergroup significance compared to day 4 of pregnancy and between sequential day (*p-value b 0.001, n.s. indicates no significant difference and p-value N 0.05).

On day 5 of pregnancy, the lamina densa of luminal epithelial cells adjacent to the attached blastocyst, appeared less uniform, morphologically frayed (Figure 2.2 C) and slightly but significantly thicker (78 nm median, p-value < 0.001) (Figure 2.2 D) compared to the BM of the day 4 luminal epithelium (65 nm median) (Figure 2.2 D). Following implantation and decidualization on day 6 of pregnancy, the densa surrounding decidual cells was uneven and discontinuous, with variable widths but median thickness similar to that of day 5 (78 nm median) (Figure 2.2 C-D). As the decidua matured, through days 7 and 8 of pregnancy, the lamina densa presented with median thicknesses two-fold greater than preimplantation epithelial BM (120 nm and 138 nm medians, respectively) and greater variability of widths (Figure 2.2 C-D). Medians were determined by pooling 1300 measurements per representative day of pregnancy. Distribution curves of widths gradually shifted right and indicated thickening of lamina densa during decidualization. Also, the spread of values from the 1300 measurements expanded, which was represented by the broadened distributional curves that indicated increased variability in widths during decidualization (Figure 2.2 D). All comparisons, except between days 5 and 6, were determined to be statistically significant (p-value < 0.001) within 95% confidence intervals using non-parametric ANOVA test (Kruskal-Wallis test) to day 4 measurements and Post-hoc pairwise comparisons between groups (Figure 2.2 D).

Pericellular gaps contained thickened lamina densa with some inclusion of fibrillar collagens in day 8 decidua (Figure 2.2 C-D). Decidua adjacent to the day 8 embryo had little to no distinguishable BM (data not shown). In summary, through examination of ultrastructure changes, the lamina densa was found to be associated with the development of the decidua accumulated progressively, initially presenting with a frayed and discontinuous morphology followed by a thickening in pericellular spaces around decidual cells.

Tensile strength of uterine tissues during early pregnancy

The findings of thickened BM in decidual tissue prompted an investigation of the tensile strength of the pre- and post-implantation tissues. Atomic force microscopy (AFM) was applied to tissue sections representing days 4 and 8 of pregnancy. Sample tension is determined using AFM by dragging a microscopic, flexible cantilever across the surface of the tissue section. The interaction between the sample and cantilever leads to bending of the cantilever that is measured by laser light that is focused on the cantilever tip. Changes in the cantilever position cause alterations in laser reflection that is detected by a photodiode that determines force (Newton, N) that is used to calculate Young's elastic modulus (N/m^2= pascals, Pa) (Binnig & Quate, 1986; Santos & Castanho, 2004). Using atomic force microscopy, tensile strength was found to increase from an average of 15 kPa in preimplantation endometrial tissues to 28 kPa in postimplantation decidual tissues (Figure 2.3). This trend of increased force resistance aligns in sequence with a previous study that showed early decidual tissue (approximately day 6 of pregnancy) to have a tensile strength averaging 23 kPa (Hiramatsu *et al.*, 2013). These results revealed that tissue stiffening coincides with BM thickening during early pregnancy.

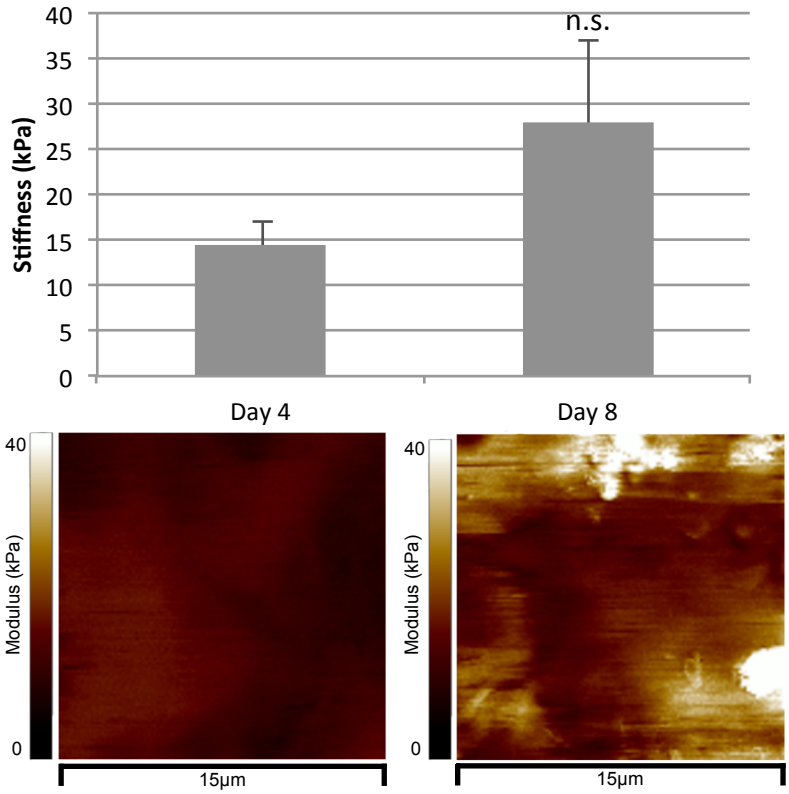


Figure 2.3: Atomic force microscopy (AFM) images and tensile strength measurements of endometrium and deciduum during early pregnancy. (top) Quantification of tensile strength near lumen on day 4 and within decidua near the embryo on day 8 of pregnancy. Bars represent mean and error bars represent SEM. (Bottom) AFM scan overview in 15µm^2 areas in representative endometrial and decidual tissues, with tensile modulus scale ranging from zero kPa (black) to 40kPa (white). n=2 Day 4 samples and n=3 Day 8 samples. p-value > 0.05, therefore results were not statistically significant (n.s.) (I acknowledge the efforts of Jon "JR" Peacock that assisted with this pilot study and Dr. David Merryman that made it possible).

Peroxidasin gene expression and protein localization with collagen IV in the uterus during early pregnancy

Recent studies have demonstrated the importance of peroxidasin in tissue development and integrity in several organisms (Nelson *et al.*, 1994; Bhave *et al.*, 2012; Fidler *et al.*, 2014; Yan *et al.*, 2014). However, the regulation of peroxidasin during pregnancy is unknown. To determine whether peroxidasin mRNA expression is altered in preparation for blastocyst attachment and/or during decidualization, expression levels of *Pxdn*, peroxidasin encoding gene, were quantified in whole uterine horns or implantation sites from days 1 through 8 of pregnancy. *Pxdn* expression gradually reduced during days 1 through 3, and then increased markedly to near days 1 and 2 levels by day 5 of pregnancy. Following a significant drop in *Pxdn* levels between days 5 and 6 of pregnancy, levels remained unchanged from days 6-8 (Figure 2.4 A). These results revealed *Pxdn* levels are up-regulated in the uterus during embryo attachment that occurs midway between days 4 and 5 of pregnancy in mice (Paria *et al.*, 1999a), but compartmentalized production was undetermined.

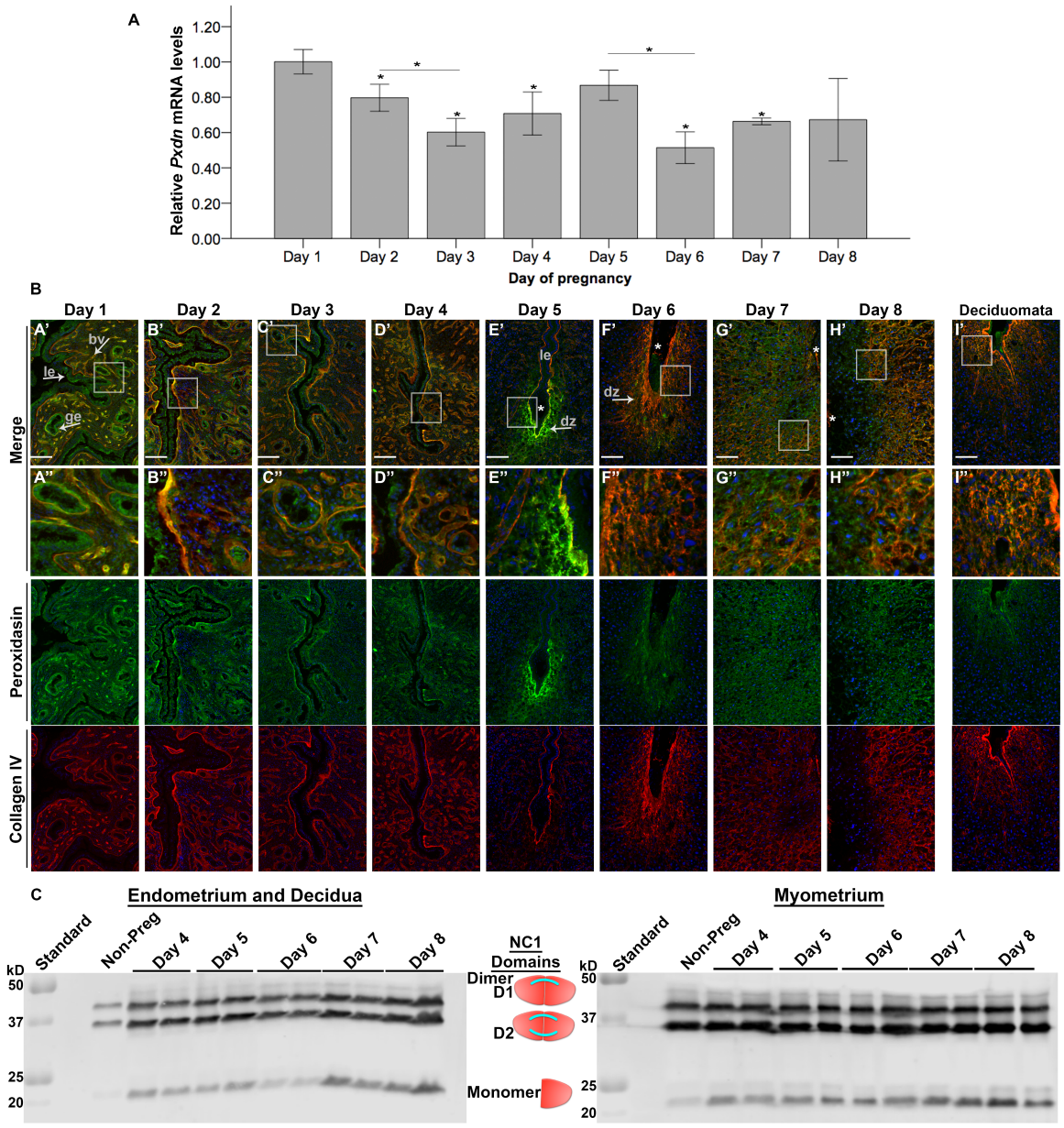


Figure 2.4: Peroxidasin and collagen IV expression and localization during early pregnancy. A) Uterine mRNA expression of peroxidasin (Pxdn). Bars represent mean and ±SEM (*p-value ≤ 0.05). B) Double immunofluorescence detection of collagen IV and peroxidasin in mouse uterus during early pregnancy. Images are oriented near the center of the uterus cross-section with collagen IV in red, peroxidasin in green, and nuclei in blue. Colocalization of collagen IV and peroxidasin is indicated by orange/yellow color. Asterisk (*) indicates the location of the embryo. Scale bar = 100 μm. Inset (White Square; 140 μm^2 region) is a representative of the field showing merge of collagen IV and peroxidasin in the second row. Le, luminal epithelium; ge, glandular epithelium; bv, blood vessels; dz., decidual zone. C) Immunoblot of collagen IV NC1 domains for crosslinking analysis (non-pregnant (Non-Preg) and day 4–8 endometrial explants and myometrium. Bands at approximately 37 kDa represent crosslinked NC1 domains, and bands at approximately 23 kDa represent monomeric NC1 domains.

Therefore, the localization of peroxidasin with collagen IV was examined in the endometrium where robust tissue changes occur during early pregnancy. Using co-immunofluorescence, it was determined if expression changes contributed to global distribution or compartmentalized production of peroxidasin and if it was incorporated into BMs. Peroxidasin was localized to all areas of collagen IV in addition to some distinctive patterns. During the preimplantation period (days 1 through 4), peroxidasin localization was found in the BMs of epithelium and endothelium of the endometrium (Figure 2.4 B, A'-D'). Patterns of short regions where peroxidasin appeared to concentrate along the lumen BM which was most pronounced on day 2 of pregnancy (Figure 2.4 B, B'). Cytoplasmic staining was greatest at day 1 of pregnancy and gradually diminished, first in endothelial and then in epithelial cells, until there was little or no detection on day 5 (Figure 2.4 B, A'-E').

On day 5 of pregnancy, luminal epithelial cells and stromal cells surrounding the embryo implantation site pooled pericellular peroxidasin in abundance compared to collagen IV (Figure 2.4 B, E', E"). This striking up-regulation of peroxidasin was not observed in other surrounding tissues, such as the myometrium where comparative abundance of collagen IV and peroxidasin remained relatively similar to previous days of pregnancy (Figure 2.5). In decidual tissues of days 6 through 8, peroxidasin remained localized to newly synthesized collagen IV (Figure 2.4 B, F'-H'). By day 7 and 8 of pregnancy, some cytoplasmic peroxidasin was detected in addition to BM-associated peroxidasin (Figure 2.4 B, G'-H'). The cells that were devoid of collagen IV around the day 8 embryo were positive for cytoplasmic peroxidasin (Figure 2.4 B, H').

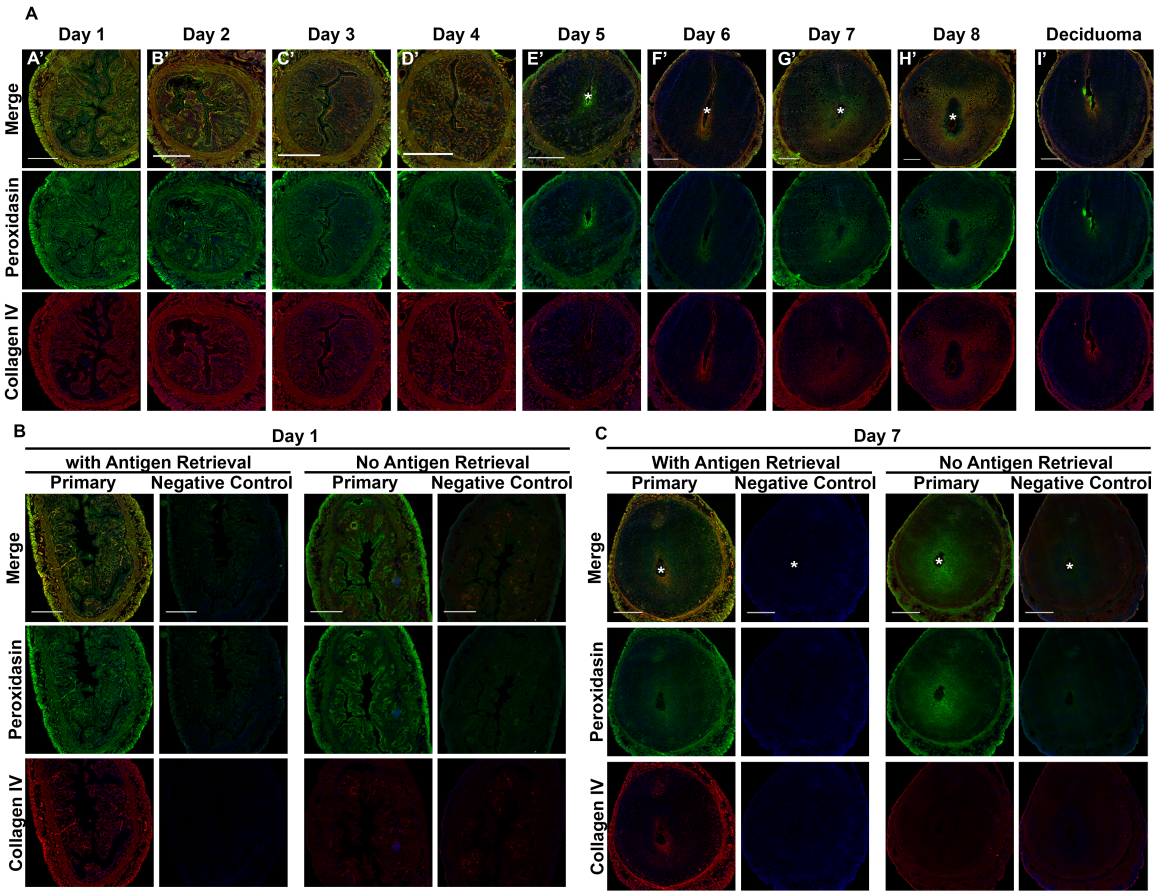


Figure 2.5: Collagen IV and peroxidasin localization during early pregnancy. A) Full cross-section of mouse uterus double immunofluorescence of collagen IV and peroxidasin during early pregnancy with collagen IV in red, peroxidasin in green, and nuclei in blue. Colocalization of collagen IV and peroxidasin is indicated by orange/yellow color. Asterisk (*) indicates the location of the embryo. Scale bar = 500 μm. Representative immunofluorescence controls preimplantation (B) and postimplantation (C).

The up-regulation of peroxidasin by blastocyst attachment poses the question of whether this physiological decidual reaction could also be induced by an experimentally controlled procedure. Embryo-induced decidualization and oil-induced deciduomata reactions involve reprogramming of tissues that progress at similar rates, exhibit equivalent alterations in expression patterns, and display comparable histological arrangement (Kennedy, 1980; Herington *et al.*, 2009). Here, day 6 deciduomata and deciduum displayed similar patterns of peroxidasin and collagen IV (Figure 2.4 B, I' and F'). Together, these results demonstrated strong temporal and spatial expression and localization patterns of peroxidasin during the periimplantation period and which preceded expression of collagen IV, and persistent localization as decidua BM assembled around cells.

Peroxidasin catalyzes the formation of sulfilimine crosslinks between adjoining protomers of collagen IV, a structural reinforcement that is essential for tissue function (Bhave *et al.*, 2012; Fidler *et al.*, 2014; McCall *et al.*, 2014).Therefore, biochemical analysis was utilized to determine whether these crosslinks form during the switch from epithelial BM of the endometrium to mesenchymal-derived BM of the decidua. Endometrial and decidual explants were subjected to collagenase digestion to excise crosslinked NC1 domains of collagen IV, followed by immunoblot assays to assess the degree of crosslinking. The immunoblots revealed 37 kDa protein bands that correspond to single (D1) and double (D2) crosslinked NC1 domains, as previously described (Fidler *et al.*, 2014), along with less intense bands around 23 kDa that correspond to non-crosslinked NC1 monomeric domains. This crosslinking pattern was common for all samples (Figure 2.4 C). Together, these data demonstrated that peroxidasin expression increased during periimplantation, localized to areas where new collagen IV was synthesized and crosslinked collagen IV across all stages of decidua development.

Phloroglucinol treatment during early pregnancy

Previously, the Hudson laboratory has demonstrated in cell lines and non-vertebrate models that peroxidasin crosslinking activity is inhibited by treatment with phloroglucinol (PHG) (McCall *et al.*, 2014; Cummings *et al.*, 2016), an antioxidant medicinal compound derived from brown algae *Ecklonia cava* (Ha *et al.*, 2013). However, toxicology research data have revealed that administration of PHG during mammalian pregnancy has no deleterious effects on fetal or maternal outcomes (Committee for veterinary medicinal products phloroglucinol trimethylphloroglucinol, 1995; Lacroix *et al.*, 2011), and has even been utilized as an antispasmodic during labor in some countries (Tabassum *et al.*, 2005). However, the impact of PHG on decidua BM development remains unknown. Given our evidence that inhibition of the formation of sulfilimine crosslinks disrupts physiology, we sought to test the null hypothesis that PHG has no significant impact on BM crosslinking during decidualization. To address this hypothesis, mice were treated with PHG or sham (diluent only) twice daily on days 5 through 8 of pregnancy then examined for potential anatomical anomalies and BM localization or sulfilimine crosslinking disruptions (Figure 2.6 A). Uterine horns displayed no anatomical differences in appearance or number of implantation sites between treatment groups beyond normal animal-to-animal variance (Figure 2.6 B). Localization of collagen IV and laminin were similar throughout the day 8 embryos, decidua, and all other tissues in both PHG and sham-treated mice (Figure 2.6 C). Some differences in embryo morphology were attributed to plane-of-section through the implantation site and section collection artifacts. Finally, the extent of sulfilimine crosslinks in decidual BMs was not impacted by PHG treatments (Figure 2.6 D). Counter-intuitively, monomeric domains of collagen IV appeared to slightly decrease with PHG treatments (Figure 2.6 D), but observations were not replicated (data not shown). Together, these data suggests that PHG does not inhibit sulfilimine crosslinking activity or BM protein localization during assembly of BMs for decidual tissues.

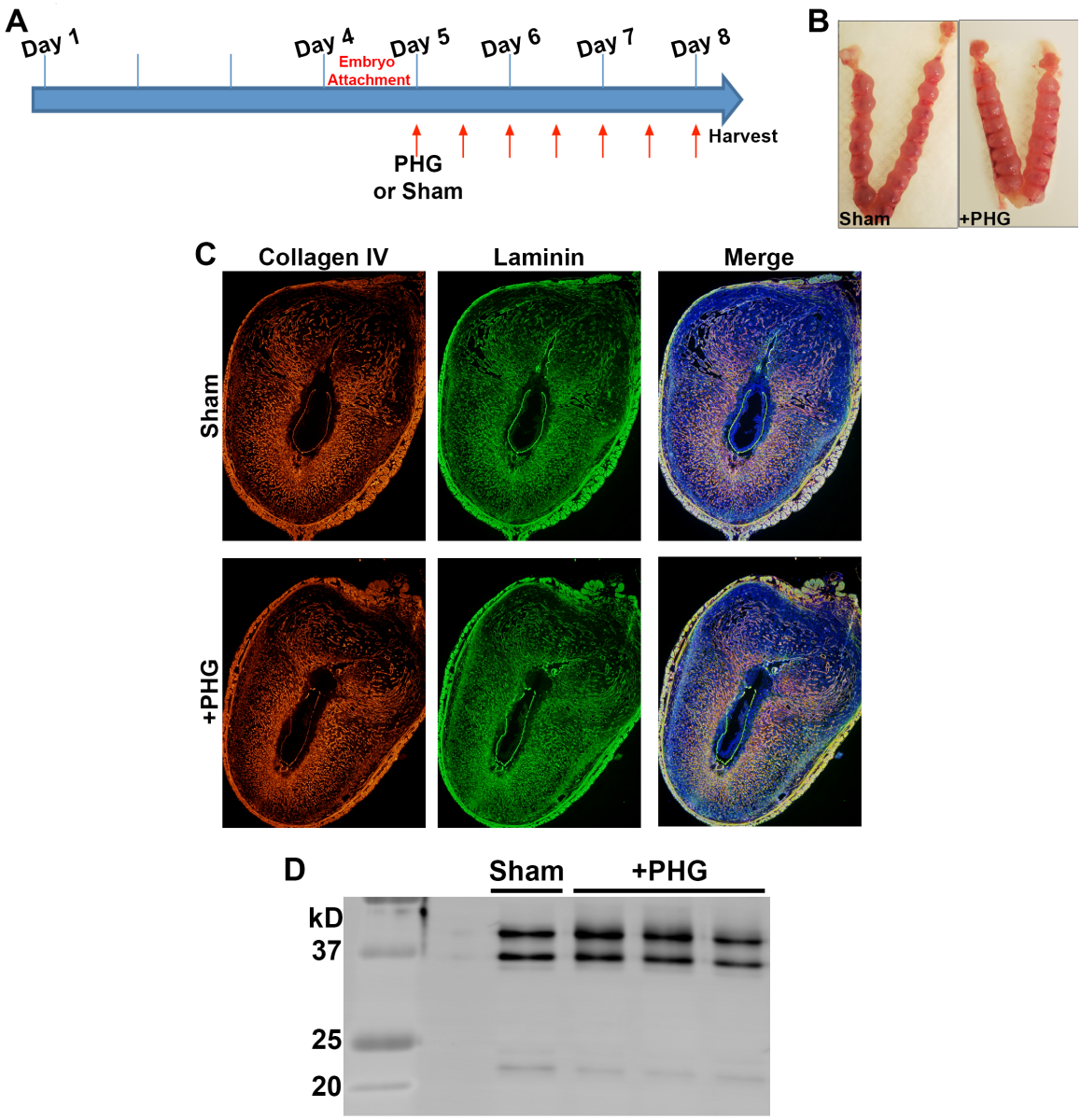


Figure 2.6: Phloroglucinol (PHG) treatment during early pregnancy. A) Schematic of treatment regimen with PHG or Sham (controls treated with diluent) every 12 hours days 5 through 8 of pregnancy. B) Representative whole uterine horns from sham and PHG treated mice on day 8 of pregnancy. C) Collagen IV and laminin localization and (D) immunoblot of collagen IV NC1 domains for crosslinking analysis from implantation sites on day 8 of pregnancy following sham and PHG treatments.

Goodpasture antigen-binding protein (GPBP) encoding gene expression and protein localization during early pregnancy

Expression of the GPBP encoding gene (*Col4a3bp*) gives rise to multiple isoforms, GPBP-1 and GPBP-2 (previously called GPBP and GPBPΔ26). One isoform, GPBP-1, preferentially localizes to the extracellular matrix. The alternatively spliced isoform, GPBP-2 also known as CERT, is predominately cytosolic where it functions in intraorganelle lipid transport and vesicular transport to the plasma membrane (Raya *et al.*, 1999; Revert *et al.*, 2008; Revert-Ros *et al.*, 2011). First, to determine whether any GPBP was expressed, mRNA expression levels were quantified during early pregnancy and decidua development. *Col4a3bp* expression followed bimodal temporal expression pattern during early pregnancy with the lowest expression during the periods of uterine receptivity and implantation, days 4 through 6 of pregnancy. After blastocyst implantation, *Col4a3bp* levels showed a gradual increase from days 5 through 8 of pregnancy (Figure 2.7 A). These findings suggested that expression of *Col4a3bp* is reduced during the initiation of blastocyst implantation.

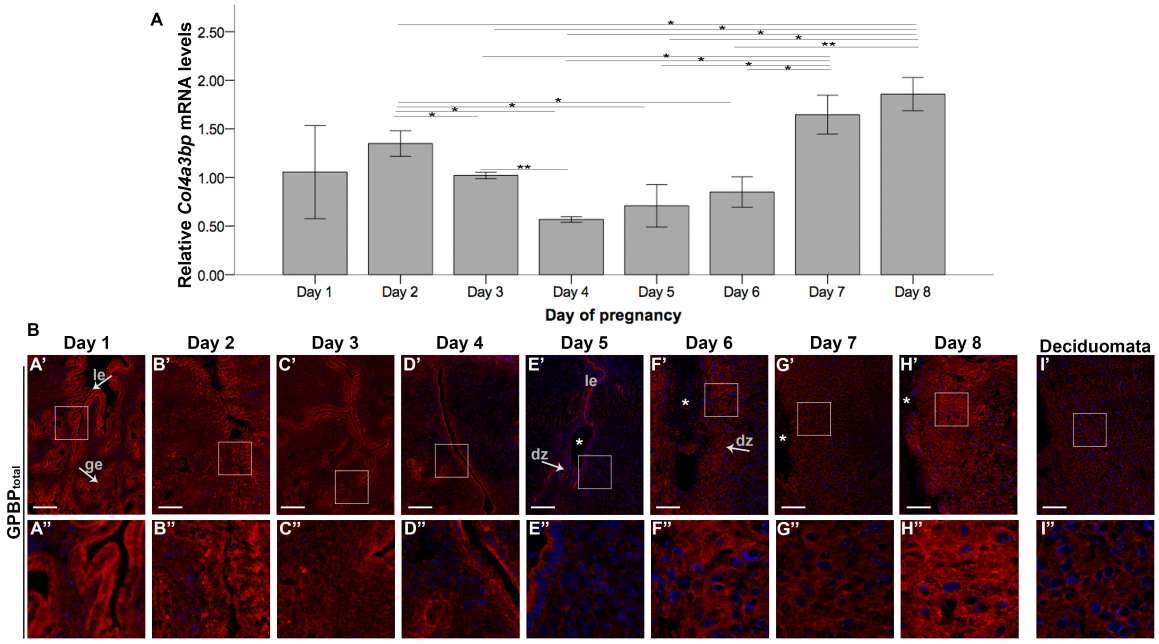


Figure 2.7: Expression and localization of GPBP in the uterus during early pregnancy. A) Uterine mRNA expression of GPBP (Col4a3bp). Bars represent mean and ±SEM (* p-value ≤ 0.05, ** p-value ≤ 0.001). B) Immunofluorescence detection of GPBP in mouse uterus during early pregnancy. Images are oriented near the center of the uterus cross-section with GPBP in red and nuclei in blue. Asterisk (*) indicates the location of the embryo. Scale bar = 100 µm. Inset (White Square; 140 μm^2 region) is a representative of the field showing localization of GPBP in the second row. Le, luminal epithelium; ge, glandular epithelium; bv, blood vessels; dz., decidual zone.

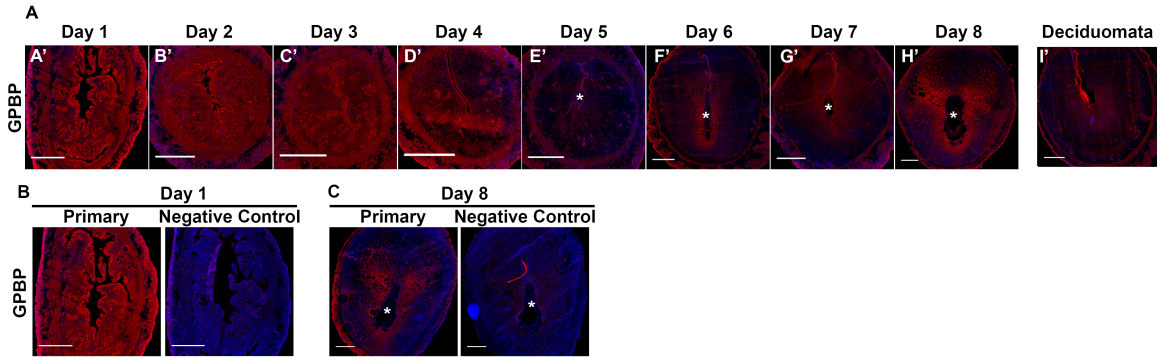


Figure 2.8: Localization of GPBP during early pregnancy. A) Full cross-section of mouse uterus immunofluorescence of GPBP during early pregnancy with GPBP in red and nuclei in blue. Asterisk (*) indicates the location of the embryo. Scale bar = 500 µm. Representative immunofluorescence controls preimplantation (B) and postimplantation (C).

Next, the localization of GPBP protein was examined by immunofluorescence using mAB N26 that detects of both GPBP-1 and GPBP-2 isoforms. On day 1 of pregnancy, GPBP was intensely localized in luminal and glandular epithelial cells with little or no localization detected in the stroma. The protein was predominantly localized to apical and basal plasma membrane of epithelial cells and showed diffuse cytoplasmic localization. Over the next few days (days 2 through 4), intracellular GPBP in the stroma and glands was decreased. However, a specific pattern of cellular localization was observed in the luminal epithelial cells at this time (Figure 2.7 B, A'-D', and 2.8). By day 2 of pregnancy, membranous localization was receding and was being replaced by dense puncta oriented to the basal regions of the cells. Further changes were observed on day 3 of pregnancy with localization of GPBP to the lateral and apical regions of epithelial cells (Figure 2.7 B, B'-C'). On days 4 and 5 of pregnancy, GPBP was primarily localized to the apical membrane of luminal epithelial cells, with little stromal localization (Figure 2.7 B, D'-E').

As decidualization progressed, the decidual zone surrounding the embryo showed strong GPBP localization from days 6 through 8 of pregnancy. Localization of GPBP on these days was observed at the cell borders and within the cytoplasm of decidual cells (Figure 2.7 B, F'-H'). This increased localization temporally coincided with BM thickening (Figure 2.2 C-D) and increased GPBP mRNA expression (Figure 2.7 A). By day 8 of pregnancy, layers of decidual cells juxtaposed to the embryo were devoid of significant levels of GPBP localization, suggesting that GPBP was primarily expressed by decidual cells encapsulated by collagen IV (Figure 2.7 B, H'). Accumulation of GPBP in the artificially-induced deciduomata displayed similar production by decidual cells as decidualization induced by the implanted blastocyst (Figure 2.7 B I'). Collectively, the data revealed that expression and localization of both isoforms, GPBP-1 and GPBP-2, are down-regulated during uterine receptivity. However, it was unclear if one or both isoforms were amplified during decidualization, a feature that coincides with decidua BM thickening.

Localization patterns of GPBP-1 and laminin in the uterus during early pregnancy

Previous studies have revealed that GPBP-1 is associated with the extracellular matrix of kidney (Raya *et al.*, 1999). To determine if our findings of pericellular GPBP (Figure 2.7 B) were associated with GPBP-1 translation and localization to basement membrane in the uterus, a novel monoclonal antibody (mAb e11-2) that recognizes the 26-residue region present in GPBP-1 but not in GPBP-2 (Figure 2.9 A) was used in immunofluorescence. Particular attention was focused on days 1 through 3 and days 6 through 8 since higher levels of expression and detection of GPBP were observed during this time frame. The detection of GPBP-1 showed similar localization patterns as detection of one or both GPBP isoforms (Figure 2.9 B and 2.7 B). GPBP-1 was primarily localized to epithelium day 1 through 3 and decidual zones days 6 through 8. Cytoplasmic puncta were observed on day 2 followed by apical localization on day 3 (Figure 2.9 B, A'-C'). At that time, basal GPBP-1 was colocalized with laminin, a biomarker of BM, patterns concentrated to the uppermost side of the basement membrane but not intensely throughout (Figure 2.9 B, A"-C"). Laminin and GPBP were colocalized in myometrium and perimetrium throughout all pre- and postimplantation days of pregnancy (Figure 2.10). Pericellular GPBP-1 colocalized with laminin in the decidual zone days 6 through 8, with days 7 and 8 showing the strongest detection of GPBP-1 (Figure 2.9 B, D'-F'). Different sides of individual cells had comparatively unequal distribution of GPBP-1 and laminin, according to relative intensities of colocalized regions (Figure 2.9, D"-F"). These decidual tissues had no presence of collagen IV alpha 3 (Figure 2.11). Thus, the uterine BM-associated patterns of GPBP-1 were achieved through interactions with other BM components, such as previously described bindings with other chains of collagen IV and laminin (Mencarelli *et al.*, 2012). Our findings of GPBP-1 immunofluorescence show that it 1) is the predominant isoform of GPBP in the uterus during early pregnancy, 2) localizes to endometrial and decidual BMs, and 3) has varied distribution around individual decidual cells.

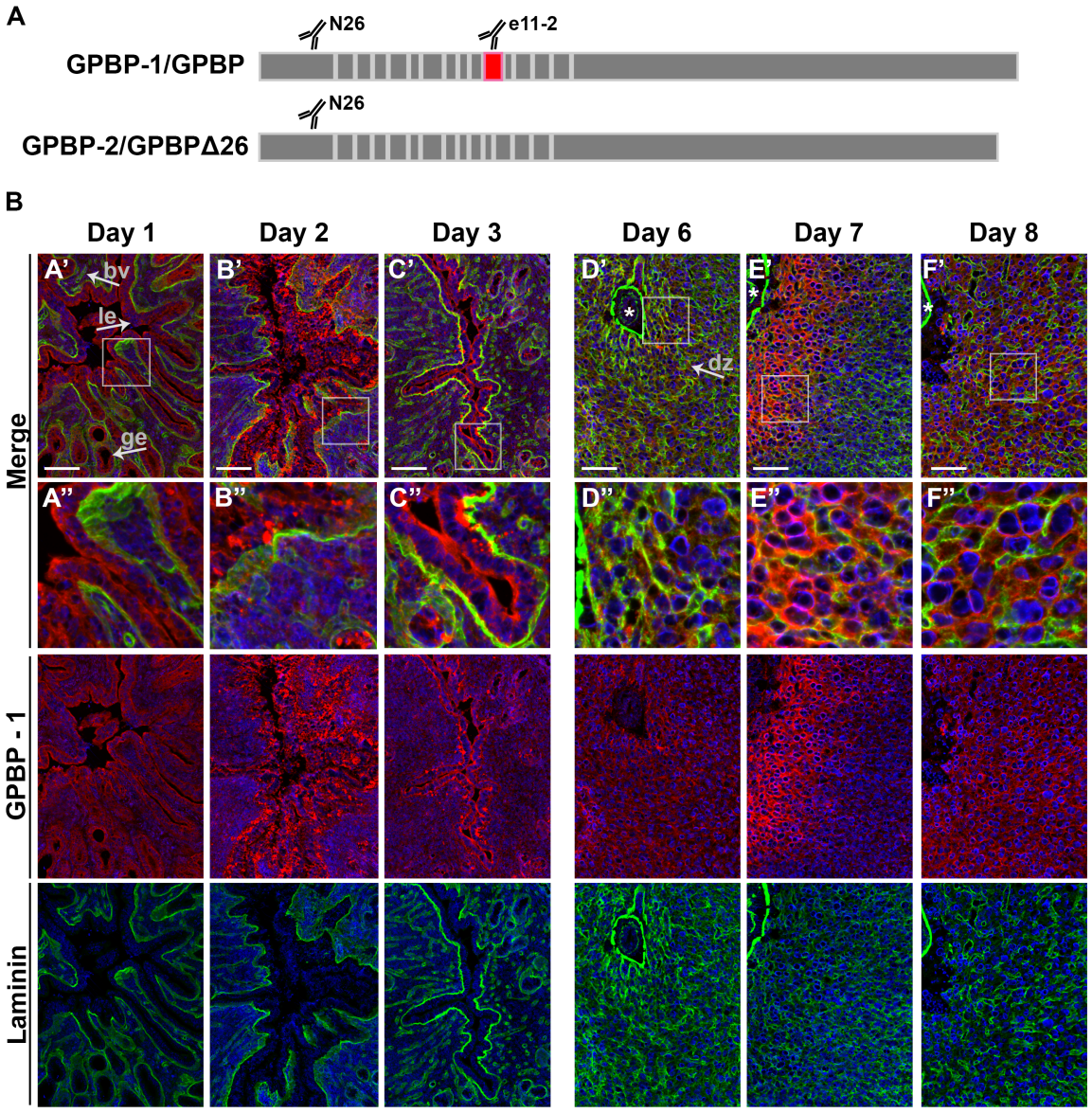


Figure 2.9: Laminin-1 and GPBP-1 isoform localization during early pregnancy. A) Diagram depicting areas of GPBP isoforms that mAb N26 and e11-2 specifically bind. The N26 antibody recognizes all isoforms of GPBP while the e11-2 antibody detects the 26-amino acid region that is encoded by exon 11 (shown as the red block), which is not present in GPBP-2 also called GPBPΔ26 or CERT. B) Double immunofluorescence detection of GPBP-1 and laminin in mouse uterus during early pregnancy. Images are oriented near the center of the uterus cross-section with GPBP-1 in red, laminin in green, and nuclei in blue. Colocalization of GPBP-1 and laminin is indicated by orange/yellow color. Asterisk (*) indicates the location of the embryo. Scale bar = 100 μm. Inset (White Square; 140 μm^2 region) is a representative of the field showing merge of GPBP-1 and laminin in the second row. Le, luminal epithelium; ge, glandular epithelium; bv, blood vessels; dz., decidual zone.

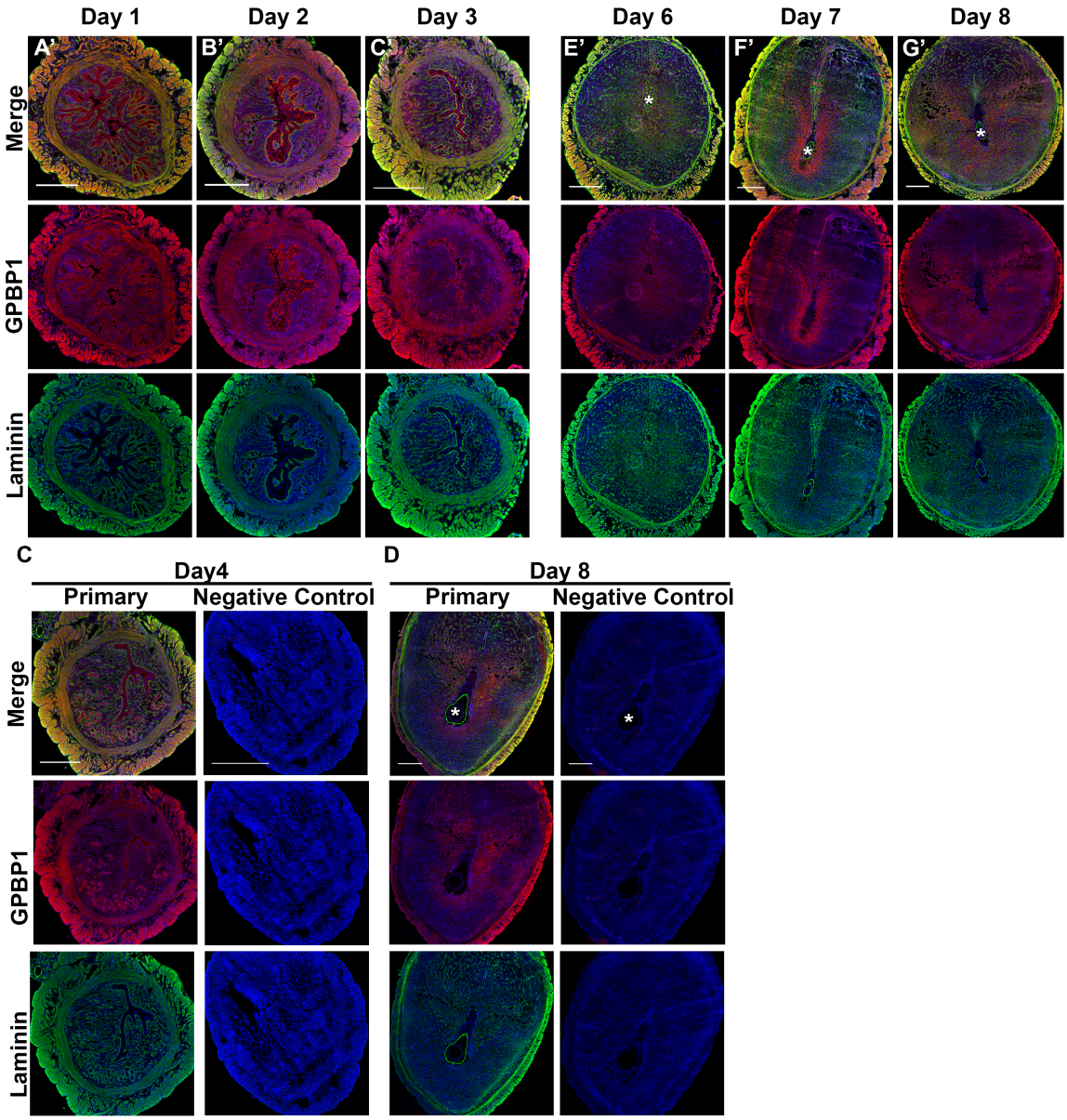


Figure 2.10: Laminin-1 and GPBP-1 isoform localization in uterine tissues during early pregnancy. A) Diagram depicting areas of GPBP isoforms that mAb N26 and e11-2 specifically bind. The N26 antibody recognizes all isoforms of GPBP while the e11-2 antibody detects the 26-amino acid region that is encoded by exon 11 (shown as the red block), which is not present in GPBP-2 also called GPBPΔ26 or CERT. B) Full cross-section of mouse uterus double immunofluorescence of GPBP-1 and laminin during early pregnancy with GPBP-1 in red, laminin in green, and nuclei in blue. Colocalization of GPBP-1 and laminin is indicated by orange/yellow color. Asterisk (*) indicates the location of the embryo. Scale bar = 500 μm. Representative immunofluorescence controls preimplantation (C) and postimplantation (D).

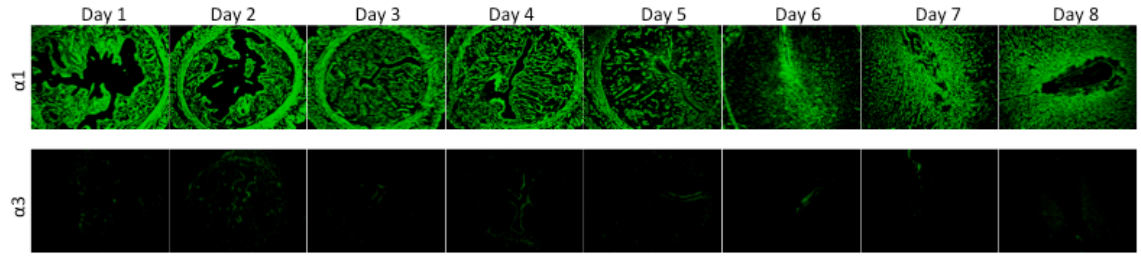


Figure 2.11: Collagen IV alpha 1 and alpha 3 chains localization patterns during early pregnancy. Immunofluorescence detection of collagen IV using chain specific antibodies against alpha 1 and alpha 3 chains (green). Images collected using 4x magnifying objective.

Knockout of GPBP1 during early pregnancy

To determine whether elimination of GPBP1 impacted BM assembly during early pregnancy, implantation from GPBP1 knockout (KO) mice were assessed on day 8 of pregnancy. These mice were designed to specifically investigate roles of GPBP1 while leaving GPBP2 intact by targeted deletion of exon 11 of *Col4a3bp* gene using Cre-lox technology (Figure 2.12 A) (Revert-Ros *et al.*, 2011). Total deletion of *Col4a3bp* causes embryonic lethality (Wang *et al.*, 2009). Immunofluorescence studies using the N26 antibody revealed that GPBP was still produced at the implantation sites. However, GPBP was localized primarily to cytoplasm and pericellular patterns were punctate (Figure 2.12 B). Laminin patterns were sporadically condensed between decidua cells and had no linear colocalization with GPBP (Figure 2.12 B). Still, consistent with a previous report (Revert-Ros *et al.*, 2011), GPBP1-KO mice reproduced normally and were capable of being maintained for generations with both null alleles. In the absence of GPBP1, BM proteins were still secreted and intracellular isoforms of GPBP may have compensated for loss of extracellular GPBP. More in-depth analysis of the BM ultrastructure and integrity is needed to determine if GPBP1-KO disrupted molecular characteristics of the decidua BM. A potential approach to address this is to establish an induced deletion of all GPBP isoforms adult mice during pregnancy. This would avoid embryonic lethality from total KO while permitting the investigation of GPBP functions during pregnancy. The present data suggest that GPBP1 may have a role in the process of encapsulating decidua cells with BM, but is not required for BM production or a successful pregnancy outcome. It also supports the argument that cytoplasmic GPBP may have roles in decidualization.

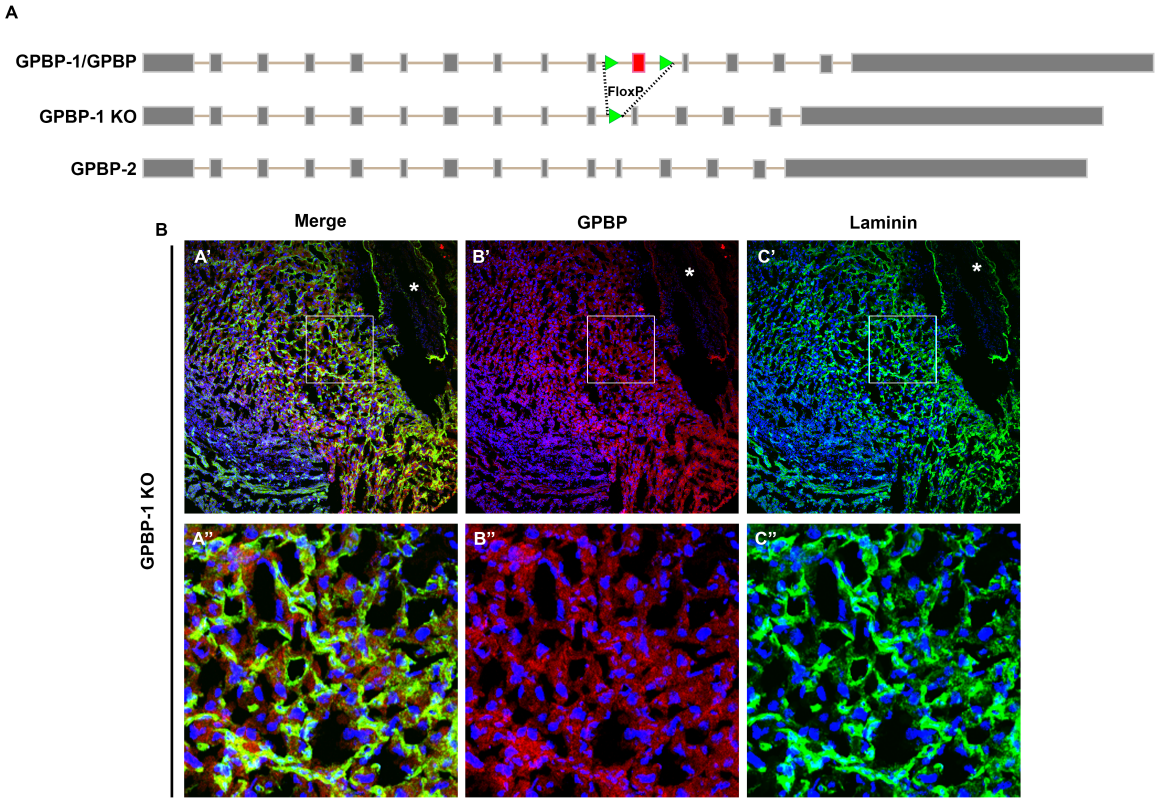


Figure 2.12: GPBP-1 knockout mice generation schematic and localization of GPBP and laminin in day 8 implantation site**.** (A) Schematic of genetic deletion GPBP1 by Floxed deletion of exon 11 of *Col4a3BP*. Grey boxes, exons; green arrowheads, flanking LoxP sites (FloxP); brown line, intron regions (not to scale). Adapted from (Revert-Ros *et al.*, 2011). (B) Co-immunofluorescence detection of GPBP (red) using an antibody toward all isotypes and laminin (green) in a representative GPBP1-KO mouse on day 8 of pregnancy. Micrographs collected with 20x objective (A'-C') and white boxes define field of view magnified in lower panels (A"-C").

Lysyl oxidase-like 2 protein localization in uterus during early pregnancy

The production of lysyl oxidase family proteins is a characteristic of matrix remodeling in both normal and pathological conditions (Grau-bové *et al.*, 2014; González-Santamaría *et al.*, 2016). Particularly, LOXL2 contributes to stability of collagens in BM-rich tissues, such as blood vessels (Bignon *et al.*, 2011; de Jong *et al.*, 2016) and corneas (Dudakova *et al.*, 2016). Evidence has revealed that in humans LOXL2 is present in term decidual tissues (Hein *et al.*, 2001), but the localization in uterine tissues during early pregnancy remain unknown. To address this, immunofluorescence detection of LOXL2 was used to investigate patterns during uterine reprogramming. LOXL2 was found to localize throughout the stroma of preimplantation endometrium with greater accumulation around glandular tissues (Figure 2.13, days 1 through 4 of pregnancy). Following implantation, LOXL2 detection is reduced throughout the endometrium on days 5 through 8 of pregnancy. On days 6 through 8, a growing presence of intensely positive LOXL2 cells appear at the interface of the embryo and uterine tissues (Figure 2.13). These data suggests that LOXL2 is not essential for decidua development but may be important for the function of trophoblastic cells at the maternal-embryo interface which is consistent with a report of up-regulation of LOXL2 human trophoblasts during early placental development (Segond *et al.*, 2013).

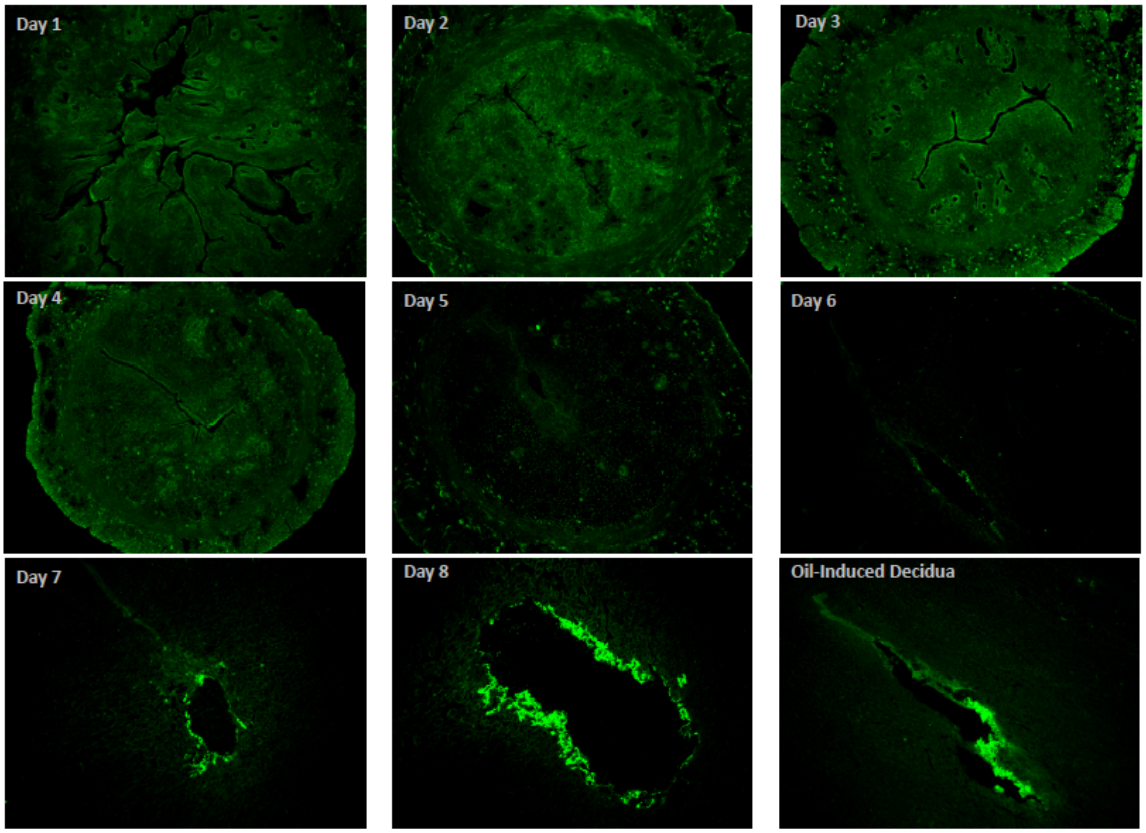


Figure 2.13: Localization of Lysyl oxidase-like 2 (LOXL2) during early pregnancy. Immunofluorescence detection of LOXL2 (green) in days 1 through 8 of pregnancy and oil-induced deciduomata. Micrographs collected using magnifying objective.

Collagen IV and laminin gene expression and protein localization patterns in the uterus during early pregnancy

Thus far, observations indicate that BMs undergo dynamic regulation are based on studies in non-vertebrate animal models and cell culture systems (Xu *et al.*, 2009; Isabella & Horne-Badovinac, 2015; Morrissey & Sherwood, 2015). In the present study, we examined uterine tissues as our model system because BM proteins, collagen IV and laminin, have been shown to localize in BMs in the endometrium before implantation and in mature decidua several days after implantation (Wewer *et al.*, 1985; Farrar & Carson, 1992; Diao *et al.*, 2011; Oefner *et al.*, 2015). These pre- and postimplantation BMs associate with different tissues that occupy an overlapping anatomical space through tissue reprogramming, yet differ in fine details. However, the dynamic transition of these components upon embryo implantation and initial triggering of decidualization is unknown. We, therefore, sought to analyze mRNA expression levels of collagen IV (collagen IVα1 and collagen IVα2) and laminin (laminin γ1) by qPCR in the uterus throughout uterine reprogramming. The expression levels of *Col4a1*, *Col4a2* and *Lamc1* remained relatively constant from days 1 through 4 (Figure 2.14 A). However, *Col4a2* levels showed significant up-regulation over *Lamc1* in day 5 implantation sites compared with their preimplantation uterine levels (days 1 through 4). While the levels of both genes for collagen IV chains showed no significant differences from days 5 through 8, *Lamc1* levels showed a steady increasing trend from days 5 through 8 (Figure 2.14 A). These findings are consistent with a previous study that demonstrated the relative expressions of genes encoding collagen IV and laminin in mouse uterus during early pregnancy using Northern and slot blot analysis (Farrar & Carson, 1992). Despite these compelling findings, definitive differences in protein localization of collagen IV and laminin have not been demonstrated, particularly during the period when expression seems to diverge with the incident of blastocyst attachment and early decidualization. We, therefore, applied co-immunofluorescences to examine the compartmental distribution associated with the differential expression of collagen IV and laminin proteins.

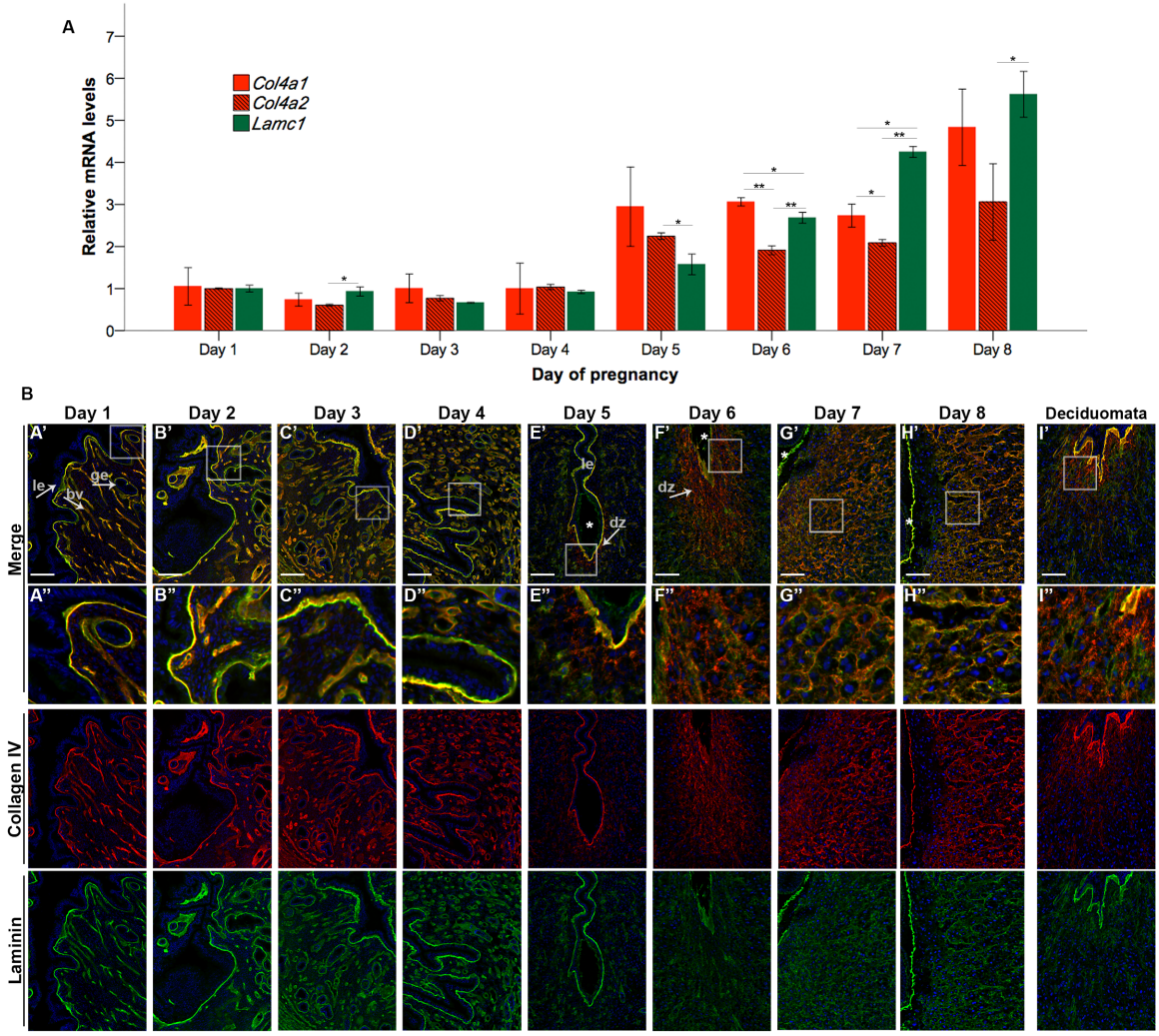


Figure 2.14: Collagen IV and laminin expression and localization during early pregnancy. A) Uterine mRNA expression of collagen IV (Col4a1 and Col4a2) and laminin (Lamc1). Bars represent mean and ±SEM (* p-value ≤0.05, ** p-value ≤0.001 B) Double immunofluorescence detection of collagen IV and laminin in mouse uterus during early pregnancy. Images are oriented near the center of the uterus cross-section with collagen IV in red, laminin in green, and nuclei in blue. Colocalization of collagen IV and laminin is indicated by orange/yellow color. Asterisk (*) indicates the location of the embryo. Scale bar = 100 µm. Inset (White Square; 140 μm^2 region) is a representative of the field showing merge of collagen IV and laminin in the second row. Le, luminal epithelium; ge, glandular epithelium; bv, blood vessels; dz., decidual zone.

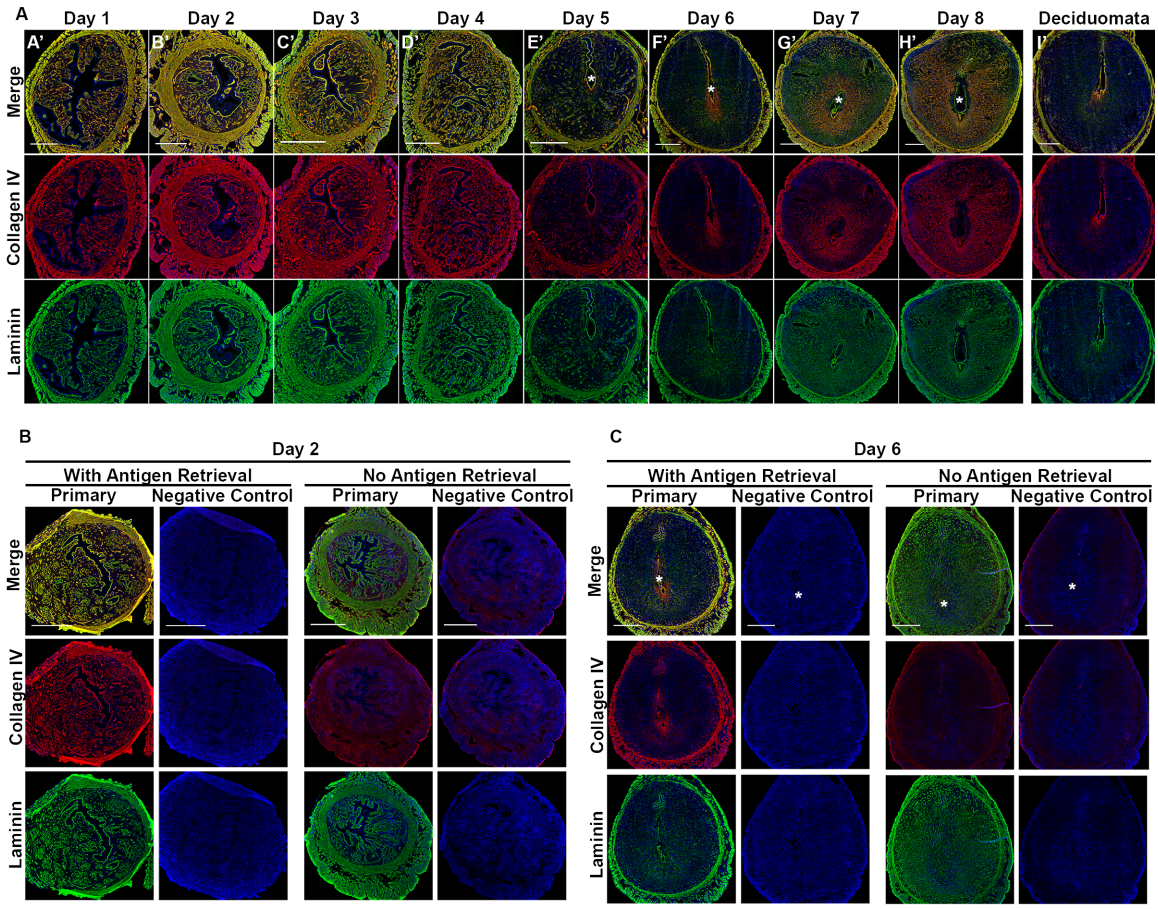


Figure 2.15: Collagen IV and laminin localization in uterine tissues during early pregnancy. A) Full cross-section of mouse uterus double immunofluorescence of collagen IV and laminin during early pregnancy with collagen IV in red, laminin in green, and nuclei in blue. Colocalization of collagen IV and laminin is indicated by orange/yellow color. Asterisk (*) indicates the location of the embryo. Scale bar = 500 μm. Representative immunofluorescence controls preimplantation (B) and postimplantation (C).

During the preimplantation period (days 1 through 4), collagen IV and laminin were colocalized exclusively beneath the BM of cells of the luminal epithelium, glandular epithelium and blood vessels in the endometrium. As expected, no collagen IV or laminin localization was detected in stromal cells at this time (Figure 2.14 B, A'-D'). Following blastocyst attachment, collagen IV and laminin production were colocalized underneath the luminal epithelium and glandular epithelium in day 5 implantation sites. Notably, the implanted blastocyst was positive for laminin, but not for collagen IV. Additionally, we noticed for the first time weak detection of collagen IV and laminin throughout the stromal area (Figure 2.14 B, E'). Higher magnification images of the area beneath the attached blastocyst revealed punctate collagen IV deposition in the absence of laminin around decidual cells situated adjacent to the luminal epithelium (Figure 2.14 B, E").

In the day 6 implantation site, punctate collagen IV staining surrounding decidual cells was observed with broader distribution into the growing decidual zone with limited detectable laminin. However, some peripheral stromal cells appeared to be positive for laminin (Figure 2.14 B, F' and 2.15). These observations demonstrated that collagen IV preceded laminin expression and production in early differentiating stromal cells. On day 7 of pregnancy, laminin was colocalized with pericellular collagen IV puncta and the BM appeared to assemble into improved compact borders surrounding decidual cells (Figure 2.14 B, G'). By day 8, collagen IV and laminin were colocalized and encapsulated individual decidual cells and distributed throughout the entire stromal space from near the embryo toward the myometrium (Figure 2.14 B H' and 2.15). At this time, a few layers of cells juxtaposed to the embryo were consistently devoid of collagen IV and laminin staining. In the myometrium, collagen IV and laminin colocalized stainings were observed as intense positive immunofluorescence around the muscle cells (Figure 2.15). We observed the similar positive production of collagen IV and laminin in deciduomata tissue (Figure 2.15 B, I'). Our results suggest that collagen IV deposition within pericellular spaces of the transitioning decidual zone may aid in the distinction of early decidual cells from stromal cells.

Localization of CD31 in uterine tissues during early pregnancy

Previously, decidual cells have been described as epithelioid because the mature cells exhibit several characteristics that are associated with epithelial cells, including basement membrane and cell-cell junction assembly (Parr *et al.*, 1986; Damjanov & Wewer, 1991). These are also characteristics of endothelial cells, yet other endothelial-like properties have not been examined in decidual tissues. To address this, localization of CD31, a biomarker of endothelium that is also known as platelet/endothelial cell adhesion molecule (PECAM), was examined in endometrial and decidual tissues during early pregnancy. On days 3 and 4 of pregnancy, CD31 was highly concentrated in vasculature networks (Figure 2.16 A"-B"), while epithelium, stroma, and avascular myometrium showed no localization (Figure 2.16 A'-B'). On day 7 of pregnancy, CD31 was dispersed throughout the decidual tissues with diffuse localization at pericellular areas and within the cytoplasm of some cells (Figure 2.16 C' and C"). On day 8 of pregnancy, decidual CD31 increased and concentrated at pericellular areas compared to day 7 decidua (Figure 2.16 D"). The decidual tissues that were positive for CD31 did not display characteristic organization of vasculature of cellular monolayers or tubular-like branching (Figure 2.16 C' and D'). These findings demonstrate that decidual tissues display endothelial-like properties by localizing CD31 to pericellular spaces but do not form vasculature structures.

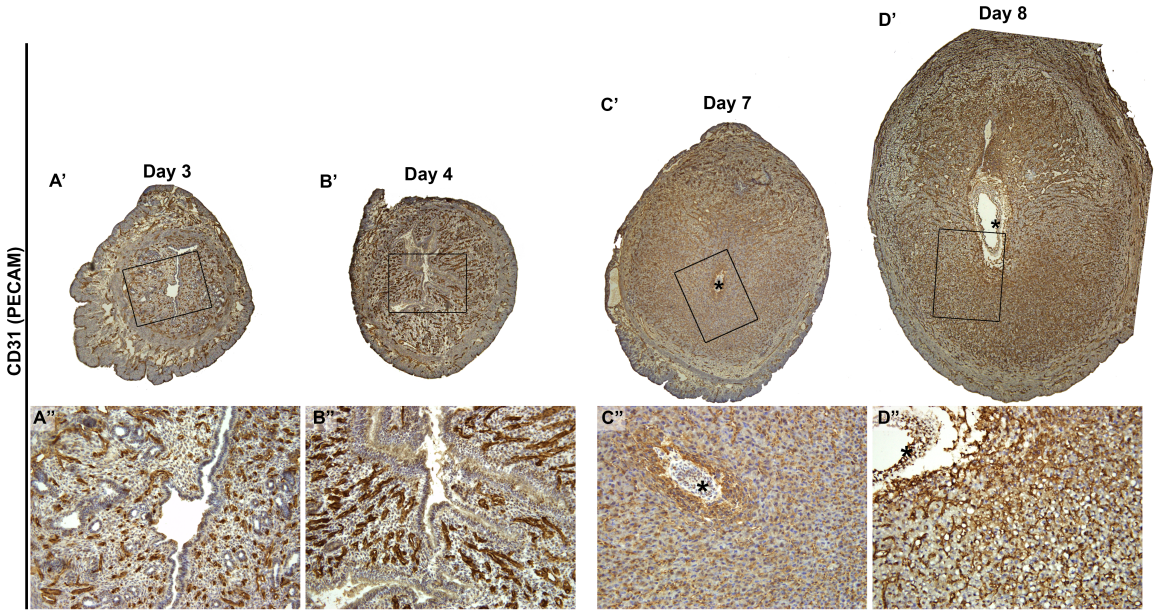


Figure 2.16: Localization of CD31 in uterine tissues during early pregnancy. The endothelial biomarker, CD31/PECAM (brown) in preimplantation endometrial tissues on days 3 and 4 of pregnancy (A'-B') and in postimplantation decidual tissues on days 7 and 8 of pregnancy (C'-D') viewed with 4x objective. Black boxes indicate field-of-view enlarged in lower panels (A"-D"). Asterisks mark the location of the embryo.

Discussion

Our findings of distinct spatial and temporal localization and expression patterns of peroxidasin, GPBP, LOXL2, collagen IV, and laminin within the reprogramming uterine tissues during early pregnancy suggest that these molecules are important players in the dynamic reorganization and functional changes that occur during decidua development. Our findings indicate that these ECM proteins possess both individual and integrated properties as components of BM scaffolds. Moreover, our findings provide further evidence for the classification of peroxidasin and GPBP as components of the BM.

A potential dual function for peroxidasin in early pregnancy

We observed two novel distribution patterns of peroxidasin in association with the developing decidua. Peroxidasin was produced prior to collagen IV and then localized adjacent to stromal cell BM following embryo attachment, presumably, to support continual sulfilimine crosslinking of collagen IV during deposition. We observed several instances of peroxidasin localized to tissue regions absent of BM. These findings suggest that in pregnancy peroxidasin may have functions beyond a role in BM reinforcement. For example, peroxidasin generates intermediate products that have the potential to protect from infection at highly vulnerable stages in pregnancy. Low concentrations of hypohalous acids (HOBr and HOCl) produced by peroxidasin have been shown to have antimicrobial functions. Exposure of *Escherichia coli* cultures to purified peroxidasin in the presence of its enzymatic substrates, H_2O_2 and chloride, results in the destruction of the bacteria (Li *et al.*, 2012). Possibly, these antimicrobial mechanisms of peroxidasin may contribute to native defense against microbial infections at the time of implantation and during early embryo development, thus protecting against the potential for pregnancy failure (King *et al.*, 2007). Notably, several microbes that disrupt decidua function, including the human papillomavirus (HPV) and *Staphylococcal aureus*, have derived mechanisms that may dock microbes to or destroy BMs (Steukers *et al.*, 2012), further emphasizing the benefits of localization of an antimicrobial producing agent, such as peroxidasin, to decidual BMs.

This is of particular interest when considering the milieu of the embryonic environment where the cells typically responsible for eliminating pathogens, such as dendritic cells and macrophages, have restricted mobility and functionality in the decidua (Burrows *et al.*, 1995; Chakraborty & Pulendran, 2009; Collins *et al.*, 2009; Yoshinaga, 2012; Hsu & Nanan, 2014).

The lack of response to PHG treatments on decidual BM assembly and function raises the potential for undiscovered other mechanisms that are naturally present for early pregnancy success. Basement membrane dynamics involves a balance of assembly and destruction of components. Although the Hudson laboratory regularly uses PHG as an inhibitor of BM assembly, PHG has reported therapeutic potential as an inhibitor of matrix metalloproteinase (MMPs), enzymes that degrade BMs (Piao *et al.*). During decidualization, PHG did not destabilize BM assembly through sulfilimine crosslink impairment and it may also inhibit BM destruction. In this scenario, decidual BM production would progress normally with PHG having some protective effects, similar to other MMP inhibitors that aid in this balance (Amano *et al.*, 2005; Anumba *et al.*, 2010).

Expression and Localization of GPBP potentially regulate BM thickening during decidualization

We found decidual GPBP was expressed after collagen IV and laminin are deposited. It was up-regulated as decidua BM was forming which suggests it may act as a key component in the assembly of new basement membranes in these adult tissues. We found the distribution of GPBP in BMs to vary in small patches around decidual cells that coincide with a variably progressive thickening of BM ultrastructure around these cells. Similarly, overexpression of GPBP has been previously shown to induce renal BM thickening in a mouse model of autoimmune pathogenesis (Revert *et al.*, 2007). Compositionally distinct nanoscale patterns of BMs can control communication between adjacent cells. For example, BMs at neuromuscular junctions differ from BMs along the rest of the muscle and regulate synaptic signaling that is essential for

neuronal control of muscles (Sanes, 2003) and BM pliability that orchestrates transmigration of individual cells during development (Kelley *et al.*, 2014). Thus, BM thickening and GPBP expression may be important contributors of the normal function of the decidua. We found that some BM was assembled and early pregnancy progressed even in the absence of GPBP-1, suggesting that cytoplasmic GPBP may also function in BM assembly and compensate for the loss of pericellular GPBP.

A previous study showed that forces exerted by decidual tissues functions in shaping the early embryo by facilitating embryo elongation and formation of the distal visceral endoderm (Hiramatsu *et al.*, 2013). This tensile strength is comparable to soft tissues, such as striated and myocardial smooth muscles, which are almost entirely comprised of specialized cells encapsulated in BM (Butcher *et al.*, 2009). Such tissues are designed to endure and respond to the greatest native forces challenging the corpus. We found that decidual tissues have even greater tensile strength during maturation through day 8 of pregnancy. We presume this is in part attributed to the thickening of BM. This notion is further supported by previous findings that associate progressive thickening of embryonic retinal BM with a substantial increase in tensile strength (Candiello *et al.*, 2007).

Basement membrane in preimplantation uterus

Our findings that collagen IV and laminin were colocalized to regions underlying polarized epithelial cells of the lumen and glands, and endothelial cells of blood vessels in the preimplantation mouse uterus, was an expected observation since these proteins are the most abundant BM components and classically colocalize in BMs throughout the body (Yurchenco, 2011). However, a significant finding of the present study was the unique expression and localization patterns of peroxidasin and GPBP in uterine epithelial cells prior to implantation. GPBP is a protein with multiple isoforms that function both at extracellular and intracellular compartments (Mencarelli *et al.*, 2010). Both intracellular and pericellular localization of this

protein in days 1 and 2 of pregnancy suggests it may serve in dual roles in intraorganelle protein transport for plasma membrane transformation (Murphy, 2004) and/or in the molecular organization of BM components (Revert *et al.*, 2007), respectively.

We also found peroxidasin to have intracellular localization and pericellular colocalization with collagen IV in the preimplantation uterine epithelial cells, which suggests active turnover and application of the enzyme in BM reinforcement by sulfilimine crosslinking. Peroxidasin is a specific peroxidase critical for the maintenance of epithelial tissue integrity across several species and organ systems (Bhave *et al.*, 2012). Perturbation of peroxidasin function yields epithelium disorganized and dysfunctional (Fidler *et al.*, 2014; McCall *et al.*, 2014; Yan *et al.*, 2014). Therefore, it is likely that peroxidasin reinforces the collagen IV network of BM scaffolds to support epithelial cell architecture in the endometrium. In the endometrium, BM interfaces the epithelium and the stroma thus mediating communication between these compartments by its known functions of engaging cell-matrix signaling and modulating chemokine signaling forming cytokine gradients and acting as growth factor reservoirs (Friedl & Alexander, 2011). The importance of the communication between these tissue compartments is represented in the mouse models that show a failure of blastocyst implantation or decidualization in the absence of endometrial glands (Filant & Spencer, 2013) or luminal epithelium (Lejeune *et al.*, 1981). In this regard, the presence of BM within the preimplantation endometrium has importance in governing for uterine receptivity, blastocyst implantation, and decidualization.

Lysyl oxidase-like 2 (LOXL2) was found to accompany preimplantation remodeling rather than postimplantation decidualization. Other lysyl oxidase family members may function in the assembly of decidual BMs, such as LOXL4 that has the capacity to also enhance collagen IV assembly (Grau-bové *et al.*, 2014). In this regard, the tailoring of BMs for decidua functions differs in from BM assembly during angiogenesis and epithelial functions (Bignon *et al.*, 2011; Kim *et al.*, 2011). It remains to be determined whether the biased production of certain LOX proteins leads to essential intricate differences in BM assembly.

Collective functions of BM components during decidualization

In response to blastocyst implantation, we observed collagen IV is distinctively associated with stromal cells surrounding the implanted blastocyst independent of laminin localization indicating that collagen IV may be important for early alterations of these cells. Current dogma argues that laminin is the pivotal BM component produced by cells forming new BMs (Sasaki *et al.*, 2004; Hohenester & Yurchenco, 2013). However, most studies contributing to this perception focus on embryo development and do not address BM production in adult tissues. Our observations herein suggest that the order of collagen IV and laminin expression may be tissue and context specific.

One of the hallmarks of decidualization is the proliferation of stromal cells prior to the transition to decidual cells (Zhang & Paria, 2006). Studies have demonstrated that collagen IV and laminin are required for cell proliferation and/or differentiation in epithelial, endothelial, and some mesenchymal cells such as muscle cells (Hagios *et al.*, 1998; Boonen & Post, 2008; Hynes, 2009; Yurchenco, 2011). Our results suggest that decidual cells are perhaps another cell type reliant on these BM properties. Epithelial-mesenchymal transition (EMT) is considered to be a fundamental event during embryogenesis, while a reverse cell fate (mesenchymal-epithelial transition, MET) was also described during cellular reprogramming (Li *et al.*, 2010). Following blastocyst implantation, stromal cells surrounding the implanted blastocyst undergo polyclonal decidualization which is crucial to sustaining pregnancy since epithelial cells surrounding the blastocyst disappear by apoptosis (Zhang & Paria, 2006). Several studies have provided evidence that decidual cells are perhaps epithelial-like cells since stromal cells loose mesenchymal proteins like snail and vimentin (Zhang *et al.*, 2013b) and gain epithelial proteins like E-cadherin, zonula occludens-1 (ZO-1) and cytokeratin (Paria *et al.*, 1999b) during stromal to decidual transition. Consistent with these findings, we report mesenchymal-derived decidual cells formed basement membrane in their pericellular spaces with up-regulated peroxidasin, GPBP, collagen IV, and laminin. In addition to representing a hallmark of epithelial-like differentiation, this present study revealed that decidual cells also exhibit some endothelial-like behaviors by

expressing endothelium cell adhesion marker CD31. The interplay of cell-matrix interactions and inclusion of BM-bound chemokines and growth factors, such as bone morphogenic proteins (BMPs), transforming growth factor-beta (TGFβ), heparin-binding epidermal growth factor-like growth factor (HB-EGF), and fibroblast growth factor 10 (FGF10), may modulate endometrial stromal cell proliferation and other functions that are epithelial or endothelial-like (Paizis *et al.*, 1998; Reddi, 2000; Lim *et al.*, 2002; Lim & Wang, 2010; Friedl & Alexander, 2011).

We also found BM to encapsulate stromal cells early in decidualization but noted continuity of BM structure was altered with the appearance of cell-cell junctions as decidual cells matured. It is known that cell-cell junctions are features of decidua establishment and function as macromolecular barriers between the mother and embryo which can block penetrance of IgG that can elicit unwanted immune rejection of the embryo and add rigidity to the tissue (Tung *et al.*, 1986; Bany & Hamilton, 2011). A recent study on cardiomyocytes, a mesenchymal-derived cell encapsulated by BM, showed a similar pericellular BM that underwent alterations for the mediation of cell junction formation (Yang *et al.*, 2014a). Perhaps decidua BM also mediates tight junction formation and functions as an additional selective barrier and contributes to rigidly while junctions are established.

Pregnancy and pseudo-pregnancy BM production response convergence

We found that embryo-induced and oil-induced decidualization produced similar distributions of BM proteins peroxidasin, GPBP, collagen IV, and laminin within similar time frames. Thus, our findings demonstrate commonalities in the mechanisms of uterine reprogramming that are triggered by natural embryonic implantation and pseudo-pregnancy. Further studies that compare responses to these stimuli will give insights into the mechanisms whereby the embryo triggers BM changes.

Conclusion

In summary, we show that peroxidasin, GPBP, LOXL2, collagen IV, and laminin are expressed in temporal and spatial patterns during uterus reprogramming, suggesting individual functions of these components and collective functions as components of BMs (Figure 2.14). The observed matrix modulation is consistent with the emerging concept that BMs are more than static scaffolds, and they are dynamic constituents in biology that influence cell fate, behavior, and tissue stability and function (Isabella & Horne-Badovinac, 2015; Morrissey & Sherwood, 2015). Our findings provide evidence that peroxidasin and GPBP are critical ECM components for embryo implantation and decidua development and supportive evidence for their classification as components of the BM. Furthermore, mouse embryo implantation can serve as a tractable model for the exploration of the functions of BM proteins during early pregnancy and human uterine physiology (Wewer *et al.*, 1985; Dockery *et al.*, 1998; Lim & Wang, 2010; Ramathal *et al.*, 2010). These findings may be clinically relevant since studies have suggested that uterine BM properties may be disrupted in patients that have pregnancy complications and high fail rate linked to spontaneous abortion (Iwahashi *et al.*, 1996), diabetes (Yurchenco & Patton, 2009; Roy *et al.*, 2010), malnutrition (McCall *et al.*, 2014), and smoking (Dempsey & Benowitz, 2001).

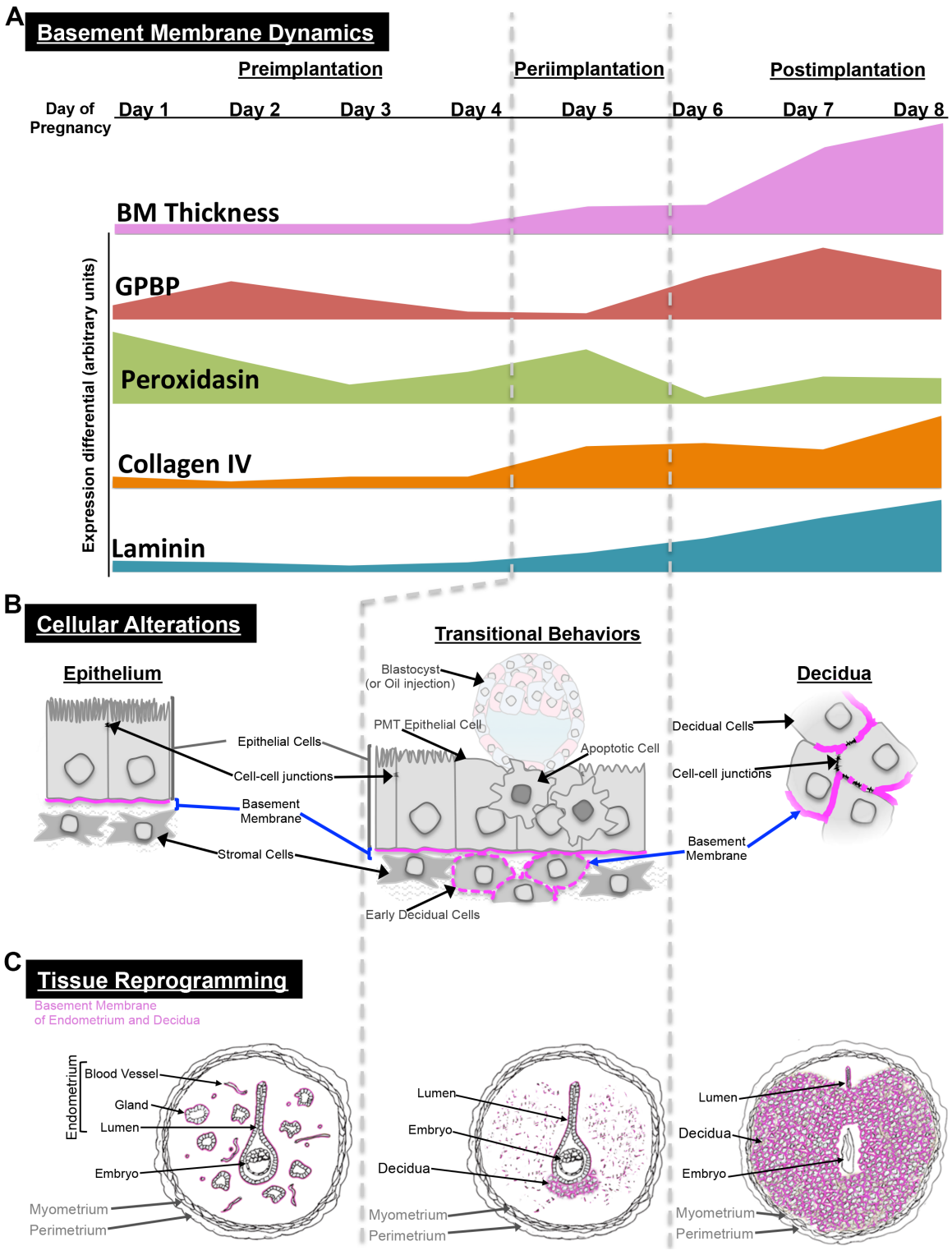


Figure 2.17. Schematic model of BM components and cellular dynamics during uterine tissue reprogramming for early pregnancy. Dynamics exhibited between days 1–4 for preimplantation, day 5 for periimplantation, and days 6–8 for post implantation. A) Basement membrane protein

dynamics of ultrastructure thickening and mRNA expression changes (differential is relative for individual gene minimum and maximum expression levels throughout days 1–8 of pregnancy); fluctuations of expression levels are similarly mirrored in protein distribution. B) Cellular alterations incorporate different localization patterns of BM during the transition from epithelium to decidua. PMT, plasma membrane transformation. C) Tissue reprogramming involves destruction and development of tissues supported by BMs, endometrium and decidua, respectively. Basement membrane represented by pink patterns. Dashed gray line demarcates BM protein, cellular, and tissue alterations that occur during periimplantation, also known as the window of implantation.

Experimental Procedures

Animals

Sexually mature CD1 mice were used for all studies (Charles River Laboratories, Crl:CD1(ICR), Wilmington, MA, USA). Female mice were bred overnight with fertile males of the same strain and the presence of vaginal plug the following morning indicated day 1 of pregnancy (Zhang & Paria, 2006). On days 1 through 4 of pregnancy, whole uterine horns were harvested. Implantation sites on day 5 of pregnancy were identified after intravenous injections of 1% Chicago Blue 6B dye (Sigma, St. Louis, MO) in saline (0.1 mL/mouse) (Huet & Dey, 1987). On days 6 through 8 of pregnancy, implantation sites were collected by visual identification. All samples were collected on the morning of the determined day of pregnancy between 0830-0930 hours, snap frozen in Freeze'It (Fisher Scientific, Waltham, MA), and stored at -80ºC until further processing. To induce artificial decidualization, referred to as deciduomata, the uterine horn was injected intraluminally with 20 µl of sesame oil on the morning of day 4 of pregnancy eliciting a hormonally primed inflammatory decidual reaction without embryo attachment (Kennedy, 1980; Herington *et al.*, 2009). Embryos were expelled from the uterine horn or do not survive the oil injection (Lei *et al.*, 2015). Artificially-induced decidual tissues were collected for immunofluorescence studies 48 hours post injection on day 6. For phloroglucinol (PHG) treatment studies, PHG was dissolved in 70% ethanol with 30% saline and warmed to 37ºC, and PHG was omitted for sham treatments. Mice were injected with 150 µl PHG at 250mg/mL every 12 hours. All animal procedures were conducted in accordance with guidelines and standards of the Society for the Study of Reproduction (SSR) and approved by Vanderbilt University's Institutional Animal Care and Use Committee (IACUC).

Histochemistry and immunohistochemistry staining **(published in (Jones-Paris *et al.*, 2016a))**

Uterine samples were embedded in Optimal Cutting Temperature (OCT) compound (Sakura Tissue-Tek, Torrance, CA), cryosectioned (Leica Biosystems, Buffalo Grove, IL), and 12µm sections were collected onto Superfrost Plus slides (Fisher Scientific, Waltham, MA). Samples were processed and stained with hematoxylin and eosin (H&E) using routine methods at the Vanderbilt University Medical Center Translational Pathology Shared Resource (TPSR, Nashville, TN). Briefly, samples were carefully acclimated to room temperature, washed with phosphate-buffered saline (PBS) for 3 minutes to remove OCT, fixed with 10% neutral buffered formalin for 3 minutes, and rinsed with running water. Staining was carried out by incubating in hematoxylin and then eosin by dipping 15 times in each dye, rinsing with running water between dyes. Sections were then dehydrated in 3 exchanges of ethanol and 2 exchanges of xylene and mounted in non-aqueous media with a coverslip.

Samples were processed and stained with CD31 antibodies and 3,3′-Diaminobenzidine (DAB)-conjugated secondary antibodies using routine methods at the Vanderbilt University Medical Center Translational Pathology Shared Resource (TPSR, Nashville, TN).

Transmission Electron Microscopy (TEM)

Fresh uterine tissues were immediately fixed in 5 mL of 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer (w/v) for 2 hours at room temperature followed by an additional 24 hours at 4ºC and were transported to Vanderbilt's Cell Imaging Shared Resource for further processing. Tissues were rinsed with 0.1 M cacodylate buffer, fixed in 1% osmium tetraoxide for 1 hour and rinsed again with 0.1 M cacodylate buffer at room temperature. Subsequently, the samples were dehydrated in ascending grades of ethanol and passed through 2 exchanges of pure propylene oxide (PO). Samples were next infiltrated with several exchanges of resin and PO as follows: 25% Epon 812 epoxy resin (ER): 75%PO (v/v), 50%ER: 50%PO twice, 75%ER:

25%PO, and 100% ER twice. The samples were incubated several hours with fresh 100% ER and then placed at 60ºC for 48 hours for polymerization (McCall *et al.*, 2014). Ultra-thin sections (70-80 nm) were cut from the regions of interest using an ultramicrotome (Leica ultracut, Buffalo Grove, IL) and mounted on 300-mesh copper grids. The sections were stained at room temperature with 2% uranyl acetate (w/v) for 15 minutes followed by Reynold lead citrate for 10 minutes.

Two ultra-thin sections per group were examined in a Philips T-12 electron microscope (FEI, Hillsboro, OR), and 11,000x- 30,000x micrographs were collected with AMT CCD and Gatan CCD camera systems. Particular attention was given to basement membrane material identified as the lamina densa in pericellular space, adjacent to cells, with some clear linearity. Basement membranes were marked by 100nm dots using paint tool in Photoshop (CS6, Adobe). These measurements were arrayed along the lamina densa orthogonally to relative regional x-axes at 100 nm intervals (between dot markers) using line measurement function in FIJI (1.49q, ImageJ). Membrane thickness was quantified across 11 to 15 fields of view at 11,000x or 15,000x and pooled by groups for analysis of 1,300 measurements.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA from uterine tissues (whole uterine horns (days 1 through 4) and implantation sites (days 5 through 8) was prepared using guanidine isothiocyanate-phenol-chloroform extraction method (Wang *et al.*, 2004). Extraction was inclusive of all cell types. Other data has been included to assess the compartmental localization of proteins that result from the translation of the expressed mRNA. DNase-treated (Bio-Rad, Hercules, CA) RNA (2.5 µg) was reverse transcript using SuperScript III and the included oligo(dT) primers per manufacturer's instructions (Life Technologies, Grand Island, NY). The cDNA (1 µL) was amplified in 20 µl total volume using iQ SYBER Green Supermix (Bio-rad, Hercules, CA) and specific primers for *Col4a1* (collagen IV, α1 encoding gene)*, Col4a2* (collagen IV, α2 encoding gene)*, Lamc1* (laminin γ1 subunit encoding

gene), *Pxdn (*peroxidasin encoding gene*), Col4a3bp (*all isoforms of GPBP encoding gene), and *Rpl7* (ribosomal protein L7 encoding gene) (Table 1). The following qPCR protocol was used: 95°C for 3 min followed by 40 cycles of 95°C for 10 seconds and 60ºC for 30 seconds. All reactions were run in triplicates. Data was collected on a CFX96 system with CFX Manager software (version 3.1, Bio-rad, Hercules, CA) and was analyzed by 2^(-delta delta Ct) method for fold change. Data was normalized to *Rpl7*, a commonly used reference gene.

Table 1: Primer sequences used for qPCR.

Gene	Accession number	Primer sequence		Product size (base pairs)	Source/ Reference
Col4a1	NM_009931.2	Sense 5'-3' Antisense 5'-3'	CAG ATG ACC CAC TGT GTC CC ACC GCA CAC CTG CTA ATG AA	286	Primer Blast (NIH/ NCBI)
Col4a2	NM_009932.3	Sense 5'-3' Antisense 5'-3'	AGG ACC TAG GAC TGG CAG G GGG CAG TGG GGT ATA GAG GT	253	Primer Blast (NIH/ NCBI)
Col4a3bp	NM_001164222.1	Sense 5'-3' Antisense 5'-3'	CTT GCG TAG ACA TGG CTC AA CCC TCT GAA GCT CAT CCT TG	212	Primer Blast (NIH/ NCBI)
Lamc1	NM_010683.2	Sense 5'-3' Antisense 5'-3'	CAG CGA GAC CAC TGT GAA GT CGT CTC ACA GAA CTG TCC CC	262	Primer Blast (NIH/ NCBI)
Pxdn	NM_181395.2	Sense 5'-3' Antisense 5'-3'	GGC GGA AAG CAC TAA GTG TA GTT GCC GCC GTG AGA TTC	218	Primer Blast (NIH/ NCBI)
Rpl7	M29016	Sense 5'-3' Antisense 5'-3'	TCA ATG GAG TAA GCC CAA AG CAA GAG ACC GAG CAA TCA AG	246	Zhao et al. 2000 (Zhao *et al*., 2000)

Immunofluorescence

Frozen tissues were warmed to -20ºC, embedded in Optimal Cutting Temperature (OCT) compound (Sakura Tissue-Tek, Torrance, CA) and sectioned (12 µm) in a cryostat (Leica Biosystems, Buffalo Grove, IL). Sections were collected on Superfrost Plus slides (Fisher Scientific, Waltham, MA), air-dried and fixed in -20ºC acetone for 10 minutes. Slides were washed several times with phosphate-buffered saline (PBS) (Corning, Corning, NY) and PBS/0.2% Tween (Sigma, St. Louis, MO). Slides to be stained with collagen IV antibody (JK2) were treated with dissociation buffer consisting of 6M urea in 0.1 M glycine buffer (pH 3.0) (Ninomiya *et al.*, 1995) followed by several washes with PBS and PBS/0.2% Tween. Laminin and peroxidasin immunodetection patterns were minimally influenced by dissociation treatment rendering co-immunostaining with collagen IV antibody possible (Jones-Paris *et al.*, 2016b). Immunodetection of GPBP and colocalization with laminin was achieved without the dissociation step because GPBP antibodies used in this study failed to show specific staining in sections treated with the dissociation buffer. All sections were preincubated with 10% normal serum from goat or horse (Invitrogen, Grand Island, NY) for 1 hour at room temperature to avoid nonspecific binding of antibodies prior to applying primary antibodies. The following primary antibodies were used for antigen detections: rat anti-collagen IV NC1 (1:500 dilution, JK2, were from Y. Sado, Shigei Medical Research Institute, Okayama, Japan (Ninomiya *et al.*, 1995; Sado *et al.*, 1995)), rabbit anti-laminin (1:50 dilution for tissues not treated with dissociation buffer and 1:500 dilution for those that were pre-treated with dissociation buffer, ab11575, Abcam, Cambridge, MA), rabbit anti-peroxidasin (1:250 dilution, were from G. Bhave, Vanderbilt University, Nashville, TN (Bhave *et al.*, 2012)), Alexa546 conjugated mouse anti-GPBP (1:50 dilution, mAb N26 to the N-terminal serine-rich domain conserved across all GPBP isoforms in several species (Granero-Moltó *et al.*, 2008), from Fibrostatin, SL, Valencia, Spain), Alexa546 conjugated mouse anti-GPBP-1 (1:50 dilution, mAb e11-2 to the 26-amino acid residue of exon 11 not present in GPBP-2, also called CERT (Revert-Ros *et al.*, 2011), from FIbrostatin, SL, Valencia, Spain), and rabbit anti-LOXL2 (1:250 dilution, ab96233, Abcam, Cambridge, MA). The secondary antibodies used for immunofluorescence detection were: Alexa555 goat anti-rat (1:200 dilution, ab150166, Abcam)

and Alexa488 goat anti-rabbit (1:200 dilution, ab150081, Abcam). All primary antibodies were diluted in PBS/0.1% Tween and 5% normal goat serum. Sections were incubated with these antibodies overnight at 4°C in a humidified chamber and then washed three times with PBS/0.2% Tween. Negative control samples were processed similarly to experimental samples but without the inclusion of primary antibodies. Diluted secondary antibodies were applied to sections for 1 hour at room temperature. After several washes, 1 μM Hoechst fluorescent dye was applied (10 minutes) for labeling cell nuclei. Following washes in PBS/0.2% tween and PBS, sections were mounted in Prolong Gold (Life Technologies, Grand Island, NY) and cured at room temperature for 2 days. Images at 0.323 μm/pixel resolutions were captured utilizing the Ariol SL-50 or Apiro Versa 200 automated fluorescent microscopes with a 20x objective (Leica Biosystems, Buffalo Grove, IL) housed in the Vanderbilt Digital Histology Shared Resource. Image linear brightness and contrast were adjusted for visual comparison using FIJI (1.49q, ImageJ, NIH, Bethesda, Maryland) controlling for within set staining and to maintain consistent intensities relative to representative controls (see Fig.4-7 in (Jones-Paris *et al.*, 2016b)).

Isolation of Collagen IV NC1 Hexamers

Uterine endometrial tissues from days 4 through 8 were separated from the myometrium casing by firm rolling pressure along the uterine horn. Both endometrial and myometrium samples were snap frozen in Freeze'It (Fisher Scientific; Waltham, MA). Frozen samples were added to preweighed 1.5 mL microcentrifuge tubes containing 100 μL of isolation buffer [50 mM Hepes (pH 7.5), 10 mM $CaCl_2$, 1 mM PMSF, 5 mM benzamadine-HCl, 25 mM 6-amino-n-hexanoic acid]. A micropestle was used to disperse each sample; residual tissue was washed from the micropestle with 200 μL of isolation buffer and added to the sample. The suspension was homogenized by sonication in an ice water bath for approximately 5 minutes followed by centrifugation at 10,000 × g for 15 minutes. The soluble fraction was discarded and the mass of the remaining insoluble material was measured and recorded. Two milliliters of isolation buffer containing 0.1 mg mL^{-1} bacterial collagenase (Worthington Biochemical; Lakewood, NJ) was added to the samples per

gram of pellet obtained. The sample was briefly mixed by vortex and subjected to sonication to loosen the pellet. Samples were allowed to digest at 37ºC with shaking for 24 hours. The solubilized NC1 fraction was collected in the supernatant after centrifugation at 14,000x g for 30 minutes. The isolated fraction was used for immunoblot analysis (Fidler *et al.*, 2014).

Immunoblot

Collagen IV NC1 hexamers were analyzed by SDS/PAGE in 12% (wt/vol) *bis*-acrylamide mini-gels with Tris-Glycine-SDS running buffer. Ten microliters of each sample were loaded using 4x LDS sample buffer (Bio-rad, Hercules, CA). Each sample was derived from individual mice and the loading order was consistent between endometrium and myometrium. After electrophoresis under non-reducing conditions, proteins were transferred to 0.2µm pore sized PVDF membrane (Bio-rad, Hercules, CA) (Fidler *et al.*, 2014). Immunodetection was carried out per manufacturer recommendations (LI-COR, Doc #988-13627, Lincoln, NE). Immunoreactivity and detection were achieved with primary antibody JK2 (1:2500) and secondary antibody goat anti-rat IRDye 680LT (1:20,000; LI-COR 926-68029, Lincoln, NE). Immunoreactivity was quantified by near-infrared signals detected using Odyssey Classic (LI-COR, Lincoln, NE, provided by Vanderbilt University Initiative for Maximizing Student Diversity shared equipment) and signals were analyzed using Image Studio Lite (LI-COR, Lincoln, NE).

Statistics

Statistical analysis and graphing were performed using SPSS Statistics (Version 22, IBM). For qPCR data analysis, independent variables two-tailed T-test was used with equal variance not assumed. For TEM data analysis, non-parametric independent-samples tests, Kruskal-Wallis and Median tests, were performed along with posthoc pairwise comparisons of distributions. The significance level was set at p-value ≤ 0.05.

CHAPTER III

Emergence of basement membrane peroxidasin and Goodpasture-antigen binding protein in early epithelial development

Introduction

During preimplantation development, the embryo starts as a single cell that undergoes waves of symmetrical cell division forming a cluster of 8-16 cells called a morula. Few reports have localized basement membrane proteins laminin, collagen IV, and fibronectin to morula stage embryos by immunodetection (Adamson & Ayers, 1979; Leivo *et al.*, 1980). The cells of the morula are totipotent, capable of differentiating into all embryonic and extraembryonic tissues. Asymmetric cell division and expression of cell-type-specific genes, including Oct4 and Cdx2, trigger the formation of 3 pluripotent cell populations: the extraembryonic trophectoderm (TE) and primitive endoderm (PE), and embryonic epiblasts (EPI) (Bedzhov *et al.*, 2014). In early tissue genesis, major components of BM, laminin and collagen IV, are expressed as cells differentiate and organized to establish the distinct germ layers that give rise to all tissues. During the blastocyst stage (embryonic day 4 (E4.0) on day 4 of pregnancy in mice), laminin is localized in and around cells of the inner cell mass (ICM) to establish the niche that gives rise to pluripotent epiblast cells. Following implantation, the E5.5 epiblasts self-organize the initial layer of polarized epithelium through gastrulation into a cup-like structure called the egg cylinder comprised of laminin and collagen IV (Figure 3.1).

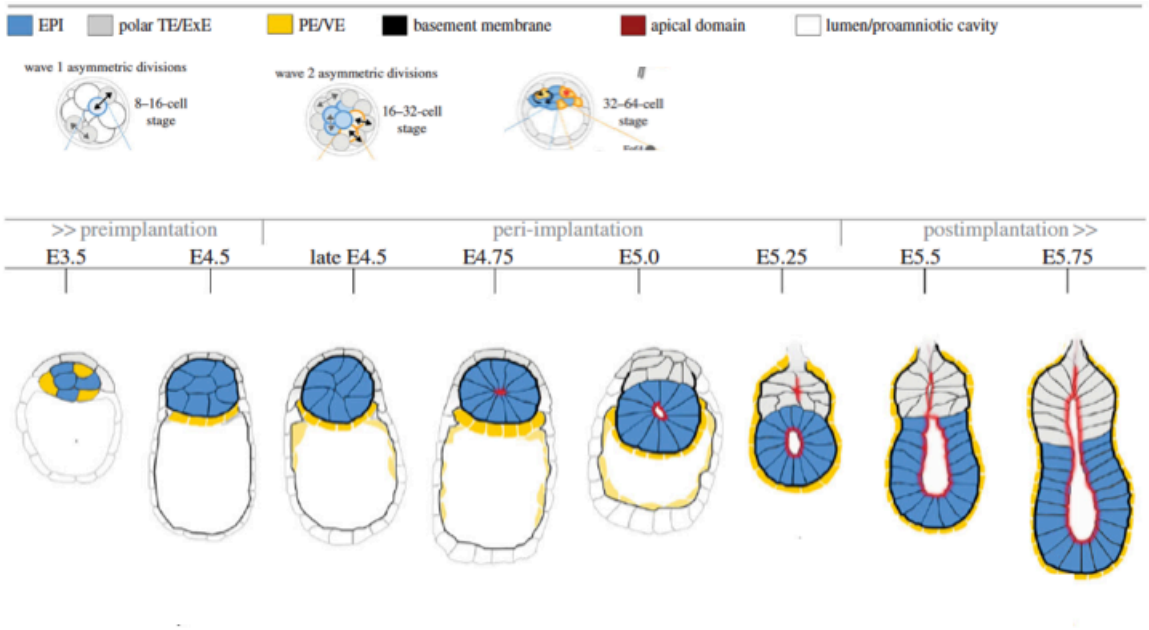


Figure 3.1: Schematic of basement membrane orientation during early morphogenesis of mouse embryo. Heavy black line illustrates the location of BM proteins such as collagen IV or laminin. Adapted from (Bedzhov *et al.*, 2014).

The integration of BM proteins is essential for embryonic development. Genetic and molecular perturbation of these components or cell receptors that bind the components leads to embryonic lethality, which has been reported in laminin, collagen IV, and integrin beta 1 knockout (KO) models (Hynes, 1992; Fässler & Meyer, 1995; Miner *et al.*, 2004; Pöschl *et al.*, 2004). Some early functions of individual BM components are unclear because overlapping functions act as a fail-safe that permits embryo development through the egg cylinder phase to ~E9.5-10.5 for many KO models of BM components. Nevertheless, the expression of major BM components denotes a biological demand for their presence. Putative BM proteins peroxidasin and GPBP are gaining increasing attention for their influence on BM functions in dynamic tissue physiology, but their expression and localization in early stage embryogenesis is unknown.

Peroxidasin reinforces BMs by catalyzing the formation of sulfilimine crosslinks throughout the collagen IV lattice (Bhave *et al.*, 2012; Fidler *et al.*, 2014; McCall *et al.*, 2014). Peroxidasin expression was first discovered in post-blastoderm *Drosophila melanogaster* embryos following gastrulation (Nelson *et al.*, 1994). Recently, peroxidasin expression was identified at similar stages of development in *Danio rerio* and *Nematostella vectensis* in studies that provide evidence for evolutionary conservation of peroxidasin functions (Hynes, 2012; Fidler *et al.*, 2014). In mice and humans, genetic mutation of peroxidasin has been reported to cause disorganized BM assembly that leads to severe embryonic eye defects and blindness (Khan *et al.*, 2011; Yan *et al.*, 2014). Its expression in mammalian development aside from ocular genesis and prior to E9.5 has not been described.

Goodpasture antigen-binding protein (GPBP) localizes to cells and BMs of tissue that are derived from all germ layers, including the brain, kidney, and lung tissues (Raya *et al.*, 1999). This suggests expression is not restricted by lineage commitment. Localization and function differences of the protein have been attributed to splice variants GPBP1 and GPBP2, full-length GPBP and GPBPΔ26 that has less binding capacity to ECM (Granero-Moltó *et al.*, 2008). Knockout of the gene responsible for the expression of both intracellular and extracellular GPBP, known as either the *Col4a3bp* or *Cert* gene, causes growth retardation that was notable by

dwarfed E7.5 embryos. Ultimately, the genetic defect leads to survival decline at E11.5 due to failed organogenesis was presumably from metabolic dysfunction, although similar stages of decline have been attributed to failure BMs in critical organs (Wang *et al.*, 2009). Knockdown of full-length GPBP alone also leads to organogenesis defects in *Mus musculus* and *Danio rerio* but is not lethal (Granero-Moltó *et al.*, 2008; Revert-Ros *et al.*, 2011). Although GPBP overexpression has been associated with ultrastructural alterations of BMs, extracellular matrix integrity was not considered or examined during early embryogenesis of these models (Revert *et al.*, 2007).

Peroxidasin and GPBP are expressed during embryo development and localize to BMs of adult tissues. However, their expression and localization during the emergence of BMs for pre- and peri-implantation embryos is unknown. In the present study, we sought to determine the dynamic regulation of peroxidasin and GPBP during the earliest stages of tissue genesis by examining mouse embryonic expression and protein localization by ultrasensitive quantitative polymerase chain reaction (qPCR) and immunofluorescence detection. Programming of embryonic stem (ES) cells requires stringent timing, localization and regulation of protein expression and interactions to enable differentiation and arrangement of tissues. These findings provide novel evidence that BM proteins peroxidasin and GPBP are dynamically regulated during initial assembly extracellular scaffolds by embryonic progenitor cells.

Results

Peroxidasin is expressed following gastrulation

Peroxidasin gene, *Pxdn*, expression was examined by qPCR of single cell to blastocysts embryos. Maternal mRNA degrades after 2-4-cell stage. Embryonic expression of *Pxdn* was not detectable through blastocyst development (Figure 3.2). Next, peroxidasin protein localization in E4.0 (morula) to E7.0 (egg cylinder) was examined by immunofluorescence on *in utero* embryos. Concurrent with our qPCR findings, peroxidasin was not detected in the embryo on day 4 or 5 of pregnancy at the morula and blastocyst stages (Figure 3.3). Peroxidasin was localized to

invaginated epiblasts layers of E6.0 and E7.0 embryos. Localization was primarily associated with basement membranes marked by collagen IV, and little cytoplasmic localization was detected in the embryos at these time points.

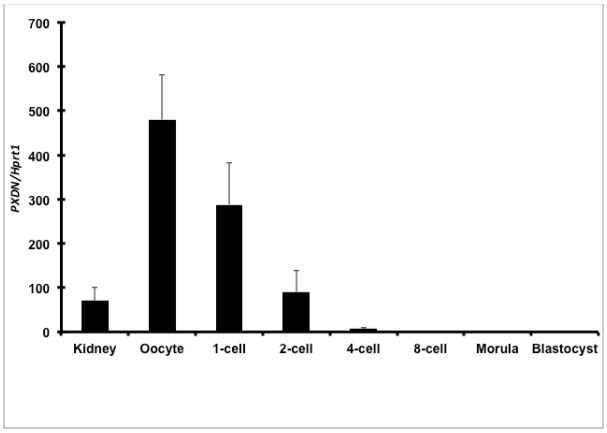


Figure 3.2: Expression of peroxidasin in ex utero early stage mouse embryos. Quantitative PCR for PXDN, peroxidasin encoding gene. Bars represent mean, and error bars represent SEM.

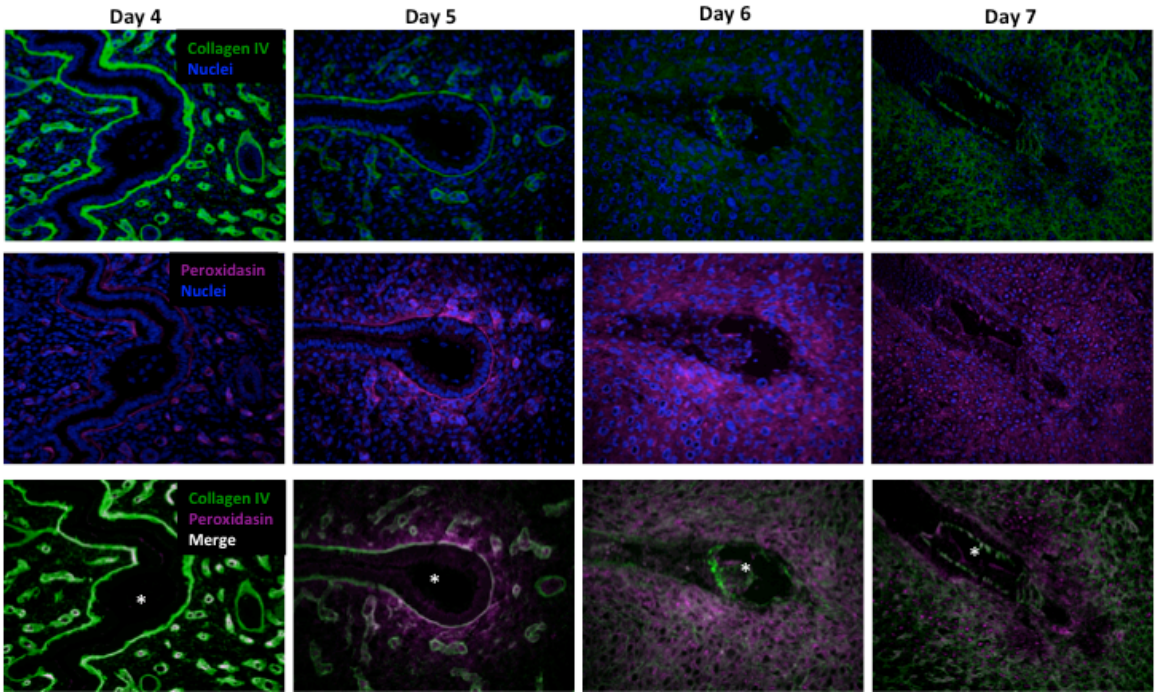


Figure 3.3: Dual detection of collagen IV and peroxidasin by immunofluorescence of *in utero* embryos days 5-8 pregnancy. Asterisks (*) indicate the location of the embryos.

We further examined peroxidasin expression during the morphogenesis from blastocyst to gastrula using *in vitro* cultures of the embryos. Blastocyst at E4.5 attached to fibronectin-coated chamber slides and the ICM arranged into a rosette-like structure after 48-hours in culture (Figure 3.4 A). At 72-hours in culture, hollowed lumen structures were observed. Collagen IV was localized to the 48-hour rosette-like ICM (Figure 3.4 B). Peroxidasin colocalized with collagen IV in the 72-hour cultured embryo lumen (Figure 3.4 C).

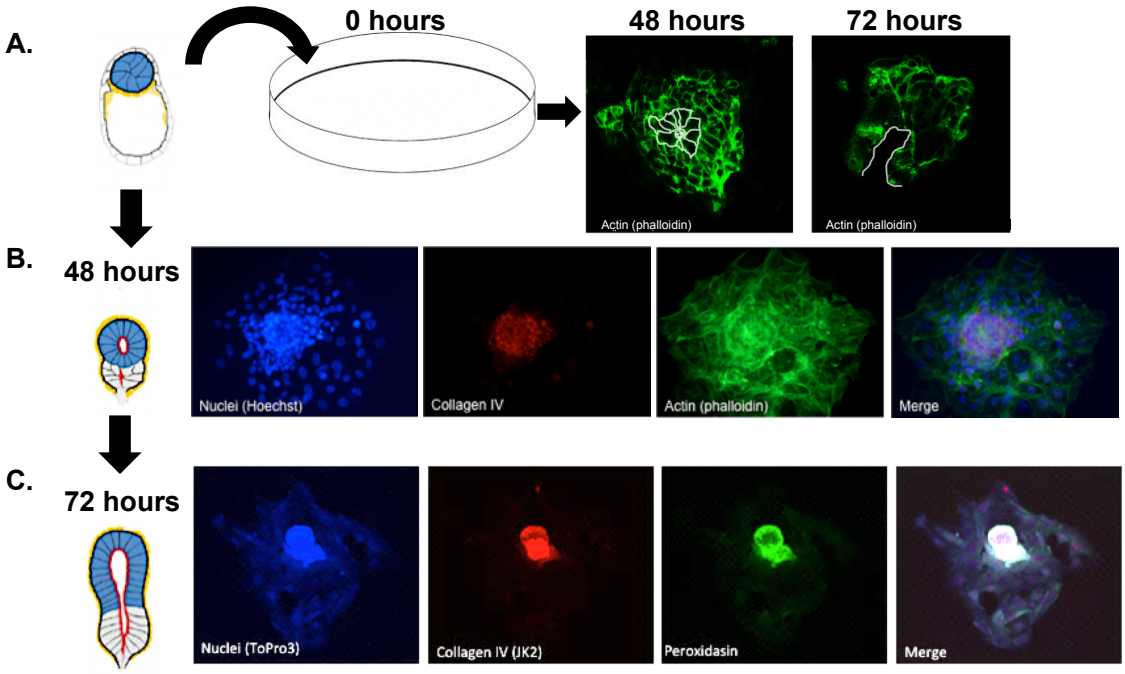


Figure 3.4: Immunofluorescence micrographs of *in vitro* mouse embryos. A) *Confocal* microscopy of in vitro cultured embryos during inner cell mass morphogenesis. At 48-hours in culture, cells organized into rosette-like arrangements. At 72-hours, small lumens were developed. B) Widefield microscopy of collagen IV localization in rosette-like arranged inner cell mass cells of 48 hour in vitro cultured embryo. C) Confocal microscopy dual immunofluorescence of collagen IV and peroxidasin localization in lumen organized cells of 72-hour in vitro cultured embryo.

Peroxidasin Crosslinks Collagen IV in Early Embryos

The colocalization of peroxidasin and collagen IV led us to explore sulfilimine crosslinking status to determine if peroxidasin had enzymatic action toward collagen IV lattices at this early stage of development. By immunoblotting for collagen IV non-collagenous domain (NC1) in BM material isolated from *ex utero* embryos, sulfilimine crosslinks were detected using previously described methods (Fidler *et al.*, 2014). No monomeric or crosslinked collagen IV nor laminin were detected on days 4 and 7 (Figure 3.5). At these stages, the embryos were considerably smaller than E8.0 and E10.0 embryos (data no shown). The lack of detectable matrix could be attributed to too small of sample size for detection using this method, which provided inconclusive crosslinking status for these particular days. Currently, immunoblot of collagenase treated samples is the most sensitive method for detection of sulfilimine crosslinks. However, innovative technologies are emerging for comparative analysis that included gold-tagged compounds that link to specific uncrosslinked sites of molecules within samples and are then detected by scanning electron microscopy. This technology has been applied to detect changes in potential disulfide crosslinking sites (Ilani *et al.*, 2013). However, there is no compound for sulfilimine crosslink sites. Embryos that had more tissue mass yielded more conclusive results. Bands at 37 kilodaltons (kD) were detected at E8.0 and E10.0 indicating the presence of sulfilimine crosslinked on NC1 domains. High molecular weight laminin was also detected at those time points (Figure 3.5). The presence of sulfilimine crosslinks in embryos undergoing early organogenesis led us to explore if crosslinks were present in diverse adult organs. Using similar methods as before, we detected sulfilimine crosslinked NC1 domains in adult organs representing all germ layers: esophagus and intestine derived from endoderm, tail and eyes derived from ectoderm, and heart, kidney and tongue derived from mesoderm (Figure 3.5).

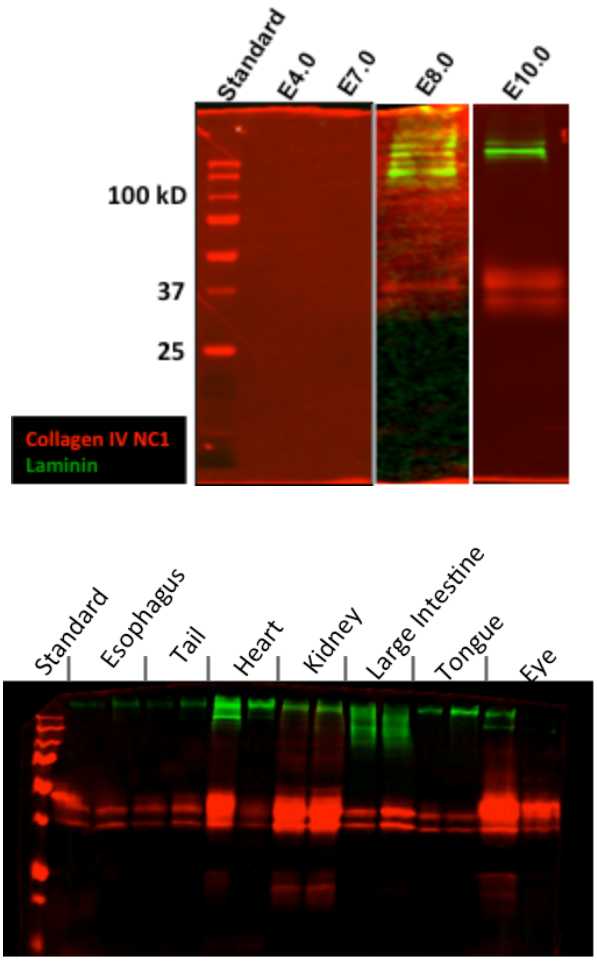


Figure 3.5 Immunoblot of collagen IV NC1 and laminin from ex utero early embryos and adult tissues. (Top)Crosslinked collagen IV NC1 (37kD) is observed in Day 8 and Day 10 embryos. Day 8 image signal threshold was narrowed for visualization of faint bands over background, but bands are specific. (Bottom) Immunoblot of basement membrane collagen IV NC1 domains and laminin in adult tissues that derive from all germ layers.

***Goodpasture antigen-binding protein (GPBP)* is expressed during early embryogenesis**

We employed immunofluorescence to determine if early stage embryos produce GPBP during tissue genesis. Blastocyst ICM had faint detectable GPBP on day 5 of pregnancy (Figure 3.6). By day 6 of pregnancy, GPBP localization increased to levels comparable to that of the surrounding decidua. In this E6.0 embryo, GPBP was localized to all embryo cells with subcellular localization in the cytoplasm and pericellular areas. On days 7 and 8 of pregnancy, GPBP detection returned to faint levels throughout the embryos (Figure 3.6).

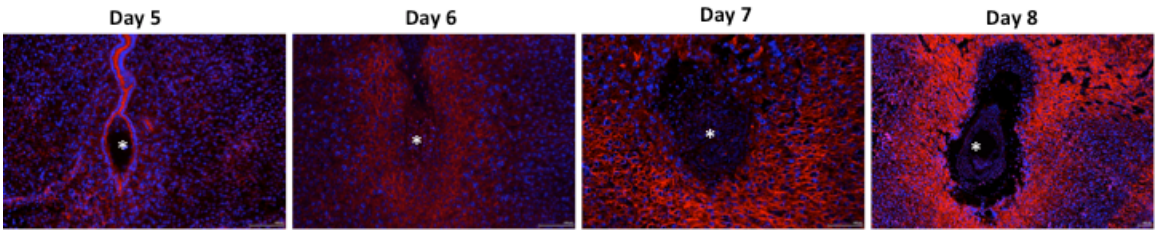


Figure 3.6 Detection of GPBP by immunofluorescence of *in utero* embryos days 5-8 pregnancy. Asterisks (*) indicate location of embryo.

Embryonal Epithelial Cells regulate Peroxidasin and GPBP in Basement Membranes

The expression of peroxidasin and GPBP in embryonic BMs offered the opportunity to further explore the dynamics of BMs in early epithelial development. However, *in utero* and embryo outgrowth studies were met with challenges that can only be rectified with extensive sampling and experimental design optimization. Embryos investigated *in utero* are the ideal setting to study embryo development, assessment is limited to terminal time points and is not readily manipulated without targeting the mother. While embryo outgrowth cultures make it possible to directly monitor progression of development and direct delivery of experimental agents, we and other investigators have found that approximately 30% of embryos arrange to form lumen-like structures in standard conditions (data not shown) (Bedzhov & Zernicka-Goetz, 2014). This low yield is partially attributed to the critical need for maternal forces of the decidua that promote egg cylinder formation (Hiramatsu *et al.*, 2013). We advanced our current study beyond those particular model limitations by implementing experiments using the PFHR9 cell line. These cells epithelial-like embryo cells derived from a benign teratocarcinoma of endodermal cells that robustly produce BM and are a reliable system to study mechanisms embryonic BM assembly (Chung *et al.*, 1977; Kramer *et al.*, 1984; Parry *et al.*, 1987; Przyborski *et al.*, 2004; Fidler *et al.*, 2014; McCall *et al.*, 2014).

With confocal microscopy, we found regulation of BM tool kit proteins in embryonal epithelial-like PFHR9 cell cultures. Recent discoveries in the Hudson laboratory have demonstrated that BM assembly is responsive to alterations that span from genetic knockout of crosslinking enzymes to the sophisticated requirement of ionic elements bromine (Br) and chlorine (Cl) for intra- and intermolecular assembly of collagen IV protomers (Bhave *et al.*, 2012; McCall *et al.*, 2014; Cummings *et al.*, 2016). Here, PFHR9 BM networks were examined in low-Cl conditions. Colocalization of laminin and peroxidasin to collagen IV networks was significantly higher in low-Cl matrices than controls, yet relative signal intensity was not altered (Figure 3.7). The localization and intensity patterns of GPBP were not impacted by Low-Cl conditions (Figure 3.8). This indicated that Low-Cl conditions shift the position of components in the BM network.

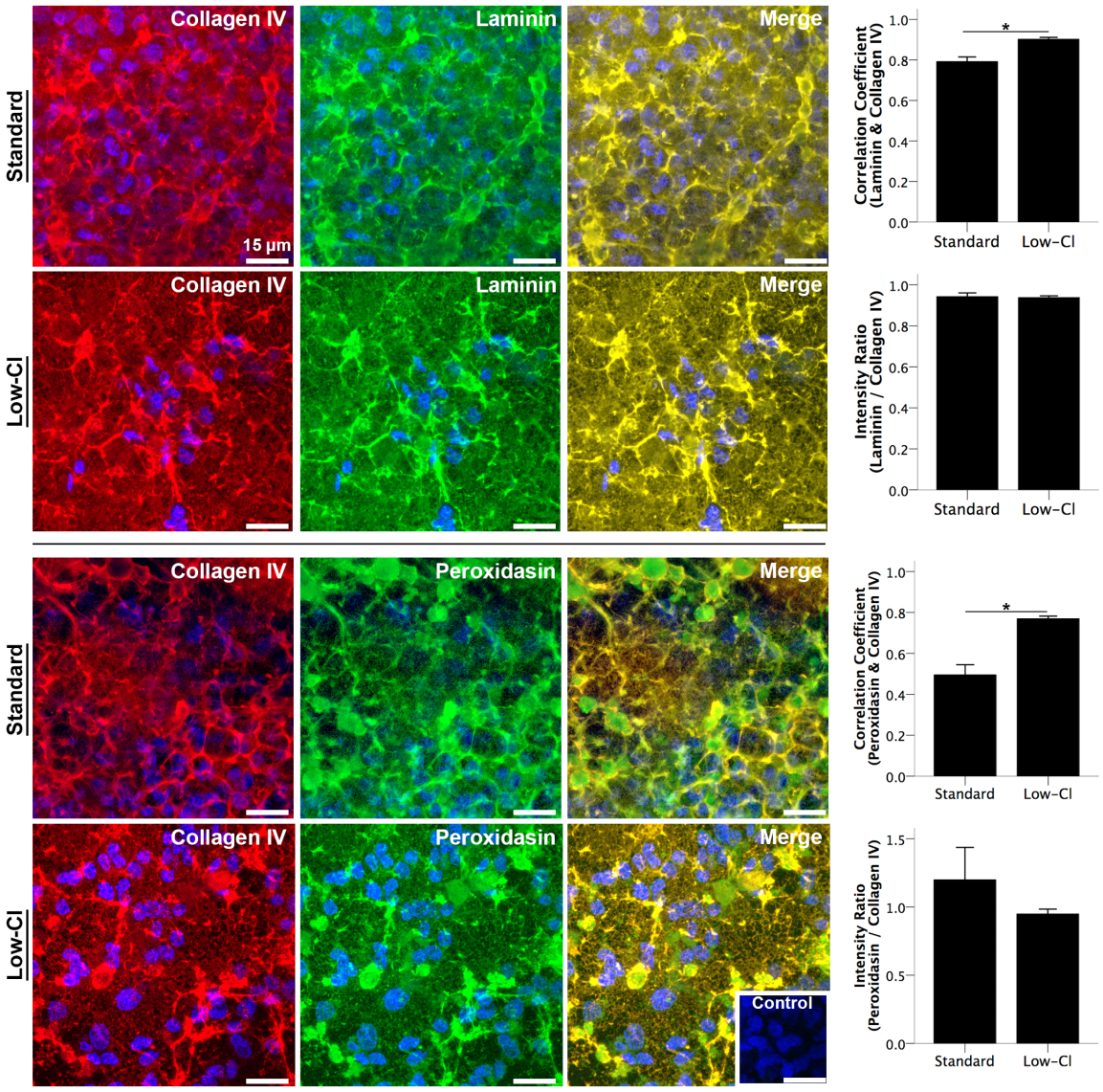


Figure 3.7: Disorganization of Collagen IV-Rich PFHR9 Matrix Under Low-Cl Conditions. (A-C) Confocal microscopy reveals that Low-Cl conditions stimulate the enhanced localization of laminin and peroxidasin to collagen IV in PFHR9 matrix. (A) Maximum intensity projection of immunofluorescent confocal z-stacks (7.2µm projections) of matrix after 5 days in standard media or Low-Cl media; collagen IV (red), laminin (green) or peroxidasin (green), merge where colocalization is yellow/orange, and nuclei/Hoechst stain (blue). Secondary antibody only negative control inset. Bar=15 µm. (B) Collagen IV and laminin or (C) collagen IV and peroxidasin colocalization analysis by Pearson's correlation coefficient (1.0= perfect colocalization), and ratio of green/red signal per field-of-view. (B, n=8; C, n=4). *p<0.001. This figure has been published in a manuscript co-authored by Jones-Paris (Cummings *et al.*, 2016).

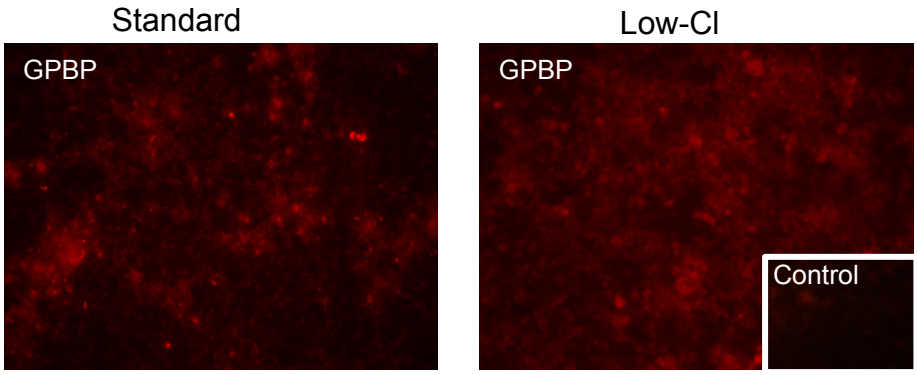


Figure 3.8: Organization of GPBP in PFHR9 Cells Under Low-Cl Conditions. (A-C) Widefield fluorescent microscopy reveals that Low-Cl conditions have no effect on localization of GPBP in PFHR9 matrix and cells. Immunofluorescence detection of GPBP after 5 days in standard media or Low-Cl media; GPBP (red) and nuclei/Hoechst stain (blue). Secondary antibody only negative control bottom right inset.

Discussion

Peroxidasin is a BM component during epithelial development

We provide evidence that peroxidasin colocalized with BM collagen IV during the initial arrangement of epithelial tissue in embryos. Peroxidasin expression originates in beneath endodermal cells of the gastrula where it establishes sulfilimine crosslinks in newly synthesized BM. This corroborates previous findings of peroxidasin having roles in the establishment of tissue form and function during comparable stages of development in *Drosophila melanogaster* (Nelson *et al.*, 1994), *Nematostella vectensis* and *Danio rerio* (Fidler *et al.*, 2014). Peroxidasin catalyzes the formation of sulfilimine crosslinks in BMs at the foremost stages of organogenesis and in diverse adult tissues. These findings suggest sulfilimine crosslinks may be necessary for early BM stability that promotes ICM involution, tissue morphogenesis, and physiology.

The concentrated localization of peroxidasin in between the emerging germ cell layers suggests particular function in proximal communication amongst cells. In *Drosophila melanogaster*, peroxidasin function modulates BM thickness by regulating compactness of collagen IV network through crosslinks (McCall *et al.*, 2014). The thickness of early embryonic BMs requires mechanistic constriction for paracrine signaling. Thickened BMs induced by mutation of Smad4 of the TGF-beta pathway caused aberrant embryogenesis stemming from disrupted reciprocal signaling between the visceral endoderm (VE) and epiblasts (Costello *et al.*, 2009). Similarly, peroxidasin localization to endoderm BMs may restrict thickness by consolidating collagen IV networks for appropriate paracrine signaling between the VE and epiblasts.

The function of peroxidasin is not limited to crosslinking. The enzymatic activity of peroxidasin involves consumption of H_2O_2 by the peroxidase domain (Nelson *et al.*, 1994). Blastocyst morphogenesis requires balanced H_2O_2 levels that mediate apoptosis for development (Pierce *et al.*, 1991). Thus, sulfilimine crosslinking by peroxidasin can incidentally tune H_2O_2-driven apoptosis in blastocysts.

Expression of GPBP is Limited During Early Epithelial Genesis

We localized GPBP to pluripotent cells undergoing organogenesis. GPBP expression was limited to intracellular compartments, with no localization to pericellular or extracellular regions. This exclusion of BM localization could have an opposing effect as the presence of peroxidasin. Studies have shown that overexpression of GPBP can lead to BM thickening (Revert *et al.*, 2007). Therefore, GPBP may be inherently silenced to prevent thickening of the nascent BM to support communication between the VE and epiblasts. The intracellular presence of GPBP suggests early embryogenesis has bias toward non-BM associated functions of GPBP. This may include plasma membrane construction during cell expansion (Revert *et al.*, 2008) and accommodation of metabolic demands by mitochondrial support during rapid growth (Wang *et al.*, 2009).

Conclusion

Basement membrane components provide the initial framework for tissue arrangement in embryos. The findings of our study establish peroxidasin and GPBP as components that are expressed during epithelial genesis and are spatially and temporally modulated during development. These findings provide a foundation to further elucidate the function of these proteins in early embryo physiology and pathology. Furthermore, studies based on these finding may translate to mechanisms that regulate adult tissue morphogenesis and pathology, such as pubescent ductal growth and breast cancer progression which have been shown require appropriate BM assembly in *in vitro* and *in vivo* studies (Nelson & Bissell, 2006; Daley & Yamada, 2013).

Experimental Procedures

Animals

Sexually mature CD1 mice were used for all studies (Charles River Laboratories, Crl:CD1(ICR), Wilmington, MA, USA). Female mice were bred overnight with fertile males of the same strain and the presence of vaginal plug the following morning indicated day 1 of pregnancy (Zhang & Paria, 2006). On days 1-4 of pregnancy, whole uterine horns were harvested. Implantation sites on day 5 of pregnancy were identified after intravenous injections of 1% Chicago Blue 6B dye (Sigma, St. Louis, MO) in saline (0.1 mL/mouse) (Huet & Dey, 1987). On days 6-8 of pregnancy, implantation sites were collected by visual identification. All samples were collected in the morning of the determined day of pregnancy between 0830-0930 hours. Whole embryos were isolated from uterine horns by flushing out pre-implantation E4.0 embryos or microdissection to remove surrounding uterine and extraembryonic tissues for crosslinking analysis. For similar analysis, various adult organs were collected from non-pregnant healthy female mice. Tissues were snap frozen in Freeze'It (Fisher Scientific, Waltham, MA), and stored at -80ºC until further processing. All animal procedures were conducted in accordance with ethical guidelines and standards and were approved by Vanderbilt University's Institutional Animal Care and Use Committee (IACUC).

Isolation of total RNA from ex utero early mouse embryos

Uteri were flushed with 37^{o}C M2 media (M7167, Sigma-aldrich, St. Louis, MO) to and embryos were rapidly collected prior to implantation, days 1 through 4.5 of pregnancy. At least 10 embryos were pooled into Eppendorf tubes and quickly frozen by dipping the tube in Freeze'it. After freezing, embryos were suspended in 50μL of phenol-SDS buffer (0.5% SDS, 25mM EDTA, 75mM NaCl, pH 8.0, plus phenol 1:2 v/v). The mixture was vortexed, centrifuged 5 minutes at 13,000RPM, then the bottom layer was collected and transferred to a new tube. *Escherichia coli*

ribosomal RNA (20μg, Boehringer-Mannheim) was added a controlled RNA contaminant for later visualization of the RNA pellet. The mixture was homogenized by repeatedly passing through a 27-guage needle on a 1-cc syringe and vortexed for 2 minutes, then the it was chilled on ice 5 minutes and centrifuged for 10 minutes at 4^{o}C at maximum speed. The organic phase (bottom) was discarded and the remaining phases were reextracted with equal volumes phenol:chloroform (1:1 v/v) by vortexing and chilling on ice 5 minutes then centrifugation. The clear aqueous phase was transferred to a new tube and 3 volumes of 4M ammonium acetate was added to precipitate the RNA. The sample was incubated at -20^{o}C for 45 minutes then RNA precipate was collected by ultracentrifugation at 50,000g for 30 minutes at 4^{o}C (TL-100, Beckman Instruments, Brea, CA). The RNA pellet was washed in 85% ethanol, then centrifuged and ethanol was aspirated. The RNA pellet was dissolved in 10μL nuclease free water and 2.5μL was used for PCR.

Widefield and Confocal Microscopy

In utero embryo section images were captured using a Olympus DP72 camera on a Olympus Bx51 microscope with a 20x objective supported by Cell Sens Standard v1.11 software (Olympus Corporation of the Americas, Center Valley, PA). Confocal images were captured using overlapping sections through a consistent volume between comparable samples using an LSM 710 META microscope equipped with a 64x objective (Zeiss, Thornwood, NY). Voxel colocalization was quantified with Imaris software (v.7.6.0, Bitplane, South Windsor, CT). Field-of-view intensity was quantified using ImageJ (v.2.0.0-rc-46/1.50g, Bethesda, Maryland). Projection images and channel merges were rendered also using ImageJ.

Isolation of Collagen IV NC1 Hexamers

Frozen samples were added to preweighed 1.5 mL microcentrifuge tubes, at least 20 embryos were pooled for *ex utero* embryo immunoblot. One hundred microliters of isolation buffer [50 mM Hepes (pH 7.5), 10 mM CaCl2, 1 mM PMSF, 5 mM benzamadine-HCl, 25 mM 6-amino-n-hexanoic acid] was added to each sample. A micropestle was used to disperse each sample; residual tissue was washed from the micropestle with 200 µL of isolation buffer and added to the sample. The suspension was homogenized by sonication in an ice water bath for approximately 5 minutes followed by centrifugation at 10,000 × g for 15 minutes. The soluble fraction was discarded and the mass of the remaining insoluble material was measured and recorded. Two milliliters of isolation buffer containing 0.1 mg mL^{-1} bacterial collagenase (Worthington Biochemical; Lakewood, NJ) was added to the samples per gram of pellet obtained. The sample was briefly mixed by vortex and subjected to sonication to loosen the pellet. Samples were allowed to digest at 37ºC with shaking for 24 hours. The solubilized NC1 fraction was collected in the supernatant after centrifugation at 14,000x g for 30 minutes. The isolated fraction was used for immunoblot analysis (Fidler *et al.*, 2014).

Fluorescent Immunoblot

Collagen IV NC1 hexamers were analyzed by SDS/PAGE in 12% (wt/vol) *bis*-acrylamide mini-gels with Tris-Glycine-SDS running buffer. Ten microliters of each sample were loaded using 4x LDS sample buffer (Bio-rad, Hercules, CA). For *ex utero* E4.0 and E7.0 embryos, total isolated sample was loaded. After electrophoresis under non-reducing conditions, proteins were transferred to 0.2µm pore sized PVDF membrane (Bio-rad, Hercules, CA) (Fidler *et al.*, 2014). Immunodetection was carried out per manufacturer recommendations (LI-COR, Doc #988-13627, Lincoln, NE). Immunoreactivity and detection were achieved with primary antibodies JK2 and anti-laminin (1:2500) and secondary antibodies goat anti-rat IRDye 680LT and 800LT (1:20,000;

LI-COR, Lincoln, NE). Immunoreactivity was quantified by near-infrared signals detected using Odyssey Classic and signals were analyzed using Image Studio Lite (LI-COR, Lincoln, NE).

Embryo outgrowth

Blastocysts were flushed from uteri using M2 media, as mentioned above. Then embryos were individually transferred to Advanced DMEM/F12 (Thermofisher, Waltham, MA) in 4-well glass bottom chamber culture slides pre-coated with 0.5mg/mL fibronectin in PBS (Sigma-Aldrich, St. Louis, MO). The embryo cultures were maintained in a humidified incubator at 37^{o}C were the trophoblasts attached and embryos continued to grow. Chamber slides containing embryos were rinsed with PBS at determined time points and processed for immunefluoresce in a manner similar to frozen sections, described above.

PFHR9 Cell Cultures **(published in (Cummings *et al.*, 2016))**

Two-hundred fifty thousand cells were seeded per 1.7cm^{2} well of 4-chamber glass slides (Lab-Tek, Thermo Fisher Scientific, Waltham, MA) in standard DMEM (Gibco, Thermo Fisher Scientific) or low-chloride media. After 48 hours, the cells were maintained 5 days in media supplemented with 50µg/mL ascorbic acid with media changes every 24 hours. Subsequently, the chamber walls were removed and matrix and adherent cells were processed directly on the slide for immunohistochemistry. Following a brief wash with phosphate-buffered saline (PBS) (Corning, Corning, NY), the cells were snap-frozen then processed for immunofluorescence in a manner similar to previously mentioned frozen tissue sections.

CHAPTER IV

Dynamic regulation of peroxidasin and Goodpasture-antigen binding protein during extraembryonic tissue development

Introduction

Basement membranes (BMs) function to partition microenvironments that require tight regulation of molecular exchange across tissues. The BM scaffolds are composed of an arrangement of several proteins that form stable supramolecular structures, including collagen IV that establishes the lattice within BMs. Peroxidasin and Goodpasture-antigen binding protein (GPBP) are among proteins with emerging roles in active BM functionality. These proteins are emerging as critical components for the structural integrity and physiological functions of intestine and kidneys, organs recognized for the precise exchange of nutrients and waste. Peroxidasin stabilizes BMs by enzymatically reinforcing collagen IV network with covalent sulfilimine crosslinks. Disruption of peroxidasin function causes tearing of gut mucosa in *Drosophila melanogaster* (McCall *et al.*, 2014). The overexpression of GPBP is correlated with autoimmune reactions targeted toward augmented and dysfunctional BM in glomeruli in mouse model kidneys (Revert *et al.*, 2007). Extraembryonic tissues that control the exchange of molecules between fetus and mother, specifically the placenta and fetal membranes (FMs), rely on BMs for functionally (Figure 4.1) (Elad *et al.*, 2014). However, spatiotemporal expression of peroxidasin and GPBP during the development of these tissues is unknown.

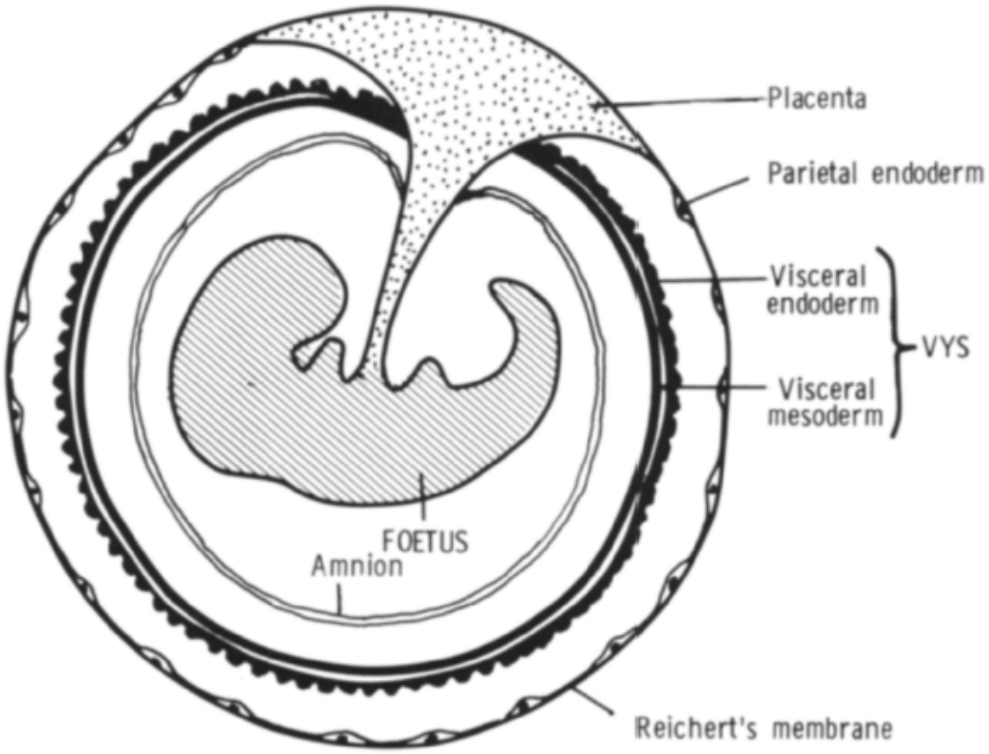


Figure 4.1. Diagram of late gestation embryo and extraembryonic membranes. Adapted from (Adamson & Ayers, 1979).

The fetal membrane (FM) tissue consists of an amnion and chorion layer that is sandwiched between amniotic fluid and trophoblast cells embedded in the maternal decidua (Figure 4.1). A major constituent of the chorion is the integration of ECM components that form a multilayered, thick (3-8μm) BM structure known as Reichert's membrane (Quondamatteo, n.d.; Inoue *et al.*, 1983; Malak *et al.*, 1993; Strauss, 2013). Reichert's membrane is composed of several classical BM proteins, collagen IV, laminin, and nidogen, (Smith & Strickland, 1981; Hogan *et al.*, 1984; Saghizadeh *et al.*, 2013) (Figure 4.1 and 4.2).

The FMs are the first embryo-derived fetomaternal barrier. The development of these tissues initiate at the posterior region of the gastrula, ~7.5 days post coitum (dpc) in mouse (Pereira *et al.*, 2011). Collagen IV expression has been identified in this region as a major ECM factor with putative function in the establishment of the niche for stems cells that build the FMs (Leivo *et al.*, 1980; Mikedis & Downs, 2009). As development progresses, FMs take shape as the avascular sac-like membranous tissue sheathing the embryo throughout midgestational development, ~8.5 dpc – ~12.5 dpc (Pereira *et al.*, 2011). As the embryo grows and expands, it retains amniotic fluid that allows for animated motions of the embryo appendages and cushions it from maternal tissue tension during rapid size growth.

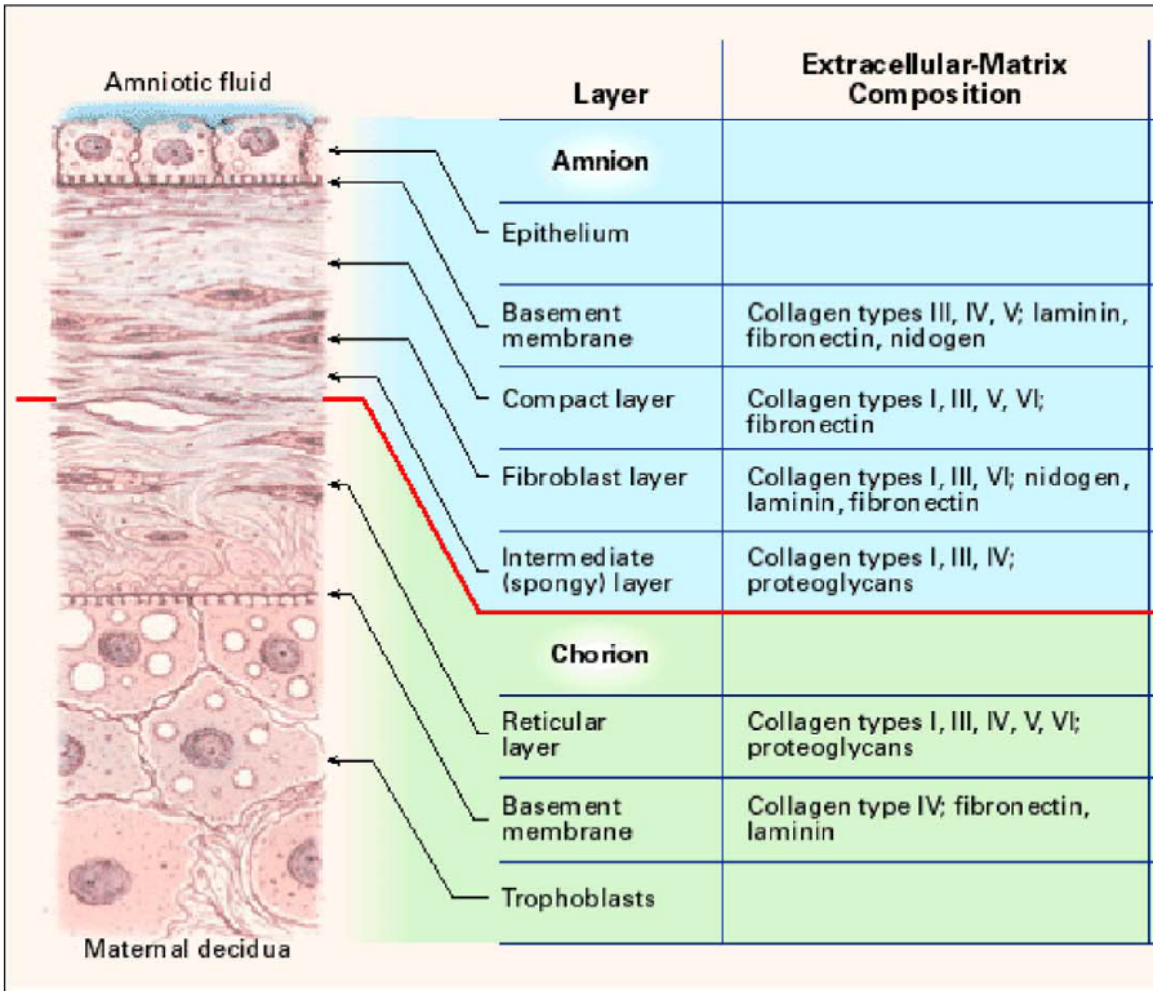


Figure 4.2: Diagram of arrangement and diversity of extracellular matrix and cellular components in fetal membranes. Adapted from (Strauss, 2013).

Disruptions of expression and biosynthesis of BM proteins are associated with FM rupture during development in animal models (Miner *et al.*, 2004; Pöschl *et al.*, 2004). The loss of molecular integrity in the BM and cessation of transmembrane signals to amnion epithelial is attributed to the tearing of membranes, fetal contact with maternal blood, and embryonic fatality (Williamson *et al.*, 1997; Nagai *et al.*, 2000; Miner *et al.*, 2004; Pöschl *et al.*, 2004; Strauss, 2013; Pokidysheva *et al.*, 2014). Human single nucleotide polymorphisms (SNPs) and dietary deficiencies in elements necessary for collagen IV biosynthesis have been identified as risk factors for premature rupture of membranes (clinically referred to as PROMs) (Casanueva *et al.*, 2005; Stuart *et al.*, 2005; Romero *et al.*, 2010). Three percent of pregnancies are complicated by PROMs, which amounts to about 150,000 annual incidences in the United States (Jazayeri, 2016). The onset of PROMs leads to rapid increase in patient and fetal morbidity by causing hypertension, respiratory distress syndrome, chorioamnionitis, abruption placentae, and occasionally fetal death (Medina & Hill, 2006). Although peroxidasin and GPBP are increasingly associated with similar physiological properties of BM barrier function integrity, their localization to developing FM has not been addressed.

The outermost layer of FMs interfaces embryonic trophoblast cells that invade into maternal decidua for the establishment of the placenta. These cells originate as outgrowths of the blastocyst trophectoderm, the single layer of cells opposing the inner cell mass, and are responsible for embryo attachment ~5 dpc (Gardner, 1989; Paria *et al.*, 2001a; Minas *et al.*, 2005; Zhang *et al.*, 2013a). The controlled migration of these cells is attributed to regulated responses to growth factors, cytokines, and ECM. Trophoblasts exhibit a balance of binding and degradation of ECM proteins, especially BM proteins (Sutherland *et al.*, 1988; Fisher *et al.*, 1989; Das *et al.*, 1994; Kimber, 2000; Pfarrer *et al.*, 2003; Klaffky *et al.*, 2006). By 8.0 dpc, the degradation of collagen IV and laminin in part creates a BM void around the embryo. Regulation of trophoblast invasion is essential for placental development and proper conjoining with the uterus. The placenta takes form, at 12.5 dpc in mouse, and functions as the portal for the exchange of oxygen, nutrients, and wastes for the embryo via a bed of blood vessels linked to maternal blood

flow (Gude *et al.*, 2004). The arrangement of the placenta is built BM scaffolds that underlie the labyrinth of blood vessels (Thomas & Dziadek; Kitaoka *et al.*, 1996).

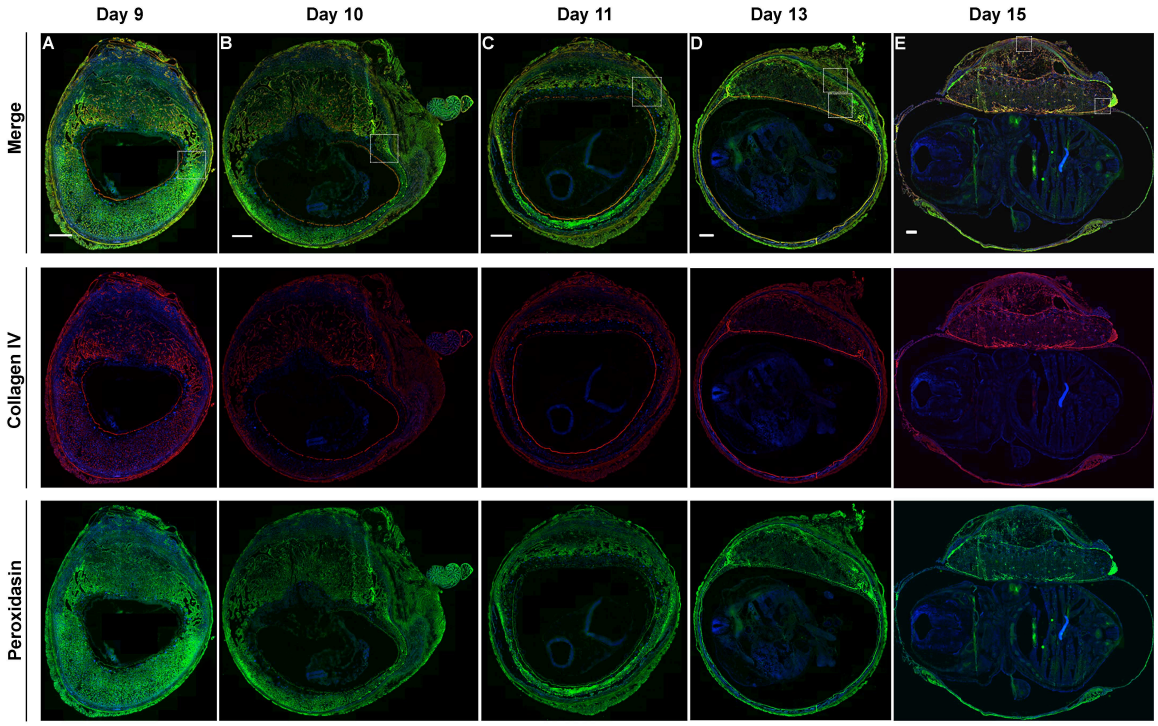
Evasion of trophoblast migratory regulation can lead to placenta accreta, the abnormal invasion and adhesion of placental villus cells beyond decidua and into myometrium or surrounding tissues (Goh & Zalud, 2015; Mogos *et al.*, 2015). Placenta accreta can have deleterious effects on both mother and fetus that require medical care that significantly increases hospitalization cost, including blood transfusion following peripartum hemorrhage, urinary and gastrointestinal injury surgery, and intensive care unit admission (Mogos *et al.*, 2015). The etiology of placenta accreta is unknown. A fraction of the incidence has been attributed to uterine tissue scarring from prior cesarean deliveries. Notably, ECM alterations are a major contributing factor in wound healing and cell behaviors following scarring (Wells *et al.*, 2015). Until recently, no studies had suggested ECM molecules have a function in the pathology of placenta accreta (Haimov-Kochman, 2002; Borbely *et al.*, 2014). However, many reports propose ECM function in preeclampsia (Bieglmayer *et al.*, 1986; Furuhashi *et al.*, 1994; Corrêa *et al.*, 2012; Páez *et al.*, 2013; Yong *et al.*, 2015; Nandi *et al.*, 2016), a placental pathology wherein blood vessels develop abnormally causing reduced fetal-maternal exchange that triggers the release of toxic chemokines into circulation (Sibai *et al.*, 2005). Putative BM proteins, peroxidasin and GPBP, have not been examined during placenta development nor associated-pathologies.

Development of extraembryonic tissues, particularly FMs and placenta, is essential for reproductive success. Although BMs are identified scaffolds within these tissues, their involvement in development and pathophysiology remains unclear. Moreover, the molecular composition of the BMs is unknown, especially in light of emerging BM proteins. In the current study, we find novel spatiotemporal localization of peroxidasin and GPBP expression during the development of extraembryonic tissues in a mouse model of normal pregnancy. These findings set the foundation for further studies in pathological conditions, including PROM, placenta accreta, and preeclampsia.

Results

Localization of peroxidasin and collagen IV in extraembryonic basement membranes

Peroxidasin and collagen IV were localized to tissues at every time point examined but with different relative levels throughout the course of development. In day 9 implantation site, peroxidasin and collagen IV displayed nearly identical patterns of localization in Reichert's membrane (Figure 4.3). This colocalization persisted through day 15 of pregnancy (Figure 4.3). Peroxidasin levels appeared relatively higher than collagen IV in most other tissues day 9-13 of pregnancy. However, on days 10 and 11 trophoblasts adjacent to the fetal membrane contained cytoplasmic peroxidasin, while absent for collagen IV and ECM-localized peroxidasin (Figure 4.3). The immature placenta of the day 13 embryo had the greatest localization of peroxidasin along the edges proximal to the embryo that interfaced maternal tissues. By day 15 of pregnancy, collagen IV and peroxidasin colocalized with relatively similar levels throughout all placental tissues (Figure 4.3). Maternal tissues were also highly positive for peroxidasin and collagen IV localization. Decidua, myometrium, and perimetrium had high detection of peroxidasin throughout days 9-13 of pregnancy (Figure 4.3). Notably, on day 11 of pregnancy, decidua cells interfacing Reichert's membrane distal to the embryo had concentrated localization of peroxidasin (Figure 4.3). These results demonstrate the temporal regulation of peroxidasin in BMs containing collagen IV throughout the development of Reichert's membrane, placenta development, and uterine reprogramming to accommodate the growing embryo.



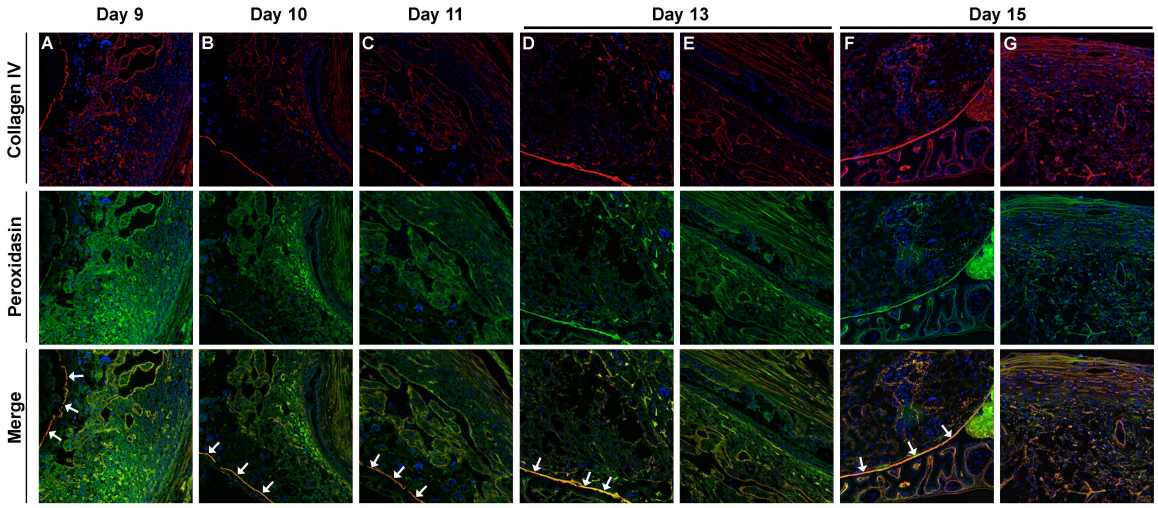


Figure 4.3. Immunofluorescence detection of peroxidasin and collagen IV during midgestation development of extraembryonic tissues. Day 9- Day 15 of pregnancy (A-E respectively) collagen IV (red) and peroxidasin (green), colocalization at relatively similar intensities (orange/yellow), and nuclei (blue). Scale bar= 500μm. Inset white box indicates region displayed magnified in lower panels (A'-E"). Arrows indicate location of Reichert's membrane in higher magnification merged images.

Localization of GPBP in extraembryonic basement membranes

The development of extraembryonic tissues was accompanied by localization of GPBP that varied in specific tissues over the examined time frame. Little to no GPBP was detected in Reichert's membrane day 9-11 of pregnancy (Figure 4.4). On days 13 and 15, GPBP was localized a dense layer of cells at the amniotic border that is potentially associated with Reichert's membrane (Figure 4.4). Placental tissues positively expressed GPBP with relatively unchanged relative levels days 13 and 15 of pregnancy (Figure 4.4). Localization of GPBP in maternal tissues was consistently detected in decidua, myometrium, and perimetrium. Similar to our observations of peroxidasin, GPBP had concentrated localization in decidua cells distal to the embryo on day 11 of pregnancy (Figure 4.4). Together, these results demonstrate the localization of GPBP in diverse extraembryonic and maternal tissues throughout midgestational pregnancy.

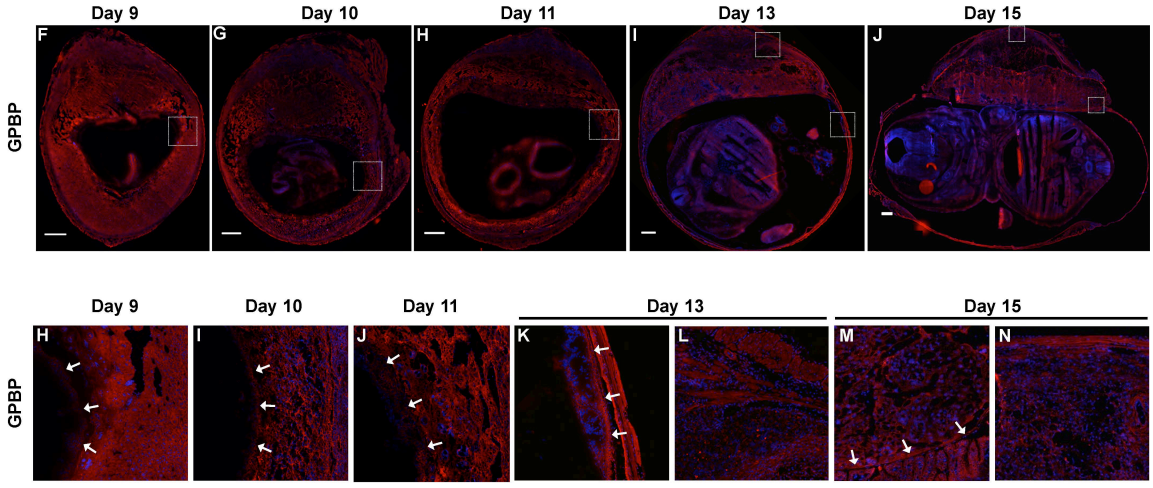


Figure 4.4: Immunofluorescence detection of GPBP during midgestation development of extraembryonic tissues. Day 9- Day 15 of pregnancy (A-E respectively) GPBP (red), and nuclei (blue). Scale bar= 500μm. Inset white box indicates region displayed magnified in lower panels (A'-E").

Discussion

Basement membranes are the integral ECM of fetal membranes and the placenta development and function. This is the first study to examine putative BM components peroxidasin and GPBP during the development of these extraembryonic tissues. Our findings revealed the components are spatially and temporally regulated in extracellular matrix in fetal membranes, placenta, and decidua during midterm pregnancy. Immunolocalization showed expression of peroxidasin and GPBP in specific tissue compartments. Reichert's membrane, decidua, and placenta were positive for peroxidasin during earlier days of extraembryonic tissue development and remodeling. In contrast, Reichert's membrane and placenta had low detectable levels of GPBP until later examined days. Both peroxidasin and GPBP were highly detectable in decidual tissues on day 9-10 and had concentrated localization in layers nearest the embryo on day 11 of pregnancy.

These dynamic patterns of peroxidasin and GPBP are suggestive of diverse requirements for BM functions as well as intracellular roles. Though the cellular mechanisms that regulate these patterns and the physiological responses they trigger can be elucidated with future experimental evidence, the temporal context of the appearance of these proteins indicates potential roles in BM scaffold establishment, cell invasion, and tissue maintenance. In particular, the colocalization of peroxidasin with collagen IV suggests sulfilimine crosslinks are incorporated as these tissues develop. Previously, peroxidasin was noted as a component for early *Drosophila melanogaster* (Nelson *et al.*, 1994) and *Danio rerio* (Fidler *et al.*, 2014) embryo development and crosslinks had been identified in term placenta (Bhave *et al.*, 2012), but this is the first description of localization in Reichert's membrane and early placental structures. In these BMs, peroxidasin can act as a safeguard against fetal membrane tearing by establishing a tissue resilient against embryo-initiated forces. This is corroborated by studies that show perturbation of peroxidasin leads to tissue tearing with disrupted BMs in intestinal tissue, another tissue required to bear regular biomechanical strain. The expression of peroxidasin in early placental development

further validates key roles in BM assembly for tissues reliant on BM scaffolds (Bhave *et al.*, 2012; McCall *et al.*, 2014). Peroxidasin is highly turned over during this window of tissue dynamics and development, as indicated by it excessive localization over collagen IV levels that is normalized in mature placenta. The distinct intracellular localization on peroxidasin in trophoblasts absent of collagen IV suggests non-ECM-associated roles. Dysregulation of reactive oxygen species (ROS) in developing placentas can cause dysfunctional endothelium that leads to pre-eclampsia (Schneider *et al.*, 2015). The expression of peroxidasin during early placenta development may provide protection against excessive ROS by its enzymatic conversion of ROS into hypohalous acids (Hammer *et al.*, 2001; Bhave *et al.*, 2012; McCall *et al.*, 2014).

The pericellular and cytoplasmic localization of GPBP in extraembryonic and decidual tissues is reflective of its complex multifunctionality (Raya *et al.*, 1999). We have recently found the increased expression of GPBP in decidua to correlate with progressive BM thickening around individual decidual cells in early pregnancy, days 4 through 8. The sustained expression of GPBP in decidual cells observed in this study days 9 through 11 of pregnancy is presumably continuation of BM thickening. The day 11 compartmentalized localization of both GPBP and peroxidasin in decidua cells distal to placenta development suggests collective function. Properties of BMs are known to have a strong impact on cell migratory behaviors. Peroxidasin and GPBP specific localization to may establish a higher crosslinked and thicker BM with anti-migratory effects, in turn biasing migration toward single sided placenta development (placenta discoidalis), unlike the banded (placenta zonaria) or multifocal (placenta cotyledonaria) placentas of non-murine and non-human species (Ferner & Mess, 2011; Lobo *et al.*, 2016). The delayed detection of GPBP in fetal membranes suggests a non-essential role in early assembly of Reichert's membrane but a potentially important role in later maintenance and integrity. In the placenta, GPBP is present throughout the placental bed and is relatively unchanged during development. In this context, additional to ECM function, GPBP may function in cell metabolism through mitochondrial support as the cells are required to expend energy for abundant migration, proliferation, and differentiation required for development of the placenta over a brief window of time (Wang *et al.*, 2009).

In homeostatic tissues, peroxidasin and GPBP are difficult to detect and have unclear functions at basal levels. In our model, we found up-regulation of both during development of extraembryonic tissues and decidua maintenance. Pregnancy elicits physiological stressors and tissue dynamics that demand spatiotemporal up-regulation of peroxidasin and GPBP. Their specific expression patterns are indicative that they are players to be considered in the manifestation of mid-term pathologies, including fetal membrane rupture, placental accerta, and pre-eclampsia.

Experimental Procedures

Experimental procedures were carried out in the same manner as those described in Chapter II except at different time points.

Animals

Sexually mature CD1 mice were used for all studies (Charles River Laboratories, Crl:CD1(ICR), Wilmington, MA, USA). Female mice were bred overnight with fertile males of the same strain and the presence of vaginal plug the following morning indicated day 1 of pregnancy (Zhang & Paria, 2006). Implantation sites on days 9, 10, 11, 13, and 15 were collected by visual identification. All samples were collected in the morning of the determined day of pregnancy between 0830-0930 hours, snap frozen in Freeze'It (Fisher Scientific, Waltham, MA), and stored at -80ºC until further processing. All animal procedures were conducted in accordance with guidelines and standards of the Society for the Study of Reproduction (SSR) and approved by Vanderbilt University's Institutional Animal Care and Use Committee (IACUC).

Immunofluorescence

Frozen tissues were warmed to -20ºC, embedded in Optimal cutting temperature (OCT) compound (Sakura Tissue-Tek, Torrance, CA) and sectioned (12 µm) in a cryostat (Leica Biosystems, Buffalo Grove, IL). Sections were collected on Superfrost Plus slides (Fisher Scientific, Waltham, MA), air-dried and fixed in -20ºC acetone for 10 minutes. Slides were washed several times with phosphate-buffered saline (PBS) (Corning, Corning, NY) and PBS/0.2% Tween (Sigma, St. Louis, MO). Slides to be stained with collagen IV antibody (JK2) were treated with dissociation buffer consisting of 6M urea in 0.1 M glycine buffer (pH 3.0) (Ninomiya *et al.*, 1995) followed by several washes with PBS and PBS/0.2% Tween. Peroxidasin immunodetection patterns were minimally influenced by dissociation treatment rendering co-immunostaining with collagen IV antibody possible. Immunodetection of GPBP was achieved without the dissociation step because GPBP antibody used in this study failed to show specific staining in sections treated with this buffer. All sections were preincubated with 10% normal serum from goat or horse (Invitrogen, Grand Island, NY) for 1 hour at room temperature to avoid nonspecific binding of antibodies prior to applying primary antibodies. The following primary antibodies were used for antigen detections: rat anti-collagen IV NC1 (1:500 dilution, JK2, were from Y. Sado, Shigei Medical Research Institute, Okayama, Japan), rabbit anti-peroxidasin (1:250 dilution, were from G. Bhave, Vanderbilt University, Nashville, TN), and Alexa546 conjugated mouse anti-GPBP (1:50 dilution, N26 to all GPBP isoforms, were from Fibrostatin, Valencia, Spain). The secondary antibodies used for immunofluorescence detection were: Alexa555 goat anti-rat (1:200 dilution, Abcam ab150166, Abcam) and Alexa488 goat anti-rabbit (1:200 dilution, Abcam ab150081, Abcam). All primary antibodies were diluted in PBS/0.1% Tween and 5% normal goat serum. Sections were incubated with these antibodies overnight at 4°C in a humidified chamber and then washed three times with PBS/0.2% Tween. After several washes, 1 µM Hoechst fluorescent dye was applied (10 minutes) for labeling cell nuclei. Following washes in PBS/0.2% tween and PBS, sections were mounted in Prolong Gold (Life Technologies, Grand Island, NY) and cured at room temperature for 2 days. Images at 0.323 µm/pixel resolutions were captured utilizing the Ariol SL-50 automated fluorescent microscope with 20x objective (Leica

Biosystems, Buffalo Grove, IL) housed in the Vanderbilt Digital Histology Shared Resource. Image linear brightness and contrast were adjusted for visual comparison using FIJI (1.49q, ImageJ, NIH, Bethesda, Maryland) controlling for within set staining and to maintain consistent intensities relative to representative controls.

Chapter V

Conclusions and future implications

Conclusions

The works in this dissertation confirm the hypothesis that BM structure and components are dynamically regulated during uterine, embryonic, and extraembryonic tissue development for pregnancy. These studies have made significant advancements in the understanding of BM spatial and temporal patterns of rapid assembly *in vivo*. Over the course of assembly, individual components are regulated with a precision that was only observed by tracking microenvironmental changes. Here, discoveries further established peroxidasin and Goodpasture-antigen binding protein (GPBP) as BM components and provided evidence for additional roles proposed in the following discussion.

Multiple roles for peroxidasin during pregnancy

Reinforcement of Collagen IV during the Development of Tissues during Pregnancy

Throughout this dissertation, novel data revealed that peroxidasin is a component of basement membranes in developing of uterine, embryonic, and extraembryonic during pregnancy. In chapter 2, peroxidasin colocalized to collagen IV in both endometrial epithelial tissues and decidual tissues. The production of peroxidasin ahead of collagen IV during early decidualization supported continual sulfilimine crosslinking during secretion of collagen IV. To our knowledge, this is the second account peroxidasin secretion preceding production of basement membrane components that undergo rapid consolidation. It was first described in early embryogenesis of *Drosophila melanogaster* during hemolymph morphogenesis where hemocytes produce peroxidasin around stage 10 of development which is successively localized to BMs by stage 15 (Nelson *et al.*, 1994). In chapter 3 of this dissertation, peroxidasin concentrated to the BM of the first layers of epithelial-like cells, underlying layers of epiblast cells during morphogenesis *in utero* and *in vitro*. In chapter 4, peroxidasin was ubiquitously present in BMs

during the development of the fetal membranes and placenta in mid-late stages of pregnancy. The consistent localization of peroxidasin to BMs of diverse tissues further establishes it as an important BM component presumably for the formation of sulfilimine crosslinks in the collagen IV lattice.

The question remains, is peroxidasin secretion rate-limiting for sulfilimine crosslinking or can collagen IV-bound or circulating peroxidasin compensate for localized decreased cellular secretion? In adult tissues, peroxidasin expression levels are limited unless the tissues are injured or undergoing pathological progression (Péterfi *et al.*, 2009). During pregnancy, specialized tissues are rapidly developed that incorporate peroxidasin and sulfilimine crosslinks into BMs. In order to further understand how peroxidasin secretion rates mediate collagen IV reinforcement, *in vitro* culture studies may elucidate cellular and molecular mechanisms that maintained a pace for continual crosslinking that support tissue-genesis during pregnancy. For example, pulsed induction of peroxidasin expression followed by inhibition of expression in a cell line, such as PFHR9 cells or primary decidua cells, may determine the duration of crosslinking activity following expression. Biochemical studies with purified peroxidasin and collagen IV have demonstrated that sulfilimine crosslinks can be formed in spite of cellular activities (Bhave *et al.*, 2012). However, the rate of peroxidasin expression required for crosslinking in an environment of continual collagen IV production is unknown. Results of those experiments may be relevant for understanding peroxidasin secretion *in vivo* during pregnancy and other physiological or pathological processes involving BM production, such as angiogenesis and fibrosis.

In addition to BM-associated patterns, a few observations were made where peroxidasin was distinctly localized to cells absent of collagen IV or laminin. Results described in chapter 2 showed that on day 5 of pregnancy peroxidasin was concentrated to the compartment of cells that surrounded the implantation site. Since there was relatively little collagen IV localized to that area at that time, these observations suggest that peroxidasin could have other important roles during the initial contact between the blastocyst and maternal tissues. Some of these implantation sites supportive roles are considered here by taking into account the uterine milieu during periimplantation, products of peroxidasin activity, and functions of related enzymes at that site.

Non-crosslinking roles of peroxidasin at embryo-maternal interface during early pregnancy

Peroxidasin is a member of a peroxidase-cyclooxygenase superfamily of proteins that are heme-containing metalloproteinases which are capable of reducing hydrogen peroxide to water and hypohalous acids (Soudi *et al.*, 2012). The proteins of this superfamily carry multiple enzymatic domains for crosslinking activities, oxidoreductase activities, and prostaglandin (PG) synthesis (Zamocky *et al.*, 2008; Soudi *et al.*, 2012). Peroxidasin oxidoreductase activity is the intermediate step during the sulfilimine crosslink formation (Bhave *et al.*, 2012; McCall *et al.*, 2014). This involves peroxidasin reducing hydrogen peroxide (H_2O_2) by cleaving the peroxidic bond then incorporating electrons from essential halides (bromide and chloride, Br^- or Cl^-) that form hypohalous acid products (HOBr and HOCl) (Zamocky *et al.*, 2008). Hypohalous acids are antimicrobial agents that produced by several related peroxidases involved in the innate immune system. In response to foreign material (e.g. pathogens and allogenic cells), leukocytes invade the site of contamination and release myeloperoxidase (MPO) and eosinophil peroxidase (EPO) that catalyze hypohalous acids production that the kill pathogens (Zamocky *et al.*, 2008; Soudi *et al.*, 2012). During pregnancy, uterine tissue restricts immune cell invasion to permit receptivity of the semi-allogenic blastocyst (Chakraborty & Pulendran, 2009; Collins *et al.*, 2009). The immune privileged implantation site may benefit from the strong presence of peroxidasin at the implantation site. The uterus is highly susceptible to bacterial infection during implantation and reports have demonstrated that hypohalous acids produced by peroxidasin destroy bacteria *in vitro* (Li *et al.*, 2012). The low concentrations of hypohalous acids are safe for the surrounding tissues and presumably for the embryo. Future studies may address potential the antimicrobial role of peroxidasin by introducing a low-grade infection with attenuated *Escherichia coli* into control and peroxidasin knockout mice during periimplantation. The weakened *Escherichia coli* may elicit an immune reaction from the presence of the pathogen without triggering major responses to endotoxin (Lei *et al.*, 2015). Results anticipated from this study will demonstrate if peroxidasin kills microbes at the implantation site and is required for early pregnancy success.

Cyclooxygenases (COXs), also known as prostaglandin endoperoxide H synthases (PGHSs), are relatives of peroxidasin and are members of the heme-peroxidase family (Zamocky *et al.*, 2008). These peroxidases catalyze uterine PG synthesis that is required for fertility and periimplantation events, including implantation and decidualization (Paria *et al.*, 2001b; Reese *et al.*, 2001; Zhang *et al.*, 2014). Particularly, COX-2 is essential for blastocyst attachment (Lim *et al.*, 1997). In mice, COX-2 is often considered a biomarker of normal implantation identified by a distinct pattern that switches along the antimesometrial-mesometrial axis of the uterine lumen between days 4 and 6 of pregnancy. On day 5 of pregnancy, COX-2 is detected in implantation sites as intense staining restricted to antimesometrial luminal cells and subepithelial stromal cells (Dey *et al.*, 2004; Chakrabarty *et al.*, 2007). This distinctive pattern resembles the striking localization patterns of peroxidasin in implantation sites on day 5 of pregnancy described in this dissertation. The common patterns of COX-2 and peroxidasin during implantation imply that peroxidasin may have similar roles as COX-2. Since PG synthesis functions of peroxidasin are not established, the next logical study will be to determine *in vitro* if peroxidasin can synthesize PGs from substrates, such as arachidonic acid (Ricciotti & FitzGerald, 2011). Together, many enzymatic activities of peroxidasin have functions during pregnancy. Future studies, such as those described here, are necessary for a determination of which roles are required for pregnancy (Figure 5.1).

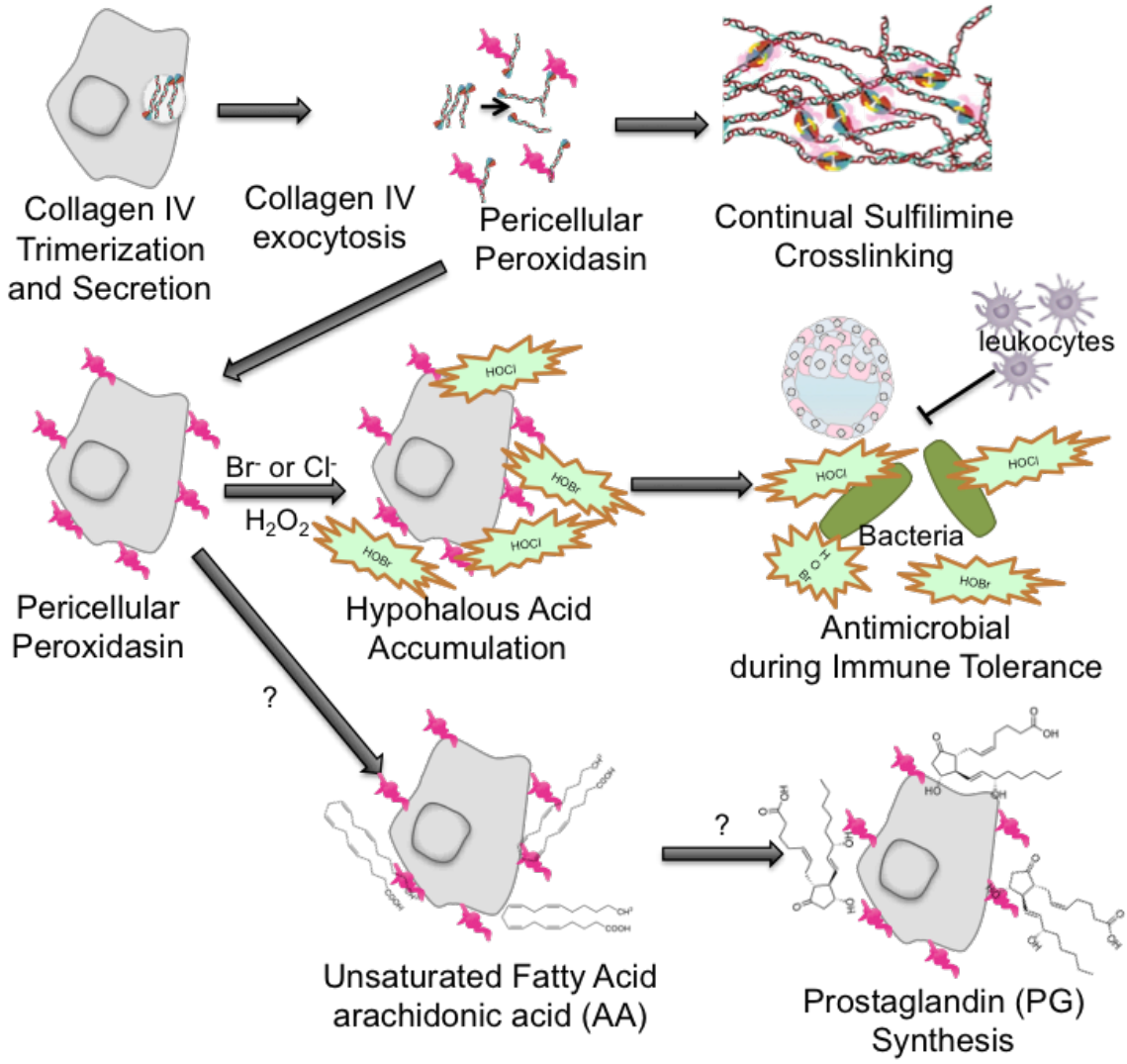


Figure 5.1: Schematic model of potential functions of peroxidasin during pregnancy. In this dissertation, evidence was provided that suggests in function for sulfilimine crosslinking of collagen IV in BMs. Distinct patterns of peroxidasin at the implantation site imply additional roles, including antimicrobial defense and PG synthesis.

Extracellular and intracellular roles for GPBP during pregnancy

The studies described in this dissertation revealed that GPBP expression levels and localization patterns were regulated temporally in early pregnancy and were limited to placental extraembryonic tissues in mid-late pregnancy. In during early pregnancy, extracellular GPBP was localized to the BMs of developing decidual cells. Increased expression and localization of GPBP correlated temporally with thickening of decidual BMs. Genetic disruption of GPBP1 isoform resulted in a more discontinuous pattern of BM laminin. Moreover, extracellular GPBP was localized around cells in the developing placenta but not in fetal membranes. These data suggest that extracellular GPBP may have roles in patterning BMs with variable thicknesses and is not ubiquitous to all assembling BMs. Structural variability of BMs has the potential to modulate migration of cells, such as placental trophoblasts, by creating regions of lesser resistance to transverse or penetrate (Kelley *et al.*, 2014). The thinner regions of BM may also promote the establishment of cell-cell junctions in the decidua tissue that forms important macromolecular barriers that can prevent maternal antibodies from reaching the embryo (Tung *et al.*, 1986; Yang *et al.*, 2014a). These proposed micro-patterning functions could be further investigated by patterning of BM produced by GPBP overexpressing and null cells then analyzing the matrix thickness and composition by electron and confocal microscopy. Additionally, these cells could be used to produce BMs in transwells, and then once the original cells are removed, trophoblast or other migratory cells could be seeded and 3-D cell migration studies would determine the impact of extracellular GPBP matrix modifications on migration.

In the preimplantation uterus, data revealed that GPBP was localized to the apical region of the cytoplasm and plasma membrane in luminal cells on days 2 through 4 of pregnancy. These data suggests GPBP intracellular roles may be important during luminal alterations that permit embryo receptivity by day 5 of pregnancy. Intracellular GPBP functions in lipid transport that facilitates the remodeling of plasma membranes (Raya *et al.*, 1999; Revert *et al.*, 2008; Revert-Ros *et al.*, 2011). Prior to embryo attachment, uterine luminal epithelium alters apical plasma membrane properties that result in the transient loss of microvilli and the support of

blastocyst attachment (Murphy, 2004). GPBP facilitate the apical remodeling of luminal epithelial cells for blastocyst receptivity. Future studies could determine if apical GPBP alters epithelial plasma membranes by experimentally forced trafficking of GPBP to apical or lateral or basal regions of the cell by linking its expression to proteins in the phosphatase and tensin homolog (PTEN) and phosphoinositide kinase (PIK) pathways (Devergne *et al.*, 2014; Peng *et al.*, 2015). Organoid cultures of luminal or intestinal cells form microvilli *in vitro* could be used to determine if apical GPBP is linked to alterations in microvilli length or presence (Lancaster & Knoblich, 2014). Anticipated results from this study could provide a further understanding of mechanisms that may link apical GPBP to preimplantation plasma membrane transformation of uterine luminal cells. Together, data in this dissertation provide insights that may lead to future studies of extracellular and intracellular GPBP functions during pregnancy.

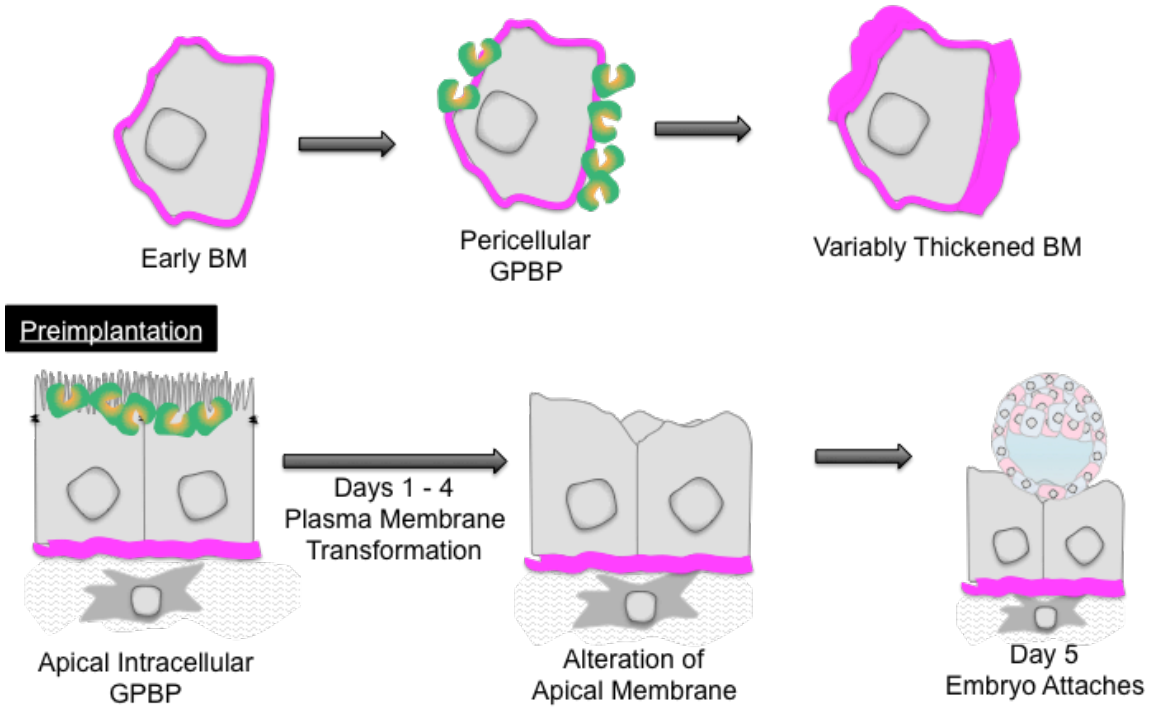


Figure 5.2: Schematic model of potential functions of GPBP during pregnancy. In this dissertation, evidence was provided that suggests extracellular localization of GPBP that correlates to thickened BM and intracellular localization that correlates to plasma membrane remodeling of preimplantation uterine luminal cells.

Implications in aspects of cancer biology

Though particular attention was given to female reproductive biology in this dissertation, these findings provide fundamental information for better understanding of cancers that mimic mechanisms associated with pregnancy. Peritoneal carcinomatosis (PC) is a subset of metastatic disease that has gross and histological similar morphological characteristics to ectopic decidual growths (Kondi-Pafiti *et al.*, 2005; Adhikari & Shen, 2013; Abramowicz *et al.*, 2014). Approximately 50% of patients with invasive gastric carcinoma and approximately 40% of patients with recurrent colorectal carcinoma develop PC (Lu *et al.*, 2010). Patients with PC have poor prognostic outcomes with a median survival estimate of 1 – 6 months (Montori *et al.*, 2014). Typical treatments involve surgical removal of visible tumors within the abdomen followed by delivery of highly concentrated, heated chemotherapy directly to the peritoneal tissues during surgery and raise 5-year survival rates to 76% of patients (Lu *et al.*, 2010; Montori *et al.*, 2014). In rat models of PC, inhibition of matrix metalloproteinase that target basement membranes was shown to prevent the growth of PC and improve survival (Aparicio *et al.*, 1999). With these findings and observations discussed in this dissertation, data suggests that BMs may have fundamental roles in regulating behaviors of cells in decidual tissues and decidual-like PC lesions.

Similar to putative functions of BMs in pregnancy, BMs of metastases influence ligand binding, spatial expansion, cellular organization, environmental plasticity, and chemokine gradients. Several reports have paralleled mechanisms of immune regulation, cell migration, and tissue proliferation involved in pregnancy with those that promote malignant progression (Pitelka *et al.*, 1980; McKay *et al.*, 1992; Murray & Lessey, 1999; Nerenberg *et al.*, 2007). Future studies are necessary to establish parallels in BM functions. Comparative analysis and functional studies of PC and decidualization may provide a foundation for understanding the common or distinct function of BMs in these physiological and pathological states. A model system has been recently introduced that could be utilized to test this notion. Imano et al. established a mouse model of PC by using mechanical abrasion to induce stromal reaction of the peritoneum that mimics decidua with cuboidal epithelioid-like cells and promotes metastatic tumor cell propagation (Imano *et al.*,

2013). Further investigations of the BM dynamics using this model coupled with physiological studies of uterine decidualization may provide knowledge that leads to discoveries of therapeutic targets and development of treatments that improve outcomes for patients with PC.

Altogether, findings and future studies described in this dissertation may provide valuable advances in the fields of matrix biology, reproductive biology, and cancer that lay the foundation for medical innovation.

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